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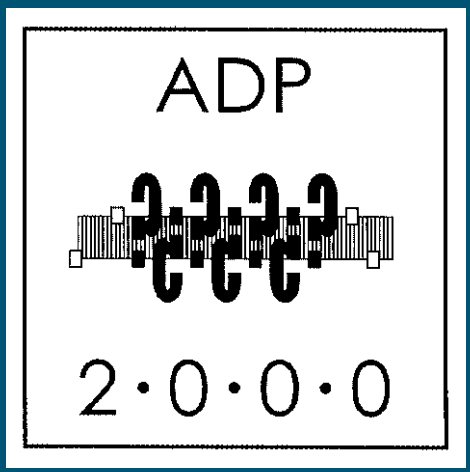
volume 85, The Platelet ADP Receptors supplement


## THE PLATELET ADP RECEPTORS
## Biochemistry, physiology, pharmacology and clinical aspects

*Chairmen:*
*M. Cattaneo, C. Gachet*

*La Thuile, March 29-31, 2000*



Haematologica

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3. The Royal Marsden Hospital Bone-Marrow Transplantation Team. Failure of syngeneic bone-marrow graft without preconditioning in post-hepatitis marrow aplasia. Lancet 1977; 2:242-4.

4. Anonymous. Red cell aplasia [editorial]. Lancet 1982; 1:546-7.

5. Karlsson S, Humphries RK, Gluzman Y, Nienhuis AW. Transfer of genes into hemopoietic cells using recombinant DNA viruses [abstract]. Blood 1984; 64(Suppl 1):58a.

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9. DuPont B. Bone marrow transplantation in severe combined immunodeficiency with an unrelated MLC compatible donor. In: White HJ, Smith R, eds. Proceedings of the third annual meeting of the International Society for Experimental Hematology. Houston: International Society for Experimental Hematology; 1974. p. 44-6.

10. Bieber MM, Kaplan HS. T-cell inhibitor in the sera of untreated patients with Hodgkin's disease [abstract]. Paper presented at the International Conference on Malignant Lymphoma Current Status and Prospects, Lugano, 1981:15.

11. Worwood M. Serum ferritin. In: Cook JD, ed. Iron. New York: Churchill Livingstone; 1980. p. 59-89. (Chanarin I, Beutler E, Brown EB, Jacobs A, eds. Methods in hematology; vol 1).

12. Ranofsky AL. Surgical operation in short-stay hospitals: United States-1975. Hyattsville, Maryland: National Center for Health Statistics; 1978. DHEW publication no. (PHS) 78-1785, (Vital and health statistics; series 13; no. 34).

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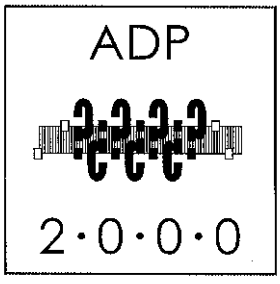


# THE PLATELET ADP RECEPTORS

BIOCHEMISTRY, PHYSIOLOGY, PHARMACOLOGY AND CLINICAL ASPECTS

Promoted by ETRO
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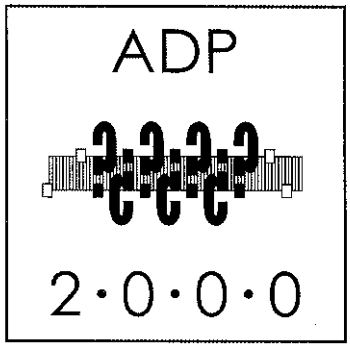


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**Haematologica** 2000; 85 (The Platelet ADP Receptors supplement):1-2

# Foreword

Adenosine-5'-diphosphate (ADP) was recognized as an inducer of platelet aggregation in the early sixties.[1,2] Although itself a weak platelet agonist, ADP plays a key role in platelet function because, when it is secreted from the platelet dense granules where it is stored, it amplifies the platelet responses induced by other platelet agonists.[3] The amplifying effect of ADP on platelet aggregation may account for the critical role played by ADP in hemostasis and in the pathogenesis of arterial thrombosis, which is documented by a number of observations: 1) pharmacologic inhibition of ADP-induced platelet aggregation decreases the risk of arterial thrombosis;[4] 2) patients lacking releasable ADP in granule stores or with congenital abnormalities of the platelet ADP receptors have a bleeding diathesis;[5-7] 3) CD39, the endothelial cell ecto-ADPase, is a critical component in the regulation of thrombogenesis.[8,9]

Despite the early recognition of ADP as a platelet aggregating agent, the molecular basis of ADP-induced platelet activation is only beginning to be understood. Biochemical, pharmacologic and clinical studies led to the proposal of a model of three purinergic receptors contributing separately to the complex process of ADP-induced platelet aggregation: the $P2X_1$ ionotropic receptor responsible for rapid influx of ionized calcium into the cytosol, the $P2Y_1$ metabotropic receptor responsible for mobilization of ionized calcium from internal stores which initiates aggregation, and an as yet unidentified P2Y receptor coupled to adenylyl cyclase inhibition (named in different ways by different authors: P2cyc, $P2Y_{ADP}$, $P2T_{AC}$), which is essential for the full platelet aggregation response to ADP, although no causal relationship exists between adenylyl cyclase inhibition and platelet aggregation.[3,10-13] It is probable that this as yet unidentified receptor is the molecular target of the ADP-selective antiaggregating drugs, ticlopidine and clopidogrel. In addition, it is probably defective in patients with a bleeding diathesis that is characterized by selective impairment of platelet responses to ADP.[6,7,14,15] On the other hand, studies with $P2Y_1$ receptor-deficient mice clearly demonstrated the critical role of this receptor in hemostasis and in thrombosis[16,17]. Despite the recent progresses in the understanding of the mechanisms involved in ADP-induced platelet responses, many issues are still unknown or remain controversial: 1) the role of $P2X_1$ receptor in platelet function; 2) the molecular identity of P2cyc; 3) the nature of the effector(s) in the Gi pathway of ADP-induced platelet aggregation, to name but a few.

In consideration of the very important recent achievements and the rapid evolution of this area of research we thought that time was ripe for organizing a meeting in which some of the most distinguished scientists in this research field could gather to exchange their experiences and to clarify the state of the art and future perspectives. The meeting took place in La Thuile, a small village in the Italian Alps, which provided a beautiful frame to what proved to be a very stimulating and interesting scientific meeting.

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## References

1. Gaarder A, Jonsen J, Laland S, Hellem A, Owren PA. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. Nature 1961; 192:531-2.
2. Gaarder A, Laland S. Hypothesis for the aggregation of platelets by nucleotides. Nature 1964; 202:909-11.
3. Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. Arterioscler Thromb Vasc Biol 1999; 19:2281-5.
4. Quinn MJ, Fitzgerald DJ. Ticlopidine and clopidogrel. Circulation 1999; 100:1667-72.
5. Bennett JS. Hereditary disorders of platelet function. In: Hoffman R, Benz EJ Jr, Shattil SS, Furie B, Cohen HJ, Silberstein LE, McGlave P, eds. *Hematology. Basic Principles and Practice*. New York, NY: Churchill Livingstone; 2000; 2154-72.
6. Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. Blood 1992; 80:2787-96.
7. Nurden P, Savi P, Heilmann E, et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. J Clin Invest 1995; 95:1612-22.
8. Marcus AJ, Broekman MJ, Drosopoulos JHF, et al. The endothelial cell ecto-APDase responsible for inhibition of platelet function is CD39. J Clin Invest 1997; 99: 1351-60.
9. Enjyoji K, Sévigny J, Lin Y, et al. Targeted disruption of CD39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation. Nature Med 1999; 9:1010-7.
10. Daniel JL, Dangelmaier CA, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. J Biol Chem 1998; 273: 2024-9.
11. Hechler B, Léon C, Vial C, et al. The $P2Y_1$ receptor is necessary for ADP-induced platelet aggregation. Blood 1998; 92: 152-9.
12. Geiger J, Honig-Liedl, Schanzenbacher P, Walter U. Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. Eur J Pharmacol 1998; 351:235-46.
13. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci USA

1998; 95:8070-4.
14. Hechler B, Eckly A, Ohlmann P, Cazenave JP, Gachet C. The $P2Y_1$ receptor, necessary but not sufficient to support full ADP-induced platelet aggregation, is not the target of the drug clopidogrel. Br J Haematol 1998; 103: 858-66.
15. Léon C, Vial C, Gachet C, et al. The $P2Y_1$ receptor is normal in a patient presenting a severe deficiency of ADP-induced platelet aggregation. Further evidence for a distinct P2 receptor responsible for adenylyl cyclase inhibition. Thromb Haemost 1999; 81:775-81.
16. Fabre JE, Nguyen M, Latour A, et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. Nature Med 1999; 5:1199.
17. Léon C, Hechler B, Freund M, et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y1 receptor null mice. J Clin Invest 1999; 104:1731.

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# HISTORICAL OVERVIEW OF THE ROLE OF PLATELETS IN HEMOSTASIS AND THROMBOSIS

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ABSTRACT

**Platelets were discovered by G. Bizzozero in 1882 and *rediscovered* in the 1960s after many decades of oblivion. Interestingly enough, their role was initially more clearly associated with thrombosis than with hemostasis. For many years a serious unresolved problem was that the clotting time was normal even in severe thrombocytopenia. The concept of coagulation as an enzymatic cascade had not yet been elaborated. During the 1960s, the interest of many experts moved from the interaction of platelets with the process of blood coagulation to the interaction of these cells with the vascular wall (adhesion) and each other (aggregation). The discovery of the role of ADP as the principles of platelet aggregation stimuli was rapidly followed by other important discoveries such as the aggregating properties of collagen and thrombin, the *release reaction*, the metabolism of arachidonic acid, and the inhibitory effect of aspirin. The use of aspirin as a potential antithrombotic drug has made the history of clinical trials in the last 30 years. The last two decades have been characterized by an explosion of cell and molecular biology approaches. There are presently people who study platelet signal transduction or platelet-leucocyte interactions but who know almost nothing about hemostasis or thrombosis! This is due not only to the intrinsic limitations of the biological approach but also to the progressive recognition of the role of platelets in other physiopathologic and clinical conditions such as inflammation, cancer growth and dissemination, and organ transplant rejection. Overlooked for more than two centuries after the microscope was made available to hematologists, considered as an artifact or a Cinderella, the platelet has mainly been considered in the past 30 years as a dangerous cell to be inhibited by (ever more expensive) drugs. But the taming of the shrew is far from being achieved.**


Key words: platelets, hemostasis, thrombosis, history

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*There is no platelet history. There are only facts and experiences that deserve narration.*

*This review is neither a systematic or critical analysis nor a research into documented past events, but rather a personal account of imaginary or real happenings that contributed to my past.*

## In the beginning ...

«The existence of a constant blood particle, differing from red and white blood cells, has been suspected by several authors for some time».

With this simple opening statement, in 1882 Giulio Bizzozero started his paper on a new blood particle and its role in thrombosis and blood coagulation.[1] One will be surprised by the fact that hemostasis was not mentioned (unless incidentally) in Bizzozero's work. Bizzozero quoted Zahn's[2] observation that following incision of a vessel wall, bleeding was not arrested by coagulation of extravasated blood, but rather by formation of a white thrombus at the site of the lesion.

However, in a footnote at the end of his monumental paper, Bizzozero mentions with astonishment that Professor Hayem in Paris had claimed «to have discovered that the thrombotic mass, responsible for hemostasis after injury to blood vessels, is formed by accumulation and mutual fusion of its hematoblasts». «The phenomenon which Hayem claims as his own discovery» – Bizzozero states – «i.e. events leading to thrombus formation ... were observed and published by myself already several months earlier». Thus Bizzozero considered the role of platelets (or hematoblasts) to be the same both in the arrest of hemorrhage and in thrombus formation. No doubt, Bizzozero did not recognize platelets as a factor in hemorrhagic conditions such as purpura. Instead he stated that «one may assume that their increased number alters the conditions of blood circulation». It was only in 1883 – the year after Bizzozero's publication – that Krauss, in his inaugural dissertation in Heidelberg, mentioned that his chief, Dr. Brohme, had noted a marked decrease of platelets in the blood of children with purpura hemorrhagica.[3] It was, however, Hayem who firmly established the relationship of platelets to purpura a few years later.[4]

Both Bizzozero and Hayem presented evidence that platelets (or hematoblasts) participate in the early phase of blood coagulation, as they observed that fibrin strands appear at loci where platelets adhere and undergo morphologic changes. «The more rapid coagulation of blood, flowing from a wound which had been open for some time, is probably due to the presence of aggregates of blood platelets which have formed after the time of the first incision, on the surface of vascular lesions and the margins of incision».[1] Both investigators concluded that platelets supply a factor needed in the clotting reaction. However, the observation that the clotting time was normal even in severe thrombocytopenia led many other investigators to conclude that platelets are not necessary in the coagulation of blood[5] although Roskam[6] had already described in 1923 the presence of fibrinogen on the platelet surface, suggesting that the fibrinogen-fibrin transition on the platelet surface might be of importance. According to Quick,[7] it was his prothrombin consumption time test that supplied a clear answer to the role of platelets in coagulation. This procedure, indeed, showed that even when the clotting time is normal, the prothrombin consumption is markedly reduced when normal plasma depleted of platelets is clotted. Mixed experiments with platelet-poor normal plasma and platelet-rich plasma from patients with hemophilia A suggested that a clotting principle in platelets (surface phospholipids) reacting with a plasma factor lacking in hemophilia (factor VIII) accelerates the *intrinsic* coagulation process.

## From Cinderella to royal prominence

Despite the excellency of these and other contributions, the platelet remained for many years the neglected stepchild in the family of blood cells. Finally, around 1960, the platelet emerged from the Cinderella stage to that of royal promince.[8] The work by Hugues and Bounameaux in Roskam's group in Liège[9] showed that the collagen component of connective tissue leads to platelet adhesion and aggregation culminating in *viscous metamorphosis*. Some other important discoveries were made at about the same time: the isolation and description of a contractile protein resembling actomyosin in platelets,[10] and the recognition that adenosine-5'-diphosphate (ADP) is a potent inducer of platelet aggregation.[11]

This latter finding was originally based on the liberation of platelet aggregating material from red cells (factor R of Hellem).[12] This only attained its full significance with the observation that platelets themselves, under the influence of a suitable agent such as thrombin, release enough of this dinucleotide to induce their own aggregation.[13] The recognition of this phenomenon remains a most important step in the understanding of the mechanism of platelet aggregation, which accordingly appeared as a self-perpetuating process.[14] In the early Sixties, Born and Cross[15] and O'Brien[16] described an optical platelet aggregation test, roughly based on the decrease of optical density of a platelet suspension corresponding to platelet clump formation by a given stimulus. Very soon, this appeared to be an easy and practical method and platelet aggregation could be studied in dozens of laboratories all around the world. Now, about 40 years later, the molecular basis of ADP-induced platelet activation is only beginning to be understood and a model of three purinergic receptors, each contributing separately to ADP-induced mechanisms has been proposed.[17]

## Meeting with aspirin

In the years 1967-1968, aspirin and the platelet met each other officially for the first time and a never-ending story was begun. In reality, already in the fifties French investigators[18,19] had observed that aspirin, in relatively small doses, resulted in a prolongation of bleeding time. They also noted that this effect was exaggerated in patients who had underlying bleeding disorders. These clinical observations were confirmed in the USA by Quick[20] who also made the important observation that, unlike aspirin, sodium salicylate had no effect on bleeding time. Weiss and Aledort[21] first showed that prolongation of the bleeding time by aspirin (3 grams/day for two and a quarter days!) was associated with a marked impairment of collagen-induced platelet aggregation. By constrast, aspirin ingestion did not inhibit ADP-induced aggregation. Sodium salicylate failed to prevent platelet aggregation either by collagen or ADP. Other groups[22-24] confirmed and extended the original findings of Weiss and Aledort. The general conclusion was that aspirin – possibly by a poorly defined platelet membrane stabilizing effect[25] – inhibited the platelet release reaction. The effects of aspirin ingestion occurred very rapidly but were of long duration (4 to 7 days), suggesting an irreversible damage to platelet population, which persisted until the affected platelets had been replaced by a sufficient number of new platelets. The critical role of the acetyl group in the aspirin effect was also rapidly singled out. Altogether, these findings reasonably explained the mild hemostatic defect produced by aspirin and indicated that it should be avoided in patients whom control of hemostasis could be a problem.

«The Antichrist» – says William in *The Name of the Rose* – «can be born from piety itself, from excessive love of God or of the truth, as the heretic is born from the saint and the possessed from the sear».[26] No surprise, therefore, that a more intriguing outcome of these studies was the possibility that, by inhibiting platelet aggregation, aspirin might be a useful antithrombotic agent. Platelet aggregates may form on collagen fibers which are exposed after the vascular intima has been broken. If aspirin was capable of inhibiting collagen-induced platelet aggregation, might it also prevent arterial thrombus formation? It was soon realized that this question could only be answered by clinical trials.[27] In the early seventies, the opinion was prevalent that anticoagulant drugs – though effective in the management of *venous* thromboembolism – had not produced any significant effect on the overall morbidity and mortality from the complications of *arterial* disease, such as myocardial infarction and stroke. Researchers such as the Canadian group of J.F. Mustard[28] were reasoning that «assuming that thrombosis is involved in the death of patients with vascular disease who die from strokes or

myocardial infarction ..., the rationale behind the use of anticoagulant drugs in conditions where the initial accumulation of a platelet mass is the primary event in thrombus formation, is open to serious question». The case for testing aspirin in the prevention of myocardial infarction and other arterial occlusion disease became therefore very strong, although «aspirin is a drug that any idiot can buy in any quantity he chooses and take for whatever condition he chooses».[29] Strangely enough, aspirin was probably first tested in Europe as a prophylactic measure in post-operative venous thromboembolism![30] The results of this Medical Research Council of England's trial were negative. We had to wait until the late eighties to be assured, by one of the largest and most complex meta-analyses performed in the past decade, that aspirin (and other antiplatelet drugs) were effective in the secondary prevention of different ischemic arterial diseases such as myocardial infarction and stroke and were possibly effective in the primary reduction of non-fatal vascular events in healthy subjects.[31]

## Development of platelet pharmacology

But let's go back to the end of the sixties, when many different inhibitors of platelet function had already been described. In that period, two review articles were published which collated most of the information available on platelet inhibitors.[32,33] Both articles made a distinction between inhibitors of ADP-induced aggregation and inhibitors of the platelet release reaction. Table 1 sets out the classification of anti-platelet compounds presented in these two review articles. On the basis of the evidence available in 1970, ADP appeared to be the principal mediator of platelet aggregation in physiological conditions. It is interesting to note that aspirin, dipyridamole and sulfinpyrazone, the first three drugs tested in large clinical trials for thrombosis prevention were identified as anti-platelet compounds between 1965 and 1968: they were all three already in clinical use for other indications and for many years no «new» antiplatelet compound came to the stage of clinical investigation. A few months after the publication of both review articles[32,33] and of a book discussing the background for a clinical trial of aspirin in the prevention of stroke,[27] a group of three articles[34-36] in Nature New Biology reported that aspirin blocked the production of $PGE_2$ and $PGF_{2a}$ in human platelets (and other experimental systems) and proposed – after more than 70 years of clinical use of this drug – that prostaglandin inhibition might explain some or even all pharmacologic properties and clinical effects of aspirin (and of all other non-steroidal anti-inflammatory drugs). The pharmacology of the anti-platelet drugs available in the late eighties (Table 2) with a historical review of the data and the concepts underlying their use was discussed in a chapter of a successful book.[37] The interested reader will find there are several topics of some historical interest in the context of the present paper, the discovery of platelet and vascular arachidonic acid metabolism as a fashionable target for all anti-platelet drugs, the so called aspirin dilemma and its solution, the disappointment with the thromboxane A2-synthase inhibitors and sulfinpyrazone, the liaison between dipyridamole and adenosine, the development of ticlopidine as a mimic of Glanzmann's thrombastenia and many other intriguing observations. I shall recount here only some details of the aspirin dilemma.

## Intermezzo: «The aspirin dilemma»

As already mentioned, on the basis of the knowledge available in the early seventies concerning the action of aspirin on platelets many clinical trials using aspirin as an antithrombotic agent in the secondary prevention of myocardial infarction and of cerebrovascular complications were initiated. However, very soon the discovery of $PGI_2$, a potent antiaggregating and vasodilating agent produced by vascular cells via the cyclo-oxygenase-initiated metabolism of arachidonic acid,[38] cast serious doubt on the usefulness of aspirin as an antithrombotic drug. The simultaneous inhibition of $TxA_2$ and $PGI_2$ synthesis could have been the reason for the disappointing results of early clinical trials on the antithrombotic effect of aspirin; failure of clinical trials still in progress was also anticipated. It was even shown that animals treated with high doses of aspirin, which inhibited $PGI_2$ synthesis, might have an increased thrombotic tendency.[39] Moreover, humans taking high doses of aspirin exhibited a shortened bleeding time (Moncada, 1978). The assumption was made, and popularized, that to achieve antithrombotic efficacy, the inhibitory effect of aspirin on platelet cyclo-oxygenase should be retained, while that on the vascular enzyme should be minimized. Many clinicians were fascinated by this *aspirin dilemma* and urged pharmacologists to solve it rapidly. Several experimental approaches were therefore adopted to estimate the dose of aspirin which suppresses the synthesis of thromboxane $A_2$ but not of prostacyclin. The initial approach was based on the assumption that the platelet enzyme would be more sensitive to aspirin than the vascular enzyme.[41] Consequently, *low* dose aspirin was expected to achieve *biochemical selectivity* as only platelet cyclo-oxygenase would be affected. Although studies *in vitro* comparing platelets with cultured human endothelial cells, showed that aspirin exerted a similar inhibitory profile,[42] the search for the lowest active dose of aspirin was intense; all attempts using *single* oral doses of aspirin failed to dissociate significantly the drug's pharmacological effects on platelets and vascular cells, both in experimental animals[43] and in man.[44] *Biochemical selectivity* of aspirin was achieved for the first time in rats in a rather unusual way: an animal made thrombocytopenic by antiplatelet antibodies was exchange-transfused with blood from another animal pretreated wich aspirin a few hours before (in order to allow complete elimination of the intact drug from the peripheral circulation). The recipient rat had therefore *aspirinated* platelets but *non-aspirinated* vessel walls. Notwithstanding this pharmacologic success, the bleeding time of the animals did not change significantly.[45] *Biochemical selectivity* was more easily demonstrated by administration of repeated small doses of aspirin.[46] This was explained by the fact that platelet cyclo-oxygenase, once irreversibly acetylated

**Table 1. Inhibitors of platelet aggregation *in vitro* as classified by Mustard and Packham[32] and de Gaetano *et al.*[33]**

| *Mustard and Packham* | *de Gaetano, Vermylen and Verstraete* |
|---|---|
| I. Inhibitors of ADP-induced aggregation | I. Inhibitors of the aggregating effect of ADP |
| a Inhibitors with structural similarities to ADP | 1. *Synthetic inhibitors* |
| b Inhibitors that bind calcium | |
| c Inhibitors that affect the platelet membrane | a Calcium-chelating agents |
| - Sulfydryl group inhibitors | b Arginine and guanidine derivatives |
| - Antihistamines | c Sulfydryl (dipyridamole and congeners, glycerylguaiacolate, nialamide ...) |
| - Local anesthetics | |
| - Antidepressants and tranquillizers | 2. *Biological inhibitors* |
| - Heparin | |
| - Fibrinogen degradation products | a Adenosine and analogous substances |
| d Factors influencing platelet metabolism or contractile protein | b Prostaglandins (PGE1, through adenyl cyclase?) |
| e Miscellaneous (dextran, clofibrate...) | c Fibrin(ogen) degradation products |
| II. Inhibitors of platelet release reaction | II. Inhibitors of release of platelet ADP (inhibitors of 'release reaction') |
| a Chelators of divalent cations | 1. *Synthetic inhibitors* |
| b Metabolic inhibitors | |
| c Adenine compounds | a Acetylsalicylic acid and other anti-inflammatory agents (sulphinpyrazone...) |
| d Prostaglandin E1 | b Antidepressant drugs |
| e Colchicine | c Miscellaneous (dextran, clofibrate...) |
| f Methylxanthines | |
| g Imipramine and amytriptiline | 2. *Biological inhibitors* |
| h Orthophosphonates | |
| i Salicylaldoxime | a Serotonin |
| j Adrenergic alpha-receptor antagonists | b Heparin |
| k Non-steroidal anti-inflammatory drugs and related compounds (sulphinpyrazone...) | |
| l Phosphatidyl and sulfated polysaccharides | |
| m Heparin and sulfated polysaccharides | |
| n Glucosamine | |
| o Dipyridamole and related compounds | |
| p Fibrinogen degradation products | |

by aspirin, could not be replaced as long as the affected platelets remained in circulation. As a consequence, the effects of single, partially effective doses of aspirin could be expected to accumulate – and this, in fact, occurred. However, the lack of effect of small dose aspirin treatment on vascular $PGI_2$ generation was less uniformly accepted. For instance, cumulative inhibition of $PGI_2$ synthesis measured on vascular segments was reported after repeated low doses of aspirin.[47] One point of debate was that suppression of platelet $TxA_2$ biosynthesis might not necessarily result in inhibition of platelet function *in vivo*. Evidence for an inhibitory effect of repeated low doses of aspirin on platelet function was provided by Weksler *et al*.[47] and De Caterina *et al*.[48] studying platelet aggregation induced by *single* aggregating stimuli (agonists). However, when *pairs* of agonists (such as PAF and adrenaline) were used to induce platelet aggregation, repeated low doses of aspirin appeared to be no longer effective.[49,50] The *low-dose aspirin* concept, though still debated at the experimental level, and before being evaluated in controlled clinical trials, received an enthusiastic reception from many clinicians. They were fascinated not only by the apparent simplicity of this pharmacologic approach, but welcomed the foreseeable reduction, or even disappearance, of side effects (mainly gastrointestinal) related to the chronic intake of relatively high doses of aspirin. This widespread attitude (at least in Italy) was soon supported by the results of six controlled clinical trials showing a dose-unrelated beneficial effect of aspirin in the secondary prevention of mortality in patients with myocardial infarction.[51] The dose-unrelated beneficial effect of aspirin was confirmed in patients with unstable angina.[52,53] To understand the clinical problem of the lack of dose-response relationship of aspirin better, some groups became interested in the possible effects of salicylate – this metabolite has a longer plasma half-life than the parent molecule and may accumulate during repeated drug administration. The importance of plasma salicylate levels in regulating the interaction between aspirin and cyclo-oxygenase[54] suggested that better knoweledge of the pharmacokinetics of aspirin and salicylate might help resolve the *aspirin dilemma*. The pharmacokinetics of aspirin have been given little consideration in thrombosis prevention trials. The necessity to consider the pharmacokinetics of aspirin was strengthened by the observation that serum $TxB_2$ generation was suppressed, even when there was no

**Table 2. Inhibitors of platelet aggregation available in 1987.[37]**

I. Drugs interfering with arachidonic acid metabolism
- Cyclo-oxygenase inhibitors
  - aspirin
  - sulfinpyrazone
- Thromboxane TxA2-synthase inhibitors
  - imidazole
  - dazoxiben
- Prostaglandin endoperoxide/$TxA_2$ receptor antagonists
  - SQ 29548
  - SKF 88046

II. Drugs increasing c-AMP levels
- Prostacyclin ($PGI_2$) and stable $PGI_2$ analogues
  - epoprostenol
  - carbacyclin
  - iloprost
- $PGD_2$
- Dipyridamole

III. Drugs interfering with adenosine
- Dipyridamole and pyrimido-pyrimidine derivatives

IV. Drugs interfering with fibrinogen binding
- Ticlopidine
- RGD peptides and derivatives
- Some snake venoms

V. Drugs interfering with serotonin (5HT)
- Ketanserin

VI. Drugs interfering with platelet activating factor (PAF)
- CV 3988
- Kadsurenone
- BN 52021

VII. Drugs interfering primarily with platelet-unrelated mechanisms
- β blockers
- Calcium antagonists ($Ca^{++}$ channel blockers)
- Antithrombin drugs

detactable aspirin in the peripheral blood in subjects taking oral aspirin.[55-58] It was suggested that pre-systemic circulation first-pass deacetylation of aspirin within the entero-hepatic circulation was responsible for the low (or absent) peripheral drug levels (Figure 1). Thus, platelets passing through the gut capillaries could be acetylated by aspirin before reaching the systemic circulation, resulting in suppression of serum $TxB_2$ generation, and the extent to which peripheral vascular cyclo-oxygenase might be affected could merely reflect the amount of intact aspirin which escaped first-pass metabolism (as well as hydrolysis by plasma esterases). The *sparing* of vascular cyclo-oxygenase after oral (compared with intravenous) administration of the same dose of aspirin was clearly shown in rats.[59,60] It now appears that the concepts of *low* and *high* doses of aspirin, and of its *biochemical selectivity* in relation to platelet and vascular cyclo-oxygenase, are relative rather than absolute, and require to be qualified in relation to the drug's pharmacokinetics. The aspirin dilemma was solved finally by determining the optimal conditions for presystemic acetylation of platelet cyclo-oxygenase in patients at risk for thrombosis. In young healthy subjects high-dose aspirin (650mg x 2) and indobufen (200mg x 2) – a cyclo-oxygenase inhibitor unrelated

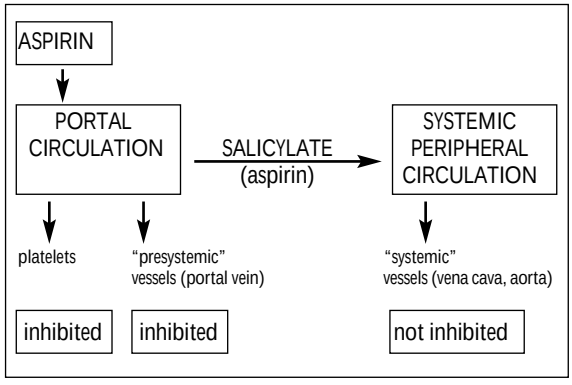


**Figure 1. Scheme of the "first-pass" de-acetylation of acetylsalicylic acid after oral ingestion of aspirin.**

to salicylate – significantly inhibited serum $TxB_2$ generation and the rise in tissue plasminogen activator actively induced by venous occlusion, without affecting the pre-occlusion values. In contrast, salicylate (569mg x 2, a dose equimolar to 650mg x 2 of aspirin) did not affect the fibrinolytic response. Moreover, low-dose aspirin (20mg x 7 days) while reducing serum $TxB_2$ generation by about 90%, did not modify the increased fibrinolytic response to venous occlusion.[61] The hypothesis that the rise in fibrinolytic activity occurring during this hypoxemic challenge is mediated by local generation of vascular $PGI_2$ was clearly demonstrated both in humans[62] and in experimental animals.[63]

Thus, any dose of aspirin which *spares* vascular cyclo-oxygenase activity would leave intact not only the antiaggregating (i.e. $PGI_2$) but also the fibrinolytic potential of the vessel wall. The aspirin dilemma could therefore have wider implications than simply the platelet-oriented $TxA_2$-$PGI_2$ balance.

## The Knights of the Round Table

There may be moments in our life when we are either strongly convinced about something old or desperately looking for something new. In these very moments, either very near to or very far from us, something is happening that will dramatically change the rest of our lives. But quite rarely are we aware that the still unknown truth – within one hour or ten years – will not allow us to think in the same way every again. In September 1970, a Round-the-Table Conference on Normal and Modified Platelet Aggregation was held in Leuven, Belgium.[64] The intention was to assemble some European scientists who had contributed significantly to the rapidly developing field of platelet aggregation and allow these workers to discuss a number of open questions. It may be of interest in this context to read the 15 questions asked by the Organizers (Table 3).

Among the 46 participants, almost all the historical contributors to platelet and hemostasis history in Europe were present, e.g. G.V.R. Born, K. Breddin, J. Caen, A.S. Douglas, R. Gross, R.M. Hardisty, H. Holmsen, J. Hugues, M.J. Larrieu, Y. Legrand, E.F. Lüscher, J.R. O'Brien, H. Poller, A. Sharp, J.J. Sixma, J.W. ten Cate, J. Vermylen and M. Verstraete. The Ital-

ians present were S. Coccheri, M.B. Donati, G. Leone, P.M. Mannucci, C. Praga and the author of this chapter. The structure of the meeting was rather unusual, as for each question prepared by the Organisers, there were two or three short introductory answers, followed by a lively and free discussion. Going now through these discussions may give the reader a unique flavor of what was the platelet and its role in hemostasis and thrombosis in Europe three decades ago. To the question «In which clinical conditions would pharmacologic inhibition of platelet aggregation be useful?», the answers were: cancer, hypertension, chronic glomerulonephritis, diabetes with thrombotic tendency, primary pulmonary hypertension... Summarizing recent data on Glanzmann's thrombasthenia, J. Caen stated that this is «the most clearly defined disorder of hemostasis» yet «one does not know why the thrombasthenic palatelets do not aggregate in the presence of ADP». «We have» – concluded Caen – «many new findings on thrombasthenic platelets, but we do not know what is or are the underlying anomalies responsible for the absence of platelet aggregation in this disease». Four years later, Nurden and Caen[65] made the seminal observations on platelet membrane glycoproteins which provided the basis for the tremendous development of our knowledge on hemostasis and thrombosis prevention.[66,67]

**Table 3. Discussion to a panel of European experts of platelets in 1970.[64]**

Question N. 1
A. Which hypothesis on platelet aggregation by ADP would seem to be most plausible?
B. Are cofactors of a protein nature involved in normal platelet aggregation?
Question N. 2
What does the optical platelet aggregation test actually measure?
Question N. 3
Which physical or chemical alterations of the platelet surface are provoked by different aggregating substances?
Question N. 4
Role of the release reaction in platelet aggregation
Question N. 5
How is platelet aggregation linked with increased availability of platelet factors 3 and 4?
Question N. 6
Does rapid disaggregation following ADP-induced aggregation have any significance?
Question N. 7
Are comparable results obtained with different "collagen" preparations?
Question N. 8
Is aggregation by collagen and thrombin the consequence of ADP release only?
Question N. 9
Do immunologic reactions provoke or modify the release reaction?
Question N. 10
How does adenosine inhibit platelet aggregation?
Question N. 11
Cyclic AMP, prostaglandins and platelet aggregation
Question N. 12
Significance of congenital or acquired modifications of platelet aggregation
Question N. 13
Inhibition of platelet aggregation by chemicals and drugs
Question N. 14
In which clinical conditions would pharmacologic inhibition of platelet aggregation be useful?
Question N. 15
Does an impaired release reaction really cause a haemorrhagic diathesis?

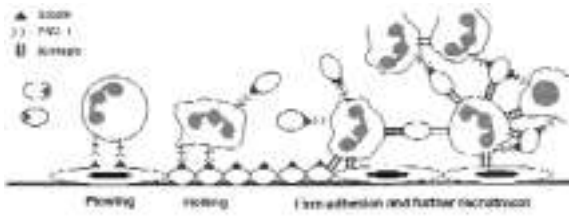

**Figure 2. Hypothetical sequence of interactions between PMN leukocytes and activated platelets or injured endothelial cells. Reprinted from ref. 70, with permission.**

**Table 4. Platelets. A multidisciplinar approach.[68]**

*Table of contents*

I. Introduction (platelet physiology, morphology, biochemistry, metabolism. Species specificities. Platelet-drug interactions)
II. Platelets, endothelium, smooth muscle cells
III. Platelets and inflammation
IV. Platelets and immunological reactions
V. Platelets and synaptosomes
VI. Platelets and tumor cells

## The other face of the moon

Possibly due to a continuous intellectual orientation towards America «buscando el oriente por el ponente», I was always attracted by the other face of the moon... In 1977 an International Symposium[68] was organized in Florence to discuss the platelet as a model of other cells and to evaluate its possible role in physiopathologic phenomena not directly related to hemostasis and thrombosis. Table 4 reports the titles of the six sessions of that Symposium. Now, many years after that Florence meeting, I am personally no longer directly engaged in platelet research, but younger people at the Mario Negri Sud research institute are actively involved in a new fascinating chapter of platelet function, namely the complex interaction of activated platelets with white cells (both polymorphonuclear and lympho-monocytes) and of activated leukocytes with platelets, the whole picture being taken – in flowing conditions – on the background of endothelial cells.[69,70] I shall therefore close this chapter with a sketch («la Fantasie au pouvoir») presented a few months ago by Chiara Cerletti at the ISTH Washington Congress.[70] Whether the supposed new thrombogenic role of platelets summarized in Figure 2 is of any clinical relevance will only be revealed in another historical overview, some time from now.

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## References

1. Bizzozero J. Über einen neuen formbestandteil des blutes und dessen rolle bei der thrombose und blutgerinnung. Virchow's Arch Path Anat Physiol Klin Med 1882; 90: 261-332.
2. Zahn FW. Untersuchungen über thrombose, bildung der thromben. Virchow's Arch Path Anat 1875; 62:81-124.
3. Krauss E. Über purpura. Inaug Diss Heidelberg, 1883.
4. Hayem G. Leçons sur les maladies du sang. Masson, Paris, 1900.
5. Mac Kay W. The blood platelets: its clinical significance. Quart J Med 1930; 24:285-93.
6. Roskam J. Contribution à l'étude de la physiologie normale et pathologique du globulin (plaquettes de Bizzozero). Arch Int Physiol 1923; 20:240-9.
7. Quick AJ. Studies on the enigma of the hemostatic dysfunction of hemophilia. Am J Med Sci 1947; 241: 272-80.
8. Quick AJ. Bleeding problems in clinical medicine. W.B. Saunders Co., Philadelphia, 1970.
9. Roskam J, Hugues J, Bounameaux Y. L'hémostase spontanée. Etude synthetique et analytique. J Physiol 1961; 53:175-83.
10. Bettex-Galland M, Lüscher EF. Thrombostenin, a contractile protein from blood platelets. Biochem Biophys Acta 1961; 49:536-44.
11. Gaarder A, Laland S. Hypothesis for the aggregation of platelets by nucleotides. Nature 1964; 202:909-11.
12. Gaarder A, Jonsen J, Laland S, Hellem A, Owren PA. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. Nature 1961; 192:531-2.
13. Haslam RJ. Role of adenosine diphosphate in the aggregation of human blood platelets by thrombin and by fatty acids. Nature 1964; 202:765-7.
14. Holmsen H, Day HJ, Stormorken H. The blood platelet release reaction. Scand J Haematol 1969; (Suppl 8).
15. Born GVR, Cross MJ. The aggregation of blood platelets. J Physiol 1963; 168:178-95.
16. O'Brien JR. Platelet aggregation. Part II. Some results from a new method of study. J Clin Pathol 1962; 15: 446-9.
17. Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. Arterioscler Thromb Vasc Biol 1999; 19:2281-5.
18. Beaumont JL, Caen J, Bernard J. Influence de l'acide acetyl salicylique dans les maladies hémorrhagiques. Sang 1956; 27:243-8.
19. Blatrix C. Allongement du temps de saignement sons l'influence de certain médicaments. Nouv Rev Franç Hémat 1963; 3:346-51.
20. Quick AJ. Salicylates and bleeding: the aspirin tolerance test. Am J Med Sci 1966; 252:265-9.
21. Weiss HJ, Aledort LM. Impaired platelet-connective tissue reaction in man after aspirin ingestion. Lancet 1967; 2:495-7.
22. Zucker MB, Peterson J. Inhibition of adenosine diphosphate-induced secondary aggregation and other platelet functions by acetylsalicylic acid ingestion. Proc Soc Exp Biol Med 1968; 127:547-51.
23. O'Brien JR. Effect of salicylates on human platelets. Lancet 1968; 1:1431.
24. Evans G, Packham MA, Nishizawa EE, Mustard JF, Murphy EA. The effect of acetylsalicylic acid on platelet function. J Exp Med 1968;128:877-94.
25. de Gaetano G, Donati MB, Vermylen J. A simple method to study the "stabilising" effect of chemicals and drugs on the platelet membrane. Thromb Res 1972; 1:631-6.
26. Eco U. The name of the rose. Martin Secker and Warburg Ltd, 1983, London (Picador edition) p. 491.
27. Fields WS, Hass WK (Eds): Aspirin, platelets and stroke-background for a clinical trial. Warren H Green, Inc, St Louis, Missouri, USA, 1971.
28. Evans G, Mustard JF, Packham MA. Thromboembolism. In: Fields WS, Hass WK, eds. Aspirin, platelets and stroke-background for a clinical trial. Warren H Green, Inc., St. Louis, Ms, USA, 1971. pp. 59-73.
29. Dikens ML. Discussion. In: Fields WS, Hass WK, eds. Aspirin, platelets and stroke-background for a clinical trial. Warren H Green, Inc, St Louis, Missouri, USA, 1971, p. 133.
30. O'Brien JR. Anti-inflammatory drugs and the prevention of thrombosis. Acta Med Scand 1971; suppl 525:211-13.
31. Antiplatelet Trialist' Collaboration. Secondary prevention of vascular disease by prolonged antiplatelet treatment. Br Med J 1988; 296:320.
32. Mustard JF, Packham MA. Factors influencing platelet function: adhesion, release, and aggregation. Pharmacol Rev 1970; 22:97-187.
33. de Gaetano G, Vermylen J, Verstraete M. In: Ambrus, JL, ed. Hematologic Reviews. Marcel Dekker, New York, 1970. p. 205.
34. Smith JB, Willis AL. Aspirin selectively inhibits prostaglandin production in human platelets. Nature New Biol 1971; 231:235-7.
35. Ferreira SH, Moncada S, Vane JR. Indomethacin and aspirin abolish prostaglandin release from the spleen. Nature New Biol 1971; 231:237-9.
36. Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. Nature New Biol 1971; 231:232-5.
37. de Gaetano G, Bertelé V, Cerletti C. Pharmacology of antiplatelet drugs. In: MacIntyre DE, Gordon JL, eds. Platelets in biology and pathology III. Elsevier, Amsterdam, 1987; 515-73.
38. Moncada S, Gryglewski R, Bunting S, Vane JR. An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. Nature 1976; 263:663-5.
39. Kelton JG, Hirsh J, Carter CJ, Buchanan MR. Thrombogenic effect of high-dose aspirin in rabbits. Relationship to inhibition of vessel wall synthesis of prostaglandin I2-like activity. Clin Invest 1978; 62: 892-5.
40. O'Grady J, Moncada S. Aspirin: a paradoxical effect on bleeding-time. Lancet 1978; 2:780.
41. Baenziger NL, Dillender MJ, Majerus PW. Cultured human skin fibroblasts and arterial cells produce a labile platelet-inhibitory prostaglandin. Biochem Biophys Res Commun 1977; 78:294-301.
42. Jaffe EA, Weksler BB. Recovery of endothelial cell prostacyclin production after inhibition by low doses of aspirin. J Clin Invest 1979; 63:532-5.
43. Villa S, Livio M, de Gaetano G. The inhibitory effect of aspirin on platelet and vascular prostaglandins in rats cannot be completely dissociated. Br J Haematol 1979; 42:425-31.
44. Masotti G, Galanti G, Poggesi L, Abbate R, Neri

Serneri GG. Differential inhibition of prostacyclin production and platelet aggregation by aspirin. Lancet 1979; 2:1213-7.
45. Dejana E, Barbieri B, de Gaetano G. "Aspirinated" platelets are hemostatic in thrombocytopenic rats with "non-aspirinated" vessel walls--evidence from an exchange transfusion model. Blood 1980; 56:959-62.
46. Patrignani P, Filabozzi P, Patrono C. Selective cumulative inhibition of platelet thromboxane production by low-dose aspirin in healthy subjects. J Clin Invest 1982; 69:1366-72.
47. Weksler BB, Tack-Goldman K, Subramanian VA, Gay WA Jr. Cumulative inhibitory effect of low-dose aspirin on vascular prostacyclin and platelet thromboxane production in patients with atherosclerosis. Circulation 1985; 71:332-40.
48. De Caterina R, Giannessi D, Bernini W, et al. Selective inhibition of thromboxane-related platelet function by low-dose aspirin in patients after myocardial infarction. Am J Cardiol 1985; 55:589-90.
49. Cerletti C, Carriero MR, de Gaetano G. Platelet-aggregation response to single or paired aggregating stimuli after low-dose aspirin. N Engl J Med 1986; 314: 316-8.
50. Di Minno G, Silver MJ, Murphy S. Monitoring the entry of new platelets into the circulation after ingestion of aspirin. Blood 1983; 61:1081-5.
51. Braunwald E, Firedewald WT, Furberg CD. Proceedings of the workshop on platelet-active drugs in the secondary prevention of cardiovascular events. Circulation 1980; 62 (suppl VI): 1-135.
52. Lewis HD Jr, Davis JW, Archibald DG, et al. Protective effects of aspirin against acute myocardial infarction and death in men with unstable angina. Results of a Veterans Administration Cooperative Study. N Engl J Med 1983; 309:396-403.
53. Cairns JA, Gent M, Singer J, et al. Aspirin, sulfinpyrazone, or both in unstable angina. Results of a Canadian multicenter trial. N Engl J Med 1985; 313:1369-75.
54. de Gaetano G, Cerletti C, Dejana E, Latini R. Pharmacology of platelet inhibition in humans: implications of the salicylate-aspirin interaction. Circulation 1985; 72:1185-93.
55. Ali M, McDonald JW, Thiessen JJ, Coates PE. Plasma acetylsalicylate and salicylate and platelet cyclooxygenase activity following plain and enteric-coated aspirin. Stroke 1980; 11:9-13.
56. Siebert DJ, Bochner F, Imhoff DM, et al. Aspirin kinetics and platelet aggregation in man. Clin Pharmacol Ther 1983; 33:367-74.
57. Pedersen AK, FitzGerald GA. Dose-related kinetics of aspirin. Presystemic acetylation of platelet cyclooxygenase. N Engl J Med 1984; 311:1206-11.
58. Cerletti C, Latini R, Dejana E, et al. Inhibition of human platelet thromboxane generation by aspirin in the absence of measurable drug levels in peripheral blood. Biochem Pharmacol 1985; 34:1839-41.
59. Cerletti C, Gambino MC, Garattini S, de Gaetano G. Biochemical selectivity of oral versus intravenous aspirin in rats. Inhibition by oral aspirin of cyclooxygenase activity in platelets and presystemic but not systemic vessels. J Clin Invest 1986; 78:323-6.
60. Gambino MC, Passaghe S, Chen ZM, et al. Selectivity of oral aspirin as an inhibitor of platelet vs. vascular cyclooxygenase activity is reduced by portacaval shunt in rats. J Pharmacol Exp Ther 1988; 245:287-90.
61. de Gaetano G, Carriero MR, Cerletti C, Mussoni L. Low dose aspirin does not prevent fibrinolytic response to venous occlusion. Biochem Pharmacol 1986; 35:3147-50.
62. Bertelé V, Mussoni L, Pintucci G, et al. The inhibitory effect of aspirin on fibrinolysis is reversed by iloprost, a prostacyclin analogue. Thromb Haemost 1989; 61: 286-8.
63. Iacoviello L, De Curtis A, D'Adamo MC, et al. Prostacyclin is required for t-PA release after venous occlusion. Am J Physiol 1994; 2662:H429-34.
64. Vermylen J, de Gaetano G, Verstraete M, eds. Round-the –Table Conference on normal and modified platelet aggregation. Acta Medica Scandinavica (Suppl 525), 1971.
65. Nurden AT, Caen JP. An abnormal platelet glycoprotein pattern in three cases of Glanzmann's thrombasthenia. Br J Haematol 1974; 28:253-60.
66. Coller BS. Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. J Clin Invest 1997; 99: 1467-71.
67. The SYMPHONY Investigators. Comparison of sibrafiban with aspirin for prevention of cardiovascular events after acute coronary syndromes: a randomised trial. Lancet 2000; 355:337-45.
68. de Gaetano G, Garattini S (Eds): Platelets: a multidisciplinary approach. Raven Press, New York, USA, 1978.
69 Cerletti C, Evangelista V, Molino M, de Gaetano G. Platelet activation by polimorphonuclear leukocytes: role of cathepsin G and P-selectin. Thromb Haemost 1995; 74:218-23.
70. Cerletti C, Evangelista V, de GaetanoG. P-selectin-beta 2-integrin cross-talk: a molecular mechanism for polymorphonuclear leukocyte recruitment at the site of vascular damage. Thromb Haemost 1999; 82:787-93.

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# HISTORICAL OVERVIEW OF THE ROLE OF ADP IN PLATELET FUNCTION

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My discussion will cover some aspects of the interaction between ADP and blood platelets, starting in 1961 when I first became involved with this topic. I shall take a personal approach, and concentrate on those aspects with which I have been most intimately associated. This will allow me a chance to pay tribute to the many brilliant and creative scientists with whom it has been my privilege and pleasure to collaborate. Since my days as an apprentice in Gustav Born's laboratory in London I have been convinced that the action of ADP on platelets is mediated by a receptor of some sort and a constant thread of interest for me has been to find out more about this interaction, and eventually to identify the receptor protein. In this endeavour there have been some failures and some successes, but I doubt whether there are many who still regard the non-receptor theories as competitive. I look forward to the day that the receptor is eventually cloned and I am able to see if some of the predictions that I have made on the basis of indirect evidence prove to be true.

My first encounter with a platelet was the result of a friendship between my then boss, W.F.J.Cuthbertson, for whom I was working at Glaxo Labs, and Soren Laland, who was Professor of Biochemistry at Oslo University. He persuaded Cuthbertson to send me to Vienna, to a meeting of the European Society for Haematology in 1961, where his student, Anna Gaarder was to describe their work on the identification of Hellem's "factor R" extracted from red cells, which made platelets stick to glass beads. The full paper,[1] identifying the factor as ADP appeared shortly after and generated a flurry of excitement, particularly among a group in London including Gustav Born at the Royal College of Surgeons and Helen Payling-Wright, herself one of the pioneers of attempts to quantify platelet adhesion to glass. Unfortunately, Anna was killed shortly afterwards in an automobile accident and I never had the opportunity to get to know her.

I was then sent to Paul Owren's lab at the Rikshospitalet in Oslo to learn Hellem's technique of pumping blood through a column of glass beads and counting the platelets before and after the passage. About this time broke the affair that tainted Owren's reputation. As a wealthy and influential figure in Norwegian scientific politics, he had used his pull to promote the use of unsaturated fats as a dietary supplement on the basis of experiments that claimed to show that eating these fatty acids reduced the tendency of platelets to adhere to glass. These experiments proved unreproducable, and it was eventually decided that the results were due to deficiencies in the technique employed, which was counting the platelets by eye – before the introduction of the Coulter Counter – with no attempt to conceal the identity of the samples. The experiments were performed in the main by a clinician with little training in scientific method, who was ultimately used a scapegoat for what to me seemed clearly the responsibility of Hellem and, ultimately, of Owren.

Back in England I struggled for a while with counting millions of platelets, until a couple of papers appeared at about the same time describing the aggregometer. One was from Born[2] and the other from John O'Brien in Portsmouth.[3] Their descriptions were eerily similar, and there was a distinct suspicion of hanky panky. Relations between the two were not very cordial thereafter. I was thrilled at the idea of being able to study platelets without all that counting, and set out to build my own aggregometer.[4] I also introduced a couple of improvements, which have since become standard - a water jacket to maintain the temperature at 37°, and a stirring device that permitted continuous recording of the optical density. Both Born and O'Brien had used converted spectrophotometers and had to stop the stirrer to take manual readings. When I showed my device to Born and his colleague Michael Cross, they were pleased and invited me to come to the Royal College for a couple of months on loan from Glaxo, to which Cuthbertson agreed. While I was there Michael was tragically killed in an air crash while lecturing in the United States. This left open a position at the College for someone interested in working on platelets, and it was offered to me along with the opportunity to work for a Ph.D.

My thesis was to investigate the metabolism of ADP and other nucleotides in blood which I had begun to study at Glaxo with Dennis Ireland,[5] so I taught myself how to prepare radioactive nucleotides using the exchange reaction catalyzed by phosphoglycerate kinase. At this time Richard Haslam spent some time at the Lab, between finishing his D Phil in Hans Krebs' Lab in Oxford, and resuming his work at ICI in Cheshire. He showed an interest in my studies and together we showed that in shed blood, the most significant route for the degradation of ADP was through the action of adenylate kinase released from

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red cells[6]. I also showed, using $^{32}P$ labeled ATP that there is an enzyme in plasma that cleaves nucleotides at the $\alpha$–$\beta$ pyrophosphate bridge giving AMP and pyrophosphate from ATP, and AMP and phosphate from ADP.[7]

At this time I was joined by Gordon Roberts, an accomplished pharmacologist and we set ourselves to study the recently discovered interaction of platelets with adrenaline and serotonin and the phenomenon of secondary aggregation, which we showed was correlated with the release of ADP from the platelets themselves. When in Oxford, Born had studied the uptake and storage of radioactive serotonin by platelets with Hugh Blashko, whose main interest was the release of catacholamines from the adrenal medullary chromaffin cells, and we had access to the techniques involved. Release of ADP was correlated with release of stored serotonin and of certain lysosmal enzymes.[8,9] Frazer Mustard, who lured Richard Haslam to Macmaster University in Ontario, were he remains to this day, has shown that secondary aggregation by ADP is an *in vitro* artifact of the reduction of calcium ion concentration due to the use of citrate as anticoagulant. However it has been a very fruitful phenomenon to study, as it led in part to the discovery of the mechanism of action of aspirin by Smith and Willis.[10]

Born had shown that adenosine and 2-chloroadenosine were antagonists of the aggregation of platelets by ADP.[2] The close chemical similarity of agonist to antagonist led him to propose the receptor hypothesis for the action of ADP, with the nucleosides acting as competitors for binding. However there were two things about this hypothesis that in my opinion did not fit well. The potency of the inhibitors increased with time during incubation with platelets, and with human platelets adenosine proved much more active than AMP, a compound even more similar to the agonist. When Sattin and Rall showed that adenosine can raise cyclic AMP levels in brain slices,[11] Brian Smith and I decided to measure cyclic AMP in platelets and eventually showed not only that adenosine raises platelet cAMP, but that ADP and adrenaline could both dramatically antagonize this effect.[12]

At the same time I was continuing to collaborate with Haslam, although it now meant travelling to Cheshire whenever we wanted to do an experiment. Our objective was to prove the receptor hypothesis by measuring the binding of high specific activity $^{32}P$ labeled ADP using centrifugation through silicone oil with a special swing-out head I had built for the Eppendorff centrifuge. Earlier attempts by Born and others using ADP labeled with $^{14}C$ or $^{3}H$ were defeated by the rapid dephosphorylation of ADP in plasma and the equally rapid uptake of the labeled adenosine produced, and its incorporation into intracellular adenine nucleotides.[13] Our technique allowed us to estimate the number of binding sites as less than 1,000 per platelet. This result suggested that to make this measurement either an improvement in the technique or the use of a higher affinity ligand would be necessary. Eventually both were employed.

These experiments were interrupted when in 1971 I emigrated to the States. At that time the receptor theory for the action of ADP on platelets was in competition with the proposal that ADP and calcium ions formed a physical bridge between the cells,[14] and the theory that ADP formed either a source of energy for a reaction catalyzed by nucleoside diphosphate kinase,[15] or as the recipient of a phosphate group donated by an exofacial kinase.[16] No new evidence supported the receptor theory, no other instances were known of nucleotides acting through a receptor mechanism and Born's suggestion that adenosine was a competitive antagonist was discredited. Our work on the inhibition of adenylate cyclase stongly suggested a receptor dependent mechanism, but its relevance to the induction of aggregation was not clear. Haslam had shown that, contrary to Salzman's suggestion,[17] inhibition of the cyclase was of itself insufficient to cause aggregation.[18]

New evidence came from work done by Donald Macfarlane, completing his thesis in Born's lab. He showed that freshly purified ATP was a strictly competitive inhibitor of aggregation with an apparent Ki of 20 $\mu$M. When Donald joined me in Philadelphia, we set out to show that ATP was also a competitive inhibitor of the effect of ADP on adenylate cyclase and on platelet shape change, and that it was specific for ADP, having no ability to inhibit other aggregating agents other than collagen.[19] These results stimulated us to resume the search for a receptor. It was clear by that time, largely due to the work of Helen Maguire and her colleagues in Australia,[20] that whereas almost all of the modifications of the ADP molecule that had been tried led to a reduction in affinity, substitution in the 2- position of the purine ring actually increased affinity. Donald then set out to synthesize 2-azido ADP, on the theory that this molecule would act as a photoaffinity probe with which the receptor could be identified. In this we failed, though the compound was as active as ADP as an aggregating agent and more active as an inhibitor of adenylate cyclase. Also we were able to estimate the number of receptors by measuring equilibrium binding of the $^{32}P$ labeled compound.[22]

When 2-azido ADP was irradiated with UV light it, as expected, underwent a profound chemical change, most probably with the intermediate formation of a reactive nitrene radical. In the presence of platelets several membrane proteins were covalently labeled, but in no case could we demonstrate protection by either ADP or ATP. We attempted to explain this failure by proposing that when ADP or its analogues are bound to the receptor, it is oriented such that the 2-position is held out of reach of the receptor. This would be consistent with the observation of Graham Jamieson that a bulky spin label attached at the 2-position still gave an active compound.[21] It also suggested that we might have more success with a compound in which the generated nitrene was attached to ADP by means of a spacer group. In the meantime we made some 2-methylthio ADP, the most active aggregating agent known. We found that whereas it was about 20 fold more active than ADP as an aggregating agent, it was 100-fold more active as an inhibitor of the cyclase. This discrepancy suggested to us that the receptor involved in aggregation might be distinct from that coupled to the cyclase.[23]

We labeled 2-methylthio ADP with $^{32}P$ and measured its binding to platelets. The binding, which was independent of the presence of divalent cations had an equilibrium dissociation constant of 5nM, an affinity that was high enough for us to confirm this measurement dynamically by measuring dissociation and association rates. The binding affinity was closer to the apparent affinity as a cyclase inhibitor than to the apparent affinity as an aggregating agent, so we concluded that 2-methylthio ADP was binding to the cyclase coupled receptor.[23] Binding was completely blocked in the presence of the non-penetrating thiol reagent, p-chloromercuribenzene sulphonate (pCMBS), which also blocks the effect of ADP on adenylate cyclase, but does not prevent platelets from responding to ADP by changing shape, though aggregation is blocked. This was also consistent with a two receptor model. Though ADP and ATP have similar affinities for the two receptors, the 2- substituted derivatives have higher affinity for the cyclase receptor which, we postulated, probably contains a thiol group in the vicinity of the agonist binding pocket.

When Donald left for the University of Iowa to continue his medical career, I abandoned this project, regarding it as more his pigeon than mine. Also, being a lousy chemist, I was ill equipped to proceed along the lines that we had mapped out. At this time I was working closely with Ed Kirby who came to Temple from Earl Davie's lab in Seattle. Ed was a hemophiliac, severely crippled as a result, and rather naturally interested in factor VIII and its carrier protein, which turned out to be von Willebrand factor. Ed had purified von Willebrand factor from bovine blood and we found that it aggregated human platelets, without the need for ristocetin. We also found that agglutination by bovine von Willebrand factor was inhibited by prior exposure of platelets to ADP.[24] Ed was one of the most optimistic, cheerful and generous of people I have known, and when he died of hepatitis as a result of treatment of his hemophilia, a bright light went out of my life.

I was not able to get back into the hunt for the ADP receptor until Gloria Cristalli from the University of Camarino came to Philadelphia for a year with her husband and came to my lab to learn about platelets. Gloria is an expert nucleoside chemist and I encouraged her to make the compound that I had been dreaming about, 2-(p-azidophenyl) ethylthioAMP, (AzPET-AMP) which I would then phosphorylate to the corresponding ADP derivative. This she did but the phosphorylation step caused more problems than I had anticipated and none of my efforts was successful until after Gloria had left to return to Italy. She therefore missed the excitement of finally being able to label the receptor when at last I managed to produce an active compound[25]. AzPET ADP was active in the cyclase assay and as an aggregating agent and shape change inducer. The radioactive compound bound to the same number of sites – about 500 – as 2-azido ADP and 2-methylthio ADP and the binding was inhibited by ADP and ATP. When photolyzed in the presence of platelets a number of proteins were covalently labeled, with the notable exception of the dominant intracellular proteins, actin and myosin, demonstrating that the labeling was confined to exofacial proteins. A protein of roughly 43 kDa was strongly labeled and the labeling was progressively inhibited by increasing concentrations of ADP, ATP and a wide range of analogs, in direct proportion to their potency as agonists or inhibitors at the adenylate cyclase coupled receptor. The labeling was also inhibited by pCMBS, which suggested that this protein is the receptor though which ADP and its analogues inhibit adenylate cyclase. In some experiments the labeled protein appeared as a doublet, though this was attributable to splitting of the band by unlabeled actin. In a few experiments a faint doublet at 56 kDa was seen which showed a similar pattern of labeling and protection as the 43 kDa band. This may correspond to the P2y and P2x receptors now known to be present. One thing that I found surprising was the extraordinary efficiency of the labeling. Normally in comparable experiments, the reactive nitrene generated by photolysis is believed to insert into C-C or C-H bonds. However, it will react in preference with a nucleophile. Inhibition of labeling by pCMBS suggests that the efficiency of labeling might be explained by the presence of a thiol group within the range of the nitrene radical when AzPET ADP is bound to the receptor. The high efficiency of labeling suggested the possibility of using AzPET ADP as a tag for isolating and purifying the receptor protein.

These experiments using $^{32}P$ labeled ligands were very arduous to perform, owing to the short half life of the isotope and the consequent need to prepare and purify the reagent at frequent intervals. For this reason also attempts to purify enough of the protein to enable a partial sequence to be obtained proved unrewarding. This approach might prove successful if the compound could be labeled with tritium, or with a non-radioactive tag.

Finally I would like to mention a study done with Robert Colman. I was not involved in the planning of this study, which was therefore not a true collaboration. However it did show that the novel anti platelet drug clopidogrel blocks the binding of 2-methylthio ADP to platelets, suggesting that drugs of this type may act in vivo to inactivate the ADP receptor.[26] This finding could prove to have significance for the development of new therapeutic agents aimed at controlling platelet responsiveness *in vivo*.

## References


1. Gaarder A, Jonsen A, Laland S, Hellem AJ, Owren P. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. Nature 1961; 192:531-2.
2. Born GVR. Platelet aggregation and its reversal. Nature (Lond.) 1962; 194:927-9.
3. O'Brien JR. Platelet aggegation. Part II. Some results from a new method of study. J Clin Pathol 1962; 15: 452-5.
4. Cuthbertson WFJ, Mills DCB. A miniature nephelometer for the study of platelet clumping. J Physiol 1963; 168:29p.
5. Ireland DM, Mills DCB. Detection and determination of adenosine diphosphate and related substances in

plasma. Biochem J 1966; 99: 283-96.
6. Haslam RJ, Mills DCB. The adenylate kinase of human plasma, erythrocytes and platelets in relation to the degradation of adenosine diphosphate in plasma. Biochem J 1967; 103: 773-84.
7. Mills, DCB. The breakdown of adenosine diphosphate and of adenosine triphosphate in plasma. Biochem J 1966; 98: 32-33P.
8. Mills DCB, Roberts GCK.Effects of adrenaline on human blood platelets. J Physiol 1967; 193: 443-53.
9. Mills DCB, Robb IA, Roberts GCK. The release of nucleotides, 5-hydroxytryptamine and enzymes from human blood platelets during aggregation. J Physiol 1968; 195:715-29.
10. Smith JB, Willis AL. Aspirin selectively inhibits prostaglandin production in human platelets. Nature New Biol 1971; 231:235-7.
11. Sattin A, Rall TW. The effect of adenosine and adenine nucleotides on the cyclic adenosine 3',5'-monophosphate content of guinea pig cerebral cortical slices. Mol Pharmacol 1970; 6:13-23.
12. Mills DCB, Smith JB. The influence on platelet aggregation of drugs that affect the accumulation of 3'5'-adenosine monophosphate in platelets. Biochem J 1971; 121:185-96.
13. Born GVR. Uptake of adenosine and of adenosine diphosphate by human blood platelets. Nature 1965; 206:1121-2.
14. Gaarder A, Laland S. Hypothesis for the aggregation of platelets by nucleotides. Nature 1964; 202:909-10.
15. Guccione MA, Packham MA, Kinlough-Rathbone RL, Mustard JF. Reactions of $^{14}$C ADP and $^{14}$C ATP with washed platelets from rabbits. Blood 1971; 37:542-55.
16. Spaet TH, Lejnieks I. Studies of the mechanism whereby platelets are clumped by adenosine diphosphate. Thromb Diath Haemorrh 1966; 15:36-51.
17. Salzman EW. Cyclic AMP in platelet function. New Engl J Med 1972; 286:358-63.
18. Haslam RJ, Davidson MML, Desjardins JV. Inhibition of adenylate cyclase by adenosine analogues in preparations of broken and intact human platelets. Evidence for the unidirectional control of platelet function by cyclic AMP. Biochem J 1978; 176:83-95.
19. Macfarlane DE, Mills DCB. The effects of ATP on platelets: evidence against the central role of ADP in primary aggregation. Blood 1975; 46:309-20.
20. Gough G, Maguire HM, Penglis F. Analogues of adenosine 5'-diphosphate - New platelet aggregators. Influence of purine ring and phosphate chain substitutions on the platelet aggregating potency of adenosine 5'-diphosphate. Mol Pharmacol 1972; 8:170-7.
21. Robey FA, Jamieson GA, Hunt JB. Synthesis and use of a new spin-labeled analogue of ADP with platelet-aggregating activity. J Biol Chem 1979; 254: 1114-8.
22. Macfarlane DE, Mills DCB, Srivastava PC. Binding of 2-azidoadenosine [β $^{32}$P]diphosphate to the receptor on intact human blood platelets which inhibits adenylate cyclase. Biochemistry 1982; 21: 544-9.
23. Macfarlane DE, Srivastava PC, Mills DCB. 2-Methylthioadenosine [β$^{32}$P]diphosphate. An agonist and radioligand for the receptor that inhbits the accumulation of cyclic AMP in intact blood platelets. J Clin Invest 1983; 71:420-8.
24. Mills DCB, Hunchack K, Karl DW, Kirby, EP. Effect of platelet activation on the agglutination of platelets by von Willebrand factor. Mol Pharmacol 1990; 37:271-7.
25. Cristalli G, Mills DCB. Identification of a receptor for ADP on blood platelets by photoaffinity labelling. Biochem J 1993; 291:875-81.
26. Mills DCB, Puri RN, Hu C-J, et al. Clopidogrel inhibits the binding of ADP analogues to the receptor mediating inhibition of platelet adenylate cyclase. Atheroscler Thromb 1992; 12:430-6.

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# P2Y RECEPTORS

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ABSTRACT

**The current nomenclature of P2Y receptors needs revising since it encompasses genuine nucleotide receptors as well as orphan receptors mistakenly included in the P2Y family on the basis of limited homology, but in the absence of functional response to nucleotides. A revised nomenclature includes the following human subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$. These are all coupled to phospholipase C activation, the $P2Y_{11}$ subtype also being coupled to adenylyl cyclase stimulation. Although Gi is partially involved in the coupling of $P2Y_1$ and $P2Y_4$ receptors to phospholipase C, the tp2y receptor, an avian ortholog of the $P2Y_4$ receptor, is the only recombinant P2Y receptor which has so far been shown to couple to adenylyl cyclase inhibition. The so-called $P2T_{AC}$ receptor, involved in Gi-mediated inhibition of adenylyl cyclase by ADP in platelets, has so far resisted all cloning attempts. Also expressed in the hematopoietic system, the $P2Y_{11}$ receptor is involved in the cAMP-mediated effect of ATP on the granulocytic differentiation of HL-60 human promyelocytic leukemia cells, a model of neutrophil maturation.**



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## Classification of P2Y receptors

The first clonings of heptahelical P2 receptors coupled to G proteins were reported in 1993.[1,2] The old pharmacologic nomenclature of P2 receptors ($P_{2Y}$, $P_{2X}$, $P_{2U}$, $P_{2Z}$ ...) was then rapidly replaced by a new molecular nomenclature based on the existence of two families: the P2X receptors, which are ligand-gated ion channels, and the G-protein-coupled P2Y receptors.[3] The first P2Y receptors to be cloned ($P2Y_1$, $P2Y_2$) closely corresponded to receptors previously characterized by pharmacologic criteria ($P_{2Y}$, $P_{2U}$). Since then several new subtypes have been isolated by homology cloning and have been assigned a subscript on the basis of the cloning chronology. Some of the cloned receptors are genuine nucleotide receptors, while others have been mistakenly included in the P2Y family on the basis of limited structural homology, but in the absence of a demonstrated functional responsiveness to nucleotides.

## Genuine P2Y receptors

### *$P2Y_1$ receptor*

$P2Y_1$ orthologs have been cloned in various species: avian,[1,4] murine,[5] rodent,[5] bovine[6] and human.[7,8] The human gene is on chromosome 3. In all species the physiologic agonist of the receptor is ADP. ATP has a lower intrinsic efficacy than ADP: its apparent activity varies from competitive antagonism to full agonism, depending on the size of the $P2Y_1$ receptor reserve.[9,10] Substitution by a long thioether chain on the C2 position in the adenine moiety increased the agonist potency and also converted the corresponding AMP derivatives into full agonists, whereas AMP itself was totally inactive.[11-13] On the other hand adenosine bisphosphates, bearing a phosphate on the 2' or 3' position of the ribose moiety, behaved as partial agonists or competitive antagonists.[14,15] Mutagenicity studies have identified critical residues on the exofacial side of TM3 and TM7 domains.[16,17] The $P2Y_1$ receptor is coupled to phospholipase C in a pertussis toxin-independent way. Additional signaling mechanisms have been detected. In platelets, the ADP-induced shape change is mediated by the phospholipase C-coupled $P2Y_1$ receptor. Following intracytoplasmic calcium chelation by BAPTA, the shape change and associated myosin light chain

phosphorylation were delayed, but not abolished, and became sensitive to the selective Rho-dependent kinase (ROCK) inhibitor Y-27632. This suggests that the $P2Y_1$ receptor is coupled to the RhoA-p160ROCK pathway in addition to phospholipase C.[18] It remains unclear whether this coupling involves $G_{q/11}$ or $G_{12/13}$. Expression of the human $P2Y_1$ receptor in *Xenopus* oocytes led to a nucleotide-induced cation ($K^+ > Na^+$) current that was not observed following expression of the $P2Y_2$ receptor.[19] That cation current was insensitive to GDPβS, which did, however, block the $Ca^{2+}$-activated $Cl^-$ current. It was concluded that the $P2Y_1$ receptor may generate a G protein-independent ionotropic response, but the significance of this observation remains unclear. Interestingly, the sequence of the $P2Y_1$ receptor, but not of other P2Y subtypes, contains the C-terminal motif DTSL which recognizes a PDZ domain of the $Na^+/H^+$ exchanger regulatory factor (NHERF).[20] This structural feature provides a basis for G-protein-independent signaling by the $P2Y_1$ receptor. Northern blotting has revealed a rather widespread expression of the $P2Y_1$ receptor.[8] The phenotype of $P2Y_1^{-/-}$ mice was mainly characterized by decreased platelet aggregation by ADP and a resistance to thromboembolism:[21,22] this is consistent with the known expression of the $P2Y_1$ receptor in platelets, its specific involvement in the ADP-induced shape change and its co-operation with the $P2T_{AC}$ receptor in the generation of a full aggregation response to ADP.[23] No other phenotypic alteration has been found so far.

### *$P2Y_2$ receptor*

The murine,[2] rodent[24] and human[25] $P2Y_2$ receptors have been cloned and the human gene found to be localized to chromosome 11q13.5-q14.[26] The recombinant receptors are activated almost equipotently by ATP and UTP. When adequate methodologic care was taken, agonist activity of ADP or UDP could not be detected.[27] Indeed, identification of P2Y receptor agonists and characterization of their rank order of potency is complicated by several factors: cross-contamination of nucleotide preparations, degradation by ectonucleotidases, interconversion between adenine and uracil nucleotides.[28] Therefore definite conclusions can only be drawn from studies performed with HPLC-purified nucleotides, short incubation periods and tricks such as the addition of hexokinase to the medium in order to consume extracellular ATP and prevent the transphosphorylation of added UDP into UTP by uridine diphosphokinase.[29] Mutagenicity studies revealed the critical involvement of positively charged residues at the exofacial extremity of the TM6 and TM7 domains.[30] The $P2Y_2$ receptor is coupled to phospholipase C and this response is partially inhibited by pertussis toxin (see below). It is also coupled via $G_i$ to the opening of inward-rectifier $K^+$ channels and to the closure of N-type voltage-gated $Ca^{2+}$ channels.[31,32] MAP kinase activation has also been described, but is probably an indirect effect downstream of phospholipase C activation. Tissue distribution of $P2Y_2$ mRNA is widespread.[25] The $P2Y_2$ mRNA was rapidly upregulated by T-cell receptor cross-linking and glucocorticoids in rat thymocytes[33] and by G-CSF and retinoic acid in human promyelocytic leukemia HL-60 cells:[34] both effects seem to represent an immediate early gene response. In $P2Y_2^{-/-}$ mice, the stimulatory effect of nucleotides on epithelial chloride secretion was almost completely abolished in the trachea, whereas a partial response was maintained in the gallbladder and a full response in the jejunum.[35]

### *$P2Y_4$ receptors*

Human[36,37] and rat[38,39] $P2Y_4$ receptors have been cloned. The human gene is localized to chromosome Xq13. These receptors are activated by UTP, but not by UDP; the effect of ATP is species-dependent, it being a full agonist for the rat receptor and a partial agonist for the human one. Thus, the agonist profile of the rat $P2Y_4$ receptor is very similar to that of the $P2Y_2$ subtype. The *Xenopus* xp2y[40] and turkey tp2y[41] are probably orthologs of the P2Y4 receptor. They are activated by ATP and UTP, as well as by other nucleotide triphosphates. The coupling of the P2Y4 receptor to phospholipase C is partially inhibited by pertussis toxin.[42] In the rat, $P2Y_4$ mRNA was detected by RT-PCR as being positive, whereas Northern blotting gave negative results, thus suggesting that the level of expression is low.[39] Expression is higher in the neonatal rat than the adult animal. Northern blotting revealed expression of $P2Y_4$ mRNA in the human lung and in a human cell line derived from lung submucosal cells,[43] suggesting that besides the $P2Y_2$ receptor, the $P2Y_4$ subtype contributes to nucleotide control of the airways.

### *$P2Y_6$ receptor*

Rat[44] and human[45] $P2Y_6$ receptors have been cloned. The human $P2Y_6$ gene is localized to chromosome 11q13.5, close to the $P2Y_2$ gene.[26] The chicken $p2y_3$ receptor,[46] which has a similar pharmacology, is probably its avian ortholog.[47] Indeed, although the shared amino acid identity between $cp2y_3$ and $hP2Y_6$ sequences is only 60%, which is less than the identity common to chick and human $P2Y_1$ receptors (86%), this is nevertheless higher than that found between distinct subtypes (35-40%) and the 2 receptors have similar profiles of agonist potency. Furthermore, using Southern blotting and screening of genomic libraries, it was demonstrated that the human genome does not contain a receptor more homologous to the avian $p2y_3$ receptor than the $P2Y_6$ receptor.[47] The $P2Y_6$ receptor is a selective UDP receptor. The existence of pyrimidinoceptors, claimed on the basis of circumstantial pharmacologic evidence,[48] was therefore thus definitely proved by the cloning of the $P2Y_6$ receptor. ADP is a weak partial agonist for this receptor whereas ATP is completely inactive: this suggests that uracil nucleotides may play a role as intercellular messengers, independently of adenine nuleotides. The $P2Y_6$ receptor is coupled to phospholipase C in a pertussis toxin-insensitive way. Inhibition of M-type $K^+$ current has also been reported.[49] $P2Y_6$ messengers are expressed in human placenta,[45,50] spleen, thymus and peripheral blood leukocytes,[45] as well as in various immune-derived human

cell lines (Jurkat, MOLT-4, JM-1, THP-1) and in T-lymphocytes infiltrating lesions of inflammatory bowel disease.[51] Both Northern blotting and functional studies have also revealed expression in airway epithelial cells.[43, 52]

### *P2Y$_{11}$ receptor*

Among the P2Y receptors, human P2Y$_{11}$ receptor has a unique feature: the open reading frame is intron-interrupted.[53] The gene has been localized to chromosome 19p31-35. It is the only ATP-selective receptor characterized so far.[53] The structure-activity relationship is quite different from that characterizing the P2Y$_1$ receptor: indeed ADP was barely active and substitution on the C2 position of the adenine moiety reduced the potency instead of enhancing it.[54] The P2Y$_{11}$ receptor is coupled to phospholipase C activation in a pertussis toxin-insensitive way. It is unique among P2Y receptors in its ability to stimulate adenylyl cyclase, by a mechanism which appears to be direct coupling. Indeed, rapid accumulation of cAMP in response to ATP was observed following stable expression of the P2Y$_{11}$ receptor in CHO cells, which do not express an endogenous $A_2$ receptor, and this effect was not inhibited by methylxanthines, thus excluding an indirect mechanism involving ATP degradation into adenosine and activation of $A_2$ receptors.[54] Furthermore, the role of prostaglandins, kinase C or $[Ca^{2+}]_i$ was also excluded. Northern blotting revealed that this receptor is expressed in human spleen, thymus and intestine,[53] as well as in HL-60 human promyelocytic leukemia cells (see below).

## Receptors mistakenly included in the P2Y family

Other receptors have been mistakenly included in the P2Y family on the basis of sequence homology, but in the absence of a functional demonstration that they responded to nucleotides. The p2y$_7$ receptor is actually a leukotriene $B_4$ receptor.[55] The p2y$_5$, p2y$_5$-like (=p2y$_9$) and p2y$_{10}$ receptors share only about 30% amino acid identity with the genuine P2Y receptors, which is no greater than their amino acid identity with other receptor families.[56-59] More specifically, they lack positively charged amino acid residues which are present in TM6 and TM7 of genuine P2Y receptors and seem to play a role in nucleotide binding via electrostatic interaction with the negatively charged phosphate groups.[30] The p2y$_5$ and p2y$_9$ receptors are the most closely related and, therefore, putative members of a new orphan receptors family, to which p2y$_{10}$, as well as some other orphan receptors homologous to P2Y receptors, such as GPR17 and GPR55,[60] might also belong. Northern blotting had shown that the expression of the cp2y$_5$ receptor is restricted to chicken activated T-cells.[56] More recently, the hp2y$_5$ messenger was detected in a T-cell line (MOLT-4 cells) derived from a human leukemia. Expression of the hp2y$_9$ mRNA was also very restricted, since no messenger could be detected in 16 human organs.[59] Recently, expression was detected in a pre-B lymphocyte cell line (JM-1 cells). The p2y$_{10}$ messenger expression is lymphoid-restricted, occurring in spleen, thymus, immature and mature B- and T-cells. Its expression is regulated directly by the PU.1 and Spi-B transcription factors.[61] These data suggest that these receptors may have a role in the immune system. It is obvious that a fundamental revision of the nomenclature is needed in order to clarify that, at present, the P2Y family has five members and encompasses selective purinoceptors (P2Y$_1$, P2Y$_{11}$), selective pyrimidinoceptors (P2Y$_6$=cp2y$_3$) and receptors of mixed selectivity (P2Y$_2$, P2Y$_4$) (Table 1).

**Table 1. Human P2Y receptors: subtypes, preferential agonists, G protein coupling and effectors**

| *Subtype* | *Agonist* | *G protein* | *Effector* |
|---|---|---|---|
| P2Y$_1$ | ADP | $G_{q/11}$ | ↑ PLC |
| P2Y$_2$ | ATP=UTP | $G_{q/11}$ + $G_i$ | ↑ PLC |
| P2Y$_4$ | UTP | $G_{q/11}$ + $G_i$ | ↑ PLC |
| P2Y$_6$ | UDP | $G_{q/11}$ | ↑ PLC |
| P2Y$_{11}$ | ATP | $G_{q/11}$ | ↑ PLC |
| | | $G_s$ | ↑ AC |

*PLC: phospholipase C; AC: adenylyl cyclase.*

## Coupling of P2Y receptors to $G_i$ and adenylyl cyclase inhibition

All genuine P2Y receptors cloned so far (P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$, P2Y$_{11}$) are coupled to phospholipase C activation. Sensitivity to pertussis toxin is variable from one subtype to another suggesting the involvement of distinct G proteins. The inositol phosphate response mediated by P2Y$_1$,[4] P2Y$_6$ [44-62] and P2Y$_{11}$ [53] receptors was not inhibited by pertussis toxin, whereas the P2Y$_2$ [25-63] and P2Y$_4$ [42] receptors exhibited partial sensitivity. Initial studies of the recombinant P2Y$_2$ receptor revealed that pertussis toxin produced a 25-35% inhibition of the effect of maximal agonist concentrations.[25] Further studies showed that the sensitivity to pertussis toxin is critically dependent on the agonist concentration as well as on kinetics.[63] The inhibition was complete at low nucleotide concentrations, but tended to disappear at agonist concentrations inducing a maximal effect. The pertussis toxin inhibition was also greater early in the stimulation.[63] These data suggest that at least two G proteins are involved, a conclusion consistent with results obtained on native P2Y$_2$ (or $P_{2U}$) receptors. The degree of inhibition by pertussis toxin was indeed variable: partial in HL-60 cells[64] but complete in aortic endothelial cells.[65] In HEL cells, phospholipase C activation by ATP/UTP was inhibited partially by pertussis toxin and completely by a $G\alpha_{16}$ antisense.[66] In gastric smooth muscle cells, the ATP/UTP-induced activation of phospholipase C was partially inhibited by polyclonal antibodies against $G\alpha_{q/11}$ or $G\beta$, while the combination of the two antibodies produced complete inhibition.[67] Similarly a complete inhibition was obtained by combining the anti-$G\alpha_{q/11}$ antibody and pertussis toxin treatment. Results from combining antibodies against specific G proteins and phospholipase C isoenzymes led to the conclusion that the P2Y$_2$ receptor is coupled to PLC-$\beta$1 via $G\alpha_{q/11}$ and to PLC-$\beta$3 via $G\beta\gamma_{i3}$. Partial sensitivity to pertussis toxin was also a feature of the human recombi-

nant $P2Y_4$ receptor: indeed the toxin inhibited the rapid and transient peak of inositol phosphates accumulation induced by UTP, but the sustained stimulation which followed was unaffected.[42] Interestingly, many $G_{i/o}$–coupled receptors contain a threonine residue close to the junction between the third intracellular loop and the sixth transmembrane domain:[68] such a residue is present in the sequence of human $P2Y_2$ and $P2Y_4$, but not $P2Y_1$, $P2Y_6$ and $P2Y_{11}$ receptors. It would, however, be misleading to overemphasize this association: determinants of the selectivity of G-protein recognition are complex [69] and the tp2y receptor, recently shown to couple to $G_i$, does not contain such a threonine residue.[41]

Inhibition of cAMP accumulation by nucleotides has been demonstrated in several cell types, such as human platelets,[70] rat hepatocytes,[71] mouse ventricular myocytes,[72] LLC-PK1 renal epithelial cells,[73] rat C6 glioma,[74,75] rat Schwann cells[76] and rat B10 brain microvascular endothelial cells.[77] The C6 model has been studied extensively. An inhibitory effect of ADP on adenylyl cyclase was documented in cell membranes[78] and the inhibition of cAMP accumulation was abolished following cell treatment with pertussis toxin,[75] indicating that $G_i$ was involved. ADP and ATP had similar potencies, while their respective 2-methylthio derivatives were much more potent inhibitors.[75] The pharmacologic profile was quite different from that of the $P2Y_1$ receptor coupled to phospholipase C activation. For instance pyridoxal phosphate 6-azophenyl 2',4'-disulfonic acid (PPADS) was a competitive antagonist of the stimulation of phospholipase C in turkey erythrocytes, which express a $P2Y_1$ receptor, but did not affect the inhibition of cAMP accumulation in C6 glioma cells.[79] On the other hand 2-thioether derivatives of ATP, such as 2-hexylthioATP or 2-cyclohexylthioATP, were much more potent as inhibitors of adenylyl cyclase in C6 glioma cells than as activators of phospholipase C in turkey erythrocytes.[80] Following stable expression of the human $P2Y_1$ receptor in 1321N1 cells, the formation of inositol phosphates was stimulated by ADP, but no inhibition of cAMP accumulation by nucleotides was detectable,[81,82] thus demonstrating the involvement of a distinct receptor. Inhibition of cAMP accumulation in platelets exhibited similar features, except that in platelets, unlike C6 glioma and B10 endothelial cells, the inhibitory effect of ADP and 2-MesADP was antagonized rather than mimicked by the corresponding triphosphonucleotides.[83] In platelets of $P2Y_1^{-/-}$ mice, the inhibition of cAMP accumulation by ADP was maintained, whereas its effect on $Ca^{2+}$ mobilization and platelet shape change was abolished.[21,22] This result constitutes the ultimate demonstration that adenylyl cyclase inhibition by ADP is mediated by a receptor distinct and independent from the $P2Y_1$ receptor: this receptor has been provisionally called $P2T_{AC}$, $P2_{CYC}$ or $P2Y_{ADP}$.

The only P2Y subtype which has so far been demonstrated to couple to $G_i$ and adenylyl cyclase inhibition is the tp2y receptor, the probable avian ortholog of the $P2Y_4$ receptor.[41,84] This receptor is activated by purine and pyrimidine triphosphonucleotides and thus has a pharmacology completely different from that of the $P2T_{AC}$ receptor. The $P2T_{AC}$ receptor, as well as the closely related receptor expressed in rat C6 glioma or B10 brain microvascular endothelial cells, has so far resisted all cloning efforts. The hypothesis that it is actually a $P2Y_1$ gene product either modified by RNA editing[85] or associated with a specific RAMP (receptor activity modifying protein)[86] can be rejected since a typical $P2T_{AC}$ response is maintained in $P2Y_1^{-/-}$ mice. Although it is a remote possibility, one cannot entirely exclude that the $P2T_{AC}$ receptor does not belong to the family of G-protein-coupled receptors family: indeed thrombospondin activates $G_i$ via an interaction with an integrin-CD47 protein complex.[87] However it is more likely to be a member of the P2Y family distantly related to the other subtypes, in the same way as the H3 receptor has little homology with H1 and H2 subtypes.[88]

## Role of the $P2Y_{11}$ receptor in granulocytopoiesis

The discovery that, via a rise in cAMP, ATP triggers, the differentiation of HL-60 human promyelocytic leukemia cells into neutrophil-like cells is a recent one.[89-91] Differentiation was documented by the appearance of fMLP-stimulated secretion of β-glucuronidase and was accompanied by cell growth suppression. ATP increased the cAMP level in HL-60 cells more potently than ADP and AMP or adenosine did, and its action was insensitive to xanthine inhibition, suggesting the unusual involvement of a P2 receptor coupled to adenylyl cyclase stimulation. Dibutyryl-cAMP is a well-known inducer of the granulocytic differentiation of these cells and the differentiating effect of ATP was abolished by a protein kinase A antagonist.[90] The same year cloning of the $P2Y_{11}$ receptor, dually coupled to phospholipase C and adenylyl cyclase activation, was reported.[53] $P2Y_{11}$ mRNA was detected in HL-60 cells,[53] but not in mature neutrophils,[34] and the pharmacologic profile of the recombinant $P2Y_{11}$ receptor closely matched that of the stimulatory effect of ATP on cAMP in HL-60 cells.[54] In particular, in both HL-60 cells and CHO cells expressing the recombinant human $P2Y_{11}$ receptor, the rank order of potency characterizing the stimulation of cAMP by nucleotides was: ATPγS≈BzATP> dATP>ATP>ADPβS>2-MeSATP. The $P2T_{AC}$ antagonist AR-C67085X is the most potent agonist of the recombinant $P2Y_{11}$ receptor so far identified. It increases the cAMP level of HL-60 cells more potently than ATP itself.[54] Interestingly, $P2Y_{11}$ transcripts were upregulated, rapidly (within 1 hour) and independently from protein synthesis, by all the agents which induce granulocytic differentiation of HL-60 cells (DMSO, retinoic acid, G-CSF, dibutyryl-cAMP), but not by agents which differentiate them into monocytes (phorbol-12 ,13-myristate-acetate, 1,25-dihydroxy-vitamin $D_3$) [34]. The $G_s$-coupled $P2Y_{11}$ receptor seems, therefore, to be involved in hematopoiesis and might constitute a new therapeutic target in the treatment of some forms of leukemia and neutropenia.

## References


1. Webb TE, Simon J, Krishek BJ, et al. Cloning and functional expression of a brain G-protein-coupled ATP receptor FEBS Lett 1993; 324:219-25.
2. Lustig KD, Shiau AK, Brake AJ, Julius D. Expression cloning of an ATP receptor from mouse neuroblastoma cells. Proc Natl Acad Sci USA 1993; 90:5113-7.
3. Fredholm BB, Abbrachio MP, Burnstock G, et al. Nomenclature and classification of purinoceptors. Pharmacol Rev 1994; 46:143-56.
4. Filtz TM, Li Q, Boyer JL, Nicholas RA, Harden TK. Expression of a cloned P2Y purinergic receptor that couples to phospholipase C. Mol Pharmacol 1994; 46:8-14.
5. Tokuyama Y, Hara M, Jones EMC, Fan Z, Bell GI. Cloning of rat and mouse P2Y purinoceptors. Biochem Bioph Res Co 1995; 211:211-8.
6. Henderson DJ, Elliot DG, Smith GM, Webb TE, Dainty IA. Cloning and characterization of a bovine P2Y receptor. Biochem Biophys Res Comm 1995; 212: 648-56.
7. Ayyanathan K, Webb TE, Sandhu AK, Athwal RS, Barnard EA, Kunapuli S. Cloning and chromosomal localization of the human $P2Y_1$ purinoceptor. Biochem Biophys Res Co 1996; 218:783-8.
8. Janssens R, Communi D, Pirotton S, Samson M, Parmentier M, Boeynaems JM. Cloning and tissue distribution of the human $P2Y_1$ receptor. Biochem Bioph Res Co 1996; 221:588-93.
9. Hechler B, Vigne P, Léon C, Breittmayer JP, Gachet C, Frelin C. ATP derivatives are antagonists of the $P2Y_1$ receptor: similarities to the platelet ADP receptor. Mol Pharmacol 1998; 53:727-33.
10. Palmer RK, Boyer JL, Schchter JB, Nicholas RA, Harden TK. Agonist action of adenosine triphosphates at the human $P2Y_1$ receptor. Mol Pharmacol 1998; 54: 1118-23.
11. Boyer JL, Siddiqi S, Fischer B, Romero-Avila T, Jacobson KA, Harden TK. Identification of potent P2Y-purinoceptor agonists that are derivatives of adenosine 5'-monophosphate. Br J Pharmacol 1996; 118: 1959-64.
12. Halfbinger E, Major DT, Tritzmann M, et al. Molecular recognition of modified adenine nucleotides by the $P2Y_1$ receptor 1. A synthetic, biochemical and NMR approach. J Med Chem 1999; 42:5325-37.
13. Major DT, Halfbinger E, Fischer B. Molecular recognition of modified adenine nucleotides by the $P2Y_1$ receptor 2. A computational approach. J Med Chem 1999; 42:5338-47.
14. Boyer JL, Romero-Avila T, Schachter JB, Harden TK. Identification of competitive antagonists of the $P2Y_1$ receptor. Mol Pharmacol 1996; 50:1323-9.
15. Boyer JL, Mohanram A, Camaioni E, Jacobson KA, Harden TK. Competitive and selective antagonism of $P2Y_1$ receptors by N6-methyl 2'-deoxyadenosine 3',5'-bisphosphate. Br J Pharmacol 1998; 124:1-3.
16. Jiang Q, Guo D, Lee BX, et al. A mutational analysis of residues essential for ligand recognition at the human $P2Y_1$ receptor. Mol Pharmacol 1997; 52:499-507.
17. Hoffmann C, Moro S, Nicholas RA, Harden TK, Jacobson KA. The role of amino acids in extracellular loops of the human $P2Y_1$ receptor in surface expression and activation processes. J Biol Chem 1999; 274: 14639-47.
18. Paul BZ, Daniel JL, Kunapuli SP. Platelet shape change is mediated by both calcium-dependent and independent signaling pathways. Role of p160 Rho-associated coiled-coil-containing protein kinase in platelet shape change. J Biol Chem 1999; 274:28293-300.
19. O'Grady SM, Elmquist E, Filtz TM, Nicholas RA, Harden TK. A guanine nucleotide-independent inwardly rectifying cation permeability is associated with P2Y1 receptor expression in *Xenopus* oocytes. J Biol Chem 1996; 271:29080-7.
20. Hall RA, Ostedgaard LS, Premont RT, et al. A C-terminal motif found in the $\beta_2$-adrenergic receptor, $P2Y_1$ receptor and cystic fibrosis transmembrane conductance regulator determines binding to the $Na^+/H^+$ exchanger regulatory factor family of PDZ proteins. Proc Natl Acad Sci USA 1998; 95:8496-501.
21. Fabre JE, Nguyen MT, Latour A, et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in $P2Y_1$-deficient mice. Nature Med 1999; 5:1199-202.
22. Léon C, Hechler B, Freund M, et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic $P2Y_1$ receptor-null mice. J Clin Invest 1999; 104:1731-7.
23. Kunapuli SP. Multiple P2 receptor subtypes on platelets: a new interpretation of their function TiPS. 1998; 19:391-4.
24. Gödecke S, Decking UKM, Gödecke A, Schrader J. Cloning of the rat P2U receptor and its potential role in coronary vasodilation. Am J Physiol 1996; 270:570-7.
25. Parr CE, Sullivan DM, Paradiso AM, et al. Cloning and expression of a human P2U nucleotide receptor, a target for cystic fibrosis pharmacotherapy. Proc Natl Acad Sci USA 1994; 91:3275-9.
26. Somers GR, Hammet F, Woollatt E, Richards RI, Southey MC, Venter DJ. Chromosomal localization of the human P2Y6 purinoceptor gene and phylogenetic analysis of the P2Y purinoceptor family. Genomics 1997; 44:127-30.
27. Nicholas RA, Watt WC, Lazarowski ER, Li Q, Harden TK. Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, a UTP-selective, and an ATP- and UTP-specific receptor. Mol Pharmacol 1996; 50:224-9.
28. Harden TK, Lazarowski ER, Boucher RC. Release, metabolism and interconversion of adenine and uridine nucleotides: implications for G protein-couple P2 receptor agonist selectivity. TiPS 1997; 18:43-6.
29. Lazarowski ER, Homolya L, Boucher RC, Harden TK. Identification of an ecto-nucleoside diphosphokinase and its contribution to interconversion of P2 receptor agonists. J Biol Chem 1997; 272:20402-7.
30. Erb L, Garrad R, Wang Y, Quinn T, Turner JT, Weisman GA. Site-directed mutagenesis of $P_{2U}$ purinoceptors. J Biol Chem 1995; 270:4185-8.
31. Mosbacher J, Maier R, Fakler B, Glatz A, Crespo J, Bilbe G. P2Y receptor subtypes differentially couple to inwardly-rectifying potassium channels. FEBS Lett 1998; 436:104-10.
32. Filippov AK, Webb TE, Barnard EA, Brown DA. Inhibition by heterologously-expressed $P2Y_2$ nucleotide receptors of N-type calcium currents in rat sympathetic neurones. Br J Pharmacol 1997; 121:849-51.
33. Koshiba M, Apasov S, Sverdlov V, et al. Transient upregulation of $P2Y_2$ nucleotide receptor mRNA expression is an immediate early gene response in activated thymocytes. Proc Natl Acad Sci USA 1997; 94:831-6.
34. Communi D, Janssens R, Zeelis N, Robaye B, Boeynaems J-M. (unpublished data).
35. Cressman VL, Lazarowski ER, Homolya L, Boucher RC, Koller BH, Grubb BR. Effect of loss of $P2Y_2$ receptor gene expression on nucleotide regulation of murine epithelial Cl- transport. J Biol Chem 1999; 274:26461-8.
36. Communi D, Pirotton S, Parmentier M, Boeynaems

JM. Cloning and functional expression of a human uridine nucleotide receptor. J Biol Chem 1995; 270: 30849-52.
37. Nguyen T, Erb L, Weisman GA, et al. Cloning, expression and chromosomal localization of the human uridine nucleotide receptor. J Biol Chem 1995; 270: 30845-8.
38. Bogdanov YD, Wildman SS, Clements MP, King BF, Burnstock G. Molecular cloning and characterization of rat P2Y4 nucleotide receptor. Br J Pharmacol 1998; 124:428-30.
39. Webb TE, Henderson DJ, Robets JA, Barnard EA. Molecular cloning and characterization of the rat P2Y4 receptor. J Neurochem 1998; 71:1348-57.
40. Bogdanov YD, Dale L, King BF, Wittock N, Burnstock. Early expression of a novel nucleotide receptor in the neural plate of *Xenopus* embryos. J Biol Chem 1997; 272:12583-90.
41. Boyer JL, Waldo GL, Harden TK. G molecular cloning and expression of an avian G protein-coupled P2Y receptor. Mol Pharmacol 1997; 52:928-34.
42. Communi D, Motte S, Boeynaems JM, Pirotton S. Pharmacological characterization of the human P2Y4 receptor. Eur J Pharmacol 1996; 317:383-9.
43. Communi D, Paindavoine P, Place GA, Parmentier M, Boeynaems JM. Expression of P2Y receptors in cell lines derived from the human lung. Br J Pharmacol 1999; 127:562-8.
44. Chang K, Hanaoka K, Kumada M, Takuwa Y. Molecular cloning and functional analysis of a novel P2 nucleotide receptor. J Biol Chem 1995; 270:26152-8.
45. Communi D, Parmentier M, Boeynaems JM. Cloning, functional expression and tissue distribution of the human $P2Y_6$ receptor. Biochem Biophys Res Comm 1996; 222:303-8.
46. Webb TE, Henderson D, King BF, et al. A novel G protein-coupled P2 purinoceptor ($P_{2Y3}$) activated preferentially by nucleoside diphosphates. Mol Pharmacol 1996; 50:258-65.
47. Li Q, Olesky M, Palmer RK, Harden TK, Nicholas RA. Evidence that the p2y3 receptor is the avian homologue of the mammalian $P2Y_6$ receptor. Mol Pharmacol 1998; 54:541-6.
48. Seifert R, Schultz G. Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and by uracil nucleotides. TiPS 1989; 10:365-9.
49. Filippov AK, Webb TE, Barnard EA, Brown DA. Dual coupling of heterologously-expressed rat $P2Y_6$ nucleotide receptors to N-type $Ca^{2+}$ and M-type $K^+$ currents in rat sympathetic neurones. Br J Pharmacol 1999; 126:1009-17.
50. Somers GR, Bradbury R, Trute L, Conigrave A, Venter DJ. Expression of the human $P2Y_6$ nucleotide receptor in normal placenta and gestational trophoblastic disease. Lab Invest 1999; 79:131-9.
51. Somers GR, Hammett FMA, Trute L, Southey MC, Venter DJ. Expression of the $P2Y_6$ purinergic receptor in human T cells infiltrating inflammatory bowel disease. Lab Invest 1998; 78:1375-83.
52. Lazarowski ER, Paradiso AM, Watt WC, Harden TK, Boucher RC. UDP activates a mucosal-restricted receptor on human nasal epithelial cells that is distinct from the $P2Y_2$ receptor. Proc Natl Acad Sci USA 1997; 94:2599-603.
53. Communi D, Govaerts C, Parmentier M, Boeynaems JM. Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. J Biol Chem 1997; 272:31969-73.
54. Communi D, Robaye B, Boeynaems JM. Pharmacological characterization of the human $P2Y_{11}$ receptor. Br J Pharmacol 1999; 128:1199-206.
55. Yokomizo T, Izumi T, Chang K, Takuwa Y, Shimizu T. A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. Nature 1997; 387:620-4.
56. Kaplan MH, Smith DI, Sundick RS. Identification of a G protein coupled receptor induced in activated T cells. J Immunol 1993; 151:628-36.
57. Webb TE, Kaplan MG, Barnard EA. Identification of 6H1 as a P2Y purinoceptor: $P2Y_5$. Biochem Biophys Res Comm 1996; 219:105-10.
58. Herzog H, Darby K, Hort YJ, Shine J. Intron 17 of the human retinoblastoma susceptibility gene encodes an actively transcribed G-protein–coupled receptor gene. Genome Res 1996; 6:858-61.
59. Janssens R, Boeynaems JM, Godart M, Communi D. Cloning of a human heptahelical receptor closely related to the $P2Y_5$ receptor. Biochem Biophys Res Comm 1997; 236:106-12.
60. Marchese A, George SR, Kolakowski LF, Lynch KR, O'Dowd BF. Novel GPCRs and their endogeneous ligands: expanding the boundaries of physiology and pharmacology. TiPS 1999; 20:370-5.
61. Rao S, Garrett-Sinha LA, Yoon J, Simon MC. The Ets factors PU.1 and Spi-B regulate the transcription in vivo of $P2Y_{10}$, a lymphoid restricted heptahelical receptor. J Biol Chem 1999; 274:34245-52.
62. Robaye B, Boeynaems JM, Communi D. Slow desensitization of the human $P2Y_6$ receptor. Eur J Pharmacol 1997; 329:231-6.
63. Janssens R, Paindavoine P, Parmentier M, Boeynaems JM. Human $P2Y_2$ receptor polymorphism: identification and pharmacological characterization of two allelic variants. Br J Pharmacol 1999; 126:709-16.
64. Dubyak GR, Cowen DS, Meuller LM. Activation of inositol phospholipid breakdown in HL60 cells by P2-purinergic receptors for extracellular ATP. J Biol Chem 1988; 263:18108-17.
65. Motte S, Pirotton S, Boeynaems JM. Heterogeneity of ATP receptors in aortic endothelial cells. Involvement of P2Y and P2U receptors in inositol phosphate response. Circ Res 1993; 72:504-10.
66. Baltensperger K, Porzig H. The $P_{2U}$ purinoceptor obligatorily engages the heterotrimeric protein $G_{16}$ to mobilize intracellular $Ca^{2+}$ in human erythroleukemia cells. J Biol Chem 1997; 272:10151-9.
67. Murthy KS, Makhlouf GM. Coexpression of ligand-gated P2X and G protein-coupled P2Y receptors in smooth muscle. J Biol Chem 1998; 273:4695-704.
68. Liu J, Conklin BR, Blin N, Yun J, Wess J. Identification of a receptor/G-protein contact site critical for signaling specificity and G-protein activation. Proc Natl Acad Sci USA 1995; 92:11642-46.
69. Wess J. G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. FASEB J 1997; 11:346-54.
70. Cooper DMF, Rodbell M. ADP is a potent inhibitor of human platelet plasma membrane adenylate cyclase. Nature 1979; 282:517-8.
71. Okajima F, Tokumitsu Y, Kondo Y, Ui M. P2Y-purinergic receptors are coupled to two signal transduction systems leading to inhibition of cAMP generation and to production of inositol trisphosphate in rat hepatocytes. J Biol Chem 1987; 262:13483-90.
72. Yamada M, Hamamori Y, Akita H, Yokoyama M. P2-purinoceptor activation stimulates phosphoinositide hydrolysis and inhibits accumulation of cAMP in cultured ventricular myocytes. Circ Res 1992; 70:477-85.
73. Anderson RJR, Breckon R, Dixon BS. ATP receptor regulation of adenylate cyclase and protein kinase C activity in cultured renal LLC-PK1 cells. J Clin Invest 1991; 87:1732-8.
74. Pianet I, Merle M, Labouesse J. ADP and indirectly ATP are potent inhibitors of cAMP production in intact isoproterenol-stimulated C6 glioma cells.

Biochem Biophys Res Comm 1989; 163:1150-7.
75. Boyer JL, Lazarowski ER, Chen XH, Harden TK. Identification of a P2Y-purinergic receptor that inhibits adenylyl cyclase. J Pharmacol Exp Ther 1993; 267: 1140-6.
76. Berti-Mattera LN, Wilkins PL, Madhun Z, Suchovsky D. P2-purinergic receptors regulate phospholipase C and adenylate cyclase activities in immortalized Schwann cells. Biochem J 1996; 314:555-61.
77. Webb TE, Feolde E, Vigne P, et al. The P2Y purinoceptor in rat brain microvascular endothelial cells couples to inhibition of adenylate cyclase. Br J Pharmacol 1996; 119:1385-92.
78. Valeins H, Merle M, Labouesse J. Pre-steady state study of β-adrenergic and purinergic receptor interaction in C6 cell membranes: undelayed balance between positive and negative coupling to adenylyl cyclase. Mol Pharmacol 1992; 42:1033-41.
79. Boyer JL, Zohn IE, Jacobson KA, Harden TK. Differential effects of P2-purinoceptor antagonists on phospholipase C and adenylyl cyclase-coupled P2Y-receptors. Br J Pharmacol 1994; 113:614-20.
80. Boyer JL, O'Tuel JW, Fischer B, Jacobson KA, Harden TK. Potent agonist action of 2-thioether derivatives of adenine nucleotides at adenylyl cyclase-linked P2Y-purinoceptors. Br J Pharmacol 1995; 116:2611-6.
81. Schachter JB, Li Q, Boyer JL, Nicholas RA, Harden TK. Second messenger cascade specificity and pharmacological selectivity of the human P2Y1-purinoceptor. Br J Pharmacol 1996; 118:167-73.
82. Schachter JB, Boyer JL, Li Q, Nicholas RA, Harden TK. Fidelity in functional coupling of the rat P2Y1 receptor to phospholipase C. Br J Pharmacol 1997; 122: 1021-4.
83. Cusack NJ, Hourani SMO. Competitive inhibition by adenosine 5'-triphosphate of the actions on human platelets of 2-chloroadenosine 5'-diphosphate, 2-azidoadenosine 5'-diphosphate and 2-methylthioadenosine 5'-diphosphate. Br J Pharmacol 1982; 77: 329-33.
84. Boeynaems JM, Communi D, Savi P, Herbert JM. P2Y receptors : in the middle of the road. TiPS 2000; 21:1-3.
85. Burns CM, Chu H, Rueter SM, et al. Regulation of serotonin-2C receptor G-protein coupling by RNA editing. Nature 1997; 387:303-8.
86. Foord SM, Marshall FH. RAMPS: accessory proteins for seven transmembrane domain receptors. TiPS 1999; 20:184-7.
87. Frazier WA, Gao AG, Dimitry J, et al. The thrombospondin receptor integrin-associated protein (CD47) functionally couples to heterotrimeric Gi. J Biol Chem 1999; 274:8554-60.
88. Lovenberg TW, Roland BL, Wilson SJ, et al. Cloning and functional expression of the human histamine H3 receptor. Mol Pharmacol 1999; 55:1101-7.
89. Jiang L, Foster FM, Ward P, Tasevski V, Luttrell BM, Conigrave AD. Extracellular ATP triggers cyclic AMP-dependent differentiation of HL-60 cells. Biochem Biophys Res Comm 1997; 232:626-30.
90. Choi SY, Kim KT. Extracellular ATP-stimulated increase of cytosolic cAMP in HL-60 cells. Biochem Pharmacol 1997; 53:429-32.
91. Conigrave AD, Lee JY, van der Weyden L, et al. Pharmacological profile of a novel cyclic AMP-linked P2 receptor on undifferentiated HL-60 leukemia cells. Br J Pharmacol 1998; 124:1580-5.

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# LIGAND SPECIFICTY, REGULATION AND CROSS-TALK OF HUMAN PLATELET ADP RECEPTORS

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ABSTRACT

**Platelet activation and aggregation stimulated by ADP plays a key role in arterial thrombosis. Therefore, the mechanisms of human platelet activation by ADP are of considerable biochemical, pharmacologic and medical interest. Three main signaling pathways induced by ADP have been described for platelets: the activation of a ligand gated cation channel with low selectivity, the activation of intracellular calcium mobilization via activation of phospholipase C, and the inhibition of adenylyl cyclase by activation of Gi-protein. The activation of platelets by ADP ultimately results in shape change, adhesion and aggregation of the cells and secretion of vasoactive substances. Two of the ADP receptors mediating the platelet responses have been identified as being P2X1 and P2Y1 receptors respectively. The $G_i$-coupled receptor is provisionally termed P2YAC or P2cyc because it has not so far been able to identify it and its characteristics do not resemble any known purinoceptor. Though for the initial signaling the receptors could be successfully attributed to platelet function, the link to final platelet responses, such as shape change, secretion and aggregation has not yet been found. For detailed study of platelet purinergic receptor pharmacology and biochemistry, the two known receptors were cloned from human platelet RNA and stably expressed in the 1321N1 astrocytoma cell line. Pharmacologic and biochemical experiments were performed with these cells. Experiments with ADP derivatives known to be selective activators of human platelet purinergic receptors were mainly in accordance with the results obtained from platelets. The biochemical experiments were focused on the regulation of the purinergic receptors by cyclic nucleotides. The inhibitory effect of cGMP elevating agents is mediated by cGMP-dependent protein kinase (PKG) and is only directed against the P2Y1/Gq pathway but does not affect the P2X1 ligand gated calcium channel. Kinetic analysis proved that the inhibitory effect of PKG activation has two components: an inhibition of calcium influx and of calcium mobilization. Additionally, PKG activation not only inhibits sP2Y1 mediated platelet responses, but also responses by the yet unidentified P2YAC receptor. The observed inhibition of Gi-protein mediated pathways by cGMP-PK activation is probably responsible for the synergistic effects of stimulators of platelet guanylyl cyclase and adenylyl cyclase.**


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## Introduction

Receptors for a wide variety of endogenous substances and drugs are expressed on human platelets. Receptors for nucleotides, nucleosides, proteins, hormones, lipids and phospholipids, and eicosanoids are found. While some of these receptors are stimulatory receptors such as the ADP, thrombin or thromboxane receptors, others such as the adenosine or prostaglandin receptors exert inhibitory effects on platelet function. The stimulatory receptors are coupled to signaling pathways mediating the final platelet responses of adhesion, shape change, secretion and aggregation. The supergroup of purinergic receptors is subdivided into two main groups: the nucleoside receptors P1 and the nucleotide receptors P2.[1] Members of both major families of purinergic receptors are present on platelets. The P1 receptor is an inhibitory platelet receptor and activates adenylyl cyclase via $G_s$-protein upon stimulation by adenosine. The increased concentration of cAMP then leads to a protein kinase A (PKA) mediated inhibition of platelet function (Figure 1). Besides this, three different P2 receptors are found on platelets. These receptors are stimulatory in nature and sensitive to ADP.[2] Although stimulation of platelet aggregation by ADP was well established a long time ago,[3] the associated receptors remained unidentified until recently. A proposed three receptor model for platelet activation has new been confirmed in several publications.[4-6] Two of the receptors have been characterized and identified as P2X1[7] and P2Y1.[8,9] The P2X1 receptor forms a ligand gated cation channel with low selectivity;[10] the P2Y1 receptor is Gq-protein coupled and induces the release of calcium ions from dense granules into the cytosol by a phosphatidyl inositol pathway.[11] The third, as yet unidentified receptor, termed provi-

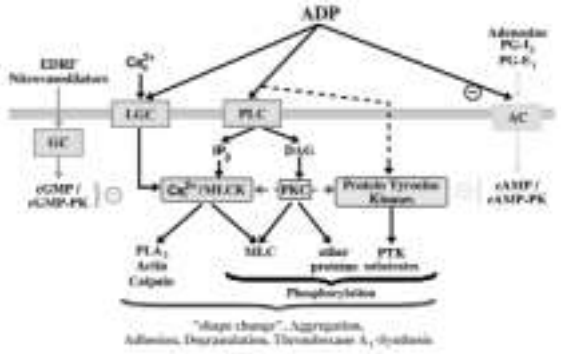


**Figure 1. Overview of human platelet signal transduction mechanisms. ADP stimulation leads to activation of three different pathways: activation of a ligand gated cation channel (LGC) inducing rapid calcium influx, stimulation of phospholipase C (PLC) and mobilization of calcium ions from intracellular calcium stores by inositol-trisphosphate ($IP_3$) and inhibition of adenylyl cyclase. The initial rise of intracellular calcium concentration further stimulates various protein kinases such as myosin light chain kinase (MLCK) or protein kinase C (PKC) as well as other calcium dependent proteins, e.g. phospholipase $A_2$ ($PLA_2$). Besides this ADP stimulates protein tyrosine kinases (PTK). In summary, these signals lead to platelet shape change, adhesion, degranulation and finally aggregation. Increased cGMP, stimulated by nitric oxide, and cAMP levels, stimulated by adenosine or prostaglandins, and subsequent activation of the respective protein kinases PKG and PKA lead to an inhibition of platelet activation.**

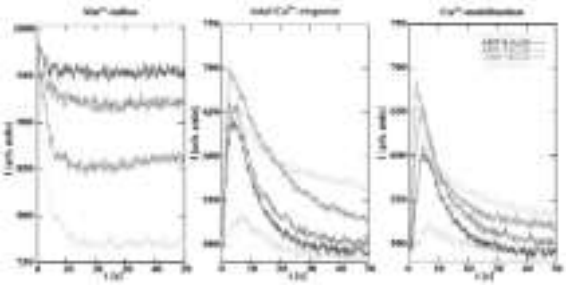

**Figure 2. Human platelet calcium responses evoked by varying ADP concentrations. Calcium responses of Fura-2 loaded human platelets to ADP concentrations ranging from 0.08 μM to 50 μM are shown. Manganese influx, representing total calcium influx, was determined in the presence of 0.5 μM $MnCl_2$. The total calcium response was measured in the presence of 1 μM $CaCl_2$, the mobilization of calcium from intracellular stores in the presence of 4 mM EGTA. The ADP was added at the concentrations indicated in the legend at 0 seconds.**

sionally P2YAC or P2cyc, is coupled via a Gi-protein to adenylyl cyclase (AC).[12] While the role of P2X1 receptor in platelet activation is still unclear, P2Y1 is obviously necessary but not sufficient for the activation of intracellular signaling pathways. Additional activation of P2YAC receptors seems to be essential for full platelet activation.[13,14]

## Purinoceptor mediated human platelet calcium responses

Human platelet intracellular calcium concentration is increased upon ADP stimulation by three different mechanisms. The initial stimulation of a ligand gated cation channel with low cation selectivity leads to a fast increase in intracellular calcium concentration.[15] This P2X1 receptor mediated calcium signal is reversible and returns back to the resting calcium concentration within seconds if no additional signal is stimulated. The second calcium response is a phospholipase C and IP3 mediated mobilization of calcium ions from platelet granules. This calcium signal is delayed with regard to the opening of the cation channel.[16] The calcium increase resulting from this process declines only slowly. While the calcium ions are released from the granules a further influx of calcium ions from the surrounding medium takes place. The biochemical mechanisms underlying this *store related* calcium influx have not yet been identified. Several experimental approaches allow the separation of the calcium elevating signals in platelets. Stopped flow fluorometry makes it possible to observe the different phases of calcium increase on the basis of their different kinetics.[16] By adding manganese ions to the platelet buffer it is possible to observe ion influx alone because the P2X1 cation channel and the store related calcium influx channel are permeable to manganese (Figure 2). Another method is based on the fact that elevated cAMP levels lead to a protein kinase A mediated inhibition of human platelet calcium mobilization and secondary influx[4]. So calcium influx via the ligand gated cation channel can be observed without being concealed by other calcium signals. Experiments with varying concentrations of ADP in the range from 0.08 to 50 μM showed that the ligand gated cation channel is activated only with low ADP concentrations (Figure 2). The secondary calcium influx could be stimulated by high concentrations of high ADP. The threshold ADP concentration for inducing this calcium influx was significantly higher than the ADP concentration stimulating maximal calcium mobilization. These data indicate that the secondary calcium influx is based on a mechanism which probably needs additional signaling, while the initial calcium responses are maximal at agonist concentrations of 2 μM.

## Correlation of platelet receptor stimulation with macroscopic phenomena

One of the most relevant questions regarding human platelet signaling is how the initiating signals, such as G-protein activation, increase of intracellular calcium concentration and adenylyl cyclase inhibition are linked to the macroscopic phenomena of platelet shape change and aggregation. We tried to elucidate these mechanisms by comparing platelet responses evoked at increasing ADP concentrations and by different ADP derivatives. While at 2 μM ADP the calcium mobilization signal and the activation of the ligand gated cation channel are maximal, platelet

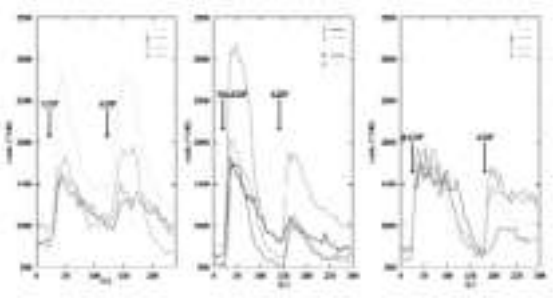

**Figure 3. Pharmacology of heterologously expressed native P2Y1 receptor in 1321N1 astrocytoma cells. The traces represent calcium responses of single 1321N1 cells transfected with native platelet P2Y1 receptor to 10 mM ADP, ATP, 1-methyl ADP and 2'-deoxy ADP respectively. The stimulants were added at the time points indicated by arrows.**

aggregation is only weak in platelet rich plasma (PRP). Apart from the 2-substituted derivatives which exhibit even stronger platelet responses than ADP there are several derivatives with stimulate only weak responses. Medium-strong agonists, e.g. ADP-βS, can stimulate all kinds of purinergic platelet responses, calcium influx, calcium mobilization, adenylyl cyclase inhibition, shape change and reversible aggregation in citrated platelet rich plasma. The weak agonist 2'-deoxy ADP only stimulates calcium mobilization and shape change but has no effect on platelet cAMP levels. Another weak agonist, 1-methyl ADP causes calcium mobilization to the same extent as ADP itself at the same concentration, but does not stimulate any other signaling pathway. This indicates that platelet shape change is not necessarily coupled to calcium mobilization, but probably needs additional activation signals.

## Pharmacology of the P2Y1 receptor

Although the P2Y1 receptor has been cloned, overexpressed in several cell types and extensively studied, the results from pharmacologic surveys are still contradictory. While in platelets ATP is unambigously a competitive antagonist of the P2Y1 receptor[11] it has been proven that in other cell types ATP may stimulate this receptor.[17] It has been claimed that ATP can act as a partial agonist of this receptor. This may explain the observation that ATP can cause calcium responses in cells in which the receptor is overexpressed. The different sensitivities of P2Y1 to ADP and ATP have also been explained by receptor heterogeneity. Small structural differences may account for different ligand binding profiles.[18] Almost all investigations on heterologously expressed P2Y1 receptors were carried out with tagged receptors. We transfected the native P2Y1 receptor without any tag in the 1321N1 astrocytoma cell line. The positive clones were selected by means of single cell calcium fluorometry. In contrast to transfection with the tagged receptor the expression level of native P2Y1 was remarkably lower. Every ADP derivative capable of stimulating P2Y1 in platelets also had a stimulating effect on the native P2Y1 receptor (Figure 3). The P2Y1 agonists 1-methyl ADP and 2'-deoxy ADP both stimulated the expressed P2Y1 receptor, whereas the calcium response was weaker than the ADP response in the astrocytoma cell line. This is in contradiction to the observation on human platelets in which these derivatives could induce calcium mobilization to almost the same extent as ADP at the same concentration. Even ATP could evoke a calcium response comparable to the calcium response induced by ADP in this cell line. Furthermore these cells lacked the fast desensitization of the P2Y1 receptor observed in platelets (Figure 3). Only for ADP itself and some ADP derivatives was the calcium response to ADP remarkably reduced in the prestimulated cells.

## Crosstalk of protein kinase G activation and purinergic receptor stimulation

The inhibition of platelet function by cGMP elevating substances is a well established fact in platelet biochemistry.[19] Initial experiments provided evidence for the inhibition of platelet aggregation by stimulation of cGMP-dependent protein kinase (protein kinase G, PKG). Yet the target of PKG activation could not be conclusively identified. In previous studies we pinpointed the effects of elevated cGMP levels in human platelets to calcium regulatory mechanisms. It could definitely be shown that only calcium release and secondary influx mechanisms were affected by PKG stimulation, but the underlying biochemical processes were still hidden from direct observation. With a new kinetic approach we tried to separate the contributing mechanisms. In the usual experiments regarding guanylyl cyclase (GC) and PKG activation effects, cells are stimulated either by nitric oxide donors or cell permeable cGMP-derivatives. In our new approach we used authentic nitric oxide dissolved in degassed water. Thus the rate limiting step of nitric oxide production in the case of nitric oxide donors or the slow diffusion of cGMP derivatives through the cell membrane, could be avoided. The effects of guanylyl cyclase activation could therefore be observed almost instantly. Addition of nitric oxide to a suspension of washed human platelets in physiologic buffer did not significantly alter intracellular calcium levels in resting platelets. However in ADP stimulated platelets intracellular calcium levels decreased almost immediately upon nitric oxide addition in the presence of calcium ions in the medium. The calcium concentration is reduced approximately to the amount obtained with ADP stimulation in the absence of extracellular calcium. When ADP and nitric oxide were added simultaneously to the platelet suspension in the presence of 1 mM $CaCl_2$ the calcium signal was nearly identical to the one caused by ADP in calcium-free medium. These results indicate that nitric oxide has a direct effect upon secondary platelet calcium influx, while P2Y1 mediated calcium mobilization remains initially unaffected. Only when the platelets are incubated with nitric oxide for a longer time does the calcium mobilization also reduce. Already after 15 seconds of preincubation with nitric oxide ADP-evoked calcium mobilization was significantly reduced. After 60 seconds incuba-

tion with nitric oxide calcium mobilization was completely diminished. These observations indicate that purinergic receptors are regulated by PKG stimulation. To obtain further proof, P2Y1 transfected 1321N1 astrocytoma cells were transfected with PKG Ib by adenoviral gene transfer. The control cells were transfected with a dysfunctional PKG Ib mutant to exclude any artifacts which might arise from the transfection procedure. After preincubation with a membrane permeable cGMP analogue the cells were stimulated with ADP and the calcium signal was monitored with fura-2 by single cell fluorometry. Despite the variation in the intensity of the calcium signals observed a clear cut difference in the calcium signal between the two groups was found. Another potential target for the regulation of platelet purinergic receptors by PKG is presumably the yet unidentified adenylyl cyclase coupled P2YAC or P2Ycyc receptor. To analyze effects of elevated cGMP levels in human platelets upon purinoceptor mediated adenylyl cyclase inhibition, washed platelets were pretreated with sodium nitroprusside (SNP), prostaglandin $E_1$ ($PG\text{-}E_1$), ADP or combinations of these substances in physiological buffers. The basal cAMP level in platelets remains essentially unchanged after SNP or ADP treatment, while $PG\text{-}E_1$ stimulated cAMP increase is remarkably reduced by SNP pretreatment, indicating an inhibition of cAMP production by GC stimulation. cAMP levels by Combinations of SNP and ADP were, therefore, be expected to cause a further reduction in cAMP levels. Surprisingly quite the reverse was observed. The cAMP level after treatment of platelets with $PG\text{-}E_1$, SNP and ADP together lies approximately between the cAMP concentration of platelets stimulated with $PG\text{-}E_1$ and SNP and the cAMP concentration from $PG\text{-}E_1$ and ADP stimulated platelets. These results indicate that there are two opposite mechanisms of cAMP regulation in platelets. The reduced cAMP levels after treatment with SNP can be explained by stimulation of cGMP-dependent phosphodiesterase PDE II leading to enhanced degradation of cAMP. In order to exclude any effects from phosphodiesterase activation or inhibition, the experiments were carried out with the selective PKG stimulator pCPT-cGMP. When platelets were treated with this substance $PG\text{-}E_1$ stimulated cAMP increase was virtually identical to that obtained in cells stimulated only with $PG\text{-}E_1$. This indicates that the lower cAMP concentration in $PG\text{-}E_1$ and SNP treated cells than in $PG\text{-}E_1$ treated cells results indeed results completely from PDE II stimulation. However, the inhibition of the ADP stimulated decrease of cAMP concentration in $PG\text{-}E_1$ treated cells by GC stimulation was also observed after PKG stimulation. These observations indicate a complex crosstalk of purinergic and cyclic nucleotide mediated signal transduction in human platelets. The inhibition of P2YAC mediated signal transduction can be clearly attributed to a PKG mediated protein phosphorylation. It has yet to be established whether the receptor itself or proteins participating in the signaling cascade are the targets of activated PKG. The coupling of cGMP and cAMP mediated pathways are supposedly the biochemical basis for the inhibition of platelets by endothelium-derived factors.[20] The synergistic effect of both prostaglandins and nitric oxide may contribute to efficient inhibition of platelets in the circulation. If both factors are absent, platelets may become more susceptible to spontaneous activation and aggregation.

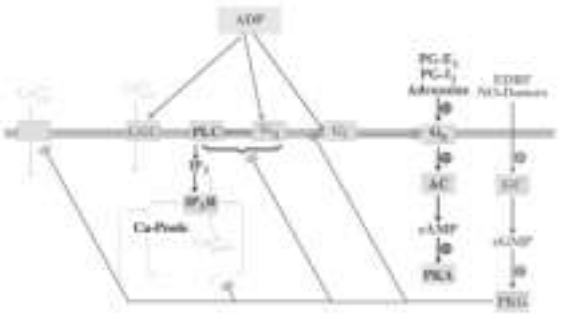

**Figure 4. Crosstalk of purinergic signaling and cGMP-dependent protein kinase. Activation of cGMP-dependent protein kinase leads to inhibition of ADP stimulated and $G_i$ mediated inhibition of adenylyl cyclase, $G_q$ mediated activation of phospholipase C, intracellular calcium stores and secondary calcium influx. ADP stimulated calcium influx through the ligand gated cation channel remains unaffected by PKG activation.**

## Summary

Investigation of the pharmacology and biochemistry of the two known platelet purinoceptors cloned from human platelet RNA and stably expressed in the 1321N1 astrocytoma cell line revealed some remarkable differences from their properties observed in platelets. Though experiments with ADP derivatives known to be selective activators of platelet purinergic receptors were in accordance with the results obtained from platelets, the inhibitory effect of ATP and the fast desensitization were not observed with the heterologously expressed receptors. The crosstalk of cyclic nucleotide signaling pathways and purinergic receptor activation were investigated both in human platelets and in the cell line transfected with the cloned receptors. Inhibition of ADP stimulated calcium increase by PKG activation results from the inhibition of two calcium elevating pathways: the store related calcium influx and P2Y1 mediated calcium mobilization (Figure 4). Besides inhibition of platelet calcium responses, cGMP also inhibits P2YAC and $G_i$-protein mediated cAMP reduction in human platelets, thus leading to reduced decrease of cAMP levels in platelets upon ADP stimulation. These mechanisms probably contribute to the efficient inhibition of platelet aggregation by cGMP elevating agents. *In vivo* these effects may be responsible for the synergism of the endothelium-derived factors, nitric oxide and prostacyclin.

## Perspective

The experiments conducted on platelets and P2Y1 transfected astrocytoma cells show that our current knowledge on the P2Y1 receptor is still unsatisfactory. Particularly the structure-activity relationship for

ADP and its derivatives and the pharmacology of the P2Y1 receptor need further investigation. Interaction of this receptor with other purinoceptors of the same or different type or G-proteins may modulate the properties of the receptor. The linkage of receptor activation to the final platelet responses is also still unresolved. The role of protein kinases, G-protein βγ subunits and small GTP binding proteins has yet to be studied in detail. How far secondary effects of platelet activation, e.g. secretion or outside-in signaling, are involved or are responsible for some of the cellular responses observed has yet to be established. Finally, the most important task remains the identification of the still elusive adenylyl cyclase coupled platelet purinoceptor.

## References


1. Fredholm BB, Abbracchio MP, Burnstock G, et al. Towards a revised nomenclature for P1 and P2 receptors. Trends Pharmacol Sci 1997; 18:79-82.
2. Mills DCB. ADP receptors on platelets. Thromb Hemost 1996; 76:835-56.
3. Born GVR. Adenosine diphosphate as a mediator of platelet aggregation in vivo: an editorial point of view. Circulation 1985; 72:741-2.
4. Geiger J, Hönig-Liedl P, Schanzenbächer P, Walter U. Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. Eur J Pharmacol 1998; 351:235-46.
5. Gachet C, Hechler B, Léon C, et al. Activation of ADP receptors and platelet function. Thromb Haemost 1997; 78:271-5.
6. Kunapuli SP. Multiple P2 receptor subtypes on platelets: a new interpretation of their function. Trends Pharmacol Sci 1998; 19:391-4.
7. MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of receptor-operated cation channels via P2X1 not P2T purinoceptors in human platelets. J Biol Chem 1996; 271:2879-81.
8. Savi P, Beauverger P, Labouret C, et al. Role of P2Y1 purinoceptor in ADP-induced platelet activation. FEBS Lett. 1998; 422:291-5.
9. Léon C, Vial C, Cazenave JP, Gachet C. Cloning and sequencing of a human cDNA encoding endothelial P2Y1 purinoceptor. Gene 1995; 171:295-7.
10. Valera S, Hussy N, Evans RJ, et al. A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. Nature 1994; 371:516-9.
11. Léon C, Hechler B, Vial C, Leray C, Cazenave JP, Gachet C. The P2Y1 receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. FEBS Lett. 1997; 403:26-30.
12. Ohlmann P, Laugwitz KL, Nürnberg B, et al. The human platelet ADP receptor activates Gi2 proteins. Biochem J 1995; 312:775-9.
13. Léon C, Hechler B, Freund M, et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y1 receptor-null mice. Clin Invest 1999; 104:1731-7.
14. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci USA 1998; 95:8070-4.
15. Mahaut-Smith MP, Sage SO, Rink TJ. Rapid ADP-evoked currents in human platelets recorded with the nystatin permeabilized patch technique. J Biol Chem 1992; 267:3060-5.
16. Rink TJ, Sage SO. Calcium signaling in human platelets. Annu Rev Physiol 1990; 52:431-49.
17. Webb TE, Simon J, Krishek BJ, et al. Cloning and functional expression of a brain G-protein-coupled ATP receptor. FEBS Lett 1993; 324:219-25.
18. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharamcol Rev 1998; 50: 413-92.
19. Geiger J, Nolte C, Sage SO, Walter U. Role of cGMP and cGMP-dependent protein kinase in nitrovasodilator inhibition of agonist-evoked calcium elevation in human platelets. Proc Natl Acad Sci USA 1992; 89:1031-5.
20. Geiger J, Nolte C, Walter U. Regulation of calcium mobilization and influx in human platelets by endothelium derived factors. Am J Physiol 1994; 267: C236-44.

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# INTERPLAY OF P2 RECEPTOR SUBTYPES IN PLATELET FUNCTION

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ABSTRACT

**During vascular injury ADP plays an important role in hemostasis by activating platelets. In platelets, the classical P2T receptor is now resolved into three P2 receptor subtypes, the P2Y1, the P2X1, and the $P2T_{AC}$ receptor, which remains to be cloned. Both pharmacologic and molecular biological approaches have confirmed the role of the P2Y1 receptor in ADP-induced platelet shape change and fibrinogen receptor activation. Sufficient pharmacologic data exist to support the notion that both P2Y1 and $P2T_{AC}$ receptors are required for complete platelet aggregation. The function of the P2X1 receptor on platelets remains elusive and yet to be determined.**



## Introduction

Platelets aggregate at the site of vascular damage to prevent bleeding but abnormal activation in the blood vessels leads to thrombosis, and thus to stroke and myocardial infarction.[1] Platelets are activated by a number of agonists, including thrombin, thromboxane, ADP, and collagen.[2] Nearly four decades ago, ADP was identified as a platelet-activating agent from erythrocytes.[3] ADP, along with ATP and serotonin, is a major constituent of the platelet dense granules and is released upon activation of platelets.[4] The importance of ADP as a platelet aggregating agent is substantiated by the observation that patients with deficiencies in storage of ADP or in ADP receptors have bleeding diatheses.[5-8]

## Nomenclature of P2 receptors

Following the introduction of the concept of receptors for extracellular nucleotides by Burnstock,[9] several physiologic effects of adenine nucleotides have been identified.[10] Receptors for nucleotides, designated P2 receptors, are divided into two classes: ligand gated ion channels (P2X) and G protein-coupled receptors (P2Y).[11] All the physiologic and intracellular signaling events triggered by ADP in platelets were attributed initially to a single cell surface receptor. Since the molecular nature of this receptor was unknown it was designated P2T (thrombocyte P2 receptor).[12] The IUPHAR has recommended that the P2T receptor be designated $P2Y_{ADP}$, indicating a G protein-coupled P2 receptor at which ADP is the most important agonist.[11] The historic studies and theories on the nature of the P2T receptor have been dealt in recent review articles including one in this issue.[13-15]

## ADP-induced intracellular signaling events in platelets

ADP, acting on cell surface P2 receptors, regulates several second messenger systems in platelets.[13-16] ADP inhibits stimulated platelet adenylyl cyclase through coupling to Gi protein, possibly $G\alpha_{i2}$,[17] and thereby decreases intracellular cAMP levels.[18] ADP also causes rapid calcium influx into platelets in the presence of physiologic calcium ion concentrations.[19,20] Even in the absence of extracellular calcium, ADP causes mobilization of intracellular calcium stores.[21] ADP activates platelet phospholipase C (PLC), resulting in inositol 1,4,5-trisphosphate formation.[22] Several investigators disputed this finding[23,27] but, recently, we have confirmed that ADP induces inositol trisphosphate formation, well correlated with mobilization of intracellular calcium stores, in human platelets.[28] In addition, ADP also causes release of arachidonic acid from membrane phospholipids through activation of phospholipase $A_2$ ($PLA_2$).[29]

## Physiologic effects of ADP on platelets

Activation of platelets by a low concentration of ADP results in shape change, in that discoid-shaped resting cells are rapidly converted to spiculated spheres.[30] Platelet shape change involves phosphorylation of myosin light chains by a calcium calmodulin kinase and rearrangement of actin-myosin filaments.[31] Higher concentrations (2-5 μM) of ADP causes platelet aggregation and granule secretion.[4,30] Platelet aggregation is due to the exposure of fibrinogen binding site on the $\alpha_{IIb}\beta_3$ integrin (fibrinogen receptor; glycoprotein IIb/IIIa).[32] ADP causes prima-

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ry aggregation which is reversible under physiological concentrations of extracellular calciu.[33] ADP-induced release of arachidonic acid and subsequently produced thromboxane $A_2$ along with granule secretion contribute to the irreversible secondary aggregation.[29] However, platelets resuspended in physiological concentrations of calcium (1-2 mM) fail to generate thromboxane $A_2$,[34] indicating a regulatory role for extracellular calcium in ADP-induced thromboxane $A_2$ generation. ADP causes release of the contents of both alpha granules and dense granules[4,30] when the extracellular calcium concentration is low.[35] ADP-induced dense granule release depends on thromboxane $A_2$, since ADP fails to release dense granule contents in aspirin-treated and washed platelets.[4] Whether ADP can cause the release of alpha granule contents directly remains controversial. Several investigators reported the direct role of ADP in the alpha granule release reactio,[13,36,37] but unpublished results from our laboratory and reports from other investigators have shown that ADP fails to expose P-selectin[38] or β-thromboglobulin[39] in aspirin-treated and washed platelets, suggesting that this event may also require thromboxane $A_2$ generation.

## A model for ADP-induced platelet activation

All the physiologic and intracellular signaling events triggered by ADP in platelets were attributed initially to a single cell surface receptor, P2T. We have resolved the P2T receptor into three components based on the effects of AR-C 66096, a potent antagonist of ADP-induced platelet aggregation, and α,β-meATP, a P2X1 receptor agonist, to distinguish the ADP-induced intracellular events.[28] AR-C 66096 blocked ADP-induced inhibition of adenylyl cyclase, but failed to inhibit ADP-mediated intracellular calcium increases, inositol trisphosphate formation, or shape change. α,β-MeATP neither affected the inositol trisphosphate formation nor stimulated adenylyl cyclase. Based on these observations we proposed the presence of three distinct P2 receptor subtypes on platelets:[28] one coupled to inhibition of adenylyl cyclase, designated $P2T_{AC}$ receptor, the second coupled to mobilization of calcium from intracellular stores through activation of phospholipase C and inositol trisphosphate formation, designated $P2T_{PLC}$, and the third an ionotropic P2X1 receptor coupled to rapid calcium influx. We have isolated a cDNA clone encoding the P2Y1 receptor from a human platelet cDNA library and demonstrated that the P2Y1 receptor is the $P2T_{PLC}$, using the P2Y1 receptor selective antagonists,[40] adenosine-3'-phosphate-5'-phosphosulfate (A3P5PS), adenosine-3'-phosphate-5'-phosphate (A3P5P), and adenosine-2'-phosphate-5'-phosphate (A2P5P).[41] Thus the concept of 'P2T' receptor[12] is resolved into three P2 receptor subtypes with distinct functions. Four other studies[42-45] independently confirmed the three-receptor model by pharmacologic approaches. Furthermore, two recent independent studies also provided support for the three-receptor model by gene disruption approaches.[46,47]

## A note on nomenclature

We have resolved the platelet ADP receptor, originally designated the P2T receptor,[12] into three subtypes with different signal transduction properties and designated the receptor on platelets mediating ADP-induced inhibition of adenylyl cyclase as $P2T_{AC}$ (a component of the P2T receptor coupled to inhibition of adenylyl cyclase and antagonized by Astra Compounds).[28,41] The rationale for this designation and deviation from the standard IUPAC nomenclature of $P2Y_{ADP}$ were discussed in our original paper.[28] Subsequently, several laboratories designated the adenylyl cyclase coupled ADP receptor on platelets differently. Hence the $P2T_{AC}$ receptor is also called P2cyc,[44] $P2Y_{ADP}$,[45] $P2Y_{AC}$,[43] 46, P2Y,[48] and P2T.[42]

## Modulators of platelet P2 receptor subtypes

Many agents have been identified as selective agonists and antagonists at the P2 receptor subtypes in platelets and can be used to delineate the function of these subtypes. Hydrolysis-resistant derivatives of ATP, e.g. AR-C 66096, have been developed as potent inhibitors of ADP-induced platelet aggregation[49] and have been shown to be selective antagonists of the $P2T_{AC}$ receptor subtype.[28] These compounds have been shown to have no effect on other subtypes when used at limited concentrations.[41] The thienopyridine derivatives, ticlopidine and clopidogrel, when administered *in vivo*, selectively abrogate ADP-induced inhibition of adenylyl cyclase and platelet aggregation[50-52] indicating that these compounds, or an active metabolite, act at the $P2T_{AC}$ receptor, but not at the P2Y1 receptor.[53] Finally, 2-methylthio-AMP (2MeSAMP) is identified as a selective antagonist of the $P2T_{AC}$ receptor.[45] Adenosine bis phosphates have been developed as selective competitive antagonists of the P2Y1 receptor[40] and these compounds have been shown to act selectively at the platelet P2Y1 receptor without any effect on $P2T_{AC}$ receptors.[41] α,β-methylene ATP (α,β-MeATP) was identified as a selective agonist on ligand gated P2X1 channels on platelets, leading to rapid influx of calcium.[20,28,41]

## Mechanism of ADP-induced platelet shape change

In the presence of extracellular calcium, α,β-MeATP, a P2X1 selective agonist, causes rapid calcium influx, but fails to elicit platelet shape change.[41] α,β-MeATP neither causes nor inhibits shape change induced by ADP,[41] suggesting that the signaling through the P2X1 receptor does not contribute to ADP-induced platelet shape change. AR-C 66096, a selective antagonist of the $P2T_{AC}$ receptor, did not inhibit ADP-induced shape change,[28] indicating that neither does the $P2T_{AC}$ receptor play any significant role in shape change induced by ADP. P2Y1 receptor selective antagonists,[40] A3P5PS, A3P5P, and A2P5P, inhibit ADP- or 2MeSADP-induced intracellular calcium mobilization and shape change in platelets.[41] The $EC_{50}$ for ADP at the cloned P2Y1 receptor is ~0.3 μM[54] which is also the dose sufficient for platelet shape change.[30] Studies with mice lacking $G_q$ revealed

that signaling through $G_q$ is essential for ADP-induced shape change.[55] All the agents that cause platelet shape change, such as thrombin, thromboxane, and serotonin, also activate PLC.[2] Hence, PLC activation is the essential step in platelet shape change. Thus the P2Y1 receptor solely mediates ADP-induced platelet shape change. The signal transduction events downstream of the P2Y1 receptor contributing to ADP-induced platelet shape change have been recently discussed.[56-58]

## Mechanism of ADP-induced platelet aggregation

What is the contribution of these three P2 receptor subtypes to ADP-induced fibrinogen receptor activation? The $P2T_{AC}$ receptor is essential for ADP-induced platelet aggregation. Selective antagonists of the $P2T_{AC}$ receptor, ATP, AR-C 66096, and 2MeSAMP have been shown to block both ADP-induced adenylyl cyclase inhibition[28,45,59] and platelet fibrinogen receptor activation.[49,59] In addition, a significant correlation was found between antagonist affinity constant values for eight nucleotide analogs, as blockers of ADP-induced aggregation and adenylyl cyclase inhibition.[60] *In vivo* administration of ticlopidine and clopidogrel results in abolishment of both ADP-induced inhibition of adenylyl cyclase and aggregation.[52] Two patients with defective ADP-induced platelet adenylyl cyclase inhibition also had abnormal aggregation suggesting that the receptor coupled to inhibition of adenylyl cyclase is essential for platelet aggregation.[7,8] Hence $P2T_{AC}$ receptor activation is required for ADP-induced platelet aggregation. The P2Y1 receptor selective antagonists, A3P5PS, A3P5P, and A2P5P, also inhibit ADP-induced human[61] and mouse[62] platelet aggregation, without affecting ADP-induced inhibition of adenylyl cyclase. Platelets from mice lacking the P2Y1 receptor failed to mobilize calcium from intracellular stores, change shape, or aggregate in response to ADP.[46, 47] Hence, intracellular signaling events from both the $P2T_{AC}$ and P2Y1 receptors are essential for ADP-induced platelet aggregation. Inhibition of signaling through either receptor, by specific antagonists, is sufficient to block ADP-induced platelet fibrinogen receptor activation. The P2Y1 receptor presumably couples to $G_q$ and causes intracellular calcium mobilization through the inositol trisphosphate pathway, and platelets from mice lacking $G_q$ fail to aggregate in response to ADP.[55] In the presence of AR-C 66096, signaling through the $P2T_{AC}$ receptor can be substituted by epinephrine acting on a2A adrenergic receptors, also coupled to $G_i$.[61] On the other hand, activation of serotonin receptors can replace signaling through the P2Y1 receptor in human,[61] rabbit,[63] or mouse[47] platelets. Moreover, this novel mechanism of ADP-induced platelet aggregation can be mimicked by co-activation of two non-ADP receptors coupled to $G_i$ and $G_q$, $\alpha_{2A}$ adrenergic receptors and serotonin receptors, respectively.[61] Thus, ADP-induced platelet aggregation results from concomitant signaling from both the $P2T_{AC}$ and P2Y1 receptors, a novel mechanism by which G protein-coupled receptors elicit a physiologic response.[61] $\alpha,\beta$-MeATP, a P2X1 selective agonist, causes rapid calcium influx in the presence of extracellular calcium, but neither causes platelet aggregation nor modulates ADP-induced platelet aggregation.[61, 64] Furthermore, selective co-activation of the P2X1 receptor and either the $P2T_{AC}$ or P2Y1 receptors also does not cause platelet aggregation.[61] Thus the P2X1 receptor mediated rapid calcium influx does not play any significant role in ADP-induced platelet aggregation. Activation of a single receptor by its agonist is believed to trigger a physiologic event, and hence, receptor subtype specific antagonists have been used to delineate the physiologic function of various receptors. The mechanism of ADP-induced platelet aggregation now suggests that some agonist-induced physiologic responses may require simultaneous activation of multiple receptor subtypes by the same agonist, resulting in converging signal transduction pathways leading to a physiologic response. Thus, conclusions derived from receptor specific antagonists may not exclude the role of another receptor subtype in an agonist-induced physiological event.

## Conclusions and future directions

Molecular mechanisms of ADP-induced platelet activation are becoming clear only now. First the resolution of the concept of a P2T receptor into three components, $P2T_{AC}$, P2Y1 and P2X1 receptors, helped to explain the intracellular and physiologic effects of ADP on platelets. The interaction of signaling events downstream of the P2Y1 and $P2T_{AC}$ receptors is a novel mechanism of physiologic response and may indeed be a general mechanism of $\alpha$IIb$\beta$3 integrin activation by all physiologic agonists. We speculate that the integrin activation on other cells also requires similar signaling mechanisms but this remains to be established. Interestingly, mouse platelets deficient in P2Y1 receptor can undergo partial aggregation in the presence of high concentrations of ADP.[47] The implications of this observation range from a fourth P2 receptor subtype on platelets to $P2T_{AC}$ receptor coupling to other G proteins. The molecular structure of the $P2T_{AC}$ receptor is not known and future investigations will depend on molecular cloning of this receptor. Selective antagonists for the P2X1 receptor need be developed to delineate the functional role, if any, of this receptor subtype in ADP-induced platelet activation. The signaling mechanisms and cascades mediated by these three receptors will provide a better understanding of ADP–mediated physiologic responses in platelets and, generally, the molecular mechanisms of agonist-induced platelet activation.

## References


1. Packham MA. Role of platelets in thrombosis and hemostasis. Can J Physiol Pharmacol 1994; 72:278-84.
2. Hourani SMO, Cusack NJ. Pharmacological receptors on blood platelets. Pharmacol Rev 1991; 43:243-98.
3. Gaarder A, Jonsen A, Laland S, Hellem AJ, Owren P. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. Nature 1961;

192:531-2.
4. Mills DCB, Rob IA, Roberts GCK. The release of nucleotides, 5-hydroxytryptamine and enzymes from human blood platelets during aggregation. J Physiol 1968; 195:715-29.
5. Holmsen H, Weiss HJ. Hereditary defect in the platelet release reaction caused by a deficiency in the storage pool of platelet adenine nucleotides. Br J Haematol 1970; 19:643-9.
6. Holmsen H, Weiss HJ. Secretable storage pools in platelets. Ann Rev Med 1979; 30:119-34.
7. Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet aggregation characterized by severe impairment of platelet responses to adenosine 5'-diphosphate. Blood 1992; 80:2787-96.
8. Nurden P, Savi P, Heilmann E, et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. J Clin Invest 1995; 95:1612-22.
9. Burnstock G. A basis for distinguishing two types of purinergic receptors. In: Straub RW, Bolis L, eds. Cell Membrane Receptors for drugs and hormones A multidisciplinary approach. Raven Press: New York, 1978. pp. 107-18.
10. Dubyak, GR, el-Moatassim C. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. Am J Physiol 1993; 265: C577-606.
11. Fredholm BB, Abbracchio MP, Burnstock G, et al. Towards a revised nomenclature for P1 and P2 receptors. Trends Pharmacol Sci 1997; 18:79-82.
12. Gordon J. Extracellular ATP: effects, sources and fates. Biochem J 1986; 233:309-19.
13. Mills DCB. ADP receptor in platelets. Thromb Haemost 1996; 76:835-56.
14. Hourani SMO, Hall DA. Receptors for ADP on human platelets. Trends Pharmacol Sci 1994; 15:103-8.
15. Gachet C, Hechler B, Leon C, et al. Activation of ADP receptors and platelet function. Thromb Haemost 1997; 77:271-5.
16. Kunapuli SP. Functional characterization of platelet ADP receptors. Platelets 1998; 9:343-51.
17. Ohlmann P, Laugwitz KL, Nuernberg B, et al. The human platelet ADP receptor activates $G_{i2}$ proteins. Biochem J 1995; 312:775-9.
18. Cooper DMF, Rodbell M. ADP is a potent inhibitor of human platelet plasma membrane adenylate cyclase. Nature 1979; 282:517-8.
19. Sage SO, Rink T. Kinetic differences between thrombin-induced and ADP-induced calcium influx and release from internal stores in fura-2-loaded human platelets. Biochem Bioph Res Co 1986; 136:1124-9.
20. MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of receptor-operated cation channels via P2X1 not P2T purinoceptors in human platelets. J Biol Chem 1996; 271:2879-81.
21. Hallam TJ, Rink TJ. Response to adenosine diphosphate in human platelets loaded with the fluorescent calcium indicator Quin 2. J Physiol 1985; 368:131-46.
22. Daniel JL, Dangelmaier CA, Selak M, Smith JB. ADP stimulates IP3 formation in human platelets. FEBS Lett 1986; 206:299-303.
23. Olbrich C, Aepfelbacher M, Siess W. Epinephrine potentiates calcium mobilization and activation of protein kinases in platelets stimulated by ADP through a mechanism unrelated to phospholipase C. Cell Signalling 1989; 1:483-92.
24. Heemskerk JWM, Vis P, Feijge MAH, Hoyland J, Mason WT, Sage SO. Roles of phospholipase C and $Ca^{2+}$ ATPase in calcium responses of single, fibrinogen-bound platelets. J Biol Chem 1993; 268:356-63.
25. Raha S, Jones GD, Gear ARL. Sub-second oscillations of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrabisphosphate during platelet activation by ADP and thrombin: lack of correlation with calcium kinetics. Biochem J 1993; 292:643-6.
26. Vickers JD, Kinlough-Rathbone RL, Packham MA, Mustard JF. Inositol phospholipid metabolism in human platelets stimulated by ADP. Eur J Biochem 1990; 193:521-8.
27. Vickers JD. ADP-stimulated fibrinogen binding is necessary for some of the inositol phospholipid changes found in ADP-stimulated platelets. Eur J Biochem 1993; 216:231-7.
28. Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli, SP. Molecular basis for ADP-induced platelet activation I: Evidence for three distinct ADP receptors on platelets. J Biol Chem 1998; 273:2024-9.
29. Macfarlane DE, Gardner S, Lipson C, Mills DCB. Malondialdehyde production by platelets during secondary aggregation. Thromb Haemost 1977; 38: 1002-9.
30. Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962; 194: 927-9.
31. Daniel JL. Myosin phosphorylation in intact platelets. J Biol Chem 1981; 256:7510-4.
32. Macfarlane DE. Agonists and receptors: adenosine diphosphate. In: Holmsen H, ed. Platelet Responses and Metabolism. Vol II: Receptors and Metabolism. CRC Press, Boca Raton, 1987. pp. 19-36.
33. Mustard JF, Perr DW, Kinlough-Rathbone RL, Packham MA. Factors responsible for ADP induced release reaction of human platelets. Am J Physiol 1975; 228: 1857-65.
34. Packham MA, Bryant NL, Guccione MA, Kinlough-Rathbone RL, Mustard JF. Effect of the concentration of $Ca^{2+}$ in the suspending medium on the responses of human and rabbit platelets to aggregating agents. Thromb Haemost 1989; 62:968-76.
35. Packham MA, Bryant NL, Guccione MA, Kinlough-Rathbone RL, Mustard JF. Effect of concentration of $Ca^{2+}$ in the suspending medium on the responses of human and rabbit platelets to aggregating agents. Thromb Haemost 1989; 62:968-76.
36. Pengo V, Boschello A, Marzari A, Baca M, Schivazappa L, Dalla Volta S. Adenosine diphosphate (ADP)-induced a-granules release from platelets of native whole blood is reduced by ticlopidine but not by aspirin or dipyridamole. Thromb Haemost 1986; 56: 147-50.
37. Rinder CS, Student LA, Bonan JL, Rinder HM, Smith BR. Aspirin does not inhibit adenosine diphosphate-induced platelet α-granule release. Blood 1993; 82: 505-12.
38. Rand ML, Perry DW, Packham MA, Gemmell CH, Yeo EL, Kinlough-Rathbone RL. Conditions influencing release of granule contents from human platelets in citrated plasma induced by ADP or the thrombin receptor activating peptide SFLLRN: direct measurement of percent release of β-thromboglobulin and assessment by flow cytometry of P-selectin expression. Am J Hematol. 1996; 52:288-94.
39. Kaplan KL, Broekman MJ, Chernoff A, Lesznik GR, Drillings M. Platelet α-granule proteins: studies on release and subcellular localization. Blood 1979; 53:604-18.
40. Boyer JL, Romeroavila T, Schachter JB, Harden TK. Identification of competitive antagonists of the P2y(1) receptor. Mol Pharmacol 1996; 50:1323-9.
41. Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation II: the P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. J Biol Chem 1998; 273:

2030-4.
42. Fagura MS, Dainty IA, McKay GD, et al. P2y(1)-receptors in human platelets which are pharmacologically distinct from P2y(ADP)-receptors. Br J Pharmacol 1998; 124:157-64.
43. Geiger J, Honigliedl P, Schanzenbacher P, Walter U. Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. Eur J Pharmacol 1998; 351:235-46.
44. Hechler B, Leon C, Vial C, et al. The P2y(1) receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. Blood 1998; 92:152-9.
45. Jantzen HM, Gousset L, Bhaskar V, et al. Evidence for two distinct G protein-coupled ADP receptors mediating platelet activation. Thromb Haemost 1999; 81:111-7.
46. Fabre JE, Nguyen M, Latour A, et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. Nat Med 1999; 5:1199-202.
47. Leon C, Hechler B, Freund M, et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. J Clin Invest 1999; 104:1731-7.
48. Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. Arterioscler Thromb Vasc Biol 1999; 19:2281-5.
49. Humphries RG, Robertson MJ, Leff P. A novel series of P2T purinoceptor antagonists: definition of the role of ADP in arterial thrombosis. Trends Phamacol Sci 1995; 16179-81.
50. Mills DCB, Puri RN, Hu CJ, et al. Clopidogrel inhibits the binding of ADP analogues to the receptor mediating inhibition of platelet adenylate cyclase. Atheroscler Thromb 1992; 12:430-6.
51. Gachet C, Cazenave JP, Ohlmann P, et al. The thienopyridine ticlopidine selectively prevents the inhibitory effects of ADP but not of adrenaline on cAMP levels raised by stimulation of the adenylate cyclase of human platelets by PGE1. Biochem Pharmacol 1990; 40:2683-7.
52. Defreyn G, Gachet G, Savi P, Driot F, Cazenave JP, Maffrand JP. Ticlopidine and clopidogrel (SR25990C) selectively neutralize ADP inhibition of $PGE_1$-activated platelet adenylate cyclase in rats and rabbits. Thromb Haemost 1991; 65:186-90.
53. Hechler B, Eckly A, Ohlmann P, Cazenave JP, Gachet C. The P2Y1 receptor, necessary but not sufficient to support full ADP- induced platelet aggregation, is not the target of the drug clopidogrel. Br J Haematol 1998; 103:858-66.
54. Schachter JB, Li Q, Boyer JL, Nicholas RA, Harden TK. Second messenger cascade specificity and pharmacological selectivity of the human P2y1-purinoceptor. Br J Pharmacol 1996; 118:167-73.
55. Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in Gaq-deficient mice. Nature 1997; 389:183-6.
56. Kunapuli SP. Molecular events in ADP-induced platelet shape change. Drug News Perspect 1999; 12: 524-8.
57. Paul BZS, Daniel JL, Kunapuli SP. Platelet shape change is mediated by both calcium-dependent and -independent signaling pathways: role of p160 ROCK in platelet shape change. J Biol Chem 1999; 274:28293-300.
58. Bauer M, Retzer M, Wilde JI, et al. Dichotomous regulation of myosin phosphorylation and shape change by Rho-kinase and calcium in intact human platelets. Blood 1999; 94:1665-72.
59. Macfarlane DE, Mills DCB. The effects of ATP on platelets: evidence against the central role of ADP in primary aggregation. Blood 1975; 46:309-20.
60. Cusack NJ, Hourani SMO. Adenosine diphosphate antagonists and human platelets: no evidence that aggregation and inhibition of adenylate cyclase are mediated by different receptors. Br J Pharmacol 1982; 76:221-7.
61. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci USA 1998; 95:8070-4.
62. Kim YB, Jin J, Dangelmaier C, Daniel JL, Kunapuli SP. The P2Y1 receptor is essential for ADP-induced platelet shape change and aggregation in mouse platelets. Platelets 1999; 10:399-406.
63. Savi P, Beauverger P, Labouret C, et al. Role of P2Y1 purinoceptor in ADP-induced platelet activation. FEBS Lett 1998; 422:291-5.
64. Savi P, Bornia J, Salel V, Delfaud M, Herbert JM. Characterization of P2x1 purinoreceptors on rat platelets-effect of clopidogrel. Br J Haematol 1997; 98:880-6.

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# ADP: AN IMPORTANT COFACTOR OF PI 3-KINASE ACTIVATION IN HUMAN BLOOD PLATELETS

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ABSTRACT

**Adenosine diphosphate (ADP), a weak platelet agonist *per se*, plays a key role as a cofactor in human blood platelet activation *in vitro* and *in vivo*. However, little is known about how a signaling pathway initiated by a specific primary agonist can be modulated by secreted ADP. Recently, we observed that although ADP by itself is a very poor activator of phosphoinositide 3-kinase (PI 3-kinase) it can play an important role as a cofactor of some platelet agonists to get an efficient synthesis of PI 3-kinase products (D3-phosphoinositides). The D3-phosphoinositides are important intracellular second messengers involved in the initiation and the temporaspatial organization of several key signaling pathways. Different PI 3-kinases have been shown to be activated in platelets and some of them are thought to play an essential role in key platelet functions. For instance, the late, integrin-dependent accumulation of phosphatidylinositol 3,4-*bis*phosphate (PtdIns(3,4)$P_2$) observed upon stimulation through the thrombin receptor PAR1, seems to be necessary for the irreversible phase of aggregation. Interestingly, secreted ADP appears to be specifically required for PAR1-induced accumulation of this phosphoinositide and irreversible platelet aggregation. From a molecular point of view, a signaling pathway initiated by the ADP receptor coupled to Gi synergizes with PAR1-dependent signaling for a significant accumulation of PtdIns(3,4)$P_2$ and the irreversible platelet aggregation. In this review, we also discuss the critical role of ADP in FcγRIIA–dependent platelet activation, possibly through modulation of the early activation of a PI 3-kinase.**



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ADP plays a key role in hemostasis and thrombosis.[1] Despite its early identification in 1961 as the first known aggregating agent, the molecular basis of ADP-induced platelet activation is only beginning to be understood. Three purinergic receptors contribute separately to the complex process of ADP-induced platelet aggregation: the P2X1 ionotropic receptor responsible for rapid influx of ionized calcium into the cytosol, the P2Y1 metabotropic receptor responsible for mobilization of ionized calcium from internal stores and an as yet unidentified P2 receptor coupled to adenylyl cyclase inhibition which is essential for the full aggregation response to ADP[2] and likely for the important cofactor effect of ADP.

## A role for PI 3-kinase in the irreversible phase of platelet aggregation induced by TRAP

The PI 3-kinases are a family of enzymes that phosphorylate the D3 hydroxyl group of phosphoinositides. These lipid kinases have been implicated in multiple biological responses such as cytoskeletal rearrangements, cellular migration, cell proliferation, protection against apoptosis or insulin-dependent metabolic processes.[3] On the basis of structural characteristics, substrate specificity and mechanism of regulation, PI 3-kinases have been divided into three main classes.[4] However, the biological functions of each PI 3-kinase are just starting to be investigated in detail. The D3-phosphoinositides generated by the various PI 3-kinases are considered as second messengers capable of binding functional protein modules such as pleckstrin homology (PH) domains[3] and by this function are able to regulate spatially and temporally specific membrane targeting of signaling proteins.[3,4] Several targets of D3-phosphoinositides have been recently identified including the serine/threonine kinases Akt, PDK or PKCξ, the tyrosine kinases of the Tec kinase family as well as exchange factors for small GTPases such as Vav or GRP1.[3] Several PI 3-kinases have been described in human blood platelets and they may be sequentially activated during platelet stimulation.[5] Although it is still difficult to assign a precise function of each platelet PI 3-kinase, it is thought that at least some of them play an important role in the platelet activation process. For instance,

an important physiologic platelet response controlled by PI 3-kinase is the irreversible phase of aggregation. In platelets stimulated by thrombin or by the thrombin receptor (PAR1) activating peptide (TRAP), the synthesis of phosphatidylinositol[3,4,5] *tris*phosphate (PtdIns(3,4,5)$P_3$) is rapid and transient, whereas PtdIns(3,4)$P_2$ accumulates upon increasing stimulation times.[6-8] Using platelets from thrombasthenic patients or RGDS-treated platelets, we have demonstrated that the synthesis of a major part of PtdIns(3,4)$P_2$ is dependent upon the engagement of $\alpha_{IIb}\beta_3$ integrin.[5,7,9] Fibrinogen binding to its receptor $\alpha_{IIb}\beta_3$ is however not sufficient *per se* to induce full activation of this pathway since aggregation is also required, as demonstrated by using thrombin-treated platelets in the absence of stirring.[7,9] The mechanisms involved in the regulation of the level of PtdIns(3,4)$P_2$ are still unclear. Several possibilities have been suggested including the hydrolysis of PtdIns(3,4,5)$P_3$ by a 5-phosphatase possibly regulated through integrin engagement,[5,10] inhibition of a PtdIns(3,4)$P_2$ 4-phosphatase[11] or activation of a C2-domain containing PI 3-kinase producing PtdIns(3)P then phosphorylated by a PtdIns(3)P4-kinase.[12] Using two structurally distinct inhibitors of PI 3-kinase (wortmannin or LY294002) Kovacsovics *et al.*[13] suggested that PI3-kinases may be involved in the irreversible phase of aggregation induced by TRAP. Moreover, a parallelism between aggregation extent and PtdIns(3,4)$P_2$ labeling was also observed in thrombin-stimulated platelets.[7] Finally, using washed platelets stimulated with ADP alone, in the presence of exogenous fibrinogen to allow $\alpha_{IIb}\beta_3$ engagement, we observed a relationship between reversible aggregation, absence of PtdIns(3,4)$P_2$ accumulation and a large reduction of the amount of myosin heavy chain and RhoA in the cytoskeleton.[14] Altogether these data suggested that irreversible aggregation may be linked to the late accumulation of PtdIns(3,4)$P_2$ in human platelets, based on these results alone, it was difficult to know whether the accumulation of this lipid is a cause or a consequence of the irreversible aggregation. Recently, we showed that PI3-kinase inhibitors, added when aggregation is at its maximum, after 2 min of TRAP stimulation, were able to induce a very rapid and dramatic decrease in the level of PtdIns(3,4)$P_2$, followed by a disaggregation of platelets.[15] These results strongly suggested a role for the late accumulation of PtdIns(3,4)$P_2$ in strengthening aggregation. The particularly active turnover of this phosphoinositide indicates that its accumulation results from sustained PI 3-kinase activation rather than inhibition of PtdIns(3,4)$P_2$ hydrolysis. The platelet disaggregation induced by PI3-kinase inhibitors is accompanied by rapid destabilization of the signaling complexes associated with the cytoskeleton and specific release of myosin heavy chains.[15] It is important to note that addition of PI3-kinase inhibitors after 2 min of stimulation by FcγRIIA cross-linking also leads to platelet disaggregation (Gratacap MP, unpublished observation). Thus PtdIns(3,4)$P_2$ appears as a central molecule of a positive feed-back loop. Indeed, aggregation is required for its production and in turn this lipid influences the strengthening and the irreversibility of aggregation. The targets of PtdIns(3,4)$P_2$ that may explain its role in the irreversible phase of aggregation are, however, still unknown.

## A key role of ADP in TRAP-induced PtdIns(3,4)$P_2$ accumulation

Interestingly, among all platelet-released substances, ADP has been shown to be selectively responsible for the stabilization of thrombin-induced platelet aggregates.[1,16-18] Indeed, ADP scavengers, like PI3-kinase inhibitors, are able to transform the classical irreversible aggregation induced by TRAP into a reversible platelet aggregation.[19] In agreement, TRAP-dependent accumulation of PtdIns[3,4] $P_2$ in human platelets is strongly and specifically impaired in the presence of the ADP scavengers.[15] In fact, TRAP or ADP alone[14] is not sufficient *per se* to induce the accumulation of PtdIns(3,4)$P_2$ but both induce reversible platelet aggegation, even in the presence of fibrinogen. The critical role of secreted ADP for the accumulation of PtdIns(3,4)$P_2$ is also observed in thrombin–stimulated platelets.[20] An exciting question is: how can a combination of these agents induce the accumulation of PtdIns(3,4)$P_2$ and irreversible platelet aggregation? The $P_2$ family of ADP receptors is composed of two classes, namely the P2X receptors, which are ligand-gated ion channels, and the P2Y receptors, which belong to the serpentine G protein-coupled receptor family.[21] In the case of platelets, the $P2Y_1$ receptor is coupled to calcium mobilization and has been shown to be responsible for ADP-induced shape change.[22-24] In addition, a not yet identified P2 receptor negatively coupled to adenylyl cyclase seems to be necessary for the completion of ADP–induced aggregation response.[22] Recently, selective antagonists and inhibitors have been developed, allowing specific discrimination between $P2Y_1$ and P2/adenylyl cyclase–dependent responses.[25,26] Adenosine 2'-phosphate 5'-phosphate (A2P5P) is a selective $P2Y_1$ antagonist [22-24,27] while AR-C66096 selectively blocks the inhibitory effect of ADP on adenylyl cyclase.[22] The pharmacology of AR-C66096 is strikingly similar to that of the antiplatelet drug clopidogrel, which inhibits selectively ADP-induced platelet aggregation by blocking the effect of ADP on adenylyl cyclase.[25] Using these pharmacologic tools, we found that ADP plays a key and specific role in the late accumulation of PtdIns(3,4)$P_2$ induced by TRAP through its receptor coupled to inhibition of adenylyl cyclase. This observation is of consequence in terms of antithrombotic pharmacology, since clopidogrel, acting through this ADP receptor, inhibits thrombosis in humans.[25] The intracellular machinery involved in this process is currently under investigation but one can speculate that, besides the inhibition of cAMP formation, the release of $\beta/\gamma$ subunits from the heterotrimeric G-protein may be critical. In this respect, an important point is to determine the type of PI3-kinase that may be regulated through the synergistic effects of TRAP and ADP. A C2 domain -containing PI3-kinase, activated by $\alpha_{IIb}\beta_3$ engagement, has recently been described in platelets.[12] This enzyme produces PtdIns(3)P, which is then phosphorylated to PtdIns(3,4)$P_2$ by a PtdIns(3)P4-kinase. This new route

could be compatible with our results. However, since ADP-dependent signaling is clearly necessary, one explanation might be that $\alpha_{IIb}\beta_3$ exposure to its ligand must reach a certain level, obtained upon addition of two weak agonists (*i.e.* TRAP and ADP), so that the formation of strong focal complexes might occur. The outside-in signaling of $\alpha_{IIb}\beta_3$ is linked to the recruitment, around the $\beta_3$ cytoplasmic tail, of signaling complexes and cytoskeletal proteins.[28] These complexes may be different according to the degree of $\alpha_{IIb}\beta_3$ activation and the mechanical strengths acting through this integrin. Myosin has been shown to interact with the tyrosine phosphorylated $\beta_3$ tail of $\alpha_{IIb}\beta_3$ *in vitro* and these tyrosine residues are indeed required for outside-in signaling and stable platelet aggregation *in vivo*,[29] possibly by controlling the extent of integrin clustering. Induction of actin-myosin contractility might be important for integrin clustering and formation of mature adhesion plaques that are linked to irreversible aggregation. Interestingly, both ADP antagonists and PI 3-kinase inhibitors are able to induce a rapid release of myosin heavy chains from the integrin cytoskeletal complexes and to destabilize the signaling machinery linked to integrin and the cytoskeleton.[15] Another possibility, based on the role of ADP in enhancing the secretion response induced by other agonists[30] could be that other adhesive receptors have to co-operate with $\alpha_{IIb}\beta_3$ for full signaling through the integrin. Consistent with this idea, a recent study suggests a modulation of $\alpha_{IIb}\beta_3$ function by thrombospondin[31] which is released upon platelet activation. It is also noteworthy that ADP is involved in platelet spreading on fibrinogen,[32-34] a mechanism that requires PI3-kinase activity.[34] The study of the molecular mechanisms involved in ADP-dependent regulation of the accumulation of PtdIns(3,4)$P_2$ in TRAP-stimulated platelets will probably bring new insights into the cross-talk between different signal transduction mechanisms that controls platelet aggregation or spreading. This synergistic effect of ADP is probably not restricted to PtdIns(3,4)$P_2$ accumulation since phospholipase D[35] and the late and sustained phosphorylation of myosin light chain (Missy K. *et al.*, submitted) are also controlled by secreted ADP in TRAP–stimulated platelets.

## ADP and PI 3-kinase are critical for platelet activation induced by FcγRIIA cross-linking

Platelets express a single class of Fcγ receptor (FcγRIIA), which is involved in heparin-associated thrombocytopenia (HIT) and possibly in inflammation.[36-40] HIT is an auto-immune, rare (2%) but severe complication of treatment with heparin. *In vitro* cross-linking of FcγRIIA by specific antibodies induces platelet secretion and aggregation.[41] Activation of FcγRIIA leads to rapid tyrosine phosphorylation of intracellular signaling proteins, activation of phospholipase C-γ2 (PLC-γ2) and calcium signaling.[42-46] We have recently shown that FcγRIIA cross-linking also induces a rapid production of PtdIns$^{3,4,5}P_3$ and a slower accumulation of PtdIns$^{3,4}P_2$. Interestingly, inhibition of PI 3-kinase by wortmannin or LY294002 fully abolished platelet secretion and aggregation, as well as PLCγ2 activation and calcium mobilization.[46,47] A PI 3-kinase (possibly PI3-kinase α) therefore plays a critical role in the early phase of platelet activation.[46,47] Interestingly, PI 3-kinase inhibition does not affect the tyrosine phosphorylation of PLC-γ2, but one of its product, PtdIns(3,4,5)$P_3$, appears to be required for activation of PLC-γ2 probably by supporting its recruitment to the membrane through binding to its N-terminal PH domain and/or its SH2 domain.[46] It is noteworthy that activation of PLC-γ2 *via* GPVI also requires the early production of PtdIns(3,4,5)$P_3$ to occur.[48] This is in sharp contrast with the above described situation in which PI 3-kinase is not necessary for the initiation of platelet activation by TRAP. This is consistent with the fact that PAR1 activates, through Gq, a PLCβ which is stimulated independently of PI 3-kinase. Interestingly, Hérault *et al*.[49] and Polgàr *et al*.[50] have shown that ADP plays a major role in platelet activation and aggregation induced by FcγRIIA cross-linking or by sera from HIT patients. Recently, we obtained pharmacologic evidence that the ADP receptor coupled to Gi was required for HIT sera or FcγRIIA clustering-induced platelet secretion and aggregation (Gratacap *et al*. submitted). These observations suggest that ADP receptor antagonists such as clopidogrel may be effective as therapeutic agents for prevention or treatment of HIT. They also raise several intriguing questions concerning the early molecular mechanisms evoked by ADP to synergize FcγRIIA -mediated platelet activation. Our data suggest that converging signaling pathways from Gi and tyrosine kinases are required for platelet secretion and aggregation induced by FcγRIIA (Gratacap MP *et al*., submitted). Preliminary results indicate that ADP does not influence the tyrosine kinase-dependent pathway initiated by FcγRIIA but is required for PLCγ2 activation and calcium mobilization. One possibility would be that ADP dependent pathway and FcγRIIA cross-linking–induced signaling converge to regulate the early production of PtdIns$^{3,4,5}P_3$ which is critical in the process of PLCγ2 activation. This attractive hypothesis is currently under investigation.

In conclusion, the recent developments in the exciting research area of ADP-induced signaling, on its own or as a cofactor of platelet activation, have already yielded surprises and suggested new hypotheses for the regulation of platelet functions. For instance, a concomitant signaling through Gi and either Gq[26] or tyrosine kinases (Gratacap MP *et al*. submitted), according to the primary agonist used, might be envisaged as a general mechanism by which efficient platelet activation and aggregation occur. Obviously, a better understanding of the molecular basis of the role of ADP as a cofactor of platelet activation should lead to new pharmacologic strategies to modulate platelet functions according to the pathologic situation.

## References

1. Born GV. Adenosine diphosphate as a mediator of platelet aggregation in vivo: an editorial view. Circulation 1985; 72:741-6.

2. Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. Arterioscler Thromb Vasc Biol 1999; 19:2281-5.
3. Toker A, Cantley LC. Signalling through the lipid products of phosphoinositide-3-OH kinase. Nature 1997; 387:673-6.
4. Vanhaesebroeck B, Leevers SJ, Panayotou G, Waterfield MD. Phosphoinositide 3-kinases: a conserved family of signal transducers. Trends Biochem Sci 1997; 22:267-72.
5. Rittenhouse SE. Phosphoinositide 3-kinase activation and platelet function. Blood 1996; 88:4401-14.
6. Sorisky A, King WG, Rittenhouse SE. Accumulation of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 in thrombin-stimulated platelets: different sensivities to $Ca^{2+}$ or functional integrin. Biochem J 1992; 286:581-4.
7. Guinebault C, Payrastre B, Racaud-Sultan C, et al. Integrin-dependent translocation of phosphoinositide 3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interactions of p85a with actin filaments and focal adhesion kinase. J Cell Biol 1995; 129:831-42.
8. Sultan C, Breton M, Mauco G, Grondin P, Plantavid M, Chap H. The novel inositol lipid phosphatidylinositol 3,4-bisphosphate is produced by human platelets upon thrombin stimulation. Biochem J 1990; 269:831-4.
9. Sultan C, Plantavid M, Bachelot C, et al. Involvement of platelet glycoprotein IIb-IIIa ($\alpha_{IIb}$-$\beta_3$ integrin) in thrombin-induced synthesis of phosphatidylinositol 3'-4'-bisphosphate. J Biol Chem 1991; 266:23554-7.
10. Giuriato S, Payrastre B, Drayer AL, et al. Tyrosine phosphorylation and relocation of SHIP are integrin-mediated in thrombin-stimulated human blood platelets. J Biol Chem 1997; 272:26857-63.
11. Norris FA, Atkins RC, Majerus PW. Inositol polyphosphate 4-phosphatase is inactivated by calpain-mediated proteolysis in stimulated human platelets. J Biol Chem 1997; 272:10987-9.
12. Zhang J, Banfic H, Straforini F, Tosi L, Volinia S, Rittenhouse SE. A type II phosphoinositide 3-kinase is stimulated via activated integrin in platelets. J Biol Chem 1998; 273:14081-4.
13. Kovacsovics TJ, Bachelot C, Toker A, et al. Phosphoinositide 3-kinase inhibition spares actin assembly in activating platelets but reverses platelet aggregation. J Biol Chem 1995; 270:11358-66.
14. Gachet C, Payrastre B, Guinebault C, et al. Reversible translocation of phosphoinositide 3-kinase to the cytoskeleton of ADP-aggregated human platelets occurs independently of Rho A and without synthesis of phosphatidylinositol (3,4)-bisphosphate. J Biol Chem 1997; 272:4850-4.
15. Trumel C, Payrastre B, Plantavid M, et al. A key role of ADP in the irreversible platelet aggregation induced by the PAR1 activating peptide through the late activation of phosphoinositide 3-kinase. Blood 1999; 94:4156-65.
16. Kinlough-Rathbone RL, Packham MA, Perry DW, Mustard JF, Cattaneo M. Lack of stability of aggregates after thrombin-induced reaggregation of thrombin-degranulated platelets. Thromb Haemost 1992; 67:453-7.
17. Cattaneo M, Canciani MT, Lecchi A, et al. Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. Blood 1990; 75:1081-6.
18. Nurden P, Savi P, Heilmann E, et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. J Clin Invest 1995; 95:1612-22.
19. Lau LF, Pumiglia K, Côté YP, Feinstein MB. Thrombin-receptor agonist peptides, in contrast to thrombin itself, are not full agonists for activation and signal transduction in human platelets in the absence of platelet-derived secondary mediators. Biochem J 1994; 303:391-400.
20. Selheim F, Idsoe R, Fukami MH, Holmsen H, Vassbotn FS. Formation of PI 3-kinase products in platelets by thrombin, but not collagen, is dependent on synergistic autocrine stimulation, particularly through secreted ADP. FEBS Lett 1999; 263:780-5.
21. North RA, Barnard EA. Nucleotide receptors. Curr Opin Neurobiol 1997; 7:346-57.
22. Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. Evidence for three distinct ADP receptors on human platelets. J Biol Chem 1998; 273: 2024-9.
23. Savi P, Beauverger P, Labouret C, et al. Role of P2Y1 purinoceptor in ADP-induced platelet activation. FEBS Lett 1998; 422:291-5.
24. Hechler B, Léon C, Vial C, et al. The P2Y1 receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. Blood 1998; 92:152-9.
25. Herbert JM, Frehel D, Kieffer G, et al. Clopidogrel, a novel antiplatelet and antithrombotic agent. Cardiovasc Drug Rev 1993; 11:180-98.
26. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci USA 1998; 95:8070-4.
27. Jantzen HM, Gousset L, Bhaskar V, et al. Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. Thromb Haemost 1999; 81:111-7.
28. Shattil SJ, Kashiwagi H, Pampori N. Integrin signaling: the platelet paradigm. Blood 1998; 91: 2645-57.
29. Law DA, DeGuzman FR, Heiser P, Ministri-Madrid K, Killeen N, Phillips DR. Integrin cytoplasmic tyrosine motif is required for outside-in $\alpha_{IIb}$-$\beta_3$ signalling and platelet function. Nature 1999; 401:808-11.
30. Cattaneo M, Lombardi R, Zighetti ML, et al. Deficiency of ($^{33}$P)2MeS-ADP binding sites on platelets with secretion defect, normal granule stores and normal thromboxane $A_2$ production. Evidence that ADP potentiates platelet secretion independently of the formation of large platelet aggregates and thromboxane $A_2$ production. Thromb Haemost 1997; 77:986-90.
31. Chung J, Gao AG, Frazier WA. Thrombospondin acts via integrin-associated protein to activate the platelet integrin $\alpha_{IIb}$-$\beta_3$. J Biol Chem 1997, 272:14740-6.
32. Haimovich B, Lipfert L, Brugge JS, Shattil SJ. Tyrosine phosphorylation and cytoskeletal reorganization in platelets are triggered by interaction of integrin receptors with their immobilized ligands. J Biol Chem 1993; 268:15868-77.
33. Gironcel D, Racaud-Sultan C, Payrastre B, et al. $\alpha_{IIb}$-$\beta_3$-integrin mediated adhesion of human platelets to a fibrinogen matrix triggers phospholipase C activation and phosphatidylinositol 3',4'-bisphosphate accumulation. FEBS Lett 1996; 389:253-6.
34. Heraud JM, Racaud-Sultan C, Gironcel D, et al. Lipid products of phosphoinositide 3-kinase and phosphatidylinositol 4',5'-bisphosphate are both required for ADP-dependent platelet spreading. J Biol Chem 1998; 273:17817-23.
35. Martinson EA, Scheible S, Marx-Grunwitz A, Presek P. Secreted ADP plays a central role in thrombin-induced phospholipase D activation in human platelets. Thromb Haemost 1998; 80:976-81.
36. Anderson CL, Chacko GW, Osborne JM, Brandt JT. The Fc receptor for immunoglobulin G (Fc gamma RII) on human platelets. Semin Thromb Hemost 1995; 21:1-9.

37. Warkentin TE, Chong BH, Greinacher A. Heparin-induced thrombocytopenia: towards consensus. Thromb Haemost 1998; 79:1-7.
38. Arnout J. The pathogenesis of the antiphospholipid syndrome: a hypothesis based on parallelisms with heparin-induced thrombocytopenia. Thromb Haemost 1996; 75:536-41.
39. Horne MK 3rd, Alkins BR. Platelet binding of IgG from patients with heparin-induced thrombocytopenia. J Lab Clin Med 1996; 127:435-42.
40. Hoylaerts MF, Thys C, Arnout J, Vermylen J. Recurrent arterial thrombosis linked to autoimmune antibodies enhancing von Willebrand factor binding to platelets and inducing Fc gamma RII receptor-mediated platelet activation. Blood 1998; 91:2810-7.
41. Anderson GP, Anderson CL. Signal transduction by the platelet Fc receptor. Blood 1990; 76:1165-72.
42. Chacko GW, Duchemin AM, Coggeshall KM, Osborne JM, Brandt JT, Anderson CL. Clustering of the platelet Fc gamma receptor induces noncovalent association with the tyrosine kinase p72syk. J Biol Chem 1994; 269:32435-40.
43. Yanaga F, Poole A, Asselin J,et al. Syk interacts with tyrosine-phosphorylated proteins in human platelets activated by collagen and cross-linking of the Fc gamma-IIA receptor. Biochem J 1995; 311:471-8.
44. Huang MM, Indik Z, Brass LF, Hoxie JA, Schreiber AD, Brugge JS. Activation of Fc gamma RII induces tyrosine phosphorylation of multiple proteins including Fc gamma RII. J Biol Chem 1992; 267:5467-73.
45. Blake RA, Asselin J, Walker T, Watson SP. Fc gamma receptor II stimulated formation of inositol phosphates in human platelets is blocked by tyrosine kinase inhibitors and associated with tyrosine phosphorylation of the receptor. FEBS Lett 1994; 342:15-8.
46. Gratacap MP, Payrastre B, Viala C, Mauco G, Plantavid M, Chap H. Phosphatidylinositol 3,4,5-trisphosphate-dependent stimulation of phospholipase C-g2 is an early key event in FcgRIIA-mediated activation of human platelets. J Biol Chem 1998; 273:24314-21.
47. Chacko GW, Brandt JT, Coggeshall KM, Anderson CL. Phosphoinositide 3-kinase and p72syk noncovalently associate with the low affinity Fc gamma receptor on human platelets through an immunoreceptor tyrosine-based activation motif. Reconstitution with synthetic phosphopeptides. J Biol Chem 1996; 271: 10775-81.
48. Pasquet JM, Bobe R, Gross B, et al. A collagen-related peptide regulates phospholipase Cγ2 via phosphatidylinositol 3-kinase in human platelets. Biochem J 1999, 342:171-7.
49. Herault JP, Lale A, Savi P, Pflieger AM, Herbert JM. In vitro inhibition of heparin-induced platelet aggregation in plasma from patients with HIT by SR 121566, a newly developed Gp IIb/IIIa antagonist. Blood Coagul Fibrin 1997; 8:206-7.
50. Polgár J, Eichler P, Greinacher A, Clemetson KJ. Adenosine diphosphate (ADP) and ADP receptor play a major role in platelet activation/aggregation induced by sera from heparin-induced thrombocytopenia patients. Blood 1998; 91:549-54.

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# CONGENITAL DISORDERS OF PLATELET FUNCTION

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ABSTRACT

**The study of inherited platelet defects has been critical in identifying important platelet receptors and characterizing their function. More recently, these studies have been extended to defects in signal transduction mechanisms. In both cases, the ability to produce knock-out mice allows the reproduction of clinical conditions and a much more detailed analysis of the defects and therefore the function of the affected molecules. Glanzmann's thrombocytopenia caused by defects in GPIIb/IIIa and Bernard-Soulier syndrome caused by defects in GPIb/IX although rare are the commonest of these inherited disorders and have been studied on a molecular level since the 1970s. Since the 1980s a wide range of other receptor defects have been recognized including two collagen receptors, α2β1 and GP VI, thromboxane receptors and ADP receptors. Several defects have been reported to affect signaling pathways though the analysis of such disorders remains difficult. A few better characterized cases exist involving defects in a PLC β2 isoform or in the Wiskott-Aldrich syndrome protein. Other congenital bleeding disorders are related to problems in storage or release from storage granules which play an important role in hemostasis. These included grey platelet syndrome and Quebec platelet disorder affecting α-granules and Hermansky-Pudlak and Chediak-Higashi syndromes affecting δ-(or dense) granules. In rare bleeding disorders the procoagulant activity of platelets is affected, concerning the exposure of negatively-charged phospholipids on the platelet surface. In Scott syndrome, the patient's platelets were unable to expose negatively charged phospholipids and, in another, Stormorken syndrome, the platelets continuously expose negatively charged phospholipids.**


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The molecular analysis of platelet defects causing bleeding disorders has been an invaluable tool in the identification of the critical platelet molecules participating in hemostasis. Since the first description of Glanzmann's thrombasthenia in 1918[1] to the more recent discoveries of patients with defects in the collagen receptor GPVI[2] and in the ADP receptor $P2_{AC}$,[3] studies on the origin of a disorder have often been a key step in establishing a function for the molecules concerned. More recently, other approaches have been used to supplement this, such as knock-out mice, but these are only possible once a molecule has been identified and characterized. Platelet related bleeding disorders have the possible advantage that they may indicate new molecules with an essential role. At first the molecules identified were membrane receptors but, more recently, with improvement in basic knowledge and in analytic techniques, other classes of molecule, such as signaling kinases, phosphatases and adapter molecules, have started to be implicated and molecules with a role in other critical platelet functions will no doubt be found as platelets from other patients are analyzed. The types of defects recognized fall into several categories; so-called "classic" or "type I" disorders, in which the causative molecule (so far generally a receptor) is totally or almost totally absent. There is then a category referred to as "type II" or "variant" disorders in which reduced expression of a normal or abnormal molecule, respectively, is observed. These categories are useful for clinical description and are also closely related to the types of molecular defects. Bleeding disorders caused by platelet defects that are well-characterized include defects in major receptors such as Glanzmann's thrombasthenia (GPIIb/IIIa) and Bernard-Soulier syndrome (GPIb/IX) as well as defects in other primary or secondary receptors, such as α2β1, GPVI, thromboxane receptors and ADP receptors. Some defects could be identified in signaling molecules downstream from the major receptors including PLCβ2 and the Wiskott-Aldrich syndrome protein (WASP). A range of bleeding disorders is caused by defects in molecules involved in the transport of substances to, or organization of, storage granules, in particular α-granules in grey platelet syndrome and Quebec platelet disorder, and dense or δ-granules in Hermansky-Pudlak and Chediak-Higashi syndromes. There are also αδ-disorders affecting both types of granule. A different category of disorder

affects the procoagulant activity of platelets, also an essential part of hemostasis. Although the defective molecule has not yet been identified, two types of disorder are known. The first is manifested as Scott syndrome, in which the exposure of negatively charged phospholipids is abnormal, leading to defective prothrombinase activity on activated platelets. The other is Stormorken syndrome in which resting platelets have exposed negatively charged phospholipids causing full procoagulant activity in the absence of agonists, surprisingly leading to a bleeding tendency. Several of these defects have been replicated in mouse models allowing a more detailed analysis of the biochemical defects and the pathology of these disorders. The possibility of making knock-out mice has widened the scope of such studies to molecules for which no human "knock-out" exists (or has not yet been found).

## Disorders of platelet membrane receptors

### *(A) Glanzmann's thrombasthenia*

Glanzmann's thrombasthenia (GT) is a disorder affecting GPIIb/IIIa.[4,5] Platelets have a deficient aggregation response to all agonists tested but agglutinate normally in response to ristocetin/von Willebrand factor. Clot retraction is absent in the classic or type I variety of the disorder but maybe only slightly affected in type II disease in which GPIIb/IIIa is present, but in reduced amounts.[6] In the classic disease fibrinogen may be completely absent from the a-granules whereas in type II disease it may be present in normal amounts.[7] The absence of aggregation in these platelets has consequences beyond simply preventing the formation of thrombi. GPIIb/IIIa has an accessory role in signaling and is critical for the development of procoagulant activity.[8] In its absence these functions are abnormal. Genetic defects in Glanzmann's thrombasthenia affect either the gene coding for the GPIIb ($\alpha_{IIb}$) or GPIIIa ($\beta_3$). In the classic disease the phenotype could be affected depending on the gene involved since $\beta_3$ is also a partner in another platelet integrin $\alpha_v\beta_3$, the vitronectin receptor.[9] Glanzmann's thrombasthenia is particularly common in populations in which consanguineous marriages are frequent either because of tradition or due to the isolation of minority groups. The GPIIb and GPIIIa genes are large and complex, with 17.2 kbp and 30 exons and 65 kbp and 15 exons, respectively.[10,11] A large number of mutations leading to amino acid substitutions, deletions, splice site mutations, and deletions have been described as well as mutations leading to a premature stop codon. These have been described in detail elsewhere in the literature.[12-19] The problems that they cause in protein folding or complex formation leading to the manifested disorders can be divided into several broad categories; deletions which affect the transmembrane region or mutations causing a frame shift and leading to a premature stop codon.[20] These cause type I disease because the affected subunit is secreted and the other subunit is not expressed. Other mutations, for example, in residues important for calcium binding domains in GPIIb,[21] affect levels of expression of the protein but do not prevent it completely, leading to type II disease. Depending whether the defect is in GPIIb[22] or GPIIIa ($\beta_3$)[23] another integrin ($\alpha_v\beta_3$) may be affected. If GPIIb is absent, levels of $\alpha_v\beta_3$ are increased, whereas if $\beta_3$ is defective both GPIIb/GPIIIa and $\alpha_v\beta_3$ can be absent.[9] Physiologic differences resulting from the absence of one or the other subunit have not yet been noted. Recently, a $\beta_3$ knock-out mouse has been described[24]. Mutations affecting disulphides in $\beta_3$ can also give rise to folding defects and to lower levels of expression in type II disease.[25] More interesting variants of Glanzmann's thrombasthenia involve mutations in the cytoplasmic domain of either subunit. In such cases the glycoproteins are often expressed in normal amounts but receptor function is affected.[26] Such variants often have normal levels of $\alpha$-granule fibrinogen suggesting that the acquisition of this protein does not require inside-out activation of GPIIb-IIIa.

### *(B) Bernard-Soulier syndrome*

Bernard-Soulier syndrome (BSS) is a disorder of GPIb/IX.[27] Patients have a prolonged bleeding time, often giant platelets, a variable but generally reduced platelet count and increased platelet turnover.[28,29] Aggregation is normal to a wide range of platelet agonists but is abnormal to von Willebrand factor (vWF) in the presence of ristocetin or botrocetin. Responses to thrombin are abnormal at low doses of thrombin but are apparently normal at higher doses.[30] Resting platelets have a higher procoagulant activity than normal but show a defective, lower expression than normal when activated.[31] The GPIb/V/IX complex consists of four subunits, GPIb$\alpha$, GPIb$\beta$, GPIX and GPV,[32,33] all belonging to the leucine-rich repeat family.[34-37] The stoichiometry of the complex appears to be 2:2:2:1 suggesting a sandwich-like structure. Defects leading to BSS have been reported in all of the genes except that for GPV. In classic BSS all four subunits are absent from the platelet surface although traces may be found in platelets after lysis with detergent. As in Glanzmann's thrombasthenia variants are known in which the glycoprotein is expressed at a lower level. Expression of GPV, or its stability at the platelet surface is dependent on expression of the rest of the complex and the stoichiometry appears to be maintained. However, it has now been shown clearly in GPV knock-out mice that neither the expression of the rest of the complex, nor its function, is affected by the lack of GPV.[38,39] Again as in Glanzmann's thrombasthenia, the phenotype is related to the type of defect. Deletions affecting the transmembrane region of GPIb$\alpha$ or mutations causing a frame-shift and leading to a premature stop codon coding for a protein lacking the transmembrane domain so that the mutant protein cannot anchor in the membrane cause the classic type of BSS and the other subunits are also absent.[40,41] In such patients as well as in the heterozygotes, the circulating levels of glycocalicin may be much higher than normal, with unknown effects. A case was also described with a mutation in the binding site for the GATA-1 transcription factor in the promoter for

GPIbβ for one allele leading to BSS because of a deletion in the other allele.[42] Likewise, point mutations which lead to severe folding problems in either GPIbα, GPIbβ or GPIX cause BSS with a strong decrease but not total absence of the subunits.[43-47] In fact it is often possible to deduce which subunit contains the mutation, based upon Western blotting or surface labeling methods as it often shows an even lower level of expression than the others. Point mutations in any of the GPIb/IX subunits which cause such marked folding problems are generally either in cysteines forming disulphide bridges or in strongly conserved residues of the leucine-rich repeats, typically leucines or asparagines. Mutation of other amino acids to cysteine can also disrupt the disulphide bridge pattern. Although folding problems in either GPIbβ or GPIX do not directly affect the GPIbα binding sites they do affect complex formation between the subunits and hence surface expression of the complex. Typical examples of such mutations are Cys209->Ser in GPIbα,[44] Asn[45]->Ser in GPIX[46,47] and Tyr88->Cys in GPIbβ.[45] In practice, relatively few mutations in GPIbβ have been detected, with mutations in GPIba and GPIX predominating. In populations of Northern and Central European origin the Asn45->Ser mutation in GPIX is particularly common and may be responsible for up to 50% cases of BSS. Finally, as in Glanzmann's thrombasthenia there are occasional mutations in which the GPIb/V/IX complex is expressed in amounts varying from strongly reduced up to normal levels but has impaired function. Several of these are mutations in the leucine-rich repeats affecting less conserved residues or a conservative mutation of a conserved residue. Thus, the Leu57->Phe mutation of GPIbα had only a slight effect on the function of the complex.[48] In the Bolzano variant the mutation is Ala156->Val in GPIbα.[49] This must have a partial effect on local folding leading to a dysfunctional expressed protein since not only is vWF binding absent but also various monoclonal antibodies fail to recognize the mutant protein; thrombin binding was, however, reported to be normal. In variant Nancy I,[50] a Leu179 of GPIbα is deleted giving partial expression of a misprocessed molecule with missing O-glycosylation and, again differences in monoclonal antibody recognition.

The fact that any glicoprotein is expressed at all suggests that in this case the deletion of one conserved leucine from a sequence of three in a row allows one of the others to partly compensate. An unusual mutation of a conserved leucine, Leu129->Pro, in GPIbα gave about 40% normal vWF binding but with 100% GPV expression implying again only a minor effect on conformation.[51] Note that this mutation lies near a β-sheet/α-helix boundary and that proline is often found at such sites. Several mutations affecting the transmembrane region or associations with other subunits allowed some expression of a correctly folded extracellular domain. Nevertheless, because the connection to the cytoskeleton or to cytoplasmic signaling molecules is abnormal the receptor does not function correctly.[45,52] Such mutations are very useful for exploring the function of the GPIb/V/IX complex.

### *(C) Platelet-type (or pseudo-) von Willebrand disease*

Platelet-type von Willebrand disease (vWD) is also a disorder of GPIb/IX but since its symptoms resemble those of type IIB von Willebrand disease it acquired this rather misleading name (also accounting for the Pseudo). In this disorder mutations in GPIb cause an enhanced interaction with von Willebrand factor.[53,54] In fact, resting platelets in this disorder can bind vWF and aggregate, unlike normal platelets. As a consequence, the plasma of these patients becomes depleted in higher multimers of vWF and the platelet/vWF aggregates are removed by the spleen. The patients may therefore show symptoms similar to the commoner type IIB vWD which is also caused by an inhanced interaction between GPIb and vWF but in which the mutation is in the vWF. Platelet-type vWD is a very rare disease but still needs to be differentiated from type IIB disease. The classic approach is by crossed aggregation experiments in which plasma or washed platelets from the patient are mixed with washed platelets or plasma, respectively, from a normal donor. The combination giving spontaneous platelet aggregation is diagnostic for the disorder, whether the platelets or vWF are abnormal. This first diagnostic step needs to be confirmed by the presence of a mutation in GPIb or vWF. Surprisingly, patients with platelet-type vWD seem to have a tendency to increased microthrombotic disease which might reflect oscillations in platelet/vWF multimer stoichiometry. In platelet-type vWD, patients with either of two different mutations (Gly233->Val and Met239->Val) within the larger loop of the GPIbα double-loops have been described.[53-55]

### *(D) Bolin-Jamieson syndrome*

This is still a poorly characterized disorder for which only three known cases have been described. The patients have a mild bleeding disorder linked to a larger form of GPIbα from one allele.[56-58] The disorder is therefore dominant. The larger form of GPIb is thought to be caused by a higher multimer form of the size polymorphism which occurs normally in the mucin-like domain. The common polymorphisms are the single (D) and double (C) copies of a 13 amino acid segment in this region, complete with O-glycosylation. Rarer forms are the triple (B) and quadruple copies (A) found in European and East Asian populations, respectively. It has been suggested that in Bolin-Jamieson syndrome a still larger version with seven copies exists.[59] Even if this explanation is correct, it still remains to be shown why this should cause a bleeding tendency. A possible explanation might be that the mixture of long and normal (C or D) forms on the platelet surface leads to a situation in which vWF can bind less well than in the normal situation with the more similar length C and D forms alone. The B and A forms are fairly rare and it is not known whether the A/C or B/D phenotypes show a less marked bleeding tendency. This disorder awaits input from expression of specific size polymorphisms of GPIb in model cells or in the GPIb knock-out

mouse. Studies on a role for size polymorphisms of GPIb in cardiovascular disease have been rather inconclusive.

### *(E) Collagen receptors*

*i) $\alpha_2\beta_1$ (or GPIa/IIa)*

Two cases of bleeding disorders related to $\alpha_2\beta_1$ have been described, both involving female patients with mild bleeding disorders.[60,61] In the first case a strong decrease but not an absence of this receptor was observed. Studies with platelets from the patient in a perfusion chamber model on stripped inverted rabbit arteries showed a much decreased adhesion of marginally activated platelets suggesting that initial adhesion via GPIb/vWF had not lead to collagen based adhesion. A second older patient described soon after had similar symptoms also accompanied by a much decreased expression of this receptor on platelets. In addition, this patient also lacked intact thrombospondin. Surprisingly, soon after, and coinciding with the patient reaching the menopause, her bleeding problem disappeared and platelet protein expression became normal. The first patient also recently passed the menopause and her condition has apparently also normalized (J.J. Sixma, personal communication). Thus, the origin of this disorder could lie in hormonal regulation of a transcription factor for the promoter of the $\alpha_2$ gene, since other $\beta_1$ integrins were not affected. Expression levels of $\alpha_2\beta_1$ are variable within a normal population and are regulated by two silent polymorphisms in the $\alpha_2$ gene.[62] Knock-out mice for the $\beta_1$ integrin subunit have been prepared but no differences in platelet phenotype have not yet been reported.[63] Knockout mice for the $\alpha_2$ integrin are currently being prepared.

*ii) GPVI*

A patient with a deficiency in GPVI was first described in 1989 in Japan[2]. The patient had a mild bleeding disorder and only the aggregation response to collagen was affected. Since then several other patients have been described, including one in whom 10% of normal levels of GPVI were expressed,[64] and another case in which despite the absence of GPVI the patient had made auto-antibodies to this receptor.[65] So far the molecular defects have not been identified but the recent cloning of GPVI[66] should allow rapid progress in this area. In future, other cases may be identified in which GPVI is normal but does not function because of defects in the Fcγ subunit or in coupling between the two.

*iii) CD36*

The role of CD36 as a collagen receptor is still controversial. Although there is some evidence that CD 36 is a collagen receptor,[67,68] the fact that about 7% of Japanese and sub-Saharan Africans as well as 0.3% of Americans lack this receptor but show absolutely no hemostatic problems, argues against an important function.[69,70] In fact, there is more evidence that CD36 functions as a scavenging receptor.[71] The molecular basis has been identified as a polymorphism in codon 90 which, if expressed, would lead to a Ser -> Pro shift.[72]

### *(F) ADP receptors*

Over the past few years several families have been described with defects in their response to ADP leading to bleeding problems.[3,73] Platelets from these patients had a normal shape change and cytoplasmic calcium signal in response to ADP, however ADP was unable to cause a reduction in cAMP levels in $PGE_1$-treated platelets. Platelets also showed almost zero binding of 2-Me-thio ADP. At present, platelets are thought to have three ADP receptors, $P2X_1$, $P2Y_1$ and P2T (also variously called $P2Y_{AC}$ or $P2Y_{CYC}$).[74] The defect in these families therefore appears to lie in the P2T receptor. Since this receptor has not yet been cloned the molecular origin is still unknown. Recently, a child was identified in Belgium with a heterozygous defect in $P2X_1$ causing a bleeding syndrome. The molecular defect was localized to a leucine deletion in one of the two transmembrane domains.[75] Since this receptor consists of three molecules forming a calcium channel the presence of one defective molecule is sufficient to prevent a channel functioning. Although there are no known cases of disease caused by a defect in the $P2Y_1$ receptor, knock-out mice for this molecule were recently prepared and had a prolonged bleeding time.[76] Thus, this receptor must also be intact for a normal platelet response to ADP.

### *(G) Thromboxane receptors*

Patients have been identified with a bleeding disorder in which the platelet response to $TXA_2$ is defective and an Arg60 ->Leu mutation was found in the $TXA_2$ receptor.[77] Platelets contain many other receptors of the seven transmembrane domain/G-protein-coupled family, including thrombin receptors PAR-1 and PAR-4, serotonin, platelet activating factor, lysophosphatidic acid, and chemokine receptors. Thus, there is still plenty of scope for explaining platelet-related bleeding syndromes.

## Disorders of signal pathways

Only a small number of hereditary bleeding disorders have so far been ascribed to defects in signaling molecules.[78] The main reason for this is that this type of molecular diagnosis remains quite difficult to perform. Signaling molecules are generally common to many types of cells and defects may not produce symptoms characteristic of a platelet defect. However, there are many patients with slight to moderate bleeding problems in whom a molecular diagnosis has not yet been made. Many mouse models from which genes for specific signaling molecules have been ablated have been prepared and have provided much insight into the roles of these genes.[79] The function of platelets has not been examined in all of these models.

### *(A) Wiskott-Aldrich syndrome*

Two forms of this disease have been described. The more severe form is an X-linked recessive disease characterized by problems of the immune system. Involvement of platelets is also indicated as the platelets are smaller than normal and function abnormally.[80]

Hereditary X-linked thrombocytopenia is a milder form affecting platelets but lacking immunological problems.[81] The gene affected in Wiskott-Aldrich syndrome codes for a 502 amino acid protein called Wiskott-Aldrich syndrome protein (WASP).[82] A wide range of defects has been found in the WASP gene causing absence or decreased expression of the protein.[83] WASP is a cytoplasmic protein which is thought to regulate actin filament assembly during platelet activation. It has adaptor protein function and contains tyrosine phosphorylation sites. Through proline-rich motifs it can also bind to signaling proteins containing SH3 domains.

#### *(B) Phospholipase C β2 isoform defect*

One patient has been described with a selective defect in a phospholipase Cβ2 isoform.[84] This leads to deficient responses to thrombin because cleavage of phosphatidylinositol to $IP_3$ and diacylglycerol is decreased. The $IP_3$ causes release of $Ca^{2+}$ from sarcoplasmic reticulum, whereas the diacylglycerol activates protein kinase C. Both of these steps are important in the signaling pathways leading to activation of GPIIb/IIIa. Consequently, this defect leads to a bleeding disorder.

#### *(C) Gα protein defect*

A patient has been described with a specific defect of Gα protein in platelets[85] resulting in a poor response to several agonists which have receptors coupled to this protein. As above, failure to activate appropriate pathways leads to inadequate activation of GPIIb/IIIa and therefore to a bleeding disorder.

### Secretion defects

Many different disorders fall within this category. Platelets contain α-granules, which are the storage site for a large group of proteins synthesized in megakaryocytes, or endocytosed from plasma, dense (δ-) granules, which contain nucleotides such as ADP and ATP, serotonin and calcium, and lysosomes which contain various enzymes. Disorders can affect one or more of these granules or the transport pathways leading to them.[86]

#### *(A) Grey platelet syndrome*

This is a mild bleeding disorder affecting the alpha granules.[87] In many patients proteins synthesized in megakaryocytes are constitutively secreted, indicating a defect in the pathway to the alpha granules. It is thought that the alpha granule membrane systems are still formed and contain typical marker proteins such as P-selectin. Many patients develop myelofibrosis because growth factors are directly secreted by megakaryocytes. Platelet aggregation responses, particularly to thrombin, are affected. α-granule contents also have a role in platelet procoagulant activity.[88]

#### *(B) Quebec platelet disorder*

This disorder has been described in two families from Quebec.[89] The α-granule proteins synthesized in the megakaryocytes are heavily degraded whereas plasma derived proteins are unaffected. Proteins affected include multimerin, von Willebrand factor and thrombospondin, all with important roles in hemostasis. A possible cause could be a defective targeting of a protease intended for lysosomes or for storage elsewhere in the α-granule.

#### *(C) Hermansky-Pudlak syndrome*

Hermansky-Pudlak syndrome is an autosomal recessive disorder characterized by oculocutaneous albinism and a bleeding tendency.[90] Lysosomal storage is defective. In platelets both lysosomes and dense granules are affected. Several varieties caused by different defects are known. A major form is common in Puerto Rico and is caused by a 16 bp duplication in the gene producing a frameshift. Another form also involving a frameshift was described in a Swiss family. The protein involved is a 79 kDa transmembrane (crossing the membrane twice) and is a component of multiple cytoplasmic organelles.[91,92] It is probably involved in organelle development.

#### *(D) Chediak-Higashi syndrome*

This is another rare autosomal recessive disorder characterized by oculocutaneous albinism, immunologic deficiency (impaired chemotaxis and bactericidal activity), neutropenia, abnormal natural killer cell function and a bleeding tendency.[93] The platelets (and other granule-containing cells) have giant inclusion bodies and organelles. The defective protein has been characterized as a cytosolic 1501 amino acid protein involved in vacuolar sorting and regulating lysosomal traffic. Overexpression in fibroblasts leads to smaller lysosomes. The protein contains hydrophobic helices and repeat motifs suggesting a role in regulating membrane-membrane interactions.[94]

#### *(E) αδ-storage pool deficiency*

In rare cases, both α- and δ-granules are affected in a disorder with autosomal dominant inheritance.[87] As might be expected, platelet aggregation is more strongly affected than in disorders of only one type of granule and adhesion may also be reduced.

### Membrane organization defects (procoagulant activity)

Formation of a stable thrombus, necessary to prevent bleeding and to initiate tissue repair, requires the generation of thrombin from plasma prothrombin via the coagulation cascade. A vital part of this process is the exposure of negatively charged phospholipids at the platelet surface.[95] In all cells, including platelets, there is a mechanism to remove phosphatidylserine, -inositol and -ethanolamine from the outer plasma membrane leaflet and transfer them to the inner leaflet. The enzyme thought to be involved and called an aminophospholipid transferase has not yet been characterized.[96] Likewise, for the programmed mixing of lipids of the inner leaflet with the outer, a specific enzyme called scramblase was postulated, then characterized.[97] Recently, there have been doubts about whether or not other molecules (flippases) are critical for this process[98] but scram-

blase was hypothesized to be possibly defective in a new class of bleeding disorder.

### *(A) Scott syndrome*

This is a rare, inherited disorder of phospholipid scrambling on the surface of blood cells including platelets.[99] The asymmetry of the lipid bilayer is maintained under conditions in which, normally, the negatively charged phospholipids are exposed on the outer surface. Because of this thrombin generation is reduced, leading to a low level of fibrin formation and poor wound closure and healing. As mentioned above the scramblase enzyme was thought to be affected in this disorder but this now seems less likely.[100]

### *(B) Stormorken syndrome*

Only one family has been found with this disorder which appears to be due to the reverse situation from that in Scott syndrome. Described in 1985, members of three generations have several health problems including a bleeding tendency.[101] A common feature is almost full procoagulant activity on resting platelets and a high number of microvesicles in plasma. Platelets showed a normal response to all agonists except collagen. It is surprising that the clinical aspect of this syndrome is not an enhanced thrombotic tendency but rather a bleeding tendency. *Ex vivo* perfusion chamber studies showed that platelet adhesion to a collagen surface was enhanced in these patients whereas thrombus growth was decreased. It is not clear why the negatively charged phospholipids are present on the outer leaflet, nor whether this reflects a defective aminophospholipid translocase nor yet a constantly active scramblase.[102]

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## References

1. Glanzmann E. Hereditäre hämorrhagische Thrombasthenie: Ein Beitrag zur Pathologie der Blutplättchen. J Kinderkr 1918; 88:113-41.
2. Moroi M, Jung SM, Okuma M, Shinmyozu K. A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. J Clin Invest 1989; 84:1440-5.
3. Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. Blood 1992; 80:2787-96.
4. Nurden AT, Caen JP. An abnormal platelet glycoprotein pattern in three cases of Glanzmann's thrombasthenia. Br J Haematol 1974; 28:253-60.
5. Phillips DR, Agin PP. Platelet membrane defects in Glanzmann's thrombasthenia. Evidence for decreased amounts of two major glycoproteins. J Clin Invest 1977; 60:535-45.
6. George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. Blood 1990; 75:1383-95.
7. Zucker MB, Pert JH, Hilgartner MW. Platelet function in a patient with thrombasthenia. Blood 1966; 28: 524-34.
8. Byzova TV, Plow EF. Networking in the hemostatic system. Integrin αIIbβ3 binds prothrombin and influences its activation. J Biol Chem 1997; 272:27183-8.
9. Coller BS, Cheresh DA, Asch E, Seligsohn U. Platelet vitronectin receptor expression differentiates Iraqi-Jewish from Arab patients with Glanzmann thrombasthenia in Israel. Blood 1991; 77:75-83.
10. Heidenreich R, Eisman R, Surrey S, et al. Organization of the gene for platelet glycoprotein IIb. Biochemistry 1990; 29:1232-44.
11. Zimrin AB, Gidwitz S, Lord S, et al. The genomic organization of platelet glycoprotein IIIa. J Biol Chem 1990; 265:8590-5.
12. Iwamoto S, Nishiumi E, Kajii E, Ikemoto S. An exon 28 mutation resulting in alternative splicing of the glycoprotein IIb transcript and Glanzmann's thrombasthenia. Blood 1994; 83:1017-23.
13. Gu J, Xu W, Wang X, Wu Q, Chi C, Ruan C. Identification of a nonsense mutation at amino acid 584- -arginine of platelet glycoprotein IIb in patients with type I Glanzmann thrombasthenia. Br J Haematol 1993; 83:442-9.
14. Kieffer N, Melchior C, Guinet JM, Michels S, Gouon V, Bron N. Serine 752 in the cytoplasmic domain of the beta 3 integrin subunit is not required for alpha v beta 3 postreceptor signaling events. Cell Adhes Commun 1996; 4:25-39.
15. Kouns WC, Steiner B, Kunicki TJ, et al. Activation of the fibrinogen binding site on platelets isolated from a patient with the Strasbourg I variant of Glanzmann's thrombasthenia. Blood 1994; 84:1108-15.
16. Lanza F, Stierlé A, Fournier D, et al. A new variant of Glanzmann's thrombasthenia (Strasbourg I). Platelets with functionally defective glycoprotein IIb-IIIa complexes and a glycoprotein IIIa 214Arg-->214Trp mutation. J Clin Invest 1992; 89:1995-2004.
17. Morel-Kopp MC, Kaplan C, Proulle V, et al. A three amino acid deletion in glycoprotein IIIa is responsible for type I Glanzmann's thrombasthenia: importance of residues Ile325Pro326Gly327 for beta3 integrin subunit association. Blood 1997; 90:669-77.
18. Newman PJ, Seligsohn U, Lyman S, Coller BS. The molecular genetic basis of Glanzmann thrombasthenia in the Iraqi-Jewish and Arab populations in Israel. Proc Natl Acad Sci USA 1991; 88:3160-4.
19. Peyruchaud O, Nurden AT, Milet S, et al. R to Q amino acid substitution in the GFFKR sequence of the cytoplasmic domain of the integrin IIb subunit in a patient with a Glanzmann's thrombasthenia-like syndrome. Blood 1998; 92:4178-87.
20. Schlegel N, Gayet O, Morel-Kopp MC, et al. The molecular genetic basis of Glanzmann's thrombasthenia in a gypsy population in France: identification of a new mutation on the alpha IIb gene. Blood 1995; 86:977-82.
21. Wilcox DA, Wautier JL, Pidard D, Newman PJ. A single amino acid substitution flanking the fourth calcium binding domain of aIIb prevents maturation of the aIIbb3 integrin complex. J Biol Chem 1994; 269:4450-7.
22. Ruan J, Peyruchaud O, Alberio L, et al. Double heterozygosity of the GPIIb gene in a Swiss patient with Glanzmann's thrombasthenia. Br J Haematol 1998; 102:918-25.
23. Ruan J, Schmugge M, Clemetson KJ, et al. Homozygous Cys542-->Arg substitution in GPIIIa in a Swiss patient with type I Glanzmann's thrombasthenia. Br J Haematol 1999; 105:523-31.
24. Hodivala-Dilke KM, McHugh KP, Tsakiris DA, et al.

Beta3-integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. J Clin Invest 1999; 103:229-38.
25. Grimaldi CM, Chen F, Scudder LE, Coller BS, French DL. A Cys374Tyr homozygous mutation of platelet glycoprotein IIIa (beta 3) in a Chinese patient with Glanzmann's thrombasthenia. Blood 1996; 88:1666-75.
26. Chen Y-P, Djaffar I, Pidard D, et al. Ser-752 --> Pro mutation in the cytoplasmic domain of integrin b3 subunit and defective activation of platelet integrin aIIbb3 (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia. Proc Natl Acad Sci USA 1992; 89:10169-73.
27. Clemetson KJ, Clemetson JM. Platelet GPIb-V-IX complex. Structure, function, physiology, and pathology. Semin Thromb Hemost 1995; 21:130-6.
28. Nurden AT. Inherited abnormalities of platelets. Thromb Haemost 1999; 82:468-80.
29. Lopez JA, Andrews RK, Afshar-Kharghan V, Berndt MC. Bernard-Soulier syndrome. Blood 1998; 91: 4397-418.
30. Jamieson GA, Okumura T. Reduced thrombin binding and aggregation in Bernard-Soulier platelets. J Clin Invest 1978; 61:861-4.
31. Bevers EM, Comfurius P, Nieuwenhuis HK, et al. Platelet prothrombin converting activity in hereditary disorders of platelet function. Br J Haematol 1986; 63:335-45.
32. Clemetson KJ, McGregor JL, James E, Dechavanne M, Luscher EF. Characterization of the platelet membrane glycoprotein abnormalities in Bernard-Soulier syndrome and comparison with normal by surface-labeling techniques and high-resolution two-dimensional gel electrophoresis. J Clin Invest 1982; 70:304-11.
33. Berndt MC, Gregory C, Chong BH, Zola H, Castaldi PA. Additional glycoprotein defects in Bernard-Soulier's syndrome: confirmation of genetic basis by parental analysis. Blood 1983; 62:800-7.
34. Lopez JA, Chung DW, Fujikawa K, Hagen FS, Papayannopoulou T, Roth GJ. Cloning of the alpha chain of human platelet glycoprotein Ib: a transmembrane protein with homology to leucine-rich alpha 2- glycoprotein. Proc Natl Acad Sci U S A 1987; 84:5615-9.
35. Lopez JA, Chung DW, Fujikawa K, Hagen FS, Davie EW, Roth GJ. The alpha and beta chains of human platelet glycoprotein Ib are both transmembrane proteins containing a leucine-rich amino acid sequence. Proc Natl Acad Sci USA 1988; 85:2135-9.
36. Hickey MJ, Williams SA, Roth GJ. Human platelet glycoprotein IX: an adhesive prototype of leucine-rich glycoproteins with flank-center-flank structures. Proc Natl Acad Sci USA 1989; 86:6773-7.
37. Lanza F, Morales M, De La Salle C, et al. Cloning and characterization of the gene encoding the human platelet glycoprotein V. A member of the leucine-rich glycoprotein family cleaved during thrombin-induced platelet activation. J Biol Chem 1993; 268:20801-7.
38. Kahn ML, Diacovo TG, Bainton DF, Lanza F, Trejo J, Coughlin SR. Glycoprotein V-deficient platelets have undiminished thrombin responsiveness and do not exhibit a Bernard-Soulier phenotype. Blood 1999; 94:4112-21.
39. Ramakrishnan V, Reeves PS, DeGuzman F, et al. Increased thrombin and responsiveness in platelets from mice lacking glycoprotein V. Proc Natl Acad Sci USA 1999; 96:13336-41.
40. Kunishima S, Miura H, Fukutani H, et al. Bernard-Soulier syndrome Kagoshima: Ser 444 --> stop mutation of glycoprotein (GP) Ibα resulting in circulating truncated GPIbα and surface expression of GPIbβ and GPIX. Blood 1994; 84:3356-62.
41. Afshar-Kharghan V, Lopez JA. Bernard-Soulier syndrome caused by a dinucleotide deletion and reading frameshift in the region encoding the glycoprotein Ib alpha transmembrane domain. Blood 1997; 90:2634-43.
42. Ludlow LB, Schick BP, Budarf ML, et al. Identification of a mutation in a GATA binding site of the platelet glycoprotein Ibβ promoter resulting in the Bernard-Soulier syndrome. J Biol Chem 1996; 271:22076-80.
43. Ware J, Russell SR, Marchese P, et al. Point mutation in a leucine-rich repeat of platelet glycoprotein Ibα resulting in the Bernard-Soulier syndrome. J Clin Invest 1993; 92:1213-20.
44. Simsek S, Noris P, Lozano M, et al. Cys209 Ser mutation in the platelet membrane glycoprotein Ib alpha gene is associated with Bernard-Soulier syndrome. Br J Haematol 1994; 88:839-44.
45. Kunishima S, Lopez JA, Kobayashi S, et al. Missense mutations of the glycoprotein (GP) Ibβ gene impairing the GPIb alpha/beta disulfide linkage in a family with giant platelet disorder. Blood 1997; 89:2404-12.
46. Wright SD, Michaelides K, Johnson DJD, West NC, Tuddenham EGD. Double heterozygosity for mutations in the platelet glycoprotein IX gene in three siblings with Bernard-Soulier syndrome. Blood 1993; 81:2339-47.
47. Clemetson JM, Kyrle PA, Brenner B, Clemetson KJ. Variant Bernard-Soulier syndrome associated with a homozygous mutation in the leucine-rich domain of glycoprotein IX. Blood 1994; 84:1124-31.
48. Miller JL, Lyle VA, Cunningham D. Mutation of leucine-57 to phenylalanine in a platelet glycoprotein Iba leucine tandem repeat occurring in patients with an autosomal dominant variant of Bernard-Soulier disease. Blood 1992; 79:439-46.
49. De Marco L, Mazzucato M, Fabris F, et al. Variant Bernard-Soulier syndrome type Bolzano. A congenital bleeding disorder due to a structural and functional abnormality of the platelet glycoprotein Ib-IX complex. J Clin Invest 1990; 86:25-31.
50. de la Salle C, Baas MJ, Lanza F, et al. A three-base deletion removing a leucine residue in a leucine-rich repeat of platelet glycoprotein Ib alpha associated with a variant of Bernard-Soulier syndrome (Nancy I). Br J Haematol 1995; 89:386-96.
51. Li C, Martin SE, Roth GJ. The genetic defect in two well-studied cases of Bernard-Soulier syndrome: a point mutation in the fifth leucine-rich repeat of platelet glycoprotein Ibα. Blood 1995; 86:3805-14.
52. Noris P, Arbustini E, Spedini P, Belletti S, Balduini CL. A new variant of Bernard-Soulier syndrome characterized by dysfunctional glycoprotein (GP) Ib and severely reduced amounts of GPIX and GPV. Br J Haematol 1998; 103:1004-13.
53. Miller JL, Cunningham D, Lyle VA, Finch CN. Mutation in the gene encoding the a chain of platelet glycoprotein Ib in platelet-type von Willebrand disease. Proc Natl Acad Sci USA 1991; 88:4761-5.
54. Russell SD, Roth GJ. Pseudo-von Willebrand disease: a mutation in the platelet glycoprotein Iba gene associated with a hyperactive surface receptor. Blood 1993; 81:1787-91.
55. Takahashi H, Murata M, Moriki T, et al. Substitution of Val for Met at residue 239 of platelet glycoprotein Ib alpha in Japanese patients with platelet-type von Willebrand disease. Blood 1995; 85:727-33.
56. Bolin RB, Okumura T, Jaimeson GA. New polymorphism of platelet membrane glycoproteins. Nature 1977; 269:69-70.
57. McGregor JL, Brochier J, Wild F, et al. Monoclonal antibodies against platelet membrane glycoproteins. Characterization and effect on platelet function. Eur J Biochem 1983; 131:427-36.

58. Meyer M, Schellenberg I. Platelet membrane glycoprotein Ib: genetic polymorphism detected in the intact molecule and in proteolytic fragments. Thromb Res 1990; 58:233-42.
59. Meyer M, Kutschner G, Beer JH, Schellenberg I. An abnormally large GPIbalpha variant resulting from an increased number of 39 bp tandem repeats detected in a patient with mild bleeding. Thromb Haemost 1999; 82:46.
60. Nieuwenhuis HK, Akkerman JW, Houdijk WP, Sixma JJ. Human blood platelets showing no response to collagen fail to express surface glycoprotein Ia. Nature 1985; 318:470-2.
61. Kehrel B, Balleisen L, Kokott R, et al. Deficiency of intact thrombospondin and membrane glycoprotein Ia in platelets with defective collagen-induced aggregation and spontaneous loss of disorder. Blood 1988; 71:1074-8.
62. Kunicki TJ, Kritzik M, Annis DS, Nugent DJ. Hereditary variation in platelet integrin alpha 2 beta 1 density is associated with two silent polymorphisms in the alpha 2 gene coding sequence. Blood 1997; 89:1939-43.
63. Brakebusch C, Hirsch E, Potocnik A, Fassler R. Genetic analysis of beta1 integrin function: confirmed, new and revised roles for a crucial family of cell adhesion molecules. J Cell Sci 1997; 110:2895-904.
64. Arai M, Yamamoto N, Moroi M, Akamatsu N, Fukutake K, Tanoue K. Platelets with 10% of the normal amount of glycoprotein VI have an impaired response to collagen that results in a mild bleeding tendency [published erratum appears in Br J Haematol 1995 Apr; 89:952]. Br J Haematol 1995; 89:124-30.
65. Sugiyama T, Okuma M, Ushikubi F, Sensaki S, Kanaji K, Uchino H. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. Blood 1987; 69:1712-20.
66. Clemetson JM, Polgar J, Magnenat E, Wells TN, Clemetson KJ. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors. J Biol Chem 1999; 274:29019-24.
67. Diaz-Ricart M, Tandon NN, Carretero M, Ordinas A, Bastida E, Jamieson GA. Platelets lacking functional CD36 (glycoprotein IV) show reduced adhesion to collagen in flowing whole blood. Blood 1993; 82:491-6.
68. Asch AS, Liu I, Briccetti FM, et al. Analysis of CD36 binding domains: ligand specificity controlled by dephosphorylation of an ectodomain. Science 1993; 262:1436-40.
69. Kehrel B, Kronenberg A, Rauterberg J, et al. Platelets deficient in glycoprotein IIIb aggregate normally to collagens type I and III but not to collagen type V. Blood 1993; 82:3364-70.
70. Daniel JL, Dangelmaier C, Strouse R, Smith JB. Collagen induces normal signal transduction in platelets deficient in CD36 (platelet glycoprotein IV). Thromb Haemost 1994; 71:353-6.
71. Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA. CD36 is a receptor for oxidized low density lipoprotein. J Biol Chem 1993; 268: 11811-6.
72. Kashiwagi H, Tomiyama Y, Honda S, et al. Molecular basis of CD36 deficiency. Evidence that a 478C-->T substitution (proline90-->serine) in CD36 cDNA accounts for CD36 deficiency. J Clin Invest 1995; 95: 1040-6.
73. Nurden P, Savi P, Heilmann E, et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function. J Clin Invest 1995; 95:1612-22.
74. Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. Arterioscler Thromb Vasc Biol 1999; 19:2281-5.
75. Oury C, Toth-Zsamboki E, Van Geet C, Nilius B, Vermylen J, Hoylaerts MF. A dominant negative mutation in the platelet P2X1 ADP receptor causes severe bleeding disorder. Important role of P2X1 in ADP-induced platlet aggregation. Blood 1999; 94:618a.
76. Leon C, Hechler B, Freund M, et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice [see comments]. J Clin Invest 1999; 104:1731-7.
77. Hirata T, Kakizuka A, Ushikubi F, Fuse I, Okuma M, Narumiya S. Arg60 to Leu mutation of the human thromboxane A2 receptor in a dominantly inherited bleeding disorder. J Clin Invest 1994; 94:1662-7.
78. Rao AK, Gabbeta J. Congenital disorders of platelet signal transduction. Arterioscler Thromb Vasc Biol 2000; 20:285-9.
79. Law DA, Nannizzi-Alaimo L, Cowan KJ, Prasad KS, Ramakrishnan V, Phillips DR. Signal transduction pathways for mouse platelet membrane adhesion receptors. Thromb Haemost 1999; 82:345-52.
80. Semple JW, Siminovitch KA, Mody M, et al. Flow cytometric analysis of platelets from children with the Wiskott-Aldrich syndrome reveals defects in platelet development, activation and structure. Br J Haematol 1997; 97:747-54.
81. Shcherbina A, Rosen FS, Remold ODE. WASP levels in platelets and lymphocytes of Wiskott-Aldrich syndrome patients correlate with cell dysfunction. J Immunol 1999; 163:6314-20.
82. Snapper SB, Rosen FS. The Wiskott-Aldrich syndrome protein (WASP): roles in signaling and cytoskeletal organization. Annu Rev Immunol 1999; 17:905-29.
83. Zhu Q, Watanabe C, Liu T, et al. Wiskott-Aldrich syndrome/X-linked thrombocytopenia: WASP gene mutations, protein expression, and phenotype. Blood 1997; 90:2680-9.
84. Lee SB, Rao AK, Lee KH, Yang X, Bae YS, Rhee SG. Decreased expression of phospholipase C-β 2 isozyme in human platelets with impaired function. Blood 1996; 88:1684-91.
85. Gabbeta J, Yang X, Kowalska MA, Sun L, Dhanasekaran N, Rao AK. Platelet signal transduction defect with Gα subunit dysfunction and diminished Gαq in a patient with abnormal platelet responses. Proc Natl Acad Sci USA 1997; 94:8750-5.
86. Lemons PP, Chen D, Whiteheart SW. Molecular mechanisms of platelet exocytosis: requirements for alpha-granule release. Biochem Bioph Res Co 2000; 267:875-80.
87. Hayward CPM. Inherited disorders of platelet α-granules. Platelets 1997; 8:197-209.
88. Alberio L, Safa O, Clemetson KJ, Esmon CT, Dale GL. Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. Blood 2000; 95:1694-702.
89. Hayward CP, Cramer EM, Kane WH, et al. Studies of a second family with the Quebec platelet disorder: evidence that the degradation of the α-granule membrane and its soluble contents are not secondary to a defect in targeting proteins to α-granules. Blood 1997; 89:1243-53.
90. Gahl WA, Brantly M, Kaiser-Kupfer MI, et al. Genetic defects and clinical characteristics of patients with a form of oculocutaneous albinism (Hermansky-Pudlak syndrome). N Engl J Med 1998; 338:1258-64.
91. Dell'Angelica EC, Aguilar RC, Wolins N, Hazelwood S, Gahl WA, Bonifacino JS. Molecular characterization of the protein encoded by the Hermansky-Pudlak syn-

drome type 1 gene. J Biol Chem 2000; 275:1300-6.
92. Oh J, Liu ZX, Feng GH, Raposo G, Spritz RA. The Hermansky-Pudlak syndrome (HPS) protein is part of a high molecular weight complex involved in biogenesis of early melanosomes [In Process Citation]. Hum Mol Genet 2000; 9:375-85.
93. Introne W, Boissy RE, Gahl WA. Clinical, molecular, and cell biological aspects of Chediak-Higashi syndrome. Mol Genet Metab 1999; 68:283-303.
94. Certain S, Barrat F, Pastural E, et al. Protein truncation test of LYST reveals heterogenous mutations in patients with Chediak-Higashi syndrome. Blood 2000; 95:979-83.
95. Bevers EM, Comfurius P, Zwaal RF. Changes in membrane phospholipid distribution during platelet activation. Biochim Biophys Acta 1983; 736:57-66.
96. Tilly RHJ, Senden JMG, Comfurius P, Bevers EM, Zwaal RFA. Increased aminophospholipid translocase activity in human platelets during secretion. Biochim Biophys Acta Bio-Membr 1990; 1029:188-90.
97. Zhou Q, Zhao J, Stout JG, Luhm RA, Wiedmer T, Sims PJ. Molecular cloning of human plasma membrane phospholipid scramblase. A protein mediating transbilayer movement of plasma membrane phospholipids. J Biol Chem 1997; 272:18240-4.
98. Menon AK, Watkins WEr, Hrafnsdottir S. Specific proteins are required to translocate phosphatidylcholine bidirectionally across the endoplasmic reticulum. Curr Biol 2000; 10:241-52.
99. Weiss HJ. Scott syndrome: a disorder of platelet coagulant activity. Semin Hematol 1994; 31:312-9.
100. Fadeel B, Gleiss B, Hogstrand K, et al. Phosphatidylserine exposure during apoptosis is a cell-type-specific event and does not correlate with plasma membrane phospholipid scramblase expression. Biochem Biophys Res Comm 1999; 266:504-11.
101. Stormorken H, Sjaastad O, Langslet A, Sulg I, Egge K, Diderichsen J. A new syndrome: thrombocytopathia, muscle fatigue, asplenia, miosis, migraine, dyslexia and ichthyosis. Clin Genet 1985; 28:367-74.
102. Solum NO. Procoagulant expression in platelets and defects leading to clinical disorders. Arterioscler Thromb Vasc Biol 1999; 19:2841-6.

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# CONGENITAL DEFECTS OF ADP RECEPTORS ON PLATELETS

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ABSTRACT

**Of at least three classes of ADP receptor on platelets, two have been cloned. The first, $P2X_1$, belongs to the ionotropic receptor family; the second, $P2Y_1$, is a seven transmembrane domain receptor associated with Gq. $P2Y_1$ is involved in the mobilization of $Ca^{2+}$ and the shape change of platelets treated with ADP. The third receptor, termed $P2T_{AC}$, is coupled to Gi and mediates inhibition of adenylyl cyclase. It is responsible for macroscopic platelet aggregation. The congenital defect described in the literature concerns this so far uncloned receptor. Patients described by Cattaneo and his colleagues, and by us, have a specifically impaired platelet aggregation to ADP. The intensity of the response is reduced and the aggregation is rapidly reversible at all doses of ADP. A receptor defect was indicated, for while epinephrine normally lowered cAMP levels of $PGE_1$-treated platelets from the patients, ADP was without effect. Another feature is a clear decrease in the number of platelet binding sites for 2-MeS-ADP, a stable analog of ADP. In contrast, shape change and $Ca^{2+}$ mobilization are unaffected, and $P2Y_1$ appears to be normally present. Interestingly, the platelet abnormalities of these patients are paralleled by those induced in normal platelets by the thienopyridine anti-platelet drugs, ticlopidine and clopidogrel. These compounds are widely used in the prevention of thrombotic syndromes in patients with atherosclerotic disease. In this review, we report the principal clinical and biological features of the inherited syndrome associated with abnormal ADP receptors.**


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## Introduction

ADP is an important platelet agonist for thrombus formation *in vivo*, being liberated not only from platelets themselves but also from damaged vascular cells at the site of vessel injury and, perhaps, from shear-affected erythrocytes.[1] At least 3 classes of ADP receptor are now known to be present on platelets, and recently two of them have been characterized and cloned. The first is $P2X_1$, an ATP-gated cation channel, first identified by whole cell patch clamp studies, which mediates $Ca^{2+}$-influx into platelets.[2,3] Somewhat surprisingly $P2X_1$, which has two transmembrane domains, appears not to be required for platelet aggregation.[4] The second receptor to be cloned is $P2Y_1$, a seven transmembrane domain protein, also present in endothelial cells and many types of tissue.[5-7] The principal responses of $P2Y_1$ engagement in platelets are $G_q$ activation, $Ca^{2+}$ mobilization from internal stores and shape change. $P2Y_1$ is coupled to phospholipase C and possibly also to the low molecular weight G protein, RhoA.[8,9] This receptor has a specific antagonist, adenosine-3'-phosphate-5'-phosphate (A3P5P).[10] Notwithstanding these advances, the congenital defect of the ADP-dependent platelet activation pathway described by Cattaneo and his colleagues[11,12] and by us[13,14] concerns another receptor yet to be cloned. This receptor has been termed $P2T_{AC}$. In this review, we analyze the results obtained in studies of this receptor and patients with a unique pathology, which confirm that a full platelet aggregation response to ADP requires signaling through at least two receptor pathways.

## The $P2T_{AC}$ receptor

The receptor responsible for macroscopic platelet aggregation induced by ADP has still not been structurally characterized. This receptor called $P2T_{AC}$ or simply $P_{2T}$ (T=thrombocyte), has been presumed but not proven to be unique to the megakaryocyte lineage. It is coupled to $G_i$ (more specifically $G_{\alpha i2}$) and receptor occupancy leads to inhibition of adenylyl cyclase.[8] A family of competitive antagonists of the $P2T_{AC}$ receptor was studied. These antagonists, modified analogs of adenosine triphosphate (ATP), have been demonstrated to reproduce inhibition of adenylate cyclase, and to inhibit platelet aggregation

induced by ADP.[10,15,16] Antagonists such as AR-C66096 and AR-C67085 are useful since they can be used to inhibit platelet aggregation *in vitro*. They therefore differ from the thienopyridines which are pro-drugs with no activity *in vitro* but which give rise to active metabolites after their metabolism in the liver.[17] AR-C66096 and AR-C67085 have a major inhibitory effect on platelet aggregation without inhibition of platelet shape change and $Ca^{2+}$-mobilization.[10,18,19] Significantly, stimulation through another seven transmembrane domain receptor coupled to $G_i$, as by epinephrine, can correct the inhibitory effect produced by the antagonist AR-C66096 on ADP-induced platelet aggregation.[20]

## The Patients

(i) *Clinical features*: the patient (ML) we studied, is a French male with a bleeding syndrome that has been principally observed after trauma and surgery. The initial diagnosis was made when he was 45 years old, after an unexplained hemoptysis.[13] The patient is now 73 years old, and he has developed no signs of atherosclerosis. His sister, now dead after suffering breast cancer, was similarly affected. The hemorrhagic syndrome was more severe in her case. She had spontaneous episodes of bleeding, menorraghia and prolonged epistaxis throughout her life. The patient (VR) reported by Cattaneo *et al.*[11] is an Italian man who was also diagnosed during adult life despite a lifelong history of excessive bleeding. Thrombin-induced clot retraction was normal in both families.

(ii) *Inheritance*: consanguinity was reported in both families, with the parents being first cousins with no history of bleeding. The daughter of patient ML and that of his affected sister have reported no excessive bleeding, although the daughter of ML had intermediate levels of binding sites for 2-MeS-ADP and appeared to be heterozygous for the disease.[13] From these observations, the inheritance of the defect may be autosomal and recessive.

(iii) *Platelet aggregation in citrated PRP*: a decreased response to ADP in citrated PRP is a common finding in clinical situations. A distinguishing characteristic of the patients described here is the very low maximal intensity of aggregation (less than 30% in citrated PRP even with 10 µM ADP) followed by a rapid and virtually complete disaggregation. Interestingly in patient ML, 100 µM ADP did induce a partial improvement although disaggregation again occurred. The response observed in ML differs from that in citrated PRP from patients with a cyclo-oxygenase defect (or those who have taken aspirin), in whom ADP induces a virtually normal first wave of aggregation even if the second wave of aggregation is inhibited.[21] Classically, in platelet storage pool diseases arising from a decreased dense granule content and/or an abnormal secretory response, the response to collagen is much reduced over a range of concentrations.[22] In both our patient and the Italian one, low doses of collagen gave a significantly reduced response. However this tended to be corrected at higher doses of collagen. A normal number of mepacrine-labeled dense bodies and normal platelet storage pools of ADP and ATP showed that the purported $P2T_{AC}$ deficiency had no influence on ADP storage or dense granule maturation.[13] The response to ristocetin was normal, although waves of aggregation and disaggregation were sometimes observed, thereby resembling the response seen in Glanzmann's thrombasthenia.[23] Epinephrine induced an almost normal aggregation in citrated PRP. Platelets from ML responded to a low concentration of TRAP (thrombin receptor activating peptide) by showing a reduced aggregation, whereas normal results were seen with high doses (such as 50 µM)[13]. Release of secreted ADP has been shown to synergize the platelet response obtained with a $TXA_2$ agonist[20] and recently also with TRAP acting through the PAR-1 receptor.[24]

(iv) *Platelet glycoproteins and fibrinogen binding*: platelets from patient ML possess a normal complement of the major membrane glycoproteins as determined by either flow cytometry or SDS-polyacrylamide gel electrophoresis.[13] In particular, GP IIb-IIIa complexes were present in usual amounts on the platelet surface and GP IIb and GP IIIa showed a normal migration and staining intensity when examined by 2-dimensional electrophoresis. Flow cytometry using FITC-fibrinogen or a rabbit anti-fibrinogen antibody showed that in unstirred suspensions ML's platelets bound small but much reduced amounts of fibrinogen and that binding occurred within one minute of adding the ADP.[13] Interestingly, the fibrinogen binding appeared not to be reversible. The use of a range of monoclonal antibodies (MoAbs) recognizing activation-dependent epitopes on GP IIb-IIIa showed only partial activation of GP IIb-IIIa complexes.[13] These antibodies included (i) PAC-1, which recognizes activated but unoccupied GP IIb-IIIa, (ii) AP-6, an anti-LIBS (ligand-induced binding site) MoAb that recognizes GP IIb-IIIa complexes after fibrinogen has bound, and F26, an anti-RIBS (receptor-induced binding site) MoAb that recognizes fibrinogen exclusively bound to GP IIb-IIIa. More information on these MoAbs and their epitopes is to be found in the paper by Nurden.[25] Typical results using PAC-1, AP-6 and 5H10, a MoAb to P-selectin,[25] are shown in Figure 1.

(v) *Combinations of agonists and washed platelets*: the results of Cattaneo *et al.*[11] suggested that the platelets of patient VR responded even less well to ADP after washing, and we have observed a similar finding for patient ML. This may be because in citrated PRP, the low levels of extracellular $Ca^{2+}$ potentiate the formation of $TXA_2$, which then acts synergistically with ADP. We therefore decided to test different combinations of agonists after the platelets in PRP had been incubated with aspirin as described by Paul *et al.*,[20] then sedimented in the presence of 0.05 U/mL apyrase, 100 nM $PGE_1$ and ACD-A prior to being resuspended in a Tyrode buffer[26] containing 0.05 U/mL apyrase to avoid ADP receptor desensitization. Experiments were performed in order to verify whether synergic activation of Gi or Gq proteins by way of other seven transmembrane domain receptors, could restore the impaired response of the patient's platelets to ADP. Thus Gi was stimulated by epinephrine, whereas Gq was tested by the addition

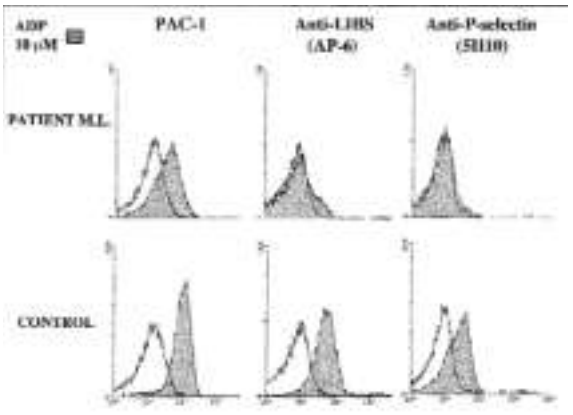


**Figure 1. Flow cytometric analysis of activation-dependent markers on GP IIb-IIIa complexes. Citrated whole blood from patient ML or a control donor (C) was added to tubes containing fibrinogen (for AP-6) and a MoAb to an activation-determinant of platelets (see Figure). Tubes were incubated for 15 min without agitation in the presence (shaded histograms) or not of 10 µM ADP prior to the addition of dichlorotriazinylamino fluorescein conjugated $F(ab')_2$ fragments of donkey anti-mouse IgM or FITC-labeled $F(ab')_2$ fragments of sheep anti-mouse IgG. After 15 min, aliquots (10,000 cells) were analyzed by flow cytometry. Further technical details are given elsewhere.[13] Results showed that GP IIb-IIIa complexes on platelets from ML showed limited activation (PAC-1) and bound little fibrinogen (AP-6). Some α-granule secretion occurred from the control platelets; this was not seen for the patient.**

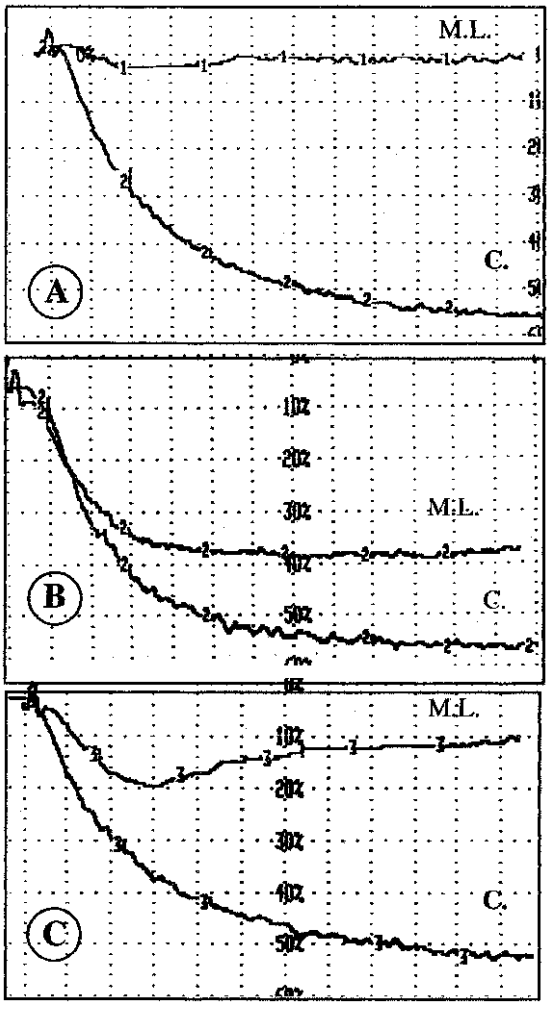


**Figure 2. Platelet aggregation patterns for the patient ML as compared with a control donor (C). Washed platelets were prepared as described by Paul *et al.*[20] and stimulated with 10 µM ADP (A), 10 µM ADP + 1 µM epinephrine (B), and 10 µM ADP + 5 µM 5-hydroxytryptamine (C). Note the substantial correction of the aggregation response of the patient's platelets by epinephrine reacting through Gi and the partial correction by 5-hydroxytryptamine reacting through Gq.**

of serotonin.[20] If the hypothesis that the defective receptor for ADP in this platelet disorder is a seven transmembrane domain receptor coupled to Gi were correct,[27, 28] then these experiments would help in clarifying the defect. Association of a low amount of epinephrine with ADP induced a substantial correction of the aggregation of washed platelets for patient ML (see Figure 2). Somewhat surprisingly, addition of serotonin to ADP also partially corrected the platelet aggregation. The fact that a major correction of the response to ADP was obtained after stimulation of Gi with epinephrine is in agreement with the presence of a genetic defect affecting $P2T_{AC}$. Nevertheless, the partial correction observed with serotonin suggests that the defect in ML's platelets may extend beyond the Gi protein-signaling pathway.

(vi) *Ultrastructural examination of ADP-induced platelet aggregates*: the characteristic morphology of a normal platelet aggregate obtained when platelets are stirred with 10 µM ADP in citrated PRP is illustrated in Figure 3. One feature is the presence of partially degranulated platelets in the center of the aggregate and a ring formed of often entirely degranulated platelets at the periphery. In contrast, aggregates of ML's platelets obtained under identical conditions were small being composed of a few loosely bound and partially degranulated platelets (Figure 4). Pseudopodia were, however, frequently seen, thereby confirming that shape change was occurring. It should be emphasized that the samples were taken at the peak of the aggregation. One possible factor in the disaggregation observed for patient ML is the absence of the ring of degranulated platelets. Secretion of adhesive proteins within the aggregates of normal donors may result in stronger contacts between platelets. Immunogold labeling with AP-6 (an anti-LIBS, see above), or with an anti-fibrinogen antibody, confirmed that activation of the GP IIb-IIIa complex after ADP stimulation was much reduced for patient ML, with there being a much lower number of contact points (and presumably fibrinogen bridges) between platelet.[13,14] Nevertheless, fibrinogen was normally localized to the α-granules in the unstimulated platelets, confirming that the purported $P2T_{AC}$ defect

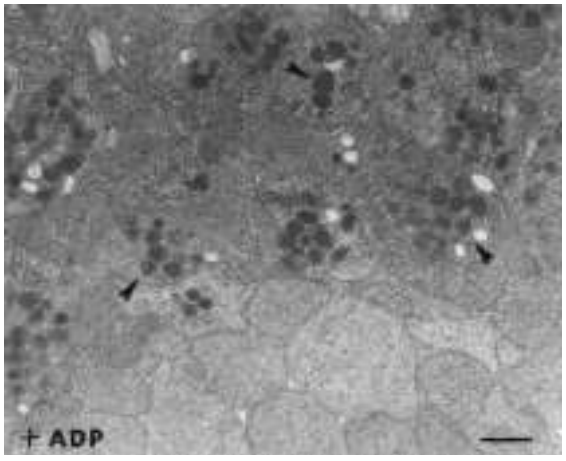


**Figure 3. Electron micrograph showing the typical ultrastructure of a platelet aggregate obtained when citrated PRP from a control subject was incubated with 10 µM ADP in a platelet aggregometer. Samples were fixed at the peak of platelet aggregation and observed by transmission electron microscopy as described elsewhere.[14] Note that platelets at the periphery of the aggregate are degranulated whereas a-granules (arrow heads) can still be seen in some platelets at the center. Bar=1 µm.**

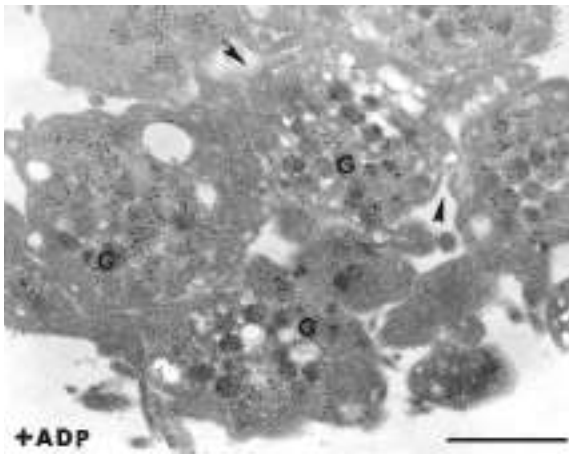


**Figure 4. Electron micrograph showing the typical ultrastructure of a platelet aggregate obtained when citrated PRP from patient ML was incubated with 10 µM ADP in a platelet aggregometer. Samples were fixed at the peak of the aggregation response. Ultrathin sections of samples subsequently embedded in Lowicryl K4M were incubated with rabbit anti-fibrinogen antibody followed by goat anti-rabbit IgG adsorbed on 5-nm gold particles.[13] Note that α-granules (G) are abundantly labeled for fibrinogen but that the platelet surface has few gold particles. Pseudopodia can be seen, but the platelets are loosely bound and have few contact points (arrow heads). Bar=1 µm.**

is of no consequence to fibrinogen uptake and storage by platelets (Figure 4). The reduced number of contact points between platelets may also help to explain why the disaggregation occurred. Interestingly, once dissociated, the patient's platelets returned progressively to a discoid shape. Much decreased fibrinogen and von Willebrand factor binding to the ADP-stimulated platelets of the Italian patient has also been reported.[11,29]

(vii) *Intracellular signaling*: the inability of ADP to lower the $PGE_1$-increased level of platelet cAMP in platelets from these patients showed that no activation of adenylyl cyclase was obtained and that signaling to Gi through $P2T_{AC}$ was not occurring. A similar abnormality was found in the patients described by Cattaneo *et al.*[11] and by us.[13] When platelets from the Italian patient were loaded with Fura 2/AM, treatment of platelets with 10 µM ADP induced an increase in $[Ca^{2+}]i$ although the intensity of the increase was somewhat lower than for control platelets. When platelets from patient ML were stimulated with ADP, $Ca^{2+}$ uptake and mobilization from internal stores were normal (JMP, unpublished results). Tyrosine protein phosphorylation in ML platelets stirred with ADP in the presence of fibrinogen was reduced with little phosphorylation of proteins of 80-85 kDa (cortactin), 100-105 kDa and 125-130 kDa.[30] The latter are aggregation-dependent phosphorylations in normal platelets and are the result of 'outside-in' signaling through GP IIb-IIIa.[31] Our results for platelets from patient ML confirm that full integrin engagement following ADP stimulation requires the $P2T_{AC}$–activation pathway. They may also signify a decreased formation of cytoskeletal complexes in ADP-activated platelets. A normal phosphorylation profile with high doses of thrombin showed that the platelet tyrosine kinases were present and Western blotting showed that all G protein subunits tested (including Gi and $G_q$) were normally located. A surprise result was the weak aggregation of the patient's washed platelets to IBOP, a $TXA_2$ analog.[30] Furthermore, tyrosine protein phosphorylation was decreased with IBOP and cortactin phosphorylation was transient. Identical results to those obtained for the patient with IBOP were seen when normal platelets were incubated with this agonist in the presence of the CP/CPK scavenging system for ADP. Both the aggregation and secretion responses, measured in flow cytometry by P-selectin expression, were decreased at lower doses of TRAP.[13,30] Recently, a role for secreted ADP in platelet aggregation induced by both U 46619 and thrombin has been confirmed.[20,24] More specifically, a role for secreted ADP (and $P2T_{AC}$) in the late activation of phosphoinositide 3-kinase has been established.[24] Thus results from our patient provide a physiologic basis showing that crosstalk between receptor systems on platelets is required for normal hemostasis and confirming the importance of the receptor pathway that is defective in this patient.

(viii) *Quantification of platelet ADP receptors*: the binding of [$^3$H] 2-MeS-ADP decreased from 836±126 molecules per platelet from control subjects to 30±17 molecules per platelet from patient ML.[13] A similar Kd was found for the patient and for the controls. For the Italian patient, comparable experiments with [$^{33}$P] 2-MeS-ADP showed a Kd = 5.4±2.1 nM with a

Bmax = 601±125 sites/platelet for controls and a Kd = 3.9±1.8 nM with a Bmax = 170±70 for the patient.[12] Thus, this patient showed a less severe decrease in the total number of binding sites. In both cases, the defect in agonist binding concerned the number of binding sites but not their affinity. The fact that the binding curves did not show two or more classes of receptor for the controls suggests that $P2T_{AC}$ and $P2Y_1$ are binding 2-MeS-ADP with similar kinetics. In experiments performed with A3P5PS, a selective and competitive antagonist of $P2Y_1$, Savi and his colleagues showed a 27% inhibition of [$^{33}$P] 2-MeS-ADP binding suggesting that P2TAC receptor density is higher than $P2Y_1$.[32] Overall, the above results provide strong evidence for deficient platelet reactivity with ADP in the respective families but do not exclude that patient ML shows additional defects to the Italian patient.

## Other ADP receptors

P2X receptors belong to the family of purinergic receptor channels. The presence of $P2X_1$ has been reported in human platelets, but its precise physiologic role in platelets is not as yet known.[2,3,33] Its structure as an ATP-gated cation channel suggests that it is involved in $Ca^{2+}$-uptake from the extracellular medium. No human pathology has so far been linked to an abnormality of this receptor.

$P2Y_1$ is a member of the seven transmembrane domain family (see Introduction). The use of A3P5P has shown that this receptor in platelets mediates ADP-dependent shape change and $Ca^{2+}$-mobilization from internal stores.[7] Two groups have produced mice 'knock-out' for $P2Y_1$.[34,35] Platelets from these mice show a severely decreased platelet aggregation to ADP, and intracellular $Ca^{2+}$-mobilization and their shape did not change. A very high concentration of ADP (100 µM) induced aggregation without shape change. Nevertheless, ADP-induced inhibition of adenylyl cyclase still occurred. The mice had no apparent bleeding tendency. As expected, aggregation was impaired with other agonists under conditions in which ADP acts synergistically. The results are therefore different from those reported for the French and Italian patients who are the subject of this review. Recently, the $P2Y_1$ receptor was shown to be normally present in the platelets of the Italian patient.[36] An interesting preliminary report concerns a patient with a mild bleeding tendency and occasional weak ADP-induced platelet aggregation.[37] In this patient ADP normally activated Gi, but induced no $Ca^{2+}$-mobilization. Significantly, mRNA for $P2Y_1$ was reduced by an estimated 75%. It will be interesting to learn more about this family. A nucleotide-binding site on GP IIb has been reported.[38] This was localized to an 18-kDa extracellular domain beginning at Tyr198. However, the significance of this finding remains obscure. Platelets from a patient with Glanzmann's thrombasthenia that lacked GP IIb and GP IIIa bound [$^3$H] 2-MeS-ADP with normal kinetics, while PCR amplification and direct sequencing of the cDNA encoding the corresponding region of GP IIb of patient ML showed no changes (J Ruan and A Nurden, unpublished finding).

## $P2T_{AC}$ - partial defects

Cattaneo *et al.*[39] have highlighted a heterogeneous group of platelet abnormalities characterized by the association of a normal primary wave of aggregation to ADP and other agonists, a normal platelet content of granule constituents, a normal production of $TXA_2$, and an intermediate number of binding sites for 2-MeS-ADP. These pathologies were called 'Primary Secretion Defects' (PSD). It was hypothesized that a partial deficiency of the ADP $P2T_{AC}$ receptor permitted primary aggregation but that release of ADP did not occur. According to their hypothesis, an initial release of ADP promotes amplification of the secretion response. In the patients, this did not occur and thus large irreversible macroaggregates did not form. The relationship between this group of patients and those described in this review in whom the ADP functional response of platelets is more severely affected is unclear. The adult daughter of patient ML also had an intermediate number of binding sites for 2-MeS-ADP and the aggregation to ADP of her platelets in citrated PRP was found to be normal although reversible platelet aggregation was once reported when she was a child. Whether the PSD patients are true heterozygotes for a $P2T_{AC}$ gene abnormality will only be answered when the gene has been identified and sequenced.

## Comparison of the platelet congenital defects with the effects of anti-platelet drugs acting against ADP receptors

Orally taken thienopyridines, ticlopidine and clopidogrel give rise to active metabolites that modify the platelet functional response in a way that is very close to that observed for patients with the congenital defects described above.[14] Thus, in both situations, there is a specifically impaired response to ADP, with a decreased intensity of platelet aggregation and aggregates that rapidly dissociate. In neither situation is shape change or $Ca^{2+}$-mobilization affected, whereas ADP-induced inhibition of adenylyl cyclase through Gi is impaired. Interestingly, clopidogrel reduced the binding of 2-MeS-ADP to rat platelets by about 70%.[12,40] However, Savi *et al.*[41] subsequently reported that clopidogrel-treated human platelets showed a more severe decrease with the results being very similar to those seen for patient ML. This apparent difference in the sensitivity of rat and human platelets to clopidogrel remains to be explained. Studies on the tyrosine phosphorylation of rat platelet proteins showed that clopidogrel inhibited the phosphorylation of several proteins in platelets stimulated by 2-MeS-ADP[40] These inhibitions paralleled the inhibition of platelet aggregation. However, the early phosphorylation of cortactin, a process associated with shape change, was not inhibited. Protein kinase C-mediated phosphorylations of plekstrin and myosin light chain kinase were little changed. As already mentioned, a series of antagonists of $P2T_{AC}$ have been developed which are active *in vitro*. They are analogues of ATP, which is a weak, non-selective but competitive $P2T_{AC}$ receptor antagonist[1]. Successive, structural modifications have resulted in molecules with high affinity for $P2T_{AC}$,

such as AR-C69931 (a therapeutically useful analog of AR-C66096), which are potent and selective antagonists.[16] When tested *in vitro* using heparinized PRP[10] or washed platelets,[42] these compounds showed marked, selective inhibition of ADP-induced aggregation.[10,15,42] Their infusion *in vivo* in the rat produced a dose-dependent inhibition of ADP-induced platelet aggregation.[43]

## Conclusions

A hereditary disorder of the platelet response to ADP has clearly been identified, but the receptor responsible for the abnormality has not yet been characterized structurally. This situation contrasts to that for $P2Y_1$; the receptor has been identified, but there is only a preliminary report of a human pathology. Thus the purported defects of $P2T_{AC}$ that are responsible for the platelet abnormalities in the rare patients described above, and which are very similar to those induced in human platelets after subjects have ingested clopidogrel or ticlopidine, require molecular confirmation. Indeed, it is not yet established whether the $P2T_{AC}$ receptor is (i) absent or present in severely decreased amounts, or (ii) it is present but functionally defective. It remains conceivable that the receptor is present but is permanently downregulated or desensitized. Whether the purported functions of $P2T_{AC}$ correspond to a single uncloned receptor or more is also difficult to determine. If $P2T_{AC}$ is a seven transmembrane receptor coupled to Gi, what is the functional role (if any) of the inhibition of adenylate cyclase? Early studies suggested that cAMP was not involved.[1] More specifically, direct inhibitors of adenylate cyclase such as SQ22536 do not induce platelet aggregation or restore the response to ADP from animals pretreated with clopidogrel.[43] An alternative explanation is that whereas the Gi $\alpha$-subunit gives a functional measure of $P2T_{AC}$ activity, the signal that leads to platelet aggregation is transmitted by the Gi $\beta\gamma$ dimer. Thus an ADP-mediated activation pathway probably remains to be identified. The involvement of another G protein cannot be excluded, although mice deficient in $G_q$ showed much more extensive platelet activation defects than those reported for the patients described here.[44] Many interesting questions wait to be resolved, not least of these being whether these patients will prove to be protected against atherosclerosis or arterial thrombosis. Significantly, the Italian patient also showed a defective shear-induced platelet aggregation,[45] confirming that ADP plays an important role in a process that may have a key role in the development of coronary artery thrombosis.

## References


1. Mills DCB. ADP receptors on platelets. Thromb Haemost 1997; 76:835-56.
2. MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of receptor-operated cation channels via P2X1 not P2T purinoceptors in human platelets. J Biol Chem 1996; 271:2879-81.
3. Sun B, Li J, Okahara K, Kambayashi JI. P2X1 purinoceptor in human platelets. Molecular cloning and functional characterization after heterologous expression. J Biol Chem 1998; 273:11544-7.
4. Takano S, Kimura J, Matsuoka I, Ono T. No requirement for P2X1 purinoreceptors for platelet aggregation. Eur J Pharmacol 1999; 372:305-9.
5. Leon C, Vial C, Cazenave JP, Gachet C. Cloning and sequencing of a human cDNA encoding endothelial P2Y1 purinoceptor. Gene 1996; 171:295-7.
6. Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. J Biol Chem 1998; 273:2030-4.
7. Hechler B, Leon C, Vial C, et al. The P2Y1 receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. Blood 1998; 92:152-9.
8. Gachet C, Hechler B, Leon C, et al. Activation of ADP receptors and platelet function. Thromb Haemost 1997; 78:271-5.
9. Boeynaems JM, Communi D, Savi P, Herbert JM. P2Y receptors: in the middle of the road. Trends Pharmacol Sci 2000; 21:1-3.
10. Jarvis GE, Humphries RG, Robertson MJ, Leff P. ADP can induce aggregation of human platelets via both P2Y1 and P2T receptors. Br J Pharmacol 2000: 129: 275-82.
11. Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. Blood 1992; 80:2787-96.
12. Gachet C, Cattaneo M, Ohlmann P, et al. Purinoceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors. Br J Haematol 1995; 91:434-44.
13. Nurden P, Savi P, Heilmann E, et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function. J Clin Invest 1995; 95: 1612-22.
14. Humbert M, Nurden P, Bihour C, et al. Ultrastructural studies of platelet aggregates from human subjects receiving clopidogrel and from a patient with an inherited defect of an ADP-dependent pathway of platelet activation. Arterioscler Thromb Vasc Biol 1996; 16: 1532-43.
15. Humphries RG, Tomlinson W, Ingall AH, Cage PA, Leff P. FPL 66096: a novel highly potent and selective antagonist at human platelet P2T-purinoreceptors. Br J Pharmacol 1994; 113:1057-63.
16. Ingall AH, Dixon J, Bailey A, et al. Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. J Med Chem 1999; 42:213-20.
17. Sharris PJ, Cannon CP, Loscalzo J. The antiplatelet effects of ticlopidine and clopidogrel. Ann Intern Med 1998; 129:394-405.
18. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci USA 1998; 95:8070-4.
19. Fagura MS, Dainty IA, McKay G, et al. P2Y1-receptors in human platelets which are pharmacologically distinct from P2YADP-receptors. Br J Pharmacol 1998; 124:157-64.
20. Paul BZ, Jin J, Kunapuli SP. Molecular mechanism of thromboxane A2-induced platelet aggregation. Essential role for $P2T_{ac}$ and $alpha_{2a}$ receptors. J Biol Chem 1999; 274:29108-14.
21. Rinder CS, Student LA, Bonan JL, Rinder HM, Smith BR. Aspirin does not inhibit adenosine diphosphate-induced platelet $\alpha$-granule release. Blood 1993; 82: 505-12.

22. Weiss HJ. Inherited abnormalities of platelet granules and signal transduction. In Colman RW, Hirsh J, Marder VJ, Salzman EW (eds) Hemostasis and Thrombosis. Basic Principles and Clinical Practice (3rd edition), JB Lippincott, Philadelphia, 1994; 673-84.
23. Nurden AT. Inherited abnormalities of platelets. Thromb Haemost 1999; 82:468-80.
24. Trumel C, Payrastre B, Plantavid M, et al. A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. Blood 1999; 94:4156-65.
25. Nurden P. Bidirectional trafficking of membrane glycoproteins following platelet activation in suspension. Thromb Haemost 1997; 78:1305-15.
26. Nurden P, Poujol C, Durrieu-Jais B, et al. Labeling of the internal pool of GP IIb-IIIa in platelets by c7E3 Fab fragments (abciximab): flow and endocytic mechanisms contribute to the transport. Blood 1999; 93: 1622-33.
27. Cattaneo M, Gachet C. ADP receptors and clinical bleeding disorders. Arterioscler Thromb Vasc Biol 1999; 19:2281-5.
28. Kunapuli SP. Multiple P2 receptor subtypes on platelets: a new interpretation of their function. TIPS 1999; 19:391-4.
29. Cattaneo M. Hereditary defect of the platelet ADP receptor(s). Platelets 1998; 9:161-4.
30. Levy-Toledano S, Maclouf J, Rosa J, et al. Abnormal tyrosine phosphorylation linked to a defective interaction between ADP and its receptor on platelets. Thromb Haemost 1998; 80:463-8.
31. Shattil SJ, Kashiwagi H, Pampori N. Integrin signalling: the platelet paradigm. Blood 1998; 91:2645-57.
32. Savi P, Beauverger P, Labouret C, et al. Role of P2Y1 purinoceptor in ADP-induced platelet activation. FEBS Lett 1998; 422:291-5.
33. Clifford EE, Parker K, Humphreys BD, Kertesy SB, Dubyak GR. The P2X1 receptor, an adenosine triphosphate-gated cation channel, is expressed in human platelets but not in human blood leukocytes. Blood 1998; 91:3172-81.
34. Leon C, Hechler B, Freund M, et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. J Clin Invest 1999; 104:1731-42.
35. Fabre JE, Nguyen M, Latour A, et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. Nat Med 1999; 5:1199-202.
36. Leon C, Vial C, Gachet C, et al. The P2Y1 receptor is normal in a patient presenting a severe deficiency of ADP-induced platelet aggregation. Thromb Haemost 1999; 81:775-81.
37. Oury C, Lenaerts T, Peerlinck K, Vermylen J, MF Hoylaerts. Congenital deficiency of the phospholipase C coupled platelet P2Y1 receptor leads to a mild bleeding disorder. Thromb Haemost (Suppl) Aug 1999; 20.
38. Greco NJ, Yamamoto N, Jackson BW, Tandon NN, Moos M Jr, Jamieson GA. Identification of a nucleotide-binding site on glycoprotein IIb. Relationship to ADP-induced platelet activation. J Biol Chem 1991; 266:13627-33.
39. Cattaneo M, Lombardi R, Zighetti ML, et al. Deficiency of (33P)2MeS-ADP binding sites on platelets with secretion defect, normal granule stores and normal thromboxane A2 production. Evidence that ADP potentiates platelet secretion independently of the formation of large platelet aggregates and thromboxane A2 production. Thromb Haemost 1997; 77:986-90.
40. Savi P, Artcanuthurry V, Bornia J, et al. Effect of clopidogrel treatment on ADP-induced phosphorylations in rat platelets. Br J Haematol 1997; 97:185-91.
41. Savi P, Heilmann E, Nurden P, et al. Clopidogrel, an antithrombotic drug acting on the ADP-dependent activation pathway of human platelets. Clin Appl Thrombosis Hemostasis 1996; 2:35-42.
42. Humphries RG, Tomlinson W, Clegg JA, Ingall AH, Kindon ND, Leff P. Pharmacological profile of the novel P2T-purinoceptor antagonist, FLP 67085 in vitro and in the anaesthetized rat in vivo. Br J Pharmacol 1995; 115:1110-6.
43. Daniel JL, Dangelmaier C, Jin J, Kim YB, Kunapuli SP. Role of intracellular signaling events in ADP-induced platelet aggregation. Thromb Haemost 1999; 82: 1322-6.
44. Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in G alpha$_q$-deficient mice. Nature 1997; 389:183-6.
45. Cattaneo M, Zighetti ML, Lombardi R, Mannucci PM. Role of ADP in platelet aggregation at high shear: studies in a patient with congenital defect of platelet responses to ADP. Br J Haematol 1994; 88:826-9.

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# HUMAN ECTO-ADPASE / CD39: THROMBOREGULATION VIA A NOVEL PATHWAY

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ABSTRACT

**Vascular injury in coronary, carotid, and peripheral arteries evokes local platelet activation, recruitment and thrombotic occlusion. Platelets are unresponsive to agonists in the presence of endothelial cells, even in the absence of eicosanoids and nitric oxide. We have characterized endothelial cell CD39 / ecto-ADPase as the prime thromboregulator. CD39 rapidly and preferentially metabolizes ADP released from activated platelets, thereby abolishing aggregation and recruitment. Our recombinant, soluble form of human CD39, solCD39, a glycosylated 66 kD protein, possesses the same enzymatic and biological properties as full-length CD39. SolCD39 blocked ADP-induced human platelet aggregation *in vitro*, and inhibited collagen- and TRAP-induced platelet reactivity. SolCD39 was studied *in vivo* in a murine stroke model driven by excessive platelet recruitment. In CD39 +/+ mice, solCD39 completely abolished ADP-induced platelet aggregation, and strongly inhibited collagen- and arachidonate-induced aggregates *ex vivo*. When administered prior to transient intraluminal right middle cerebral artery occlusion, solCD39 reduced ipsilateral fibrin deposition, decreased $^{111}$In-platelet deposition, and increased post-ischemic blood flow two-fold at 24 hr. These results were better than those obtained with aspirin. CD39 –/– mice, generated by deleting exons 4-6 (apyrase conserved regions 2-4), had normal phenotypes, hematologic profiles and bleeding times, but exhibited a decrease in post-ischemic perfusion and an increase in cerebral infarct volume as compared to genotypic CD39 +/+ controls. CD39 –/– mice, reconstituted with solCD39, had increased post-ischemic flow and were rescued from cerebral injury. We conclude that solCD39 has potential as a novel therapeutic agent for thrombotic diatheses.**



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## Introduction

Cell-cell interactions and cell-vessel wall interactions are of critical importance for hemostasis. Many of these interactions occur via *transcellular metabolism*, a locution that indicates reciprocal or collaborative metabolism of signaling molecules by different cells. This is particularly pertinent in the case of endothelial cells and platelets. We currently believe that endothelial cells downregulate platelet reactivity via at least three different pathways: a cell-associated aspirin-insensitive nucleotidase,[1] and two independent short-lived fluid-phase signaling systems - eicosanoids such as prostacyclin ($PGI_2$);[2] and the nitric oxide (NO) system.[3] In 1991, we documented that platelet reactivity remained inhibited by endothelial cells under experimental conditions which rendered NO ineffective, even when both cell types were aspirin-treated, to delete $PGI_2$ from the system. Using biochemical and functional measurement techniques, we determined that aspirin-treated human umbilical vein endothelial cells (HUVEC) inhibited platelet function *in vitro* largely via metabolism of ADP from the *releasate* generated by platelets activated by a variety of agonists. This metabolism of ADP resulted in loss of platelet activation, release, recruitment, and aggregation.[1] This paradigm of platelet inhibition is unique in that it does not interfere with platelet function except for removal of the soluble phase agonist responsible for excessive platelet activation and recruitment. Such conditions would otherwise promote thrombosis. Our data suggest that enhancing the activity of this pathway has a strong antithrombotic action, without significantly reducing the hemostatic effectiveness of platelets.

## Identification of CD39 as the endothelial cell ecto-ADPase responsible for Inhibition of platelet function

We initially identified the ability of endothelial cells to inhibit platelet reactivity via metabolism of ADP, rather than via eicosanoids or NO: aspirin-treated HUVEC were incubated with radio-labeled ADP. Under these conditions, no $PGI_2$ was formed, and any NO generated rapidly decayed or was blocked by addition of purified oxyhemoglobin. Radio-TLC was employed to separate and identify ADP and its metabolites (Figure 1). Cell-free supernatant from the incubation of HUVEC with ADP was transferred to aggregometry cuvettes containing platelet-rich

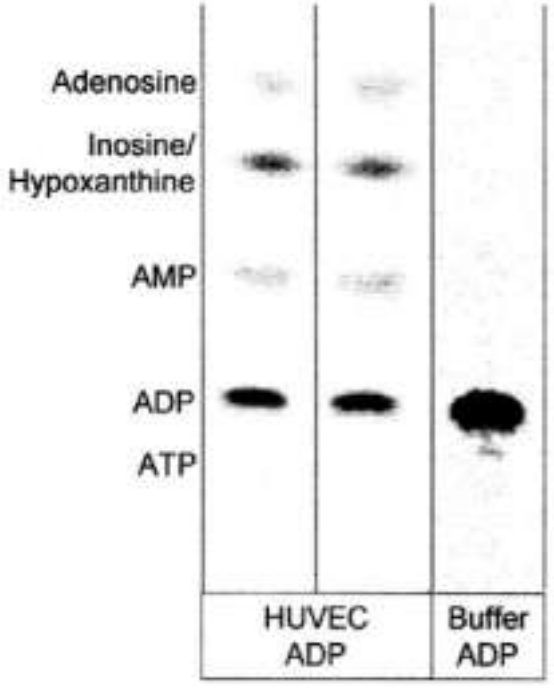


**Figure 1. UVEC metabolism of ADP. HUVEC were incubated for 5 min with 50 µM [$^{14}$C]-ADP. ADP metabolites were separated by radio-TLC. The activity of 5'nucleotidase, as well as adenosine deaminase is inferred from the rapid appearance of adenosine and inosine in these and other scans. COS cells do not possess ecto-ATPase, -ADPase or 5'nucleotidase (data not shown). Was performed with An InstantImager® (Packard Instrument Co., Meriden, CT, USA) were used for detection and quantification.**

plasma (PRP) to result in addition of 5 µM ADP if the ADP had not been metabolized. However, the data demonstrated that $^{14}$C-ADP and induction of platelet activation decreased concurrently and rapidly. In addition, AMP, accumulated transiently, was further metabolized to adenosine and then deaminated to inosine (Figure 1).[1] We next sought to identify the molecule(s) responsible for this presumed ADPase activity. Initially, we established that the HUVEC ADPase was a membrane-associated ecto-nucleotidase of the E-type.[4] Characteristics of this enzyme included Ca/Mg dependence, ineffectiveness of specific inhibitors of P-, F-, and V-type ATPases, and the capacity to metabolize both ATP and ADP, but not AMP. Such properties identified the HUVEC enzyme as an apyrase (ATP diphosphohydrolase), ATPDase, EC3.6.1.5.[4] At the time, ecto-nucleotidase research was severely hindered by difficulties in protein isolation. This was due to the low abundance of the protein(s) in a setting of high enzyme activity and their sensitivity to denaturing agents.[4-7] The HUVEC enzyme is indeed an example of this.

A new nomenclature has recently been proposed to unify usage in this rapidly evolving and broadening field.[8] According to this nomenclature, HUVEC ecto-ADPase/CD39 is human E-NTPDase-1.

In 1996, a soluble apyrase was purified from potato tubers, and its cDNA cloned.[9] Sequence analysis revealed 25% amino acid identity and 48% amino acid homology with human CD39.[9] CD39 had been cloned as a cell-surface glycoprotein,[10] expressed on activated B-cells, NK cells, and subsets of T-cells as well as on some HUVEC cell lines.[11] Nucleotidases with homology to CD39 and potato apyrase are expressed throughout nature in species as varied as the garden pea, *C. elegans* and *Toxoplasma*.[9] Interestingly, at least 4 regions within these molecules had extraordinary homology, and were designated apyrase conserved regions (ACR).[9] From these reports, we proposed that HUVEC ADPase is identical to CD39.[12] This was based on the following observations: more than 95% of the ADPase activity from an ADPase preparation purified from HUVEC membranes can be immunoprecipitated with any of several anti-human CD39 antibodies. Confocal microscopy and indirect immunofluorescence studies localized CD39 to the HUVEC cell surface. Most importantly, when we transfected COS cells with a vector containing the cDNA for either human or murine CD39, we could demonstrate expression of both CD39 and ecto-ADPase activity on the COS cell surface. Polymerase chain reaction (PCR) analyses using either authentic human CD39 cDNA or cDNA synthesized from HUVEC mRNA resulted in products of identical size for each of four different CD39-specific primer pairs. Sequencing of the PCR products confirmed their identity in each instance. The PCR products encompassed 75% of the coding region of CD39, including the original 4 ACR (apyrase domain, Figure 2). In addition, Northern analyses demonstrated that HUVEC and MP-1 cells (from which CD39 was originally cloned) contained same sized messages for CD39. Protein purification studies of ecto-ATPDases from different cell sources were reported from other laboratories as well.[13,14] Of critical importance were experiments in which COS cells transfected with human CD39 cDNA acquired the ability to block ADP-induced platelet aggregation.[12] This occurred with COS cells transfected with either human or murine CD39. Transfectants metabolized ADP to AMP within 3 minutes. These observations are especially pertinent to the time frame of events leading to formation of a hemostatic platelet plug or thrombus. We know that platelet adhesion to injured subendothelium leads to immediate release of ADP and recruitment of additional platelets to form an occlusive thrombus in less than 4 minutes. This chronology parallels the time-course we observed for platelet inhibition by CD39-expressing cells and was also commensurate with their respective ADPase activities (Figure 3). These results amplify the importance of CD39 as a thromboregulator. They also represent the first direct demonstration of a physiological function for CD39 as an ADPase, i.e. blockade of platelet responsiveness to the prothrombotic agonist ADP via its metabolism to AMP. This phenomenon might represent evolution of an endothelial mechanism targeted toward metabolism of prothrombotic platelet-derived ADP in preference to ATP, thereby controlling excessive platelet accumulation. The biological properties of CD39 suggested a novel strategy for therapeutic intervention. While aspirin treat-

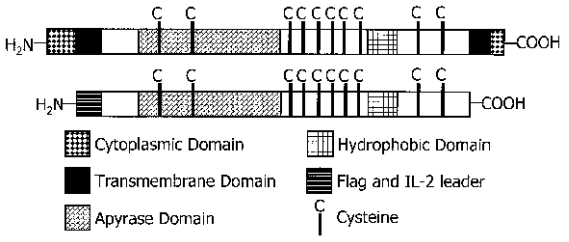


**Figure 2. Domain structure of ecto-ADPase/CD39. Two transmembrane regions are located near the amino and carboxy termini; a hydrophobic sequence is centrally located. The putative apyrase conserved region (ACR) is shown on the left side as apyrase domain, adjacent to the N-terminal portion. Cysteine residues are marked as C. The engineered soluble form of CD39, containing a Flag tag and IL-2 secretion leader, and lacking the two transmembrane regions, is presented below for comparison.[19]**

ment controls the prothrombotic action of thromboxane, it also prevents formation of the antithrombotic eicosanoid, prostacyclin, thereby limiting the effectiveness of aspirin. Aspirin is also a relatively promiscuous acetylating agent, as we demonstrated in 1970,[15] with undesirable side effects. Another endothelial thromboregulator, NO, is an aspirin-insensitive inhibitor of platelet function. However, it is inhibited *in vitro* and *in vivo* by hemoglobin following its rapid diffusion into erythrocytes,[16,17] or reaction with albumin.[18] Importantly, CD39 is aspirin-independent, and completely inhibits platelet reactivity even when eicosanoid formation and NO production are blocked. Based on the observation that ADPase/CD39 is an effective physiologic and constitutively expressed endothelial cell inhibitor of platelet reactivity, we postulated that a soluble form of the human enzyme might represent a promising new antithrombotic modality that could be evaluated *in vivo* and *ex vivo*.

## Inhibition of platelet reactivity by recombinant soluble ecto-ADPase/CD39 (solCD39)

We hypothesized that a soluble form of human CD39, retaining nucleotidase activities, could constitute a new antithrombotic agent to be administered to patients with a low threshold for platelet activation. A recombinant, soluble form of human CD39 was designed based upon the structure of CD39 (Figure 2). CD39 contains two putative transmembrane regions near the amino and carboxyl termini, respectively. These serve to anchor the native protein in the cell membrane. Modeling studies, antibody epitope analyses and sequence homology demonstrated that the portion of the molecule between the transmembrane regions is external to the cell.[10] The extracellular region contains the 4 ACR characteristic of members of the apyrase family, in concordance with the notion that the external portion of CD39 is critical for its ecto-ADPase activity. To generate a soluble form of CD39, the extracellular domain, encoding 439 amino acids, was isolated using oligonucleotide cassettes and PCR and placed

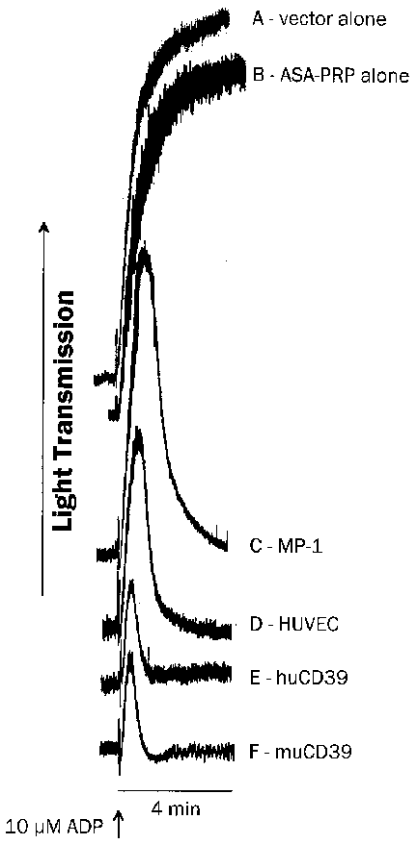


**Figure 3. Blockade and reversal of platelet aggregation to ADP by intact HUVEC, MP-1 cells (an activated B-cell-line[10]), and COS cells transfected with full-length human or murine CD39. PRP from a donor who had ingested aspirin was stimulated with 10 µM ADP (A) in the presence of COS cells transfected with *empty* vector; (B) in the absence of any additions; and in the presence of (C) MP-1 cells, (D) HUVEC, and COS cells transfected with (E) human CD39 or (F) murine CD39. Expression of CD39 led to metabolism of ADP in the platelet *releasate*, and acquisition of platelet inhibitory activity.[1]**

in a mammalian expression vector.[19] Secretion of the recombinant molecule was ensured by addition of the IL-2 leader sequence. Following transfection with this solCD39-encoding plasmid, COS cells generated levels of ATPase and ADPase activity in their conditioned medium which increased linearly with time for a 5-day period. No nucleotidase activity was generated following transfection with an empty or a truncated vector. SolCD39 was isolated from conditioned media derived from transiently transfected COS cells using immunoaffinity column chromatography using an anti-CD39 monoclonal antibody, and yielded a single ~66 kD protein with both ATPase and ADPase activities. This suggested that the molecule was properly glycosylated in this cell system, and this is supported by the remarkable stability of the enzyme both *in vitro* and *in vivo*. Incubation of the purified protein with N-glycanase to remove N-linked oligosaccharides yielded a band with the predicted mole-

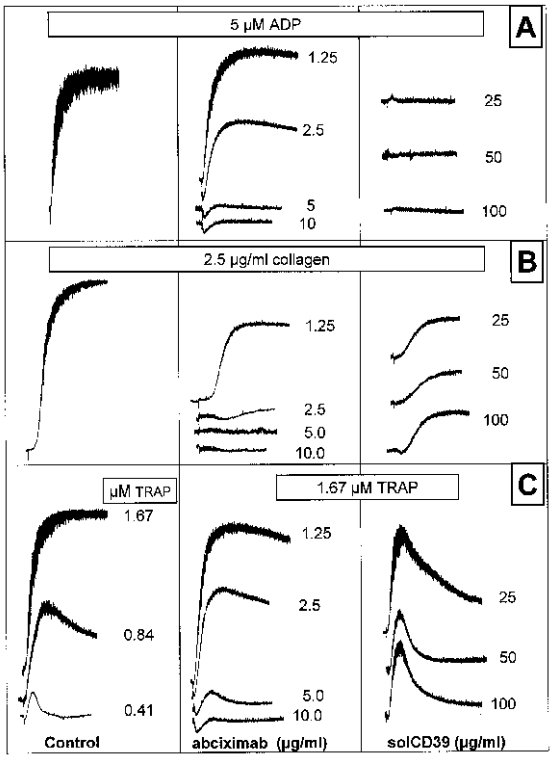


**Figure 4. Inhibition and reversal of platelet aggregation. PRP from a donor who had ingested aspirin was stimulated with 5 µM ADP, 2.5 µg/mL collagen (Chrono-Log), or TRAP6 as indicated. *In vitro* platelet responses to these agonists were strongly inhibited by both abciximab and solCD39.**

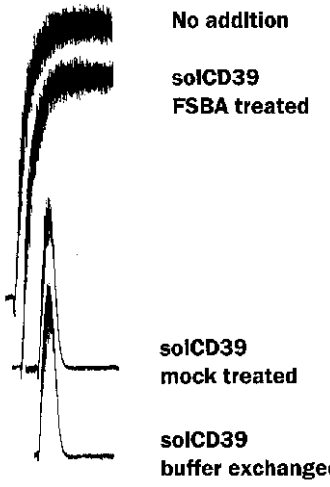


**Figure 5. SolCD39 is inactivated by FSBA (5'-p-fluorosulfonylbenzoyladenosine, an ATP analog which binds irreversibly to several ATPDases). ASA-treated PRP was incubated with 4.4 µg/mL solCD39, pre-treated with FSBA, or without FSBA (mock). Platelets were stimulated with 10 µM ADP, and the aggregation response recorded. A full aggregation response was obtained without added FSBA as well as with FSBA-treated solCD39, but mock-FSBA-treated solCD39 inhibited ADP-induced aggregation to a similar extent as untreated, buffer-exchanged solCD39.[19]**

cular weight of 52 kDa.[19] Purified solCD39 blocked ADP-induced platelet aggregation *in vitro* and inhibited collagen-induced platelet reactivity.[19] In more recent experiments, aggregation induced by the thrombin receptor activation peptide (TRAP) was also strongly blocked by CD39 (Figure 4). Based on these data we postulate that collagen and TRAP depend more on released ADP for recruitment and aggregation than previously appreciated. A modified CHO cell-based solCD39 expression system was developed to increase protein production. Such production could be maintained as a stably expressing line and could be grown in defined, serum-free medium to facilitate protein purification. Thus, the conditioned medium from these CHO cells contained 20-fold more ATPase and ADPase activity than that from COS cells.[19] Following administration of solCD39 to mice, enzyme activity was measurable for an extended period of time. The elimination phase half-life was ~2 days.[19] The ability of sol CD39 to inhibit platelet activation was due to the enzymatic activity of solCD39 and not to a *covering up* of some site for essential for platelet responsiveness to ADP. This was demonstrated by reacting solCD39 with FSBA (5'-p-fluorosulfonyl-benzoyl-adenosine), an ATP analog that blocks collagen-induced platelet activation and reacts irreversibly with ATPDases found in several cell types. Incubation of platelets with FSBA-treated sol-

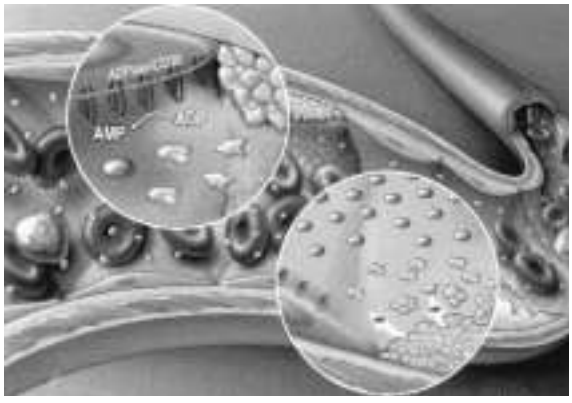


**Figure 6. Schematic depiction of thromboregulation by endothelial cell ecto-ADPase/CD39. Platelet activation on or proximal to a site of vascular injury induces release of ADP from platelet dense granules (inset lower right). Released ADP activates and thereby recruits additional platelets which have arrived into the local microenvironment in the evolving thrombus. Activation and recruitment of platelets in proximity to endothelial cells is inhibited by metabolism of released ADP to AMP by endothelial cell ecto-ADPase/CD39. CD39 does not act on the platelet *per se*, but on the platelet "releasate". These platelets then return to an unstimulated state, thereby limiting thrombus formation (inset upper left). Ecto-ADPase/CD39 has been identified and functionally characterized as a physiologic, constitutively expressed thromboregulator.[1]**

CD39 prevented inhibition of platelet reactivity to ADP.[19] The extent of inactivation of the enzymatic activity of solCD39 paralleled the loss of platelet inhibitory activity (Figure 5).[19]

## Conclusions

Our data, obtained with a novel, soluble form of recombinant human ecto-ADPase, solCD39, indicates potential for a new class of antithrombotic agent acting via metabolism of a critical mediator. SolCD39 blocks and reverses platelet activation, preventing recruitment of additional platelets into a growing thrombus. In this manner, the extent of occlusion as well as vascular wall damage during and immediately after cardio- and cerebrovascular events such as stroke, myocardial infarction, angioplasty, and stenting might largely be prevented (Figure 6). Importantly, because of its independent mode of action, solCD39 could be combined with currently utilized therapeutic agents, including heparin, aspirin, and GPIIb/IIIa antagonists. Future plans include generation of dose-response curves in mammalian stroke and coronary artery disease models and, subsequently, initiation of phase I toxicity studies in man.

## References


1. Marcus AJ, Safier LB, Hajjar KA, et al. Inhibition of platelet function by an aspirin-insensitive endothelial cell ADPase. Thromboregulation by endothelial cells. J Clin Invest 1991; 88:1690-6.
2. Marcus AJ, Weksler BB, Jaffe EA, Broekman MJ. Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. J Clin Invest 1980; 66:979-86.
3. Broekman MJ, Eiroa AM, Marcus AJ. Inhibition of human platelet reactivity by endothelium-derived relaxing factor from human umbilical vein endothelial cells in suspension. Blockade of aggregation and secretion by an aspirin-insensitive mechanism. Blood 1991; 78:1033-40.
4. Plesner L. Ecto-ATPases: identities and functions. Int Rev Cytol 1995; 158:141-214.
5. Côté YP, Filep JG, Battistini B, Gauvreau J, Sirois P, Beaudoin AR. Characterization of ATP-diphosphohydrolase activities in the intima and media of the bovine aorta. Biochim Biophys Acta Mol Basis Dis 1992; 1139:133-42.
6. Strobel RS, Nagy AK, Knowles AF, Buegel J, Rosenberg MD. Chicken oviductal ecto-ATP-diphosphohydrolase – purification and characterization. J Biol Chem 1996; 271:16323-31.
7. Vasconcelos EG, Ferreira ST, de Carvalho TMU, et al. Partial purification and immunohistochemical localization of ATP diphosphohydrolase from *Schistosoma mansoni*. Immunological cross-reactivities with potato apyrase and *Toxoplasma gondii* nucleoside triphosphate hydrolase. J Biol Chem 1996; 271:22139-45.
8. Zimmermann H, Beaudoin AR, Bollen M, et al. Proposed nomenclature for two novel nucleotide hydrolyzing enzyme families expressed on the cell surface. In: Vanduffel L, Lemmens R, eds. Ecto-ATPases and Related Ectonucleotidases. Proceedings of the Second International Workshop on Ecto-ATPases and Related Ectonucleotidases. Maastricht, The Netherlands: Shaker Publishing, 2000:1-8.
9. Handa M, Guidotti G. Purification and cloning of a soluble ATP-diphosphohydrolase (apyrase) from potato tubers (*Solanum tuberosum*). Biochem Biophys Res Commun 1996; 218:916-23.
10. Maliszewski CR, Delespesse GJ, Schoenborn MA, et al. The CD39 lymphoid cell activation antigen. Molecular cloning and structural characterization. J Immunol 1994; 153:3574-83.
11. Kansas GS, Wood GS, Tedder TF. Expression, distribution, and biochemistry of human CD39. Role in activation-associated homotypic adhesion of lymphocytes. J Immunol 1991; 146:2235-44.
12. Marcus AJ, Broekman MJ, Drosopoulos JHF, et al. The endothelial cell ecto-ADPase responsible for inhibition of platelet function is CD39. J Clin Invest 1997; 99:1351-60.
13. Kaczmarek E, Koziak K, Sévigny J, et al. Identification and characterization of CD39 vascular ATP diphosphohydrolase. J Biol Chem 1996; 271:33116-22.
14. Christoforidis S, Papamarcaki T, Galaris D, Kellner R, Tsolas O. Purification and properties of human placental ATP diphosphohydrolase. Eur J Biochem 1995; 234:66-74.
15. Al-Mondhiry H, Marcus AJ, Spaet TH. On the mechanism of platelet function inhibition by acetylsalicylic acid. Proc Soc Exp Biol Med 1970; 133:632-6.
16. Olsen SO, Tang DB, Jackson MR, Gomez ER, Ayala B, Alving BM. Enhancement of platelet deposition by cross-linked hemoglobin in a rat carotid endarterectomy model. Circulation 1996; 93:327-32.
17. Marcus AJ, Broekman MJ. Cell-free hemoglobin as an oxygen carrier removes nitric oxide, resulting in defective thromboregulation. Circulation 1996; 93:208-9.
18. Stamler JS, Jaraki O, Osborne J, et al. Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin. Proc Natl Acad Sci USA 1992; 89:7674-7.
19. Gayle RB, III, Maliszewski CR, Gimpel SD, et al. Inhibition of platelet function by recombinant soluble ecto-ADPase/CD39. J Clin Invest 1998; 101:1851-9.

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# Pharmacology of the platelet ADP receptors: agonists and antagonists

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ABSTRACT

**The effects of purine nucleotides on platelets have been known since the 1960s, when Born demonstrated aggregation induced by ADP and inhibited by ATP. However, the mechanism of action of ADP is not fully understood, and there has been controversy about the number of ADP receptors on platelets. ADP causes shape change, aggregation, mobilization of calcium from intracellular stores, rapid calcium influx and inhibition of adenylate cyclase, and the relationship between these is becoming clearer. Two cloned P2 receptors have been detected on platelets, one a cation channel ($P2X_1$), the other G protein-coupled ($P2Y_1$), and a third G protein-coupled receptor ("$P2Y_{AC}$") may also exist. The $P2X_1$ receptor is responsible for rapid calcium influx and is activated by ATP as well as by ADP, but is likely to be desensitized under normal experimental conditions and its role is uncertain. The $P2Y_1$ receptor is thought to be responsible for calcium mobilization, shape change and the initiation of aggregation, while the $P2Y_{AC}$ receptor is responsible for inhibition of adenylate cyclase and is required for full aggregation. The structure-activity relationships for agonists and antagonists at these receptors have been investigated to a limited extent, and while in general they are similar some differences do exist. In particular, 2-alkylthio-substituted analogues of ATP and AMP are selective competitive antagonists at the $P2Y_{AC}$ receptor, 3'-substituted AMP analogs are selective $P2Y_1$ receptor antagonists and the phosphorothioate analogs of ADP have a lower efficacy for the $P2Y_{AC}$ receptor than for the $P2Y_1$ receptor.**


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## Introduction

It has been known for many years that ADP aggregates platelets and that this aggregation is competitively inhibited by ATP, so these naturally-occurring nucleotides have been regarded as the archetypal agonist and antagonist respectively at platelet ADP receptors.[1,2] Indeed, the $P_{2T}$ receptor was originally defined on this basis and thought to be different from the other known P2 receptors in that ATP was an antagonist rather than an agonist.[3] The structure-activity relationships of this receptor have been extensively investigated by looking at the effect on platelet aggregation of a wide range of analogs of ADP and ATP or AMP (also a weak antagonist). In general it has been found that analogs substituted at the 2 position of the adenine ring (for example 2-methylthioadenosine 5'-diphosphate; 2-MeS-ADP) retain activity and in some cases are more potent than the unsubstituted endogenous compounds, while alterations of the ribose ring or the phosphate chain (for example replacement of a bridging oxygen with a methylene group, as in adenosine 5'-(α,β-methylene)-diphosphonate); α,β-me-ADP) in general reduce activity and substitutions at the 8 or $N^6$ positions abolish activity.[4,5] Overall these structure-activity relationships are most similar to those of the functionally-defined $P_{2Y}$ receptor,[5,6] now known to be the cloned $P2Y_1$ receptor.[7,8]

## Comparison of aggregation and inhibition of adenylate cyclase

Even in 1985 when the $P_{2T}$ receptor was so named, it was a matter of controversy whether all the effects of ADP on platelets were indeed mediated by a single receptor or whether more than one existed.[4,9,10,11] In particular, interest focused on whether the ability of ADP to inhibit adenylate cyclase was mediated by a different receptor from that by which ADP induced the functional effects of shape change and aggregation. Two lines of evidence first suggested that there might be two ADP receptors on platelets, one concerning the effects of agonists and the other the effects of non-competitive inhibitors of the effects of ADP. With regard to agonists, it was reported that two analogs of ADP substituted at the 2-position of the adenine ring, 2-azidoadenosine 5'-diphosphate (2-azido-ADP) and 2-MeS-ADP, were more potent

as inhibitors of adenylate cyclase than they were as aggregating agents.[9,11,12] Whereas ADP itself is roughly equipotent for each effect, 2-azido-ADP and 2-MeS-ADP are both about 5-fold more potent than ADP as aggregating agents but around 20 and 200 times more potent respectively than ADP as inhibitors of adenylate cyclase.[9,11] This is not true for all 2-substituted analogs of ADP, as 2-chloroadenosine 5'-diphosphate (2-chloro-ADP), like ADP, is roughly equipotent for each effect.[12] The enhanced potency of 2-azido-ADP and 2-MeS-ADP for inhibition of adenylate cyclase is not due to any other, non-receptor-mediated effect of these compounds, as they are both competitively inhibited by ATP with an apparent $pA_2$ value (a measure of antagonist affinity) similar to the $pA_2$ value of ATP for inhibiting these actions of ADP, indicating an action solely at an ADP receptor.[12] However, differences in potency for agonists are not by themselves strong evidence for different receptors, as potency reflects not only binding affinity but also the ability to activate receptors (efficacy) and coupling of a single receptor to two different effector systems with different efficiencies could explain these differences in potency. For example, cloned 5-$HT_{1A}$ receptors expressed in HeLa cells couple to both inhibition of adenylate cyclase and stimulation of phospholipase C but the coupling to adenylate cyclase appears to be stronger, so that agonists have a higher potency in this assay than if their ability to mobilize calcium is measured, and compounds which are partial agonists for inhibition of adenylate cyclase act as antagonists of the calcium response.[13] For antagonists however the $pK_B$ values obtained (estimates of affinity) were independent of the assay used, as expected because their effects reflect simply binding to the receptor independent of any effects of coupling. Differential effects for aggregation and inhibition of adenylate cyclase were also obtained with three phosphate-modifed analogs of ADP, adenosine 5'-O-(2-thiodiphosphate) (ADP-β-S) and the $R_P$ and $S_P$ diastereoisomers of adenosine 5'-O-(1-thiodiphosphate) (ADP-α-S). ADP-β-S was a partial agonist both for aggregation and for inhibition of adenylate cyclase, but its efficacy for aggregation was greater than its efficacy as an inhibitor of adenylate cyclase, as it achieved 75% and 50% respectively of the maximal response to ADP in these assays.[14] The discrepancy was even more marked for both isomers of ADP-α-S, which were partial agonists for aggregation with a similar efficacy to ADP-β-S, but actually acted as antagonists of ADP for inhibition of adenylate cyclase. The $S_P$ isomer was about five-fold more potent than the $R_P$ isomer as an aggregating agent and as an inhibitor of the effect of ADP on adenylate cyclase.[15] These results were interpreted as suggesting that both effects of ADP and of these analogs were mediated by a single receptor but that the efficacy of the analogues in inducing the two responses differed. These results, taken together with those from the 2-substituted analogs discussed above, do not fit with a model of a single receptor coupled preferentially to inhibition of adenylate cyclase,[13] as the efficacy of the phosphorothioate analogs is lower for inhibition of adenylate cyclase than for aggregation. Instead, they would support a model of *agonist trafficking*, in which different agonists can activate receptors so that they preferentially interact with a certain signaling pathway.[16] In this model too the effects of antagonists would be expected to be independent of the signaling pathway activated. With regard to inhibitors of the effects of ADP, thiol reagents such as p-mercuribenzene sulphonate were reported to inhibit the ability of ADP to inhibit adenylate cyclase but not to cause shape change or aggregation,[17] and it was suggested that they might selectively bind to an ADP receptor coupled to adenylate cyclase.[9,11] However, these compounds are clearly not competitive antagonists and indeed also block the inhibitory effect of adrenaline on adenylate cyclase,[18] suggesting that their site of action is not in fact the ADP receptor. Of more potential significance is the action of an adenosine analog with some structural similarities to ADP, 5'-fluorosulfonylbenzoyladenosine (FSBA), which has been used as an affinity reagent for nucleotide-binding sites. FSBA inhibits ADP-induced shape change and aggregation but not the effect of ADP on adenylate cyclase, and this has been taken as further evidence for the existence of two ADP receptors on platelets.[19,20,21,22,23] The 100 kDa protein labeled by FSBA has been called *aggregin* and suggested to be the receptor by which ADP induces shape change and aggregation,[22,23] although this has not been universally accepted, largely because of the rather non-specific nature of the inhibition caused by FSBA[24,25,26] and the difficulty of interpreting experiments carried out with an irreversible affinity reagent of this type.[27] An additional problem with FSBA is that it has been reported not to inhibit the increase in cytoplasmic calcium induced by ADP,[28] although this increase in calcium is believed to be intimately involved with platelet activation by all receptor agonists, not just ADP.[29] Because of the irreversible nature of these inhibitors, an attempt was made to find a number of truly competitive antagonists which could be used to investigate whether the two effects of ADP are mediated by a single receptor. ATP itself was used, together with 2-chloroadenosine 5'-triphosphate (2-chloro-ATP), adenylyl 5'-(β,γ-methylene)-diphosphonate (β,γ-me-ATP), $P^1,P^5$-diadenosine pentaphosphate ($Ap_5A$), adenosine 5'-O-(3-fluorotriphosphate) (ATP-γ-F), 2-chloroadenosine 5'-phosphorothioate (2-chloro-AMPS) and the $R_P$ and $S_P$ diastereoisomers of adenosine 5'-O-(1-thiotriphosphate) (ATP-α-S), and the competitivity of these antagonists was tested and their affinity calculated using the rigorous pharmacologic approach of Schild analysis.[30] All these eight nucleotide analogs were shown to act as competitive antagonists, and there was a significant correlation between their affinities calculated as antagonists of aggregation and as antagonists of the effect of ADP on adenylate cyclase.[10] This suggested that these two effects are mediated by a single type of receptor, although another explanation of course is that these compounds were unable to discriminate between two different receptors. The non-specific P2 antagonist suramin, which has a completely different structure, was later also shown to inhibit both these effects of ADP.[31,32] Because of the suggestion, based on the reported effect of FSBA, that ADP-induced calcium

mobilization is mediated by a separate receptor from that mediating aggregation,[28] some of the analogs mentioned above were also tested for their effects on cytoplasmic calcium, measured in washed platelets using fura-2. A good correlation was found for all agonists and antagonists tested, including suramin, 2-MeSADP, ADP-α-S and ADP-β-S, between their effects on aggregation and their effects on calcium.[31,32,33,34] This strongly suggests that the effect of FSBA on aggregation is not truly an effect via the ADP receptor, but at some site distal to activation of the receptor and activation of phospholipase C. Overall there was also a significant correlation between the effects of these compounds on calcium and their effects on inhibition of adenylate cyclase.[35] During the search for competitive antagonists for ADP-induced aggregation, the actions of a series of 2-alkylthio-substituted ATP and AMP analogs was studied, and these compounds (including 2-methylthioadenosine 5'-triphosphate; 2-MeS-ATP) were noted to be specific but apparently non-competitive inhibitors, unable to inhibit ADP-induced aggregation completely but instead resulting in a partial (approximately 50%) inhibition even at the highest concentrations used.[36] One of these AMP analogs, 2-ethylthioadenosine 5'-monophosphate (2-EtS-AMP) was investigated further, together with an analogue of 2-MeS-ATP which had been stabilized to avoid enzymic degradation to 2-MeS-ADP by the replacement of the β,γ bridging oxygen by a methylene linkage (2-methylthioadenylyl 5'-(β,γ-methylene)-diphosphonate; 2-MeS-β,γ-me-ATP).[37,38] Each of these compounds, like 2-MeS-ATP, partially inhibited ADP-induced platelet aggregation in a highly specific manner, but even at high concentrations could only achieve about 50% inhibition. However, they were each able to inhibit the effect of ADP on adenylate cyclase in an apparently competitive manner, and their $IC_{50}$ values for this were similar to their $IC_{50}$ values as inhibitors of aggregation. This suggested that they act by inhibiting only one component of the action of ADP responsible for the induction of aggregation, and that this component is inhibition of adenylate cyclase. This suggestion was strengthened by the finding that 2-MeS-β,γ-me-ATP inhibited aggregation induced by ADP-β-S (which does inhibit adenylate cyclase weakly), but not aggregation induced by ADP-α-S (which does not inhibit adenylate cyclase).[37,38] The overall conclusion from this study was *that ADP may induce aggregation by interacting with two forms of a calcium-mobilizing $P_{2T}$-purinoceptor, only one of which is coupled to inhibition of adenylate cyclase and at which alkylthio analogs of ATP and AMP are specific competitive antagonists*.[38] A series of ATP analogs structurally similar to 2-MeS–β,γ-me-ATP including 2-propylthioadenylyl 5'-(β,γ-difluoromethylene)-diphosphonate (ARL 66096) and 2-propylthioadenylyl 5'-(β,γ-dichloromethylene)-diphosphonate (ARL 67085) have been shown to inhibit ADP-induced aggregation and are being developed as antithrombotic drugs.[39,40] ARL 66096 also inhibits the effect of ADP on adenylate cyclase but has little effect on ADP-induced increases in calcium, phospholipase C activation or shape change.[41,42,43] 2-MeS-ATP also has only a weak effect on ADP-induced shape change compared with its effect on aggregation, and like its stable analogue 2-MeS-β,γ-me-ATP is a powerful inhibitor of the effect of ADP on adenylate cyclase.[44] It should be noted however that the effects of the 2-alkyl substituted compounds on ADP-induced aggregation may depend on the experimental conditions used, as 2-MeS-ATP and 2-MeS-β,γ-me-ATP are able to abolish aggregation in washed platelets and appear competitive but only cause a partial inhibition in platelets in plasma.[36, 37, 38, 39, 44]

Although in the original classification of P2 receptors 2-MeS-ATP and ATP were reported to be agonists with a potency order of 2-MeS-ATP > ATP at the functionally-defined P2Y receptor[6] and also at the cloned $P2Y_1$ receptor,[7] when carefully-purified compounds were used it was demonstrated that in fact they were antagonists, as in the platelets, and indeed the presence of the $P2Y_1$ receptor on platelets was directly demonstrated.[45,46] In this study 2-MeS-ATP had a slightly lower affinity for the cloned $P2Y_1$ receptor than did ATP, suggesting that its previously-reported higher potency as an agonist probably reflects the relative potency as agonists of their breakdown products, 2-MeS-ADP and ADP respectively. Interestingly, ARL 66096 was reported to be a very weak antagonist of the cloned $P2Y_1$ receptor,[41] suggesting that the inhibitory actions of these compounds on platelets cannot be accounted for by antagonism of the $P2Y_1$ receptor. Two ADP analogs with methylene linkages in the phosphate chain, α,β-me-ADP and adenosine 5'-(α,β-ethylene) diphosphonate (α,β-ethyl-ADP) were shown to act as antagonists for ADP-induced aggregation but to have neither agonist nor antagonist activity for inhibition of adenylate cyclase.[47] The related analogue adenosine 5'-(α,β-imido) diphosphonate (α,β-imido-ADP) was similar, having weak partial agonist activity for aggregation but again being neither an agonist nor an antagonist for inhibition of adenylate cyclase.[47] This finding also did not support the idea that both effects are mediated by a single receptor, but the very weak effects of these compounds on aggregation made it hard to investigate their mechanism of action rigorously. More usefully, a series of AMP analogs substituted on the 3' position of the adenine ring such as adenosine 3'-phosphate 5'-phosphosulfate (A3P5PS) also have differential effects on ADP-induced responses, and inhibit ADP-induced aggregation, shape change and the increase in calcium, but not the effect of ADP on adenylate cyclase.[48,49,50,51,52,53] As these compounds have been shown to be competitive antagonists at the $P2Y_1$ receptor,[54] this suggested the involvement of the $P2Y_1$ receptor only in shape change, aggregation and the activation of phospholipase C, but not in the inhibition of adenylate cyclase, and led to the development of the currently-accepted model of ADP-induced aggregation. In this model the $P2Y_1$ receptor is responsible for the activation of phospholipase C, calcium mobilization and shape change and another receptor, not yet cloned but called $P2Y_{AC}$, $P2Y_{ADP}$ or $P_{2T}$, for the inhibition of adenylate cyclase, while the activation of both are required for aggregation.[48,49,50,55] That there are indeed two G protein-coupled receptors for ADP on platelets has recently been strongly supported by the

generation of genetically-modified mice lacking the $P2Y_1$ receptor, in which shape change, calcium increases and aggregation in response to ADP were greatly reduced while the inhibition of adenylate cyclase by ADP was unaffected.[56] It is perhaps surprising that the adenylate cyclase linked $P2Y_{AC}$ receptor has so far resisted attempts to clone it, which may suggest that it has low sequence homology with the known members of the P2Y family[57] in spite of the fact that its structure-activity relationships are quite similar to those of the $P2Y_1$ receptor (see below). It clearly must be a member of the G protein-coupled P2Y family of receptors, and indeed the inhibitory effect of ADP on adenylate cyclase is thought to be via activation of the G protein $G_{i2}$.[58]

## Comparison of the $P2Y_1$ and $P2Y_{AC}$ receptors

The current model requires some re-evaluation of the known effects of agonists and antagonists on platelet function, and explains many previous findings. In particular, it means that simply looking at the effects of compounds on aggregation, although functionally important, does not give clear information as to their effects on either the $P2Y_1$ or the $P2Y_{AC}$ receptor but is a composite of the two effects. Instead, to compare effects of agonists and antagonists on the two receptors it is more informative to study increases in calcium or shape change as a measure of $P2Y_1$ activation and inhibition of adenylate cyclase for the $P2Y_{AC}$. This information is available for a few compounds only (see Table 1), but some conclusions can be drawn which give hints as to the characteristics of the $P2Y_{AC}$ receptor and how it differs from the $P2Y_1$ receptor. ADP is an agonist with equal potency and ATP an antagonist with equal affinities for both receptors, and indeed it seems that it is free unliganded form ($ADP^{3-}$ and $ATP^{4-}$ respectively) which is the active form in each case, as indicated by a comparison of shape change and adenylate cyclase inhibition in the presence and absence of divalent cations.[59] 2-MeS-ADP is around 10-fold more potent than ADP and in fact appears to act with equal potency at each receptor, which is perhaps surprising given that the original suggestion that there might be two receptors on platelets was made at least partly because its potency in aggregation was less than its potency as an inhibitor of adenylate cyclase.[11] 2-chloro-ATP is roughly 10-fold more potent as an antagonist of the effect of ADP on calcium than as an antagonist of its effect on adenylate cyclase, while $S_P$-ATP-α-S, $Ap_5A$, β,γ-me-ATP and suramin have apparently equal affinities for both receptors. The 2-alkylthio-substituted analogs, 2-MeS-ATP and 2-MeS-β,γ-me-ATP have approximately 100-fold higher affinity for the adenylate cyclase coupled receptor compared to the $P2Y_1$ receptor, while A3P5PS has no effect on the $P2Y_{AC}$ receptor at a concentration of 100 μM but inhibits shape change with a $pA_2$ value of around 6, similar to the value reported for the $P2Y_1$ receptor in turkey erythrocytes.[54] The two phosphorothioate analogs of ADP are very interesting, as they appear to have much the same affinity but different efficacies at the two receptors. ADP-β-S acts as a full agonist for shape change but is around 10-fold less potent than ADP, while it acts as a partial agonist for inhibition of adenylate cyclase but its $pD_2$ value is similar in each assay. $S_P$-ADP-α-S is also a full agonist for shape change and is almost equipotent with ADP with a $pD_2$ value of 5.9, but acts as an antagonist at the $P2Y_{AC}$ receptor, with a $pA_2$ value of 5.1, similar to that of ATP. Overall the potency order for agonists at the $P2Y_1$ receptor (judged by shape change or calcium mobilization) is 2-MeS-ADP > ADP = $S_P$-ADP-α-S > ADP-β-S, while for the adenylate cyclase coupled $P2Y_{AC}$ receptor it is 2-MeS-ADP > ADP = ADP-β-S with $S_P$-ADP-α-S being an antagonist. For antagonists there are some differences between the results obtained for shape change and for calcium mobilization, but overall the affinity order at the $P2Y_1$ receptor is $S_P$-ATP-α-S = 2-chloro-ATP = A3P5PS > 2-MeS-ATP = $Ap_5A$ = ATP = suramin > β,γ-me-ATP = 2-MeS-β,γ-me-ATP, while at the adenylate cyclase linked receptor it is 2-MeS-β,γ-me-ATP = 2-MeS-ATP > ATP = $S_P$-ATP-α-S = $Ap_5A$ = $S_P$-ADP-α-S = suramin > 2-chloro-ATP > β,γ-me-ATP >> A3P5PS. ARL 66096 is clearly similar to 2-MeS-β,γ-me-ATP in being selective for the $P2Y_{AC}$ receptor,[41,42,43] but its affinity for the two receptors has not been reported in such a way as to allow quantitative comparison. From published data it appears to inhibit the effect of 10 μM ADP on adenylate cyclase with an $IC_{50}$ value of around 30 nM giving a $pIC_{50}$ value of 7.5,[42] suggesting that it may have a higher affinity for the $P2Y_{AC}$ receptor than the 2-methylthio substituted analogs, and it had no effect on ADP-induced shape change at 10 μM,[43] suggesting that its $pA_2$ value for antagonism of the $P2Y_1$ receptor must be less than 5. As an inhibitor of ADP-induced aggregation a $pA_2$ value of around 9 has been calculated,[60] and by analogy with 2-MeS-ATP this may indeed reflect its affinity for the $P2Y_{AC}$ receptor. Indeed in general for antagonists, as expected, the observed $pA_2$ values for antagonists do reflect the higher of their affinities for the two receptors.

## Roles of the $P2Y_1$ and $P2Y_{AC}$ receptors in platelets

Because aggregation is a complex process, the results obtained using this measure of activation depend greatly on the experimental conditions used. In particular, as shown in Table 1, there are profound differences for some compounds depending on whether platelets are used in plasma or are washed and resuspended in buffer. The differences are particularly pronounced for those compounds which discriminate between the two receptors, such as the 2-alkylthio analogs and the phosphorothioate analogs. In plasma the phosphorothioate analogs ADP-α-S and ADP-β-S act as partial agonists, but in washed platelets their efficacy is greatly reduced and ADP-α-S acts as a pure antagonist. For the 2-alkylthio analogs, in plasma their inhibition is only partial whereas in washed platelets they can abolish aggregation. In both these cases their effects in washed platelets closely reflect their effects on adenylate cyclase, whereas in plasma the adenylate cyclase effect can only account for part of their action. It

**Table 1. $pD_2$ values for agonists and $pA_2$ values for antagonists for aggregation, shape change, increases in calcium and inhibition of adenylate cyclase in human platelets.**

| | *Aggregation (PRP)* | *Aggregation (washed)* | *Shape change (washed)* | *Increases in calcium (washed)* | *Inhibition of adenylate cyclase (PRP)* |
|---|---|---|---|---|---|
| *Agonists* | | | | | |
| ADP | 5.5 | 5.7 | 6.2 | 6.1 | 5.5 |
| 2-MeS-ADP | 6.5 | 7.0 | 7.2 | 7.4 | 7.5 |
| ADP-β-S | 4.7 (75%)† | < 4 | 5.2 | 5.4 (60%)† | 5.3 (50%)† |
| $S_P$-ADP-α-S | 5.4 (75%)† | Antagonist (see below) | 5.9 | 6.3 (60%)† | Antagonist (see below) |
| *Antagonists* | | | | | |
| ATP | 4.6 | 4.9 | 4.6 | 5.0 | 5.2 |
| 2-chloro-ATP | 4.1 | ND | ND | 5.6 | 4.5 |
| $S_P$-ATP-α-S | 5.4 | ND | ND | 5.9 | 5.3 |
| $Ap_5A$ | 4.5 | 4.8 | 4.6 | 5.1 | 4.8 |
| α,β-me-ATP | 4.1 | < 4 | < 4 | 4.3 | 4.2 |
| 2-MeS-ATP | $pIC_{50}$ = 6 (60%)# | 7.0 | 5.2 | 5.3? § | 7.2(washed) |
| 2-MeS-β,γ-me-ATP | $pIC_{50}$ = 6.2 (50%)# | ND | 4 (PRP)* | ND | 7.3 $pIC_{50}$ = 6.3 |
| $S_P$-ADP-α-S | Partial agonist (see above) | 5.4 | Agonist (see above) | Partial agonist (see above) | 5.1 |
| A3P5PS | ND | 5.2 | 6.0 | 5.4 | Inactive (washed) |
| Suramin | Inactive | 4.6 | 5.0 | 4.6 | 5.1 (washed) |

*Values were measured in platelets either in plasma (PRP) or washed by centrifugation and resuspended in HEPES buffered saline, as indicated. $pD_2$ = negative log of the molar concentration of agonist required to achieve 50% of the maximal response. $pA_2$ = negative log of the molar concentration of antagonist required to cause a 2-fold shift of the concentration-response curve to ADP. †Partial agonist for this effect; the maximal response as a percentage of maximal response to ADP is given in parentheses. #Partial inhibition of the response to ADP; the percentage inhibition achieved is given in parentheses. $pIC_{50}$ values were calculated from inhibition of the effects of 5 μM ADP. §The nature of the inhibition was not determined but did not appear to be simply competitive; however, this value was calculated from the shift in the concentration-response curve to ADP assuming competitive inhibition. ND: not determined. Data are taken from refs. #10, 12, 14, 15, 31, 32, 33, 36, 38, 44, 52, 59. * Hourani, Welford & Cusack, unpublished results.*

appears likely that in unwashed platelets the $P2Y_1$ receptor is capable of inducing a partial aggregation which is greatly enhanced by coactivation of the $P2Y_{AC}$ receptor, whereas in washed platelets coactivation is necessary for any aggregation to be observed. A study of platelets in plasma comparing the effects of adenosine 3'-phosphate 5'-phosphate (A3P5P, similar to A3P5PS) with those of ARL 67085 suggested that the $P2Y_1$ receptor is responsible for the initiation of a transient aggregation while the $P2Y_{AC}$ receptor determines the final extent of sustained aggregation.[61] One explanation for the difference between washed platelets and platelets in plasma is that the act of washing the platelets results in the release of ADP which selectively desensitizes the $P2Y_1$ receptor, reducing its ability to induce aggregation alone so that coactivation is now required. That the $P2Y_1$ receptor does desensitize more readily than the $P2Y_{AC}$ receptor was also suggested by the generation of genetically modified mice lacking CD39, the main vascular ATPdiphosphohydrolase. Platelets from these mice appeared to have been desensitized *in vivo* as ADP-induced aggregation was much reduced, and a selective desensitization of the $P2Y_1$ receptor was suggested because ADP-induced aggregation could be enhanced by the addition of serotonin (which activates phospholipase C) but not by adrenaline (which inhibits adenylate cyclase).[62] Another possible explanation for the difference between platelets in plasma and washed platelets is that the presence of the plasma somehow enhances the effect of the $P2Y_1$ receptor. This is an important issue because if the $P2Y_1$ receptor does readily desensitize, then even in platelet-rich plasma the observed responses to nucleotides may not be the same as *in vivo*, as the very act of taking blood for experiments will cause release of high concentrations of nucleotides[63] and and almost certainly some desensitization. This may help to explain why in studies on platelets ATP has always been observed to be antagonist, whereas there is still controversy over whether triphosphates are agonists or antagonists at the cloned $P2Y_1$ receptor.[41,45,46,64,65] Whether this is also the case for the $P2Y_{AC}$ receptor is of course unknown as this receptor has not been cloned yet.

## The $P2X_1$ receptor

The question of desensitization is also very important when considering the role of the third proposed P2 receptor on platelets, the $P2X_1$ receptor. The presence of an ADP receptor-operated channel allowing entry of calcium was first suggested from functional studies,[66,67] and the presence of a $P2X_1$ receptor has now been directly demonstrated by molecular tech-

niques.[68,69,70,71] However, the significance of this receptor is not clear, as ATP is more potent than ADP at the cloned $P2X_1$ receptor[71,72] but as discussed above agonist responses to ATP are not detected *in vitro* under the normal experimental conditions. The actions of ATP at the $P2X_1$ receptor can only be detected if the platelets are pre-treated with apyrase, presumably to remove adenine nucleotides and reverse the densitization caused by release of ATP and ADP.[73] Adenosine 5'-(α,β-methylene)-triphosphonate (α,β-me-ATP) and adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S) were also able to activate the channel, but were less potent than ADP, and AMP and UTP were inactive.[73] In a direct study of the ability of analogs to stimulate a rapid calcium influx corresponding to activation of this channel a potency order of ADP, 2-chloro-ADP, 2-MeS-ADP > ADP-β-S, α,β-me-ATP > ATP, β,γ-imido-ATP was reported, which is not identical to that expected for a $P2X_1$ receptor.[74] Again this suggests that the responses of platelets *in vivo* may be rather different from the responses observed *in vitro*. Indeed, when cells are damaged or when platelets aggregate, both of which are considered to be triggers for further agregation, both ADP and ATP will be released so one might expect that they would both cause platelet activation.

## Conclusions

Overall, the current model of platelet activation by ADP therefore includes three receptors, each with rather different structure-activity relationships, which act together to cause the functionally important response of aggregation, a crucial event in hemostasis and thrombosis. The importance of the $P2Y_1$ receptor is shown by the prolonged bleeding time and resistance to thromboembolism (induced by a mixture of collagen and ADP) in the $P2Y_1$ knockout mice;[56] the importance of the $P2Y_{AC}$ receptor is shown by the anti-thrombotic effects of the $P2Y_{AC}$-selective antagonist ARL 67085[40] and the clinical effectiveness as antithrombotic drugs of ticlopidine and clopidogrel, which are believed to down-regulate the $P2Y_{AC}$ receptor *in vivo* by some unknown mechanism.[75] The physiologic and pathologic importance of the platelet $P2X_1$ receptor has yet to be established, although investigation of the platelets from the recently-generated $P2X_1$-knockout mice[76] should prove very valuable. A fuller understanding of the ways in which these three receptors interact to control platelet function *in vivo* is essential for the future development of effective antithrombotic drugs.

## References


1. Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature 1962; 194: 927-9.
2. Macfarlane DE, Mills DCB. The effects of ATP on platelets: evidence against the central role of released ADP in primary aggregation. Blood 1975; 46:309-20.
3. Gordon JL. Extracellular ATP: effects, sources and fate. Biochem J 1986; 233:309-19.
4. Haslam RJ, Cusack NJ. Blood platelet receptors for ADP and for adenosine. In: Burnstock G, ed. Purinergic Receptors. Receptors and Recognition, Series B, Vol 12. London: Chapman and Hall, 1981. p. 221-85.
5. Hourani SMO, Cusack NJ. Pharmacological receptors on blood platelets. Pharmacol Rev 1991; 43:243-98.
6. Burnstock G, Kennedy C. Is there a basis for distinguishing two types of P2-purinoceptor? Gen Pharmacol 1985; 16:433-40.
7. Harden TK, Barnard EA, Boeynaems JM, et al. P2Y receptors. In: The IUPHAR Compendium of Receptor Characterization and Classification. London: IUPHAR Media, 1998: 209-17.
8. Boarder MR, Hourani SMO. The regulation of vascular function by P2 receptors: multiple sites and multiple receptors. Trends Pharmacol Sci 1998; 19:99-107.
9. Macfarlane DE, Mills DCB, Srivastava PC. Binding of 2-azidoadenosine [β-$^{32}$P]diphosphate to the receptor on intact human platelets which inhibits adenylate cyclase. Biochemistry 1982; 21:544-9.
10. Cusack NJ, Hourani SMO. Adenosine 5'-diphosphate antagonists and human platelets: no evidence that aggregation and inhibition of stimulated adenylate cyclase are mediated by different receptors. Br J Pharmacol 1982; 76:221-7.
11. Macfarlane DE, Srivastava PC, Mills DCB. 2-methylthioadenosine [β-$^{32}$P]diphosphate. An agonist and radioligand for the receptor that inhibits the accumulation of cyclic AMP in intact blood platelets. J Clin Invest 1983; 71:420-5.
12. Cusack NJ, Hourani SMO. Inhibition by adenosine 5'-triphosphate of the actions on human platelets of 2-chloroadenosine 5'-diphosphate, 2-azidoadenosine 5'-diphosphate and 2-methylthioadenosine 5'-diphosphate. Br J Pharmacol 1982; 77:329-33.
13. Hoyer D, Boddeke HWGM. Partial agonists, full agonists, antagonists: dilemmas of definition. Trends Pharmacol Sci 1993; 14:270-5.
14. Cusack NJ, Hourani SMO. Partial agonist behaviour of adenosine 5'-O-(2-thiodiphosphate) on human platelets. Br J Pharmacol 1981; 73:405-8.
15. Cusack NJ, Hourani SMO. Effects of RP and SP diastereoisomers of adenosine 5'-O-(1-thiodiphosphate) on human platelets. Br J Pharmacol 1981; 73:409-12.
16. Kenakin T. Agonist-receptor efficacy II. Agonist trafficking of receptor signals. Trends Pharmacol Sci 1995; 16:132-8.
17. Mill DCB, Macfarlane DE. Attempts to define a platelet ADP receptor with 203Hg-p-mercuribenzene sulphonate (MBS). Thromb Haemost 1977; 38:82.
18. Macfarlane DE, Mills DCB. Inhibition by ADP of prostaglandin induced accumulation of cyclic AMP in intact human platelets. J Cyc Nuc Research 1981; 7:1-11.
19. Bennett JS, Colman RF, Colman RW. Identification of adenine nucleotide binding proteins in human platelet membranes by affinity labelling with 5'-fluorosulfonylbenzoyl adenosine. J Biol Chem 1978; 253:7346-54.
20. Colman RW, Figures WR, Colman RF, Morinelli TA, Niewiarowski S, Mills DCB. Identification of two distinct adenosine diphosphate receptors in human platelets. Trans Assoc Am Physicians 1980; 93:305-16.
21. Mill DCB, Figures WR, Scearce LM, Stewart GJ, Colman RF, Colman RW. Two mechanisms of inhibition of ADP-induced shape change by 5'-p-fluorosulfonylbenzoyl adenosine: conversion to adenosine, and covalent modification at an ADP-binding site distinct from that which inhibits adenylate cyclase. J Biol Chem 1985; 260:8078-83.
22. Colman RW. Aggregin: a platelet ADP receptor that mediates aggregation. FASEB J 1990; 3:1425-35.

23. Colman RW. Platelet ADP receptors stimulating shape change and inhibiting adenylate cyclase. News Physiol Sci 1992; 7:274-8.
24. Morinelli TA, Niewiarowski S, Kornecki E, Figures WR, Wachfogel Y, Colman RW. Platelet aggregation and exposure of fibrinogen receptors by prostaglandin endoperoxide analogs. Blood 1983; 61:41-9.
25. Colman RW, Figures WR, Scearce LM, Strimpler AM, Zhou F, Rao AK. Inhibition of collagen-induced platelet activation by 5'-p-fluorosulfonylbenzoyl adenosine: evidence for an adenosine diphosphate requirement and synergistic influence of prostaglandin endoperoxides. Blood 1986; 68:565-70.
26. Figures WR, Scearce L, Wachtfogel Y, Chen J, Colman RF, Colman RW. Platelet ADP receptor and α2-adrenoceptor interaction. Evidence for an ADP requirement for epinephrine-induced platelet activation and an influence of epinephrine on ADP binding. J Biol Chem 1986; 261:5981-6.
27. Mills DCB. ADP receptors on platelets. Thromb Haemost 1996; 76: 835-56.
28. Rao AK, Kowalska MA. ADP-induced platelet shape change and mobilization of cytoplasmic ionised calcium are mediated by distinct binding sites on platelets: 5'-p-fluorosulfonylbenzoyladenosine is a weak platelet agonist. Blood 1987; 70:751-6.
29. Seiss W. Molecular mechanisms of platelet aggregation. Physiol Rev 1989; 69:158-78.
30. Arunlakshana O, Schild HO. Some quantitative uses of drug antagonists. Br J Pharmacol 1959; 14:48-58.
31. Hourani SMO, Hall DA, Nieman CJ. Effects of the P2-purinoceptor antagonist, suramin, on human platelet aggregation induced by adenosine 5'-diphosphate. Br J Pharmacol 1992; 105:453-7.
32. Hall DA, Hourani SMO. Effects of suramin on increases in cytosolic calcium and on inhibition of adenylate cyclase induced by adenosine 5'-diphosphate in human platelets. Biochem Pharmacol 1994; 47:1013-8.
33. Hall DA, Hourani SMO. Effects of analogues of adenine nucleotides on increases in intracellular calcium mediated by P2T-purinoceptors on human blood platelets. Br J Pharmacol 1993; 108:728-33.
34. Hourani SMO, Hall DA. ADP receptors on human blood platelets. Trends Pharmacol Sci 1994; 15:103-8.
35. Hourani SMO, Hall DA. P2T purinoceptors: ADP receptors on platelets. Ciba Foundation Symposium 1996; 198:52-69.
36. Cusack NJ, Hourani SMO. Specific but non-competitive inhibition by 2-alkylthio analogues of adenosine 5'-monophosphate and adenosine 5'-triphosphate on human platelet aggregation induced by adenosine 5'-diphosphate. Br J Pharmacol 1982; 75:397-400.
37. Hourani SMO, Welford LA, Cusack NJ. 2-MeS-AMP-PCP and human platelets: implications for the role of adenylate cyclase in ADP-induced aggregation? Br J Pharmacol 1986; 87:84P.
38. Hourani SMO, Welford LA, Cusack NJ. Effects of 2-methylthioadenosine 5'-β,γ-methylenetriphosphonate and 2-ethylthioadenosine 5'-monophosphate on human platelet activation induced by adenosine 5'-diphosphate. Drug Dev Res 1996; 38:12-23.
39. Humphries RG, Tomlinson W, Ingall AH, Cage PA, Leff P. FPL 66096: a novel, highly potent and selective antagonist at human platelet P2T-purinoceptors. Br J Pharmacol 1994; 113:1057-63.
40. Humphries RG, Robertson MJ, Leff P. A novel series of P2T purinoceptor antagonists: definition of the role of ADP in arterial thrombosis. Trends Pharmacol Sci 1995; 16:179-81.
41. Fagura MS, Dainty IA, McKay GD, et al. $P2Y_1$-receptors in human platelets which are pharmacologically distinct from $P2Y_{ADP}$-receptors. Br J Pharmacol 1998; 124:157-64.
42. Daniel JL, Dangelmaier CA, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. J Biol Chem 1998; 273:2024-9.
43. Sanderson HM, Heptinstall S, Vickers J, Lösche W. Studies on the effects of agonists and antagonists on platelet shape change and platelet aggregation in whole blood. Blood Coag Fibrinol 1996; 7:245-8.
44. Park HS, Hourani SMO. Different effects of adenine nucleotide analogues on shape change and aggregation induced by adenosine 5'-diphosphate (ADP) in human platelets. Br J Pharmacol 1999; 127:1359-66.
45. Léon C, Hechler B, Vial C, Leray C, Cazenave JP, Gachet C. The P2Y1 receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaroblastic cells. FEBS Lett 1997; 403:26-30.
46. Hechler B, Vigne P, Léon C, Breittmayer JP, Gachet C, Frelin C. ATP derivatives are antagonists of the P2Y1 receptor: similarities to the platelet ADP receptor. Mol Pharmacol 1998; 53:727-33.
47. Cusack NJ, Pettey, CJ. Effects of phosphate-modified analogs of adenosine 5'-diphosphate and adenosine 5'-triphosphate at P2T-purinoceptors mediating human platelet activation by ADP. Drug Dev Res 1996; 37:212-22.
48. Hechler B, Léon C, Vial C, et al. The $P2Y_1$ receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. Blood 1998; 92:152-9.
49. Savi P, Beauverger P, Labouret C, et al. Role of $P2Y_1$ purinoceptor in ADP-induced platelet activation. FEBS Lett 1998; 422:291-5.
50. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci USA 1998; 95:8070-4.
51. Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The $P2Y_1$ receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. J Biol Chem 1998; 273:2030-4.
52. Park HS, Tennant JP, Waktolla GF, Sarkardei S, Kass GEN, Hourani SMO. Effects of adenosine 3'-phosphate 5'-phosphosulfate on P2 receptors in platelets and smooth muscle preparations. Drug Dev Res 1998; 45:67-73.
53. Jantzen HM, Goussent L, Bhaskar V, et al. Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. Thromb Haemost 1999; 81:111-7.
54. Boyer JL, Romero-Avila T, Schachter JB, Harden TK. Identification of competitive antagonists of the $P2Y_1$ receptor. Mol Pharmacol 1996; 50:1323-9.
55. Kunapuli SP. Multiple P2 receptor subtypes on platelets; a new interpretation of their function. Trends Pharmacol Sci 1998; 19:391-4.
56. Fabre JE, Nguyen MT, Latour A, et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in $P2Y_1$-deficient mice. Nature Med 1999; 5:1199-202.
57. Boeynaems JP, Communi D, Savi P, Herbert JM. P2Y receptors: in the middle of the road. Trends Pharmacol Sci 2000; 21:1-3.
58. Ohlmann P, Laugwitz KL, Nürnberg B, et al. The human platelet ADP receptor activates $G_{i2}$ proteins. Biochem J 1995; 312:775-9.
59. Hall DA, Frost V, Hourani SMO. Effects of extracellular divalent cations on responses of human blood platelets to adenosine 5'-diphosphate. Biochem Pharmacol 1994; 48:1319-26.
60. Tomlinson W, Humphries RG, Robertson MJ, Leff P. ARL 67085 and ARL 66096 are slowly dissociating competitive P2T-purinoceptor antagonists. Br J Phar-

macol 1997; 120:131P.
61. Jarvis GE, Humphries RG, Robertson MJ, Leff P. ADP can induce aggregation of human platelets via both P2Y1 and P2T receptors. Br J Pharmacol 2000; 129: 275-82.
62. Enjyoji K, Sévigny J, Lin Y, et al. Targeted disruption of CD39/ATPdiphosphohydrolase results in disordered hemostasis and thromboregulation. Nature Med 1999; 5:1010-7.
63. Born GVR, Kratzer MAA. Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. J Physiol 1984; 354:419-29.
64. Palmer RK, Boyer JL, Schachter JB, Nicholas RA, Harden TK. Agonist action of adenosine triphosphates at the human $P2Y_1$ receptor. Mol Pharmacol 1998; 54: 1118-23.
65. Filippov AK, Brown DA, Barnard EA. The $P2Y_1$ receptor closes the N-type $Ca^{2+}$ channel in neurones, with both adenosine triphosphates and diphosphates as potent agonists. Br J Pharmacol 2000; 129:1063-6.
66. Sage SO, Mahaut-Smith MP, Rink TJ. Calcium entry in nonexcitable cells: lessons from human platelets. News Physiol Sci 1992; 7:108-13.
67. Sage SO, MacKenzie AB, Jenner S, Mahaut-Smith MP. Purinoceptor-evoked calcium signalling in human platelets. Prostag Leukotr Ess 1997; 57:435-8.
68. Vial C, Hechler B, Leon C, Cazenave JP, Gachet C. Presence of $P2X_1$ purinoceptors in human platelets and megakaryoblastic cell lines. Thromb Haemost 1997; 78:1500-4.
69. Clifford EE, Parker K, Humphreys BD, Kertesy SB, Dubyak GR. The $P2X_1$ receptor, an adenosine-triphosphate-gated cation channel, is expressed in human platelets but not in human blood leukocytes. Blood 1998; 91:3172-81.
70. Scase TJ, Heath MF, Allen JM, Sage SO, Evans RJ. Identification of a P2X1 purinoceptor expressed on human platelets. Biochem Biophys Res Comm 1998; 242:500-28.
71. Sun B, Okahara K, Kambayashi J. $P2X_1$ purinoceptor in human platelets. J Biol Chem 1998; 273:11544-7.
72. Valera S, Hussy N, Evans RJ, et al. A new class of ligand-gated ion channel defined by $P_{2X}$ receptor for extracellular ATP. Nature 1994; 371:516-9.
73. MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of receptor-operated cation channels via $P2X_1$ not $P_{2T}$ purinoceptors in human platelets. J Biol Chem 1996; 271:2879-81.
74. Geiger J, Hönig-Leidl P, Schanzenbächer P, Walter U. Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. Eur J Pharmacol 1998; 351:235-46.
75. Savi P, Nurden P, Nurden AT, Levy-Toledano S, Herbert JM. Clopidogrel: a review of its mechanism of action. Platelets 1998; 9:251-5.
76. Mulryan K, Gitterman DP, Lewis CJ, et al. Reduced vas deferens contraction and male infertility in mice lacking $P2X_1$ receptors. Nature 2000; 403:86-9.

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# PHARMACOLOGY OF AR-C69931MX AND RELATED COMPOUNDS: FROM PHARMACOLOGICAL TOOLS TO CLINICAL TRIALS

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ABSTRACT

**A pivotal role for adenosine diphosphate (ADP) in platelet activation, aggregation and arterial thrombosis was proposed almost 40 years ago. However, it is only recently that the identification of potent and selective pharmacologic agents has enabled definition of the mechanisms involved in the various platelet responses to ADP. Pharmacologic studies have identified the presence of $P2X_1$ (cation influx) and $P2X_1$ (calcium mobilization) receptor subtypes on platelets. The functional significance of the $P2X_1$ receptor remains unclear, whilst $P2X_1$ receptor activation has been linked to shape change (an early event in platelet activation), and to transient (loose) platelet aggregation. Importantly, sustained platelet aggregation is dependent on stimulation of a third subtype of P2 receptor which has a unique pharmacology and, although G-protein coupled, has been neither cloned nor identified in other cell types and, therefore, cannot be allocated a definitive home in the P2Y family. Thus, this P2 receptor can, at present, retain the historical P2T designation and is defined pharmacologically by highly potent, selective, specific and competitive P2T antagonists, such as AR-C69931MX and AR-C67085MX. In addition to their value in defining the P2 receptor pharmacology of the platelet *in vitro*, AR-C69931MX and related compounds, unlike the available $P2X_1$ and $P2X_1$ ligands, have properties suitable for probing the pathophysiologic significance of P2T receptor-mediated platelet aggregation *in vivo*. Intravenous administration of AR-C69931MX and AR-C67085MX in models of arterial thrombosis has shown that blockade of the P2T receptor confers a unique anti-thrombotic profile, characterized by anti-thrombotic efficacy superior to that of aspirin and equivalent to that of fibrinogen receptor (GPIIb/IIIa) antagonists, without the degree of compromise of hemostasis associated with the latter class of agent. Similarly, in man, potent inhibition of ADP-induced platelet aggregation by AR-C69931MX has been confirmed *ex vivo* in both healthy volunteers and acute coronary syndrome patients, with a kinetic and dynamic profile enabling a rapid degree of control of the anti-aggregatory effect with minimal effect on bleeding time. The preclinical findings described above indicate that P2T receptor-mediated platelet aggregation plays a pivotal role in experimental arterial thrombosis. An important role for this pathway in arterial thrombosis in man is suggested by clinical studies with ticlopidine and clopidogrel, agents which indirectly (as prodrugs) attenuate P2T receptor-mediated platelet aggregation, albeit to a limited extent. The ability of direct P2T receptor antagonists such as AR-C69931MX to provide full inhibition of ADP-induced platelet aggregation in man in a controllable manner and without significant compromise of haemostasis supports development of these agents as novel anti-thrombotic drugs. Experience gained with intravenous representatives of this class provides considerable encouragement for development of orally-active P2T receptor antagonists and an expectation that these will have significant advantages over other existing and emerging oral anti-thrombotic agents.**


Key words: AR-C69931MX, P2T receptor, platelets, ADP receptors

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A pivotal role for adenosine diphosphate (ADP) in platelet activation, aggregation and arterial thrombosis was proposed almost 40 years ago.[1] Over the following decades, much effort has been devoted to a deeper understanding of many aspects of ADP-induced platelet activation: its role in responses to other platelet stimuli; characterization of receptor subtype(s) involved; the signal transduction mechanisms involved; its role in platelet-mediated thrombosis; the potential of inhibitors of ADP-induced platelet activation as anti-thrombotic drugs. However, significant progress with many of these investigations has been hampered by the lack of suitable pharmacologic tools and it is only recently that the identification of potent and selective $P_{2T}$ receptor antagonists has enabled definition of the mechanisms involved in the various platelet responses to ADP. This overview summarizes some of the key advances made using these compounds.

## AR-C69931MX and related compounds

The properties of adenosine triphosphate (ATP) as a competitive antagonist of ADP-induced platelet aggregation were first described by Macfarlane and Mills in 1975.[2] This led to the original definition of

the $P_{2T}$ subtype with the (then) unique pharmacologic profile of ADP as agonist and ATP as competitive antagonist.[3] However, ATP is by definition a non-selective P2 receptor ligand, with low potency and poor metabolic stability. These properties preclude its use both as a pharmacologic tool for definitive receptor classification *in vitro* and for exploring the importance of the $P_{2T}$ receptor in thrombosis *in vivo*.

During the 1980s, significant advances were made in the identification of analogues of ATP which were resistant to breakdown by ectonucleotidases with increased affinity for the $P_{2T}$ receptor. The key structure-activity relationships (SAR) observed were the affinity-conferring properties of substituents in the 2-position of the adenine ring[4,5] and the metabolic stability afforded by α,β methylene substitution in the triphosphate component of the molecule.[6] While far from optimal in respect of $P_{2T}$ potency and selectivity, these analogs provided a valuable SAR platform for the subsequent medicinal chemical program conducted within our group. In a broad exploration of SAR at a number of P2 receptor subtypes, a significant discovery was that unprecedented affinity and selectivity for the $P_{2T}$ subtype was achieved with extended alkylthio substitutions at the 2-position of the adenine ring, resulting in identification of the potent, selective $P_{2T}$ receptor antagonists, AR-C66096MX[7] and AR-C67085MX[8] (FPL or ARL prefixes were used in early descriptions of these compounds; MX = tetrasodium salt). A continued synthesis/screening campaign led to identification of AR-C69931MX, substituted in both the 2- and 6-positions.[9] The structures of these 3 compounds, compared to that of ATP, are presented in Figure 1. All 3 compounds are potent inhibitors of ADP-induced ($P_{2T}$-mediated) aggregation of human washed platelets and show at least a 3000-fold selectivity for the $P_{2T}$ receptor over other P2 receptor subtypes ($P2YP_1$/$P2XP_1$, see below) now known to be present on platelets.

Subsequent to the initial descriptions of AR-C66096MX ($pICP_{50}$ against ADP-induced aggregation of human washed platelets = 8.2) and AR-C67085MX ($pICP_{50}$ 8.6), studies conducted by ourselves and other groups have revealed some additional properties of these compounds at high concentrations: partial $P2YP_1$ agonist behavior of AR-C66096MX in a high expression system;[10] $P2YP_{11}$ agonism with AR-C67085MX.[11] These effects are less evident with AR-C69931MX and, with its anti-aggregatory potency advantage ($pICP_{50}$ 9.4), it has become the compound of choice for ongoing investigations by ourselves and other groups.

Further experience with these compounds has also emphasized the importance of very careful definition and control of agonist/antagonist incubation conditions, particularly when conducting quantitative pharmacologic studies for receptor characterization. This reflects the observation that, while all 3 compounds are competitive $P_{2T}$ antagonists under equilibrium conditions, apparent non-competitive properties are evident under non-equilibrium conditions.[12,13] This feature is particularly evident with AR-C69931MX and can be a problem in functional studies in platelets where the tendency for spontaneous platelet activation to occur with prolonged incubation times can make it difficult to achieve true equilibrium.

## One or 2 receptors for ADP: platelet $P_2$-subtypes?

The concluding sentence in the original description of AR-C (then FPL) 66096MX read: «*Identification of FPL 66096, a $P_{2T}$-purinoceptor antagonist of unprecedented potency and selectivity, provides a novel pharmacological tool to clarify this issue further, to investigate the role of ADP in platelet aggregation produced by other agents and for use in the classification of P2-purinoceptors in general*».[7] While the main thrust of our subsequent research activities has been to evaluate the potential of $P_{2T}$ antagonists

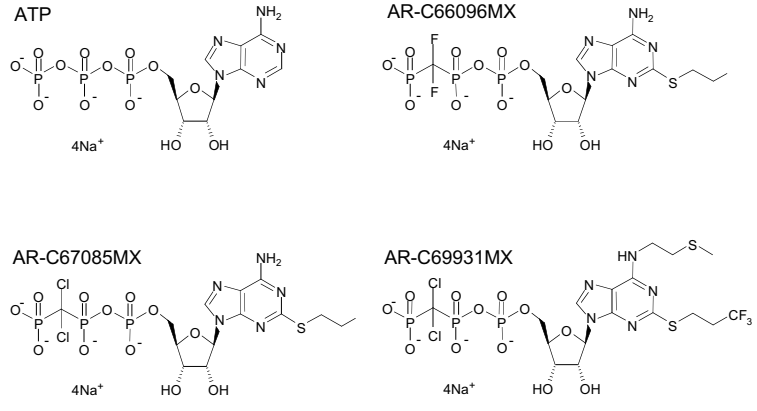


**Figure 1. Chemical structures of adenosine triphosphate (ATP), AR-C66096MX, AR-C67085MX and AR-C69931MX.**

as novel anti-thrombotic agents, arguably the most significant scientific advance arising from availability of AR-C69931MX and analogs has been resolution of the long-running debate over whether 1 or 2 subtypes of receptor are responsible for ADP-mediated platelet activation. Thus, use by a number of groups[10,14-16] of selective $P_{2T}$ and $P2Y_1$ antagonists, and/or exploitation of the susceptibility of $P2Y_1$ receptors to desensitization, has led to the now widely accepted 3 receptor model, illustrated in Figure 2.

While the functional significance of the $P2Y_1$ receptor on platelets remains unclear, $P2Y_1$ receptor activation has been linked to shape change and to transient (loose) platelet aggregation. Importantly, sustained platelet aggregation is dependent on stimulation of the third (currently uncloned) subtype of P2 receptor,[17,18] defined pharmacologically by highly selective and potent competitive antagonism by AR-C69931MX and analogues. To date, we have retained use of the term "$P_{2T}$" for this receptor, consistent with the original functional definition provided by ADP (agonist) and ATP (competitive antagonist).[2] However, it is important to note that this profile alone, in the absence of confirmation by use of one of the AR-C compounds, can no longer be considered definitive of the $P_{2T}$ subtype since recent findings indicate that, under conditions of low receptor density, this agonist/antagonist profile can apply equally to the $P2Y_1$ receptor.[10] This observation also invalidates the use of the term $P2Y_{ADP}$[19] as an alternative to $P_{2T}$ since, under the conditions pertaining at the platelet membrane, both the $P_{2T}$ and $P2Y_1$ subtypes are "ADP receptors" within the P2Y family. Other terms in common usage ("$P2T_{AC}$", "$P2Y_{AC}$", "$P2T_{CYC}$") can be considered synonymous with $P_{2T}$.

## A pivotal role for ADP in platelet activation

Early studies designed to determine the importance of released ADP in platelet responses initiated by other agonists provided conflicting results, principally due to the inadequacy of the available tools: the ADP scavenging enzyme, apyrase, or the ATP-regenerating system creatine phosphate/creatine phosphokinase.[20-22] The recent availability of selective $P_{2T}$ (and $P2Y_1$) antagonists has contributed to a growing body of evidence indicative of a central and, in some cases, permissive role for ADP in responses to other platelet stimuli. Thus, in human washed platelets in a 96-well assay format (see below for importance of defining assay system), an intact ADP/$P_{2T}$ axis is an important component of collagen-induced platelet aggregation and is essential to support aggregation to the thromboxane mimetic, U44619, or platelet activating factor (PAF).[23] This pivotal role of the ADP/$P_{2T}$ pathway has also been observed in human blood when aggregation is induced by collagen, 5-hydroxytryptamine (5HT), PAF, thrombin receptor activating peptide (TRAP), U46619 and adrenaline.[24] Although most stimuli of platelet activation cause release of ADP from dense granules it has still, until recently, been somewhat difficult to reconcile the *broad-spectrum* inhibitory profile of $P_{2T}$ antagonists with the fact that these compounds selectively target just one receptor subtype for a single platelet agonist. However, building on the model of ADP-induced platelet activation outlined in Figure 2, the initial finding that rapid and full expression of an ADP response requires activation of both $P_{2T}$ ($G_i$-coupled) and $P2Y_1$ (Gq-coupled) receptors[25] has been expanded to a working hypothesis that most platelet stimuli require

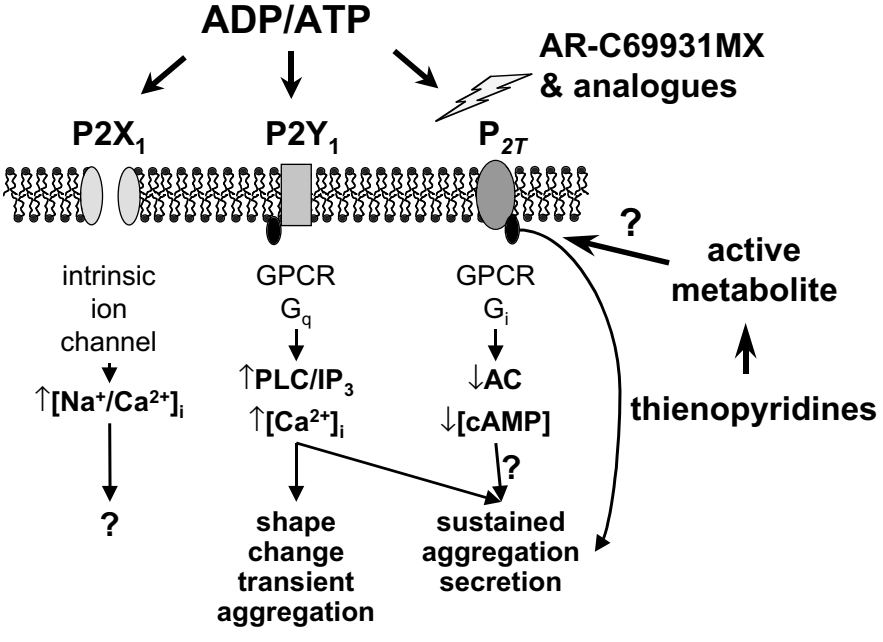


**Figure 2. The 3 receptor model of ADP-induced platelet activation indicating site of action of AR-C69931MX and analogs and of thienopyridines.**

co-activation of both the $G_i$ and $G_q$ pathways and that, certainly in the case of U46619-induced aggregation, the $G_i$ component is provided predominantly via the $P_{2T}$ receptor.[26] However, it is important to note that experimental observations and interpretation regarding the relative contribution from the $P_{2T}$ pathway can be significantly influenced by assay conditions. One example of this is that in a 96-well format with agitation by shaking of the plate, platelet aggregation induced by U46619 is absolutely dependent on an intact $P_{2T}$ pathway.[23] In contrast, with agitation by stirring in an aggregometer, the aggregation response to U46619 is only partially inhibited by a $P_{2T}$ antagonist.[7] One possible explanation for this difference is that, in the latter system, other agonists signaling through $G_i$ (eg. adrenaline) are better able to substitute for removal of the $P_{2T}$ component. It is likely that during thrombogenesis *in vivo*, the $G_i$ signal will be provided predominantly via the $P_{2T}$ pathway due to the presence of very high local concentrations of ADP.[27,28]

## ADP and other platelet responses

In addition to inhibition of platelet aggregation, significant effects of $P_{2T}$ antagonism on other aspects of platelet activation ([$^{14}$C]5HT release, P-selectin expression) have been observed following stimulation by the same range of agonists listed in the previous section.[24] The effect on P-selectin expression is particularly intriguing since, in addition to being implicated in thrombosis,[29] the platelet P-selectin/monocyte sialyl Lewis X interaction, as one mechanism of localizing inflammatory cells to the vessel wall as part of a growing thrombus, may be implicated in both acute and chronic disease progression.[30,31] Thus, it can be speculated that, by preventing this interaction, $P_{2T}$ antagonists may have disease-modifying as well as anti-thrombotic properties. In support of this possibility, results obtained in the first Phase II clinical study with AR-C69931MX showed that intravenous infusion of the P2T antagonist abolished ADP-induced platelet/monocyte conjugate formation measured *ex vivo*.[32]

## ADP in arterial thrombosis: hypothesis testing with AR-C69931MX and analogs

In addition to their value in defining the P2 receptor pharmacology of the platelet and the role of ADP in platelet activation *in vitro*, AR-C69931MX and related compounds, unlike available $P2X_1$ and $P2Y_1$ ligands, have properties suitable for probing the pathophysiological significance of $P_{2T}$ receptor-mediated platelet aggregation *in vivo*. Experience with these compounds in a number of animal models (Table 1) indicates that $P_{2T}$ receptor activation contributes significantly to arterial thrombosis and, therefore, that $P_{2T}$ antagonists have considerable potential as a novel class of anti-thrombotic agent. An important aspect of all these models is that there is no dependence on addition of exogenous ADP. Thus, they serve as true hypothesis tests for the role of endogenous ADP in thrombosis. The physicochemical properties of the triphosphate $P_{2T}$ antagonists make them suitable for parenteral administration only and the kinetic properties of AR-C69931MX and AR-C67085MX were designed to provide a rapid onset of action when administered by intravenous infusion, with a rapid reversal of effect following cessation of infusion.

Consistent with the high degree of selectivity and specificity observed *in vitro*, administration of anti-thrombotic doses of AR-C69931MX or AR-C67085MX did not produce any hemodynamic (blood pressure, heart rate) or hematologic (platelet, red and white cell counts, prothrombin time, activated partial thromboplastin time) effects *in vivo*.

**Table 1. Summary of the effects of intravenous infusion of the $P_{2T}$ receptor antagonists, AR-C69931MX and AR-C67085MX in animal models of thrombosis.**

| Model | Endpoint | $P_{2T}$ antagonist | Comparator | Main Findings | Reference |
|---|---|---|---|---|---|
| Cyclic Flow Reductions (CFR) in dog femoral artery | Inhibition of CFR *vs* prolongation of bleeding time | AR-C69931MX AR-C67085MX | Ro449883, GR144053, Aspirin | Greater anti-thrombotic efficacy *cf* aspirin. Equivalent efficacy *cf* GPIIb/IIIa antagonists with markedly greater anti-thrombotic/anti-haemostatic separation. | (9, 33) |
| Prosthetic graft in dog femoral arterial/arterial shunt | Occlusion time | AR-C67085MX | Aspirin | Significant increase in time to occlusion *cf* aspirin | (33) |
| Electrically damaged, stenosed rabbit carotid artery | Occlusion time | AR-C67085MX | Aspirin | Significant increase in time to occlusion *cf* control. Aspirin no effect. | (33) |
| tPA lysis of dog coronary artery thrombus | Coronary artery patency | AR-C69931MX | tPA + placebo | Significant improvement in post-lysis patency *cf* placebo | (34) |

## Comparison with other anti-thrombotic approaches

Having demonstrated the anti-thrombotic potential of $P_{2T}$ receptor antagonists, an important further consideration before progressing to clinical development was to be confident that this novel approach can offer some advantage compared to other established and emerging anti-thrombotic therapies. Thus, an important component of our work in animal models of thrombosis has been to obtain comparative data, with particular focus on aspirin (as the established "benchmark" anti-platelet therapy) and glycoprotein (GP) IIb/IIIa antagonists (as the key emerging therapy, particularly in the acute, intravenous setting). In studies conducted to date, AR-C67085MX has been shown to be more effective than aspirin in preventing thrombosis in the damaged, stenosed carotid artery of the rabbit and in preventing thrombotic occlusion of prosthetic (Dacron) grafts inserted in a femoral arterial-arterial shunt in the dog.[33] Of particular note is the observation that intravenous infusion of either AR-C69931MX[9] or AR-C67085MX[33] in a model of dynamic arterial thrombosis (modified from Folts,[35]) confers a unique anti-thrombotic profile, characterized by anti-thrombotic efficacy superior to that of aspirin and equivalent to that of fibrinogen receptor (GPIIb/IIIa) antagonists, without the degree of compromise of hemostasis associated with the latter class of agent. Another interesting observation in these experiments is that, for both the $P_{2T}$ antagonist and GPIIb/IIIa antagonist mechanisms, substantial (>90%) inhibition of ADP-induced platelet aggregation measured *ex vivo* is required for a full anti-thrombotic effect. While the relationship between the degree of inhibition of platelet aggregation and clinical benefit remains to be fully established in clinical practice, a recent study[36] indicates that, in patients undergoing coronary intervention, inhibition of platelet function by GPIIb/IIIa antagonists by ≤ 70% was associated with a higher risk of adverse events compared with inhibition of > 70%. The preclinical anti-thrombotic/anti-hemostatic profile observed for $P_{2T}$ receptor antagonists suggests that, with this class of agent, it may be possible to target high levels of platelet inhibition with less concern regarding bleeding complications compared to the GPIIb/IIIa antagonists. For intravenous $P_{2T}$ antagonists such as AR-C69931MX, this potential pharmacodynamic advantage is augmented by the high degree of control of effect provided by the rapid onset/offset kinetic properties.

## Comparison with thienopyridines

The foregoing discussion has highlighted the importance of the ADP/P2T axis in platelet activation and in experimental arterial thrombosis. An important role for this pathway in arterial thrombosis in man is suggested by clinical studies[37,39] with the thienopyridines, ticlopidine and clopidogrel, agents which indirectly (as prodrugs, see Figure 2) attenuate $P_{2T}$ receptor-mediated platelet aggregation, albeit to a limited extent.[40] As a result of the CAPRIE study,[37] clopidogrel is now indicated for «*the reduction of atherosclerotic events (myocardial infarction, stroke, vascular death) in patients with atherosclerosis documented by recent stroke, myocardial infarction, or established peripheral arterial disease*». However, the degree of clinical benefit observed with clopidogrel over aspirin in CAPRIE was modest (relative risk reduction of 8.7% *vs.* aspirin) and a crucial question for our program was whether "direct" acting $P_{2T}$ receptor antagonists such as AR-C69931MX have the potential to provide a greater level of efficacy. At the current stage of development, a comparison on the basis of relative clinical efficacy was not feasible but, given the potential linkage between anti-platelet and anti-thrombotic efficacy (see above), a comparison of effects of AR-C69931MX and clopidogrel on ADP-induced platelet aggregation seemed worthwhile. Using whole blood impedance aggregometry, we compared the effects of a clinically-relevant concentration of AR-C69931MX added *in vitro* with that of clopidogrel administered orally at the approved therapeutic dose (75 mg/day) to 8 healthy male volunteers for 11 days. Our findings[41] of limited inhibition *ex vivo* of ADP-induced platelet aggregation by clopidogrel (46% inhibition vs 10 mM ADP) were consistent with published data[40] and contrasted markedly with the near complete inhibition observed with the direct P2T antagonist, AR-C69931MX, added *in vitro* (97% inhibition vs 10 mM ADP). These results are also consistent with preclinical findings in the rat indicating greater anti-aggregatory efficacy of AR-C67085MX compared with ticlopidine.[42]

## Clinical experience

The attractive preclinical properties of AR-C67085MX and AR-C69931MX supported progression to clinical evaluation as a potential novel class of anti-thrombotic agent and AR-C69931MX is currently completing its phase II program.

In phase I studies, intravenous infusion of AR-C69931MX was well tolerated in healthy subjects and produced dose-related inhibition of ADP-induced platelet aggregation measured *ex vivo*.[43] Full inhibition of the aggregation response occurred at doses producing only modest prolongation of bleeding time and reversal of effects was rapid and complete within 20 min of cessation of infusion at the highest dose (4 µg/kg/min iv).

The dynamic and kinetic profile of AR-C69931MX makes it ideally suited as an anti-thrombotic for use in acute coronary syndromes. In phase II evaluation in patients with unstable angina/non Q-wave myocardial infarction[44,45] or in those undergoing coronary intervention,[46] addition of AR-C69931MX (up to 4 µg/kg/min iv) to standard therapy with aspirin and heparin (or low molecular weight heparin) has proven to be well tolerated and not associated with any significant increase in major bleeding or major adverse events.

## Summary and conclusion

Understanding of structure-activity requirements for the platelet $P_{2T}$ receptor has enabled development of a series of highly potent and selective $P_{2T}$ receptor antagonists. As such, AR-C69931MX and related compounds have proven to be valuable pharmaco-

logic tools in defining the P2 receptor pharmacology of the platelet and in defining the role of ADP in platelet activation *in vitro*. In addition, AR-C69931MX and AR-C67085MX have properties suitable for probing the pathophysiologic significance of $P_{2T}$ receptor-mediated platelet aggregation in models of arterial thrombosis *in vivo*. This has enabled demonstration that blockade of the $P_{2T}$ receptor confers a unique anti-thrombotic:anti-hemostatic profile which offers potential advantages over other anti-thrombotic approaches. Results from phase I clinical evaluation of AR-C69931MX have shown that full inhibition of ADP-induced platelet aggregation can be achieved in man in a controllable manner and without significant compromise of hemostasis. AR-C69931MX is currently in late Phase II clinical development as an acute use anti-thrombotic agent, intended for intravenous administration to patients with acute coronary syndromes being managed with or without intervention.

Experience gained with AR-C69931MX and analogs provides considerable encouragement for development of orally-active $P_{2T}$ receptor antagonists and an expectation that these will have significant advantages over other existing and emerging oral anti-thrombotic agents.

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## References

1. Gaarder A, Jonsen J, Laland S, Hellem A, Owren PA. Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. Nature 1961; 192:531-2.
2. Macfarlane DE, Mills DCB. The effects of ATP on platelets: evidence against the central role of released ADP in primary aggregation. Blood 1975; 46: 309-20.
3. Gordon JL. Extracellular ATP: effects, sources and fate. Biochem J 1986; 233:309-19.
4. Cusack NJ, Hourani SMO. Adenosine 5'-diphosphate antagonists and human platelets: no evidence that aggregation and inhibition of stimulated adenylate cyclase are mediated by different receptors. Br J Pharmacol 1982; 76:221-7.
5. Cusack NJ, Hourani SMO. Specific but noncompetitive inhibition by 2-alkylthio analogues of adenosine 5'-monophosphate and adenosine 5'-triphosphate of human platelet aggregation induced by adenosine 5'-diphosphate. Br J Pharmacol 1982; 75:397-400.
6. Welford LA, Cusack NJ, Hourani SMO. ATP analogues and the Guinea-pig Taenia coli: a comparison of the structure-activity relationships of ectonucleotidases with those of the P2-purinoceptor. Eur J Pharmacol 1986; 129:217-24.
7. Humphries RG, Tomlinson W, Ingall AH, Cage PA, Leff P. FPL 66096: A novel, highly potent and selective antagonist at human platelet P2T-purinoceptors. Br J Pharmacol 1994; 113:1057-63.
8. Humphries RG, Tomlinson W, Clegg JA, Ingall AH, Kindon ND, Leff P. Pharmacological profile of the novel P2T-purinoceptor antagonist, FPL 67085 in vitro and in the anaesthetized rat in vivo. Br J Pharmacol 1995; 115:1110-16.
9. Ingall AH, Dixon J, Bailey A, et al. Antagonists of the platelet P2T-receptor: a novel approach to antithrombotic therapy. J Med Chem 1999; 42:213-20.
10. Fagura MS, Dainty IA, McKay GD, et al. P2Y1-receptors in human platelets which are pharmacologically distinct from P2YADP-receptors. Br J Pharmacol 1998; 124:157-64.
11. Communi D, Robaye B, Boeynaems JM. Pharmacological characterization of the human P2Y11 receptor. Br J Pharmacol 1999; 128:1199-1206.
12. Tomlinson W, Humphries RG, Robertson MJ, Leff P. ARL 67085 and ARL 66096 are slowly dissociating competitive P2T-purinoceptor antagonists [abstract]. Br J Pharmacol 1997; 120(Suppl):131P.
13. Tomlinson W, Cusworth E, Midha A, Kirk IP, Humphries RG, Leff P. Characterisation of the P2T receptor antagonist properties of AR-C69931MX in human washed platelets in vitro [abstract]. Paper presented at The Platelet ADP Receptors: Biochemistry, Physiology, Pharmacology and Clinical aspects Meeting, La Thuile, Italy, 2000.
14. MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of receptor-operated cation channels via P2X1 not P2T purinoceptors in human platelets. J Biol Chem 1996; 271:2879-80.
15. Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation I. Evidence for three distinct ADP receptors on human platelets. J Biol Chem 1998; 273:2024-9.
16. Geiger J, Honig-Liedl P, Schanzenbacher P, Walter U. Ligand specificity and ticlopidine effects distinguish three human platelet ADP receptors. Eur J Pharmacol 1998; 351:235-47.
17. Jarvis GE, Humphries RG, Robertson MJ, Leff P. ADP can induce aggregation of human platelets via both P2Y1 and P2T receptors. Br J Pharmacol 2000; 129: 275-82.
18. May JA, Glenn JR, Spangenberg P, Heptinstall S. The composition of the platelet cytoskeleton following activation by ADP: effects of various agents that modulate platelet function. Platelets 1997; 7:159-68.
19. Fredholm BB, Abbrachio MP, Burnstock GB et al. Towards a revised nomenclature for P1 and P2 receptors. Trends Pharmacol Sci 1997; 18:79-81.
20. Huang EM, Detwiler TC. Reassessment of the evidence for the role of secreted ADP in biphasic platelet aggregation. J Lab Clin Med 1980; 95:59-68.
21. Morinelli TA, Niewiarowski S, Kornecki E, Figures WR, Wachtfogel Y, Colman RW. Platelet aggregation and exposure of fibrinogen receptors by prostaglandin endoperoxide analogues. Blood 1983; 61:41-9.
22. Nunn B, Chamberlain PD. Further evidence against the validity of using an ADP-removing enzyme system (CP/CPK) for demonstrating the role of secreted ADP in platelet activation. Thromb Res 1983; 30:19-26.
23. Tomlinson W, Kirk IP, Humphries RG, Leff P. P2T receptor activation by ADP: a permissive role in aggregation of human washed platelets induced by PAF or U46619 [abstract]. Paper presented at The Platelet ADP Receptors: Biochemistry, Physiology, Pharmacology and Clinical aspects, La Thuile, Italy, 2000.
24. Storey RF, White A, May JA, Newby LJ, Heptinstall S. The central role of the P2T receptor in amplification of platelet aggregation and secretion [abstract]. Thromb Haemostas 1999(Suppl):1329.
25. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced

platelet aggregation. Cell Biol 1998; 95:8070-4.
26. Paul BZ, Jin J, Kunapuli SP (1999). Molecular mechanism of thromboxane A(2)-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors. J Biol Chem 1999; 274:29108-14.
27. Born GVR, Kratzer MAA. Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. J Physiol 1984; 354:419-29.
28. Ugurbil K, Holmsen H. In: Gordon JL, ed. Platelets in biology and pathology, II. New York: Elsevier/North-Holland, 1981:147-77.
29. Ueyama T, Ikeda H, Haramaki N, Kuwano K, Imaizumi T. Effects of monoclonal antibody to P-selectin and analogue of sialyl Lewis X on cyclic flow variations in stenosed and endothelium-injured canine coronary arteries. Circulation 1997; 95:1554-9.
30. Azar RR, Waters DD. The inflammatory etiology of unstable angina. Am Heart J 1996; 132:1101-6.
31. Ross R. Atherosclerosis – an inflammatory disease. N Engl J Med 1999; 340:115-26.
32. Sanderson, HM, Storey RF, Wilcox RG, Heptinstall, S. The effects of a P2T antagonist on platelet aggregation, activation and platelet-leukocyte conjugate formation in vitro and following administration to patients with unstable angina. Thromb Haemostas 1999(Suppl):31.
33. Leff P, Robertson MJ, Humphries RG. The role of ADP in thrombosis and the therapeutic potential of P2T-receptor antagonists as novel antithrombotic agents. In: Jacobson A, Jarvis MF, eds. Purinergic approaches in experimental therapeutics. New York: Wiley-Liss Inc, 1997:203-16.
34. Wang K, Zhou X, Zhongmin Z, Topol E, Lincoff EA. Sustained coronary artery recanalization with adjunctive infusion of a novel P2T-receptor antagonist AR-C69931 in a canine model [abstract]. J Am Coll Cardiol 2000; 35(Suppl A):281.
35. Folts JD, Crowell EB, Rowe GG. Platelet aggregation in partially obstructed vessels and its elimination with aspirin. Circulation 1976; 54:365-70.
36. Steinhubl S, Talley D, Kereiakes D, et al. A Prospective Multicenter Study to Determine the Optimal Level of Platelet Inhibition With GPIIb/IIIa Inhibitors in Patients Undergoing Coronary Intervention - The GOLD Study [abstract]. J Am Coll Cardiol 2000; 35 (Suppl A):44.
37. CAPRIE Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). Lancet 1996; 348:1326-39.
38. Balsano F, Rizzon P, Violi F et al. Antiplatelet treatment with ticlopidine in unstable angina. Circulation 1990; 82:17-26.
39. Gent M, Blakely JA, Easton JD et al. The Canadian American Ticlopidine Study (CATS) in thromboembolic stroke. Lancet 1989; 2:1215-20.
40. Boneu B, Destelle G, on behalf of the Study Group. Platelet anti-aggregating activity and tolerance of clopidogrel in atherosclerotic patients. Thromb Haemostas 1996; 76:939-43.
41. Jarvis GE, Nassim MA, Humphries RG, et al. The P2T antagonist AR-C69931MX is a more effective inhibitor of ADP-induced platelet aggregation than clopidogrel [abstract]. Blood 1999; 94(Suppl 1):22a.
42. Clegg JA, Fraser-Rae L, Humphries RG, Robertson MJ. The effect of FPL 67085 on ADP-induced platelet aggregation ex vivo in the urethane-anaesthetized rat: a comparison with oral aspirin and ticlopidine [abstract]. Br J Pharmacol 1995; 114(Suppl):102P.
43. Nassim MA, Sanderson JB, Clarke C, et al. Investigation of the novel P2T receptor antagonist AR-C69931MX on ex vivo adenosine diphosphate-induced platelet aggregation and bleeding time in healthy volunteers [abstract]. J Am Coll Cardiol 1999; 33(Suppl A):225.
44. Storey RF, Oldroyd KG, Wilcox RG. First clinical study of the novel platelet ADP receptor (P2T ) antagonist AR-C69931MX, assessing safety, tolerability and activity in patients with acute coronary syndromes [abstract]. Am Heart J 1999(Suppl):3514.
45. Jacobsson F, Dellborg M, Swahn E, Wallentin L. Tolerance and safety of cangrelor, a novel purine receptor antagonist, used as a platelet aggregation inhibitor in the acute coronary syndrome [abstract]. J Am Coll Cardiol 2000; 35(Suppl A):343.
46. Weaver WD, Harrington RA, Grines CL, et al. Intravenous AR-C69931MX, a novel P2T platelet receptor antagonist in patients undergoing percutaneous coronary interventions-preliminary results from a placebo or active controlled trial [abstract]. J Am Coll Cardiol 2000; 35(Suppl A):36.

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# PHARMACOLOGY OF TICLOPIDINE AND CLOPIDOGREL

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ABSTRACT

**Large clinical trials performed with ticlopidine in patients with atherosclerotic arterial diseases have shown that it is of benefit to patients who are at high risk of vascular events and have demonstrated to be more efficacious than aspirin. The search for other active antiplatelet drugs within the original chemical class of the thienopyridines led to the discovery of a new molecule: clopidogrel. Clopidogrel is a novel ADP-selective agent whose antiaggregating properties are several times greater than those of ticlopidine and are apparently due to the same mechanism of action (i.e. inhibition of ADP binding to one of its platelet receptors). This effect has been seen in various experimental animal species as well as in healthy volunteers and in atherosclerotic patients. Of particular interest is the ability of this drug to prevent arterial as well as venous thrombosis in animals and also to reduce myointimal thickening occurring after endothelial injury of the rabbit carotid artery. Clopidogrel seems to be better tolerated than ticlopidine and, on the basis of the activity/toxicity ratio observed, is a promising compound for evaluation in atherosclerotic cardiovascular and cerebrovascular diseases. The clopidogrel anti-aggregating activity has been attributed to a recently evidenced active metabolite, generated by hepatic metabolism, which reproduces *in vitro* all the antiplatelet effects of clopidogrel. This compound irreversibly affects the not yet cloned ADP receptor P2YAC, triggering the activation of the GpIIb-IIIa complex and the platelet aggregation when co-stimulated with P2Y1. The efficacy of thienopyridines in preventing thrombosis in atherosclerotic patients demonstrates that P2YAC is a relevant target for antithrombotic drugs.**


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## Introduction

Over the last decade, considerable interest has been focused on the role of platelets and platelet inhibitor therapy in atherosclerosis-derived diseases. The well-established role of platelets in arterial thrombosis provided the rationale for the development of many drugs which inhibit platelet functions[1] and the treatment of cardiovascular diseases and, in particular, ischemic heart disease, has been unquestionably transformed by the use of anti-platelet therapy. Fortunately, there has been remarkable growth in our understanding of the molecular mechanisms of platelet aggregation and several new antiplatelet agents have recently emerged. Ticlopidine was discovered in 1972 and developed as an antithrombotic drug some years later. In 1978, ticlopidine was introduced on the market with a very narrow indication, that of extracorporeal circulation. Then, having proved its benefit, it became a useful antithrombotic drug. Ticlopidine has been shown to exhibit beneficial effects in patients with a transient ischemic attack (TIA) or a reversible ischemic neurologic deficit (RIND) or a minor stroke,[2] in patients with a recent history of a major ischemic cerebral event related to atherosclerosis[3] and in patients with peripheral vascular disease.[4] In this high-risk population, ticlopidine reduced cardiovascular morbidity and mortality by 60% whereas there was no evidence that aspirin was effective. Clopidogrel was discovered in 1986 and approved for use in western countries in 1997. This compound has been demonstrated to prevent cardiovascular death in atherosclerotic patients in CAPRIE, a randomized phase-III, triple-blinded clinical trial enroling more than 19,000 patients with atherosclerotic disease. It was shown in this trial that clopidogrel was more effective than aspirin in reducing the combined risk of ischemic stroke, myocardial infarction or vascular death and it might be used for widespread prevention of fatal or non-fatal systemic ischemic events.[5] The CAPRIE study also showed that the overall safety profile of clopidogrel was at least as good as that of medium-dose aspirin.

## Pharmacology

Ticlopidine and clopidogrel belong to the thienopyridine family of drugs. That having been said, the presence of a methoxy carbonyl group on the benzylic position in the clopidogrel molecule provides increased pharmacologic activity and gives this drug a better safety profile. Clopidogrel is an S enan-

tiomer, and when tested in animals, the corresponding R enantiomer was devoid of antithrombotic activity, indicating that this position is critical for the pharmacologic activity of the drug. The antithrombotic activity of thienopyridines has been demonstrated in several animal species and models of arterial thrombosis, some of which were insensitive to aspirin.[6-8] In various arterial-type models of thrombosis, clopidogrel exhibited a potent, dose-dependent antithrombotic activity, being approximately 50 times more potent than ticlopidine and about 100 fold more active than aspirin.[6-15] In experiments aimed at determining the role of platelets in experimental venous thrombosis, we showed that ADP-mediated platelet activation played a major role in the development of venous thrombosis under low thrombogenic conditions[16] and suggested that clopidogrel may be of therapeutic interest in pathologies involving venous thrombosis. A recent study from our laboratory[17] gave further insight into these processes, showing that clopidogrel was able to alter thrombin generation in rat platelet rich plasma, therefore confirming that ADP-induced platelet aggregation is of particular importance in the triggering of venous-type thrombosis. Since these compounds have been chosen for their ability to affect platelet aggregation when tested *ex vivo*, their antithrombotic activity has been attributed to this property but some attempts have been made to find other pharmacologic effects of thienopyridines, which could be relevant to their antithrombotic effects. These include decrease of the levels of circulating fibrinogen,[18] increase of erythrocyte filterability,[19] and stimulation of nitric oxide production.[20] However, these effects, although contributing to the overall protective effect of these drugs, seem to be of secondary interest with regard to their antiplatelet activity. The effects of ticlopidine and clopidogrel on platelet function have been extensively studied. A reduction of platelet aggregability has been reported for numerous agonists, but the effects against ADP were the most frequently observed.[6,7] Antagonism of the fibrinogen receptor Gp IIb-IIIa,[21] inhibition of the generation of prostanoids,[22] activation of adenylyl cyclase, and inhibition of phosphodiesterases[23] were first proposed as mechanisms responsible for the anti-aggregating activity of these compounds, but these hypotheses were invalidated in further studies[24,25,26,27] and it is now clearly admitted that these compounds are selective inhibitors of ADP-induced aggregation.[28]

## Mode of action of thienopyridines

ADP has been demonstrated to induce several changes in platelets, only some of which are affected by thienopyridines. In the 80s, this observation suggested that ticlopidine and clopidogrel interacted with different transduction pathways but the recent demonstration of the presence of different ADP receptor types at the platelet surface now explains the observed results. In the past, the ADP receptor had not been identified as the target of thienopyridines, but by measuring the binding of [$^{32}$P]-2-MeS-ADP to platelets, Mills *et al.* clearly demonstrated that clopidogrel treatment affected ADP receptors on human platelets.[29] This inhibitory property of clopidogrel and ticlopidine was then confirmed by us in rats.[30] At the same time, based on clopidogrel selectivity and differences in ADP affinity, the presence of two different ADP receptor types was first shown on rat platelets.[31]

### *Thienopyridine-resistant platelet purinoceptors: P2Y1*

The clopidogrel-resistant effects of ADP were attributed to platelet high affinity receptors, which were recently identified as P2Y1.[32] This receptor represents almost 20% of the receptors recognized by 2-MeS-ADP at the platelet surface. When expressed in Jurkat cells, P2Y1 behaves as a $G_q$-coupled receptor triggering the activation of inositol phosphate metabolism and cytosolic calcium increase. These effects are inhibited by A3P5P.[33] The stimulation of platelets with low ADP concentrations induces shape change, release of calcium from internal pools and IP metabolism in a P2Y1-dependent manner, as shown by the inhibition by A3P5P and the lack of effect of the thienopyridines.[32,33,34,35] Consequently, in rat platelets treated with clopidogrel, no major changes were observed in the ADP-induced phosphorylation of plekstrine (p47) or myosin light chain (p20) phosphorylation, two processes linked to P2Y1 activation by ADP.[36] Recently, two different groups have generated P2Y1-deficient mice.[37,38] These animals have pronounced defects in platelet functions including shape change, cytosolic calcium increased and IP metabolism, but also aggregation. These observations clearly indicate that the P2Y1 pathway is activated during ADP-induced aggregation, as proposed previously by several authors.[32,33,34] It should also be noted that purine dependent calcium influx has been associated with the activation of another purinoreceptor present at the platelet surface: P2X1.[34] The importance of this calcium channel in platelet activation remains controversial. We found that activation of this receptor by its major ligand $\alpha$-$\beta$ methylene-ATP neither potentiated nor attenuated ADP-induced shape-change or aggregation.[39] Similar findings were also found in human platelets,[40] but a recent report states that platelets from a subject with a defect in P2X1 failed to aggregate after an ADP challenge.[41] Clopidogrel has been shown not to affect P2X1 in rat platelets.[39]

### *Thienopyridine-sensitive platelet purinoceptor: $P2Y_{AC}$*

The thienopyridine-sensitive receptor, named P2t or $P2Y_{AC}$, whose structure has not been defined yet, down regulates adenylyl cyclase through a $G_i$-dependent pathway when stimulated by micromolar concentrations of ADP.[42] The presence of this receptor does not seem to be restricted to platelets, since ADP-dependent downregulation of adenylyl cyclase has been observed in other cells, including rat C6 glioma cells[43] and rat B10 cerebral capillary endothelial cells.[44] However, attempts to find similar receptors linked to the same kind of regulation in cells of hemapoietic lineage, including megakaryocytoblas-

tic cell lines, have not been successful.[45] The thienopyridine-related prevention of the ADP-induced inhibition of adenylyl cyclase is not directly responsible for the anti-aggregating effect of thienopyridines, since direct inhibition of adenylyl cyclase by SQ 22536 did not affect the inhibition of the ADP-induced platelet aggregation by clopidogrel.[46] This, added to the fact that adenylyl cyclase inhibition by SQ 22536 does not potentiate platelet aggregation induced by threshold concentrations of ADP, clearly indicates that cyclase downregulation is an epiphenomenon of platelet aggregation. This finding was controversially discussed by Weber[47] but confirmed recently by Kunapuli.[48] Inherited defects of this pathway have been reported by two independent groups, in both cases being strongly comparable to subjects treated by thienopyridines,[49,50] including defective binding of 2MeS-ADP, defective ADP-induced cyclase downregulation and aggregation and defective hemostasis. These observations, together with the pharmacologic effects of thienopyridines demonstrate the significance of the $P2Y_{AC}$ in platelet aggregation.

### *Purinoreceptor-dependent platelet aggregation*

Both ADP receptors have been demonstrated to act in synergy to trigger platelet aggregation, each of them being ineffective alone.[32,33,34] Therefore, the inhibition of only one pathway results in a potent inhibition of ADP-triggered platelet aggregation. This correlates perfectly with the *in vitro* effects of A3P5P, the *ex vivo* activity of thienopyridines and the defects observed in P2Y1-deficient mice and in patients with a defective ADP pathway. Gp IIb-IIIa complex activation, which allows the binding of fibrinogen to platelets has been demonstrated to be inhibited in subjects treated with thienopyridines[24,51] and in ADP pathway defective patients,[49,50] when platelet aggregation was stimulated by ADP. In the same way, by electronic microscopy, strong similarities were noted between platelet clots from clopidogrel-treated subjects and from ADP pathway defective patients,[52] showing a loose-woven structure, with only few interplatelet contacts. Activation of P2Y1 and $P2Y_{AC}$ pathways have been found to be mimicked by 5HT2A[32] and alpha1-AR respectively,[53] thus indicating that they share common elements in their transducing pathways. All the information acquired with thienopyridines as pharmacologic tools has since been confirmed with another $P2Y_{AC}$ receptor antagonist, ARL66096.[54]

## Metabolism

Ticlopidine and clopidogrel need to be administered *in vivo* to exhibit anti-aggregating activity. However, some direct effects of thienopyridines *in vitro* have been reported: inhibition of platelet aggregation,[55] inhibition of mitochondrial oxidative metabolism,[56] anti-angiogenic[57] and pro-apoptotic effects.[58] Nevertheless, these effects (most of them being observed at non-relevant doses) do not seem to account for the *ex vivo* anti-aggregant activity of these drugs, responsible for their antithrombotic properties. The anti-aggregating activity of ticlopidine only occurs after repeated oral administration,[6] whilst a similar effect is obtained approximately 2 hours after oral or intravenous administration of a single dose of clopidogrel.[7] The achievement of an anti-aggregating effect only after *in vivo* administration suggests that the thienopyridines do not act directly on platelets, and shows that an active anti-aggregant substance must be produced through a metabolic process. A study performed on clopidogrel confirmed this hypothesis.[59] We showed that the liver was the metabolic site from which the anti-aggregant activity of clopidogrel originates. This was demonstrated by functional hepatectomy, achieved by inserting a portal-jugular shunt, which abolished the anti-aggregant effect of clopidogrel and by liver perfusion studies. We further showed that the hepatic bioactivation of clopidogrel required a cytochrome P450-1A-dependent metabolism.[60] A study of the metabolism of ticlopidine resulted in the identification of about twenty separate metabolites,[8] representing approximately 30% of the initial compound, but none of them had *in vitro* activity. The other metabolites representing approximately 70% of the initial compound have not yet been identified but no study has been able to demonstrate an anti-aggregant activity in the plasma of treated subjects.[51,61] This suggests that the active metabolite(s) circulate at very low concentrations and/or may have a very short half-life/lives. Furthermore, since the platelets of clopidogrel-treated subjects remain resistant to ADP even after washing, the anti-platelet effects of clopidogrel are irreversible.[51,61] The inhibition of platelet aggregation continues after the end of treatment, and the rate at which aggregation is restored correlates closely with platelet production.[7,8] These observations suggested the presence of an active metabolite of clopidogrel, produced by the liver, acting in an irreversible manner on platelets. This compound has recently been purified and its chemical structure determined.[62] It is a thiol reactive of clopidogrel which directly targets the $P2Y_{AC}$ receptor at the ADP-binding site on platelets. This interaction is highly specific and irreversible, two features which correspond to the anti-aggregating activity observed after clopidogrel treatment.

## Conclusions

In conclusion, thienopyridines, by irreversibly antagonizing the $P2Y_{AC}$ receptor on platelets, provide long lasting protection of platelets against ADP, a key mediator of thrombosis. The selectivity of thienopyridines with regard to platelet activation by ADP has allowed the significance of the latter to be evaluated in platelet physiology, pathophysiology, hemostasis and thrombosis. These compounds have enabled the discovery of several ADP receptors present at the platelet surface and allowed several biochemical changes to be clearly attributed to one of these platelet ADP receptors. The existence of a congenital deficiency in ADP receptors, which duplicates perfectly the effects of a thienopyridine treatment, has confirmed these observations. Beyond the observed cellular events, the effects on hemostasis and thrombosis demonstrate the *in vivo* importance of the $P2Y_{AC}$ platelet purinoreceptor. Clopidogrel's

specificity for such a relevant activation pathway, associated with it good tolerance profile are indicators of its efficacy and safety, such that this drug is presently considered as the Gold Standard in secondary prevention in atherosclerotic patients.

## References


1. Anti-platelet Trialists Collaboration. Secondary prevention of vascular disease by prolonged antiplatelet therapy. Br Med J 1988; 296:320-31.
2. Hass WK, Easton JD, Adams HP Jr, Pryse-Phillips W, Molony BA, Anderson S. A randomized trial comparing ticlopidine hydrochloride with aspirin for the prevention of stroke in high-risk patients. Ticlopidine Aspirin Stroke Study Group. N Engl J Med 1989; 321: 501-7.
3. Gent M, Blakely JA, Easton JD, et al. The Canadian American Ticlopidine Study (CATS) in thromboembolic stroke. Lancet 1989; 1:1215-20.
4. Janzon L, Bergqvist D, Boberg J, et al. Prevention of myocardial infarction and stroke in patients with intermittent claudication; effects of ticlopidine. Results from STIMS, the Swedish Ticlopidine Multicenter Study. J Intern Med 1990; 227:301-8.
5. CAPRIE Steering Committee. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). Lancet 1996; 348:1329-39.
6. Maffrand JP, Defreyn G, Bernat A, Delebassée D, Tissinier AM. Reviewed pharmacology of ticlopidine. Acta Med Int Angiologie 1988; (suppl. 77):6-13.
7. Herbert JM, Frehel D, Vallée E, et al. Clopidogrel, a novel antiplatelet and antithrombotic agent. Cardiovasc Drug Rev 1993; 11:180-98.
8. Panak E, Maffrand JP, Picard-Fraire C, Vallée E, Blanchard J, Roncucci R. Ticlopidine: a promise for the prevention and treatment of thrombosis and its complications. Haemostasis 1983; 13:1-54.
9. Yao SK, Ober JC, McNatt J, et al. ADP plays an important role in mediating platelet aggregation and cyclic flow variations in vivo in stenosed and endothelium-injured canine coronary arteries. Circ Res 1992; 70:39-48.
10. Chesebro JH, Lam JYT, Badimon L, Fuster V. Restenosis after arterial angioplasty: a hemorrheologic response to injury. Am J Cardiol 1987; 60:10B-6B.
11. Herbert JM, Bernat A, Sainte-Marie M, Dol F, Rinaldi M. Potentiating effect of clopidogrel and SR46349, a novel 5HT2 antagonist on streptokinase-induced thrombolysis in the rabbit. Thromb Haemost 1993; 69:268-71.
12. Bernat A, Dol F, Herbert JM, Sainte-Marie M, Maffrand JP. Potentiating effects of anticoagulants and antiplatelet agents on streptokinase-induced thrombolysis in the rabbit. Fibrinolysis 1993; 7:23-30.
13. Yao SK, McNatt J, Anderson HV, et al. Concomitant administration of clopidogrel with tissue type plasminogen activator delays reocclusion after the lysis of thrombi in canine coronary arteries. Circulation 1990; 82:770.
14. Ross R. The pathogenesis of atherosclerosis. An update. N Engl J Med 1986; 314:488-500.
15. Herbert JM, Tissinier A, Defreyn G, Maffrand JP. Inhibitory effect of clopidogrel on platelet adhesion and intimal proliferation following arterial injury in rabbits. Artherioscler Thromb 1993; 13:1171-9.
16. Herbert JM, Bernat A, Maffrand JP. Importance of platelets in experimental venous thrombosis in the rat. Blood 1992; 80:2281-6.
17. Dol F, Gaich C, Bernat A, Mauran G, Herbert JM. Effect of clopidogrel on thrombin generation in the rat. Thromb Res 1992; 65:S160.
18. Mazoyer E, Ripoll L, Boisseau MR, Drouet L. How does ticlopidine treatment lower plasma fibrinogen? Thromb Res 1994; 75:361-70.
19. Hayakawa M, Kuzuya F. Effects of ticlopidine on erythrocyte aggregation in thrombotic disorders. Angiology 1991; 42:747-53.
20. de Lorgeril M, Bordet JC, Salen P, et al. Ticlopidine increases nitric oxide generation in heart-transplant recipients: a possible novel property of ticlopidine. J Cardiovasc Pharmacol 1998; 32:225-30.
21. Cimminiello C, Milani M, Uberti T, Arpaia G, Bonfardeci G. Effects of ticlopidine and indobufen on platelet aggregation induced by A23187 and adrenaline in the presence of different anticoagulants. J Int Med Res 1989; 17:514-20.
22. Ashida SI, Abiko Y. Mode of action of ticlopidine in inhibition of platelet aggregation in the rat. Thromb Haemost 1979; 41:436-49.
23. Bonne C, Battais E. Ticlopidine and adenylate cyclase. Agents Actions Suppl 1984; 15:88-96.
24. Gachet C, Stierlé A, Cazenave JP, et al. The thienopyridine PCR 4099 selectively inhibits ADP-induced platelet aggregation and fibrinogen binding without modifying the membrane glycoprotein IIb-IIIa complex in rat and in man. Biochem Pharmacol 1990; 40:229-38.
25. Johnson M, Heywood JB. Possible mode of action of ticlopidine: a novel inhibitor of platelet aggregation. Thromb Haemost 1979; 42:367.
26. Gachet C, Cazenave JP, Ohlmann P, et al. The thienopyridine ticlopidine selectively prevents the inhibitory effects of ADP but not of adrenaline on cAMP levels raised by stimulation of the adenylate cyclase of human platelets by PGE1. Biochem Pharmacol 1990; 40:2683-7.
27. Defreyn G, Gachet C, Savi P, Driot F, Cazenave JP, Maffrand JP. Ticlopidine and clopidogrel (SR 25990C) selectively neutralize ADP inhibition of PGE1-activated platelet adenylate cyclase in rats and rabbits. Thromb Haemost 1991; 65:186-90.
28. Cattaneo M, Akkawat B, Lecchi A, Cimminiello C, Capitanio AM, Mannucci PM. Ticlopidine selectively inhibits human platelet responses to adenosine diphosphate. Thromb Haemost 1991; 66:694-9.
29. Mills DCB, Puri R, Hu CJ, et al. Clopidogrel inhibits the binding of ADP analogues to the receptor mediating inhibition of platelet adenylate cyclase. Arterioscler Thromb 1992; 12:430-6.
30. Savi P, Laplace MCl, Maffrand JP, Herbert JM. Binding of [$^3$H]-2-methylthio-ADP to rat platelets - effect of clopidogrel and ticlopidine. J Pharmacol Exp Ther 1994; 269:772-7.
31. Savi P, Laplace MC, Herbert JM. Evidence for the existence of two different ADP-binding sites on rat platelets. Thromb Res 1994; 76:157-69.
32. Savi P, Beauverger P, Labouret C, et al. Role of P2Y1 purinoceptor in ADP-induced platelet activation. FEBS Lett 1998; 422:291-5.
33. Leon C, Vial C, Cazenave JP, Gachet C. Cloning and sequencing of a human cDNA encoding endothelial P2Y1 purinoceptor. Gene 1996; 171:295-7.
34. Jin J, Daniel JL, Kunapuli SP. Molecular basis for ADP-induced platelet activation. II. The P2Y1 receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. J Biol Chem 1998; 273:2030-4.
35. Derian CK, Friedman PA. Effects of ticlopidine ex vivo on platelet intracellular calcium mobilization. Thromb Res 1988; 50:65-76.
36. Savi P, Artcanuthurry V, Bornia J, et al. Effect of clopidogrel treatment on ADP-induced phosphorylations

in rat platelets, Br J Haematol 1997; 97:185-91.
37. Fabre JE, Nguyen M, Latour A, et al. Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y1-deficient mice. Nat Med 1999; 5:1199-202.
38. Leon C, Hechler B, Freund M, et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. J Clin Invest 1999; 104:1731-7.
39. Savi P, Bornia J, Salel V, et al. Characterization of P2X1 purinoreceptors on rat platelets: effect of clopidogrel. Br J Haematol 1997; 98:880-6.
40. Vial C, Hechler B, Leon C, Cazenave JP, Gachet C. Presence of P2X1 purinoceptors in human platelets and megakaryoblastic cell lines. Thromb Haemost 1997; 78:1500-4.
41. Oury C, Toth-Zsamboki E, Van Geet C, Nilius B, Vermylen J, Hoylaerts MF. A dominant negative mutation in the platelet P2X1 ADP receptor causes severe bleeding disorder. Important role of P2X1 in ADP-induced platelet aggregation. Blood 1999; (Suppl 618a).
42. Boeynaems JM, Communi D, Savi P, Herbert JM. P2Y receptors: in the middle of the road. Trends Pharmacol Sci 2000; 21:1-3.
43. Nicholas RA, Lazarowski ER, Watt WC, Li Q, Boyer J, Harden TK. Pharmacological and second messenger signalling selectivities of cloned P2Y receptors. J Auton Pharmacol 1996; 16:319-23.
44. Webb TE, Feolde E, Vigne P, et al. The P2Y purinoceptor in rat brain microvascular endothelial cells couple to inhibition of adenylate cyclase. Br J Pharmacol 1996; 119:1385-92.
45. Vittet D, Mathieu MN, Launay JM, Chevillard C. Platelet receptor expression on three human megakaryoblast-like cell lines. Exp Hematol 1992; 20: 1129-34.
46. Savi P, Pflieger AM, Herbert JM. cAMP is not an important messenger for ADP-induced platelet aggregation. Blood Coagul Fibrinolysis 1996; 7:249-52.
47. Weber AA, Hohlfeld T, Schror K. cAMP is an important messenger for ADP-induced platelet aggregation. Platelets 1999; 10:238-41.
48. Daniel JL, Dangelmaier C, Jin JG, Kim YB, Kunapuli SP. Role of intracellular signaling events in ADP-induced platelet aggregation. Thromb Haemost 1999; 82:1322-6.
49. Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. Blood 1992; 80:2787-96.
50. Nurden P, Savi P, Heilmann E, et al. An inherited bleeding disorder linked to a defective interaction between ADP and its receptor on platelets. Its influence on glycoprotein IIb-IIIa complex function. J Clin Invest 1995; 95:1612-22.
51. Savi P, Heilmann E, Nurden P, et al. Clopidogrel: an antithrombotic drug acting on the ADP-dependent activation pathway of human platelets. Clin Appl Thrombosis Hemostasis 1996; 2:35-42.
52. Humbert M, Nurden P, Bihour C, et al. Ultrastructural studies of platelet aggregates from human subjects receiving clopidogrel and from a patient with an inherited defect of an ADP-dependent pathway of platelet aggregation. Arterioscler Thromb Vasc Biol 1996; 16:1532-43.
53. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci USA 1998; 9: 8070-4.
54. Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular basis for ADP-induced platelet activation. I. Evidence for three distinct ADP receptors on human platelets. J Biol Chem 1998; 273: 2024-9.
55. Weber AA, Reimann S, Schror K. Specific inhibition of ADP-induced platelet aggregation by clopidogrel in vitro. Br J Pharmacol 1999; 126:415-20.
56. Abou-Khalil S, Abou-Khalil WH, Yunis AA. Mechanism of interaction of ticlopidine and its analogues with the energy-conserving mechanism in mitochondria. Biochem Pharmacol 1986; 35:1855-9.
57. Klein-Soyer C, Cazenave JP, Herbert JM, Maffrand JP. SR 25989 inhibits healing of a mechanical wound of confluent human saphenous vein endothelial cells which is modulated by standard heparin and growth factors. J Cell Physiol 1994; 160:316-22.
58. Chen WH, Yin HL, Chang YY, Lan MY, Hsu HY, Liu JS. Antiplatelet drugs induce apoptosis in cultured cancer cells. Kao-Hsiung I Hsueh Ko Hsueh Tsa Chih [Kaohsiung J Med Scien] 1997; 13:589-97.
59. Savi P, Herbert JM, Pflieger AM, et al. Importance of hepatic metabolism in the antiaggregating activity of the thienopyridine clopidogrel. Biochem Pharmacol 1992; 44:527-32.
60. Savi P, Combalbert J, Gaich C, et al. The antiaggregating activity of clopidogrel is due to a metabolic activation by the hepatic cytochrome P450 1A. Thromb Haemost 1994; 72:313-7.
61. Di Perri T, Pasini FL, Frigerio C, et al. Pharmacodynamics of ticlopidine in man in relation to plasma and blood cell concentration. Eur J Clin Pharmacol 1991; 41:429-34.
62. Savi P, Pereillo JM, Andrieu A, et al. Structure and activity of the active metabolite of clopidogrel. Thromb Haemost 1999; (Suppl ):230.

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# CLINICAL TRIALS WITH ADP RECEPTOR ANTAGONISTS

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Cardiovascular disorders represent the first cause of mortality and morbility in the western world, therefore knowledge of the mechanism leading to vascular occlusion is one of the major objectives of future research. Cardiovascular disease is essentially due to atherosclerosis of arterial wall, plaque erosion or fissuring, representing a crucial event in the formation of occlusive thrombi. Platelets play a major role in vascular occlusion because clinical trials with antiplatelet demonstrated 25% reduction in the incidence of vascular death, myocardial infarction and stroke in patients with several forms of atherosclerosis. The pathophysiologic role of platelets in vascular events is particularly evident in the acute coronary syndrome in which this drug category significantly reduces cardiovascular events. The role of platelets in the atherosclerosis progression is still controversial as only meta-analysis provides clear-cut evidence that inhibition of platelet functon is really associated with reduction of cardiovascular events.

Most trials with antiplatelet drugs, in the setting of cardiovascular events, have been done with aspirin, that inhibits cyclo-oxygenase enzyme and in turn thromboxane $A_2$, a potent aggregating and vasocostrictive substance. In the last two decades new compounds have been developed with the aim of inhibiting platelet function with alternative mechanisms of action.

Thienopyridines represent a relative novel category of drug that inhibit the aggregation of platelet induced by ADP. Ticlopidine is the first compound of this class of drug, that has been used in several settings of atherosclerosis disease. More recently clopidogel, a derivative of ticlopidine, has been developed and used for clinical purpose. This review will focus on beneficial effects of this drug category in several settings of cardiovascular disease.

### *Peripheral vascular disease (PVD)*

PVD is essentially due to atherosclerosis of peripheral vessel; however it is also characterised by high rate of mortality and morbility as a consequence of cardiovascular accidents occurring in coronary and cerebral vessel. Ticlopidine has been used in this setting with 2 aims:

1. preventing deterioration of peripheral vessels;
2. reducing cardiovascular mortality and morbility.

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As far as peripheral vascular deterioration is concerned, ticlopidine has been shown to improve walking distance in patients with claudication. In a follow-up of 21 months we also showed that patients with claudication treated with ticlopidine did not show worsering of ankle/arm pressure ratio, which is a good marker of atherosclerosis progression and complication; conversely patients treated with placebo showed a progressive decrease of this index, thus providing indirect evidence that ticlopidine could be useful to retard atherosclerosis progression.[1]

As far as the prevention of cardiovascular disease is concerned, ticlopidine has been investigated, compared to placebo-controlled groups, in claudicant patients.[2] During a follow-up of 5-6 years, ticlopidine-treated patients had a significant reduction of fatal and non-fatal events, that did not, however, reach statistical significance on an intention to treat basis; conversely, on treatment analysis demonstrated a significant reduction of cardiovascular events on ticlopidine-treated patients (Table 1).

### *Cerebrovascular disease*

Two clinical trials have been done with ticlopidine in patients with previous cerebrovascular disease (CVD) for preventing further cardiovascular events[3,4] (Table 2). In CATS study patients with definite stroke were randomly allocated to placebo or ticlopidine and followed-up for 2 years; end-points of the study was a combination of ischemic stroke, myocardial infarction and vascular death. The overall relative reduction in the risk of the major vascular outcomes was 23.3% ($p$=0.02) in the intervention to treat analysis and 30.2% ($p$=0.006) in the efficacy analysis. Clinical advantages were similar in men (–28.1%) and females (–32.4%). In the TASS patients with TIA, RIND or minor stroke were randomly allocated to ticlopidine (250 mg b.i.d.) or aspirin (650 mg b.i.d.) and followed-up for 3 years; primary end points of the study were a cluster of stroke or death. Using intention-to-treat analysis ticlopidine reduced by 21% ($p$<0.005) the risk of stroke and by 12% ($p$=0.02) the risk of non fatal stroke or vascular death when compared to aspirin. The reduction of relative risk was particularly evident in the first year of follow-up and tended to diminuish after 2 and 3 years of follow-up.

### *Unstable angina*

Efficacy of antiplatelet drugs in unstable angina has also been analyzed using ticlopidine, following the same methodologic approach used in the American study, but including male and female unstable angina patients treated with a standard therapy or a

**Table 1. STIMS.**

| | *STIMS* |
|---|---|
| Patients | Chronic peripheral arterial disease. Abnormal systolic pressure gradient between upper arm and ankle |
| Intention to treat | All randomized |
| Efficacy | All intention-to-treat within 15 days of drug discontinuation |
| Primary analysis | Intention to treat (2 tailed) |
| Primary outcome cluster | • Protocol endpoints fatal or non-fatal or myocardial infarction and stroke plus TIAs<br>• Study endpoints as above + sudden death |
| Secondary outcome cluster(s) | – |
| Study size – patient numbers | 687 patients: ticlopidine: 346, placebo: 341 |
| Mean follow-up | 5.6 years |

**Table 3. STAI.**

| | *STAI* |
|---|---|
| Patients | Unstable angina |
| Intention to treat | All randomized |
| Efficacy | All intention-to-treat treat within 28 days after drug discontinuation |
| Primary analysis | Intention to treat (2 tailed) |
| Primary outcome cluster | Fatal and non-fatal MI or vascular death |
| Study size – patient numbers | 652 |
| Follow-up | 3-6 months |

standard therapy plus ticlopidine (250 mg twice a day)[5] (Table 3).

After 3-6 months of follow-up, the study showed a significant reduction in vascular death and non-fatal myocardial infarction (46.3%) and in fatal and non-fatal myocardial infarction (53.2%). The effect appeared after 15 days of treatment and was most evident in patients with a previous history of myocardial infarction. Side effects observed in the group taking ticlopidine were most frequently gastrointestinal disturbances (9.7%) and skin rash (1.9%). Fifteen patients (4.9%) taking ticlopidine discontinued treatment, 10 because of gastrointestinal discomfort, and five for skin reactions.

These findings support the key role played by platelets in the clinical evolution of unstable angina. Comparison of risk reduction obtained with aspirin or ticlopidine clearly shows that both drugs can reduce the occurrence of serious vascular complications by about 50%. Comparison between these two drugs is necessary in order to assess their risk/benefit in unstable angina patients.

### *CAPRIE*

CAPRIE was a randomized, blinded, international trial designed to assess the relative efficacy of clopidogrel (75 mg once daily) and aspirin (325 mg once daily) in reducing the risk of a composite outcome cluster of ischemic stroke, myocardial infarction, or vascular death[6] (Table 4).

After 1-3 years of follow-up, the study showed that patients treated with clopidogrel had an annual

**Table 2. TASS and CATS.**

| | TASS | CATS |
|---|---|---|
| Patients | Stroke precursor/minor stroke | Completed stroke |
| Intention to treat | All randomized | All randomized but "truly ineligible" |
| Efficacy | All intention-to-treat except ineligibles and within 10 days of drug discontinuation | All intention-to-treat within 28 days after drug discontinuation |
| Primary analysis | Intention-to-treat (2 tailed) | Efficacy (1 tailed) |
| Primary outcome cluster | Stroke or death | Ischemic stroke, myocardial infarction (MI), vascular death |
| Secondary outcome cluster(s) | Fatal or non-fatal stroke | • Stroke, MI, death<br>• Fatal or non-fatal stroke<br>• Vascular death<br>• Death |
| Study size – patient numbers | 3069 patients<br>ticlopidine: 1529<br>aspirin: 1540 | 1,072 patients<br>ticlopidine: 531<br>placebo: 541 |
| Mean follow-up | 3.2 years | 2 years |

**Table 4. CAPRIE.**

| | *CAPRIE* |
|---|---|
| Patients | Atherosclerotic vascular disease (recent ischemic stroke, recent myocardial infarction, symptomatic peripheral arterial disease) |
| Intention to treat | All randomized |
| Efficacy | All intention-to-treat treat within 28 days after drug discontinuation |
| Primary analysis | Intention to treat |
| Primary outcome cluster | Ischemic stroke, Myocardial infarction, Vascular death |
| Secondary outcome cluster(s) | • Amputation<br>• Death |
| Study size – patient numbers | 19,185<br>Clopidogrel: 9599; Aspirin: 9586 |
| Follow-up | 1-3 years |

5.32% risk of ischemic stroke, myocardial infarction, or vascular death compared with 5.83 with aspirin. These findings demonstrate that there was a significant ($p$=0.043) relative-risk reduction of 8.7% in favor of clopidogrel (95% CI 0.3-16.5).

### *Side effects*

The most frequent side effects with ticlopidine are localized in the gastrointestinal tract and include diarrhea, nausea, dyspepsia, and vomiting. Cutaneous rash is another frequent side effect occurring in about 10% of patients. A severe side effect occurring in ticlopidine-treated patients is leukopenia; this complication is described in <2% of patients particularly in the first 4 months of follow-up. This adverse effect, however, was not observed with clopidogrel treatment, that showed a rate of neutropenia of the same magnitude (0.5%) showed by the aspirin group.

### *Conclusions*

On the basis of the above reported studies with ticlopidine and clopidogrel, it can be affirmed that thienopyridines are as effective as aspirin in reducing cardiovascular events in patients with atherothrombosis. Since these drugs affect two different pathways of platelet aggregation, their combination has been suggested as a valid approach for further preventing cardiovascular events in acute coronary syndromes. Clinical trials are in progress to answer this important clinical issue.

## References


1. Balsano F, Coccheri S, Libretti A, et al. Ticlopidine in the treatment of intermittent calaudication: a 21 month double blind trial. J Lab Clin Med 1989; 114; 84-91.
2. Janzon L, Bergqvist D, Boberg J, et al. Prevention of myocardial infarction and stroke in patients with intermittent claudication; effects of ticlopidine. Results from STIMS, the Swedish Ticlopidine Multicentre Study. J Int Med 1990; 227:301-8.
3. Gent M, Blakely JA, Easton JD, et al. The Canadian American Ticlopidine Study (CATS) in thromboembolic stroke. Lancet 1989;1:1215-20.
4. Hass WK, Easton JD, Adams HP, et al. A randomised trial comparing ticlopidine hydrochloride with aspirin for the prevention of stroke in high-risk patients. N Engl J Med 1989; 321:501-7.
5. Balsano F, Rizzon P, Violi F, et al. Antiplatelet treatment with ticlopidine in unstable angina. A controlled multicenter clinical trial. Circulation 1990; 82:17-26.
6. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet 1996; 348:1329-39.

# Closing remarks

### *Well established points*

The model of three purinergic receptors mediating all the effects of ADP on platelets, namely the ligand-gated non-selective cation channel $P2X_1$ responsible for a rapid calcium entry, the $P2Y_1$ receptor, coupled to Gq, responsible for calcium mobilization, shape change and initiation of platelet aggregation by ADP and the yet unknown P2 receptor negatively coupled to adenylyl cyclase (P2cyc), responsible for amplification and completion of the platelet response to ADP is now well established and agreed by all the investigators working in the field. Also well established, albeit less well known, are the methododologic problems in the study of platelet responses to ADP. A special homage was rendered to J. Fraser Mustard who defined factors influencing ADP-induced platelet aggregation. The role of external ionized calcium as well as of albumin in the suspensions of washed platelets, the quality of the blood samples, the choice of anticoagulants, and comparison between species, among other aspects, were discussed. Finally, there is consensus concerning the structures of the cloned P2Y receptors and the pharmacology of 5 of them: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$. The pharmacologic properties of the so-called AR-C compounds as well as of the thienopyridine compounds, selective antagonists and inhibitors of P2cyc are also clearly accepted by all although some controversies remained in terms of comparison of the two classes of drugs. Finally, congenital disorders of platelet function, among which the selective defect of ADP-induced platelet aggregation related to a P2cyc defect, were extensively reviewed.

### *New data*

The following new data were presented:

1) ADP is an important cofactor in phosphatidylinositol 3-kinase (PI-3K) activation both in the stabilization of TRAP-induced platelet aggregation and in FcγRIIa-induced platelet activation.
2) Gαi2 deficiency results in partial impairment of ADP-induced platelet activation, confirming a role for Gαi2 in ADP signaling.
3) The Gi pathway is a necessary complementary signal in platelet aggregation, independently of the starting stimulus (PKC or PLC).
4) In Gαq knockout mice, ADP can restore collagen-induced platelet aggregation and, at very high concentrations (100 μM), promotes partial aggregation in the absence of calcium signaling and shape change. Similarities of the Gαq deficient mice with the $P2Y_1$ receptor knockout mice were underlined.
5) The P2cyc receptor plays important roles in the potentiation of platelet dense granule secretion and in the exposure of phosphatidylserine and thus, probably in thrombin generation. All these points unravel the molecular mechanisms underlying the crucial role of ADP as a cofactor in all aspects of platelet activation and emphasize the involvement of the P2cyc receptor in these processes. The effects of the new AR-C compound, AR-C69931MX, a selective P2cyc receptor antagonist, globally confirm these findings since it was widely used either as a tool or as a drug both *in vitro* and *in vivo*.
6) $P2Y_1$ knockout mice are resistant to a thrombin dependent-thromboembolism model. Moreover, *in vivo* pharmacologic modulation of the $P2Y_1$ receptor with MRS2179 results in a similar resistance to acute thrombosis induced either by collagen-adrenalin or by tissue factor. Thus, the $P2Y_1$ receptor is a promising target for new antiplatelet agents. The regulation of its gene expression by thrombopoietin was also reported.
7) The well known refractory state of platelets to ADP results entirely from the selective desensitization of the P2Y1 receptor probably by internalization while the P2cyc receptor is still functional and responsive to ADP. A role for ADP in modulating platelet adhesion and limiting the expansion of the thrombus was also shown.
8) Recombinant CD39, the ectoATPDase or apyrase-like ectoenzyme, is active both *in vitro* and *in vivo* as an antiplatelet agent, and seems to be a potent and promising antithrombotic drug in stroke.

### *Controversies*

New but controversial were three reports dealing with a possible role of the $P2X_1$ receptor in platelet activation and in hemostasis. The case of a patient with a bleeding disorder that might be due to the presence of a mutated form of the $P2X_1$ receptor was described. The reasons for the discrepancy between the severity of the bleeding diathesis and the mild inhibition of platelet agregation and calcium signals reported were unclear. Two studies reporting on functional properties of the $P2X_1$ receptor, one on shape change induced by a selective $P2X_1$ agonist, one on activation of ERK/MAP kinase through the $P2X_1$ receptor, were extensively discussed and left some key questions unanswered. Further studies are certainly required to unravel the role of this receptor in platelet physiology.

### *Perspectives*

It was planned to organize a second ADP meeting in two years. The hope is the following questions will be answered by then:

What is the role of the $P2X_1$ receptor in platelet activation, hemostasis and thrombosis? The availability of $P2X_1$ knockout mice would certainly be of great help, also in consideration of the lack of appropriate selective agonists.

What is the molecular identity of the P2cyc (or $P2T_{AC}$, or $P2Y_{ADP}$) receptor? The current attempts to

identify it are expression cloning, protein purification or examination of patients' platelets. So far, no molecular structure has been proposed although it seems obvious to many of us that it should be a G-protein coupled receptor since Gαi2 is involved in this pathway.

In terms of pharmacology, the use of new drugs selective for ADP, $P2Y_1$ or P2cyc antagonists as well as recombinant CD39 should be better characterized both in animal models and in clinical studies.

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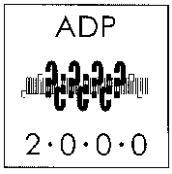
# The Platelet ADP Receptors

Oral communications and posters




## ORAL COMMUNICATIONS

### IMPAIRED PLATELET ACTIVATION IN Gαi2-DEFICIENT MICE

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Pharmacologic evidence suggests that ADP-dependent platelet aggregation requires activation of two receptors. One ADP receptor, termed P2Y1, stimulates intracellular calcium mobilization and apparently couples to Gq and other G proteins. The second ADP receptor, which is the target of the anti-thrombotic drugs ticlopidine and clopidogrel, mediates the inhibition of adenylyl cyclase and therefore likely couples to Gi or Gz proteins. However, since repression of cAMP levels is required but not sufficient for platelet aggregation, other G proteins might be critical in this signaling pathway. In the present study we have first examined the role of the Gi2 pathway in ADP- and thrombin-induced platelet activation using mice in which the gene for the α subunit of the predominant platelet Gαi subtype, Gαi2, has been disrupted. Second, the selective ADP receptor antagonist 2MeSAMP has been used to determine the contribution of released ADP activating the Gi-linked receptor in thrombin-induced platelet signaling. When the inhibition of adenylyl cyclase by ADP was compared in platelets from Gαi2-deficient and wild-type mice, a strong but incomplete impairment of cAMP signaling was observed. This supports the hypothesis that Giα2 and possibly other G proteins, e.g. Gi3 or Gz are involved in ADP-dependent cAMP signaling *in vivo*. Gαi2-deficient platelets were also partially defective in thrombin-dependent repression of cAMP levels. Since inhibition by thrombin of adenylyl cyclase in wild-type platelets was blocked by the selective antagonist 2MeSAMP, this effect is likely to be indirectly mediated by released ADP activating the Gi-linked ADP receptor. ADP-dependent aggregation was also strongly reduced with platelets from Gαi2-deficient mice, indicating a major role for this G protein subtype in platelet aggregation. Aggregation induced by threshold levels of thrombin was impaired in the Gαi2-deficient platelets and this effect was mimicked by 2MeSAMP with wild-type platelets. This suggests a role for released ADP and Gαi2 in the stabilization of thrombin-induced aggregates. By contrast, platelet shape change was not affected, indicating that Gαi2 is not required for shape change. Finally, activation of the platelet membrane integrin αIIbβ3 (GPIIb-IIIa), which is a critical prerequisite for platelet aggregation, was analyzed using FITC-fibrinogen binding and flow cytometry. Consistent with the diminished aggregation, integrin activation was severely impaired in ADP-stimulated platelets from Gαi2-deficient mice, and to a lesser degree in thrombin-stimulated mouse platelets. In conclusion, these observations suggest that, (a) Gαi2 is involved in the inhibition of adenylyl cyclase by ADP *in vivo*, (b) Gαi2 is a critical component in the signaling pathway for ADP-dependent activation of integrin αIIbβ3 resulting in platelet aggregation, and (c) thrombin-dependent repression of cAMP levels and aggregation are mediated, at least in part, by secreted ADP and the Gi2-linked ADP receptor.

### INDEPENDENT ACTIVATION OF PROTEIN KINASE C OR PHOSPHOLIPASE C CAN INDUCE PLATELET AGGREGATION PROVIDED A Gi PROTEIN-COUPLED RECEPTOR IS ACTIVATED

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Concomitant activation of two different G-protein-coupled receptors, one responsible for the activation of PLC, Gq, and the other that reduces cAMP concentration, Gi, is essential for the exposure of the fibrinogen binding site on the integrin αIIbβ3. The aim of the present study was thus to verify whether the activation of biochemical pathways downstream of Gq protein activation is sufficient in order to induce platelet aggregation provided a Gi protein-coupled receptor is activated. For this purpose we studied aggregation in a platelet suspension treated both with aspirin, to eliminate the formation of $TxA_2$, and with the ADP scavenger system creatine phosphate/creatine kinase (CP/CPK), in response to epinephrine, used as Gi activator, and to the snake venom toxin convulxin, a PLCγ2, but not Gq-protein, activator, and to phorbol myristate acetate (PMA), a PKC activator. The results obtained showed: 1) convulxin or PMA alone are not able to induce platelet aggregation; the response was obtained only if epinephrine was added concomitantly; 2) PKC inhibitor, Ro 31-8220, did not suppress platelet aggregation in response to convulxin plus ADP or epinephrine; 3) the cytosolic calcium chelator BAPTA did not inhibit the aggregometric response to the combined stimulation by convulxin or PMA plus ADP or epinephrine. These data suggest that the activation of an enzyme downstream of Gq, such as phospholipase C (PLC) or protein-kinase C (PKC), is sufficient to induce platelet aggregation provided a Gi coupled receptor is activated; both pathways involved are not dependent on enhancement of the calcium concentration. In conclusion, we demonstrated that direct Gq activation is not required for platelet aggregation.

## ADP INDUCES PARTIAL PLATELET AGGREGATION WITHOUT SHAPE CHANGE AND POTENTIATES COLLAGEN INDUCED AGGREGATION IN THE ABSENCE OF Gαq

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Platelets from Gαq knockout mice are unable to aggregate in response to physiologic agonists like ADP, thromboxane $A_2$, thrombin or collagen, although shape change still occurs in response to all these agonists except ADP. ADP-induced platelet aggregation results from simultaneous activation of the purinergic $P2Y_1$ receptor coupled to calcium mobilization and shape change and of a distinct P2 receptor, $P2_{cyc}$, coupled through Gi to adenylyl cyclase inhibition, which is responsible for completion and amplification of the response. $P2_{cyc}$ could be the molecular target of the antithrombotic drug clopidogrel and the ATP analogs ARC-69931MX, 67085 and 66096. The aim of the present study was to determine whether externally added ADP could still act through the Gi pathway in Gαq deficient mouse platelets and thereby amplify the residual responses to agonists such as collagen.

Although ADP was not able to induce platelet aggregation or an intracellular calcium rise in Gαq deficient mouse platelets, it still inhibited cAMP production to a similar extent as in wild type platelets. This effect was selectively blocked by clopidogrel or ARC-69931MX, suggesting the $P2_{cyc}$ receptor to be functional in these platelets. Collagen induced only shape change or weak aggregation and a low secretion response, whereas strong irreversible aggregation occurred when ADP and collagen were added together. Similar results were obtained using adrenaline, which suggested that restoration of the full aggregation induced by collagen was dependent on activation of the Gi pathway. The potentiating effect of ADP on collagen induced aggregation was strongly inhibited *in vitro* by the ATP analog ARC-69931MX and *ex vivo* by clopidogrel. Conversely, the potentiating effect of adrenaline was not affected by clopidogrel or ARC-69931MX, indicating that ADP was acting through the $P2_{cyc}$ receptor. Finally, in an attempt to highlight the consequences of strong activation of the Gi pathway in platelets, we added high concentrations (100 μM) of ADP or adrenaline to Gαq deficient mouse platelets. ADP (100 μM) induced the formation of small aggregates of platelets which did not change shape as observed by scanning and transmission electron microscopy. Again, this effect of ADP was inhibited by clopidogrel or ARC-69931MX, indicating that it resulted from activation of the $P2_{cyc}$ receptor. The $P2_{cyc}$ mediated aggregation was also integrin dependent, since it was inhibited by a monoclonal anti-mouse GPIIb-IIIa antibody. However, as high concentrations of adrenaline had no such impact on Gαq deficient platelets, the effects of ADP mediated by $P2_{cyc}$ did not appear to be restricted to the inhibition of adenylyl cyclase through $Gi_2$. In conclusion, the present work provides insight into the role of the $P2_{cyc}$ receptor in the unique platelet aggregatory properties of the physiologic autocrine agonist ADP.

## ADP POTENTIATES PLATELET DENSE GRANULE SECRETION INDUCED BY U46619 OR TRAP THROUGH ITS INTERACTION WITH THE $P2_{cyc}$ RECEPTOR

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*Background.* ADP is a weak agonist. As such, it does not induce secretion of the platelet dense granule constituents directly, but through the aggregation-mediated synthesis of thromboxane $A_2$ ($TxA_2$), which is greatly enhanced when $[Ca^{2+}]_{out}$ is decreased by sodium citrate. However, we recently showed that, when secretion has been triggered by an agonist, secreted ADP potentiates platelet secretion independently of aggregation and the synthesis of $TxA_2$. *Aims*. To assess which of the 3 platelet receptors for ADP ($P2X_1$, $P2Y_1$ and $P2_{cyc}$) is involved in the potentiation of platelet secretion by ADP. *Subjects.* Four normal volunteers and patient VR (congenitally deficient in the platelet $P2_{cyc}$ receptor). *Methods*. [$^{14}$C]5HT secretion was measured 2 min after the addition of U46619 (1 μmol/L) or TRAP (20 μmol/L, which stimulates the PAR1 thrombin receptor) to pre-labeled, aspirin-treated washed platelets suspensions containing 2 mmol/L $CaCl_2$ and apyrase (to prevent desensitization to ADP), which were not stirred (to prevent platelet aggregation). The effects of epinephrine (10 μmol/L) and the following compounds were investigated: AR-C69931MX ($P2_{cyc}$ antagonist, 0.1 μmol/L), MRS-2179 ($P2Y_1$ antagonist, 50 μmol/L), α,β-me-ATP ($P2X_1$ agonist, 10 μmol/L). *Results*. ADP and epinephrine, when added alone to platelet suspensions, did not induce detectable platelet secretion. The table shows the percent platelet [$^{14}$C]5HT secretion induced by U46619 or TRAP:

| | U46619 | | TRAP | |
|---|---|---|---|---|
| *Additions* | *Controls** | *V.R.* | *Controls** | *V.R.* |
| Tyrode | 41.2 | 6.9 | 38.9 | 18.5 |
| AR-C69931MX | 6.6 | 6.6 | 24.3 | 18.4 |
| MRS-2179 | 31.2 | 4.5 | 39.3 | 17.5 |
| AR-C + MRS | 5.4 | 5.9 | 24.5 | 18.7 |
| AR-C + MRS + α,β-me-ATP | 4.5 | 4.3 | 22.0 | 717.4 |
| AR-C + MRS + epinephrine | 28.8 | 25.1 | 32.8 | 25.1 |

**Values are means of 4 experiments.*

*Conclusions.* P2cyc, which is negatively coupled to adenylyl cyclase (AC) mediates the potentiation of platelet secretion by released ADP. Epinephrine, whose receptor is also negatively coupled to AC, potentiates secretion similarly to ADP. Therefore, the Gi pathway seems to be required for full platelet secretion.

## DESENSITIZATION OF THE PLATELET AGGREGATION RESPONSE TO ADP: DIFFERENTIAL DOWNREGULATION OF THE P2Y1 AND P2CYC RECEPTORS

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Platelets activated by ADP become refractory to restimulation, but the mechanism of this process is not well understood. A normal platelet response to ADP requires coactivation of the $P2Y_1$ receptor responsible for shape change and the P2cyc receptor, responsible for completion and amplification of the response.

The aim of the present study was to characterize the desensitization of platelets to ADP and to determine whether or not these two receptors are desensitized simultaneously through identical pathways when platelets become refractory to ADP.

Full inhibition of platelet aggregation in response to restimulation by ADP required the presence of ADP in the medium or use of a high concentration (1 mM) of its non-hydrolysable analog ADPβS. Platelets incubated for 1 hour at 37°C with 1 mM ADPβS and resuspended in Tyrode's buffer containing apyrase displayed a stable refractory state characterized by the inability to aggregate or change shape in response to ADP. ADPβS treated platelets loaded with fura-2/AM showed complete blockade of the calcium signal in response to ADP, whereas the capacity of ADP to inhibit $PGE_1$ stimulated cAMP accumulation in these platelets was only diminished. Consequently, serotonin was able to promote ADP induced aggregation through activation of the Gq coupled $5HT_{2A}$ receptor while adrenaline had no such effect. These results suggested that the refractory state of ADPβS treated platelets was entirely due to desensitization of the $P2Y_1$ receptor, the $P2_{cyc}$ receptor remaining functional. Binding studies were performed to determine whether the $P2Y_1$ and/or the $P2_{cyc}$ binding site was modified in refractory platelets. Using selective $P2Y_1$ and $P2_{cyc}$ antagonists (A3P5P and AR-C66096 respectively), we could demonstrate that the decrease in [$^{33P}$]2MeSADP binding sites on refractory platelets corresponded to disappearance of the $P2Y_1$ site with no change in the number of $P2_{cyc}$ sites, suggesting internalization of the $P2Y_1$ receptor. This was confirmed by flow cytometric analysis of Jurkat cells expressing an epitope-tagged $P2Y_1$ receptor, in which ADPβS treatment resulted in complete loss of the receptor from the cell surface.

Our overall results indicate that the inhibition of platelet aggregation in response to restimulation by ADP is due to full desensitization of the $P2Y_1$ receptor through its internalization, whereas activation of the $P2_{cyc}$ receptor is only weakly downregulated. Thus, the two platelet ADP receptors are differentially regulated during platelet activation, which might be of importance in hemostasis and should be kept in mind in antiplatelet therapy.

## TRANSIENT ADHESION REFRACTORINESS OF PLATELETS UNDER FLOW CONDITIONS: THE ROLE OF PARTIAL ACTIVATION AND MICROAGGREGATE FORMATION BY SUBOPTIMAL ADP CONCENTRATION

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Exposure of whole blood (WB) to subendothelial extracellular matrix (ECM) under flow conditions results in platelet adhesion followed by release reaction and aggregation of circulating platelets on the adherent platelets. The effect of released ADP on the properties of circulating non-adhered platelets was studied. WB was exposed to ECM at a high shear rate (1,300 $s^{-1}$) for 2 min (1st run); the suspension phase was transferred to a new ECM coated well for a second run (2nd run) under similar conditions. An almost complete absence of platelet adhesion to the ECM in the 2nd run was observed. This adhesion refractoriness was transient since after 10 to 20 min incubation the treated platelets regained their ability to interact with the ECM. At the refractory stage, a fraction of the platelets transiently form microaggregates in the suspension. The adhesion refractoriness was dependent on platelet activation at the 1st run as indicated by the ability of $PGE_1$ and anti GPIIb-IIIa antibody (ReoPro®) to inhibit this process. It was prevented also by addition of apyrase (ADP scavenger) suggesting a role for ADP in mediating this response. Furthermore, exposure of WB samples to a suboptimal concentration of ADP (0.75-1 µM) for 2 min resulted in a similar transient platelet adhesion refractoriness to ECM under flow conditions. FACS analysis of WB single platelets before and immediately after the 1st run on ECM or after an addition of a suboptimal concentration of ADP, revealed a transient reduction in the expression of GPIb (35 to 55%) and an increase in fibrinogen binding on platelet membrane (80 to 100%) without any change in GPIIbIIIa expression. In conclusion, activation followed by release reaction of adherent platelets on the ECM induced transient reduction in GPIb and increased fibrinogen binding associated with formation of microaggregates by circulating platelets, resulting in transient platelet adhesion refractoriness. These data suggest a role for ADP at suboptimal concentrations in modulating platelet function and limiting the expansion of the thrombus.

## P2T RECEPTOR ACTIVATION BY ADP: A PERMISSIVE ROLE IN AGGREGATION OF HUMAN WASHED PLATELETS INDUCED BY PAF OR U46619

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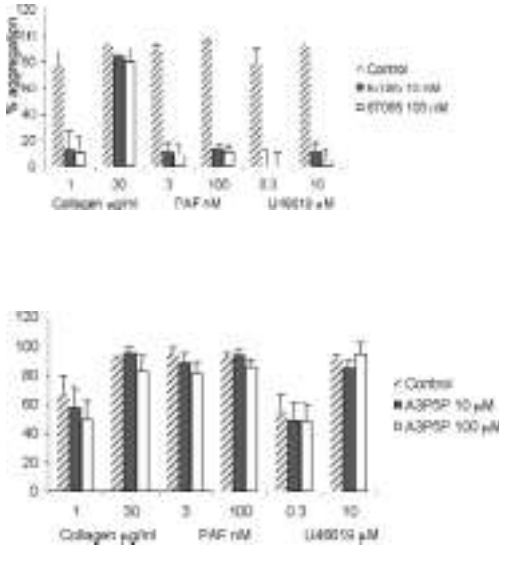
*Background.* P2 receptor-mediated platelet activation by ADP plays a major role in hemostasis and thrombosis, with full expression of the aggregation

response requiring concomitant activation of both the P2T and $P2Y_1$ receptor subtypes. While a permissive or amplifying role of ADP in platelet responses to other agonists is accepted, the relative contribution of the P2T and $P2Y_1$ receptor subtypes in this context remains unclear. *Aim.* In the present study we have used the competitive P2T antagonist, AR-C67085 (pKB 9.1), and the $P2Y_1$ antagonist, A3P5P (pKB 6.0), to assess the relative importance of ADP activation of these two receptor subtypes in aggregation induced by PAF, U46619, or collagen. *Methods*. Aggregation of human washed platelets was assessed turbidimetrically in 96 well plates as a decrease in absorbance (650 nm). Concentration/effect curves were obtained to each of the agonists in the absence (control) or presence of either AR-C67085 (10, 100 nM) or A3P5P (10, 100 µM) added 5 min before the agonist. These concentrations are 10 and 100 fold above the pKB for the respective receptors. *Results.* (% aggregation) are presented below for both a submaximal and maximal concentration of each agonist. Responses to collagen, PAF and U46619 were significantly inhibited by the P2T antagonist, AR-C67085 (Figure 1), and, in the case of the latter two agonists, this inhibition was not overcome by a 30-fold increase in the agonist concentration. In contrast, the $P2Y_1$ antagonist, A3P5P (Figure 2), at a concentration 100-fold above its pKB had no significant effect on any of the agonist responses.

*Conclusions.* These results indicate that, in this assay system, collagen-induced platelet aggregation is amplified by ADP acting via the P2T and not the $P2Y_1$ receptor. In the case of PAF- and U46619-induced aggregation, P2T receptor stimulation by ADP is an absolute requirement, indicative of a permissive role for P2T receptor activation in responses to these agonists.



## THE CENTRAL ROLE OF THE $P_{2T}$ RECEPTOR IN AMPLIFICATION OF PLATELET AGGREGATION, SECRETION AND PROCOAGULANT ACTIVITY

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*Background.* ADP plays a major role in hemostasis and thrombosis by acting as an agonist for platelet activation via $P2X_1$, $P2Y_1$ and $P_{2T}$ receptors. Previous studies both of patients with congenital bleeding disorder and of the effects of the thienopyridines, ticlopidine and clopidogrel, have suggested an important role for the $P_{2T}$ receptor in amplification of platelet aggregation and secretion. AR-C69931MX is a direct $P_{2T}$ receptor antagonist that is currently being assessed as intravenous antithrombotic therapy in patients with acute coronary syndromes. *Aim.* We aimed to study the role of the $P_{2T}$ receptor in platelet function using AR-C69931MX. *Methods.* We studied a wide range of agonists including ADP, collagen, TRAP, PAF, 5HT, epinephrine, U46619, streptokinase and some non-ionic X-ray contrast media. We used both whole-blood single-platelet counting and PRP turbidimetry to study aggregation, P-selectin expression as measured by flow cytometry to assess α-granule release, $^{14}$C-5HT release from labeled platelets to assess dense granule release, and annexin V binding and microparticle formation measured by flow cytometry to assess platelet procoagulant activity. Hirudin was predominantly used as anticoagulant, although the effect of citrate anticoagulation was also studied. The inhibitory effects of a range of AR-C69931MX concentrations were studied with blood concentrations of 100 to 1,000 nM encompassing the therapeutic concentrations achieved by AR-C69931MX infusion. The effects of aspirin were also assessed both *in vitro* and *ex vivo*. *Results*. AR-C69931MX potently inhibited ADP-induced aggregation and secretion. AR-C69931MX inhibited whole blood aggregation induced by maximal concentrations of 5HT, epinephrine and streptokinase, and submaximal concentrations of collagen, TRAP, PAF and U46619. AR-C69931MX also substantially inhibited P-selectin expression (median fluorescence) and 5HT release in response to all the agonists (including non-ionic contrast media), even at concentrations giving maximal aggregation. For example, AR-C69931MX 100 nM inhibited P-selectin expression and 5HT release in response to U46619 1 µM by 78% and 83%, respectively. In PRP, AR-C69931MX rendered aggregation induced by TRAP 20 µM reversible, in a concentration-dependent manner, with complete reversal of aggregation by AR-C69931MX 800 nM, and the pattern of inhibition of $^{14}$C-5HT release in response to ADP, collagen and TRAP was similar to that seen in whole blood. AR-C69931MX dramatically inhibited the platelet procoagulant response induced by TRAP in a concentration-dependent manner. The effects of aspirin on aggregation and secretion were much more limited: responses to collagen and streptokinase were inhibited but not responses to the other agonists.

Aspirin only inhibited ADP-induced responses when citrate was used as anticoagulant. Aspirin had no significant effect on TRAP-induced procoagulant activity. AR-C69931MX and aspirin had additive effects on collagen- and streptokinase-induced responses. The effects of the $P2Y_1$ antagonist A2P5P (30-300 μM) were also assessed, alone or in combination with AR-C69931MX, and we found that the $P2Y_1$ receptor also has an important role in determining the responses to agonists other than ADP with additive effects to those mediated by the $P_{2T}$ receptor. For example, AR-C69931MX and A2P5P inhibited collagen-induced aggregation equally with additive or synergistic effects of the combination and further additive or synergistic effects when aspirin was added. *Conclusions:* These results show the extent to which activation of the $P_{2T}$ receptor by ADP released in response to other agonists amplifies the overall response to those agonists. The role of thromboxane $A_2$, on the other hand, is more limited, particularly when studies are performed at physiologic divalent cation levels.

## A DOMINANT NEGATIVE MUTATION IN THE PLATELET $P2X_1$ ADP RECEPTOR CAUSES A SEVERE BLEEDING DISORDER

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ADP is an important platelet agonist for physiologic hemostasis. Two ADP receptors, the Gq protein-coupled $P2Y_1$ receptor, and the so-called $P2T_{AC}$ receptor, coupled to a Gi protein subtype, are both needed during ADP-induced platelet aggregation. Upon ADP binding, rapid calcium influx occurs through the platelet $P2X_1$ ligand-gated ion channel but the function of this receptor remains unclear. We report on a 6-year old patient with selective impairment of ADP-induced platelet aggregation and secretion, leading to a severe bleeding disorder. This patient had no abnormal $P2Y_1$ or $P2T_{AC}$ receptors. We show that a patient's platelets express non-functional $P2X_1$ channels due to a cell lineage-specific *de novo* mutation in one allele of the $P2X_1$ gene. This mutation leads to loss of one leucine residue in the second transmembrane domain of the $P2X_1$ receptor. Voltage-clamped HEK293 cells expressing mutated $P2X_1$ channels failed to develop a significant ATP or ADP-induced current. Furthermore, when co-expressed with the wild type receptor in Xenopus oocytes, the mutated protein exhibited a dose-dependent dominant negative effect on the normal ATP or ADP-induced $P2X_1$ channel activity. These data suggest that the patient's platelet dysfunction is due to the expression of non-functional trimeric $P2X_1$ channels in platelet membranes. Thus, we provide the first clinical evidence for a possible involvement of the ionotropic $P2X_1$ receptor during physiologic ADP-induced platelet aggregation.

## FUNCTIONAL ROLES OF $P2X_1$ PURINOCEPTORS IN HUMAN PLATELETS

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In human platelets, metabotropic $P2Y_1$ and $P2Y_{AC}$ receptors stimulate shape change and aggregation whereas the importance of ionotropic $P2X_1$ receptors during platelet activation is unclear. We investigated the functional roles of the $P2X_1$ purinoceptor using simultaneous measurements of fluorescence and light transmission in platelet suspensions. $[Ca^{2+}]_i$ was measured using fura-2 (in saline) or fluo-3 (plasma:saline mixtures). Shape change and aggregation were monitored from the transmission of 578 nm light. Platelets were prepared as described previously (*MacKenzie et al. J Biol Chem 1996; 271:2879*) except that an initial 2-3 mL of blood was discarded; blood was also drawn directly into anticoagulant to limit purinoceptor desensitization. The effects of citrate on $pH_0$ and $[Ca^{2+}]_0$ were compensated for in plasma:saline experiments. Stimulation with the P2X-specific agonist α,β-methylene ATP caused a transient increase in $[Ca^{2+}]_i$ and a delayed, transient decrease in light transmission, consistent with platelet shape change, in both saline and plasma: saline mixtures. The light transmission decrease was unaffected by Reopro (4 μg $mL^{-1)}$ and was abolished by omission of apyrase or external $Ca^{2+}$; either condition also prevented $P2X_1$ receptor-dependent calcium influx. Experiments conducted at 13°C slowed the calcium responses elicited by the metabotropic receptors compared to the ionotropic $P2X_1$ receptors, allowing further elucidation of the relative roles of these receptors in the $[Ca^{2+}]_i$ signals.

*Supported by the British Heart Foundation (FS/97052 & BS/10)*

## ADP-INDUCED ACTIVATION OF THE EXTRACELLULAR-REGULATED KINASE / MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY VIA THE IONOTROPIC $P2X_1$ RECEPTOR IN PLATELETS

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The intracellular signaling mechanisms underlying the complex process of ADP-induced platelet activation have become the focus of great interest. Exposure of platelets to ADP leads to several intracellular changes linked to three ADP receptors: 1) $P2Y_1$, responsible for the activation of phospholipase C, which process results in the release of $Ca^{2+}$ from platelet internal stores and stimulation of PKC, 2) the unknown, so-called $P2T_{AC}$ receptor, linked to the inhibition of stimulated adenylyl cyclase, and 3) the $P2X_1$ ligand-gated ion channel, causing a rapid $Ca^{2+}$ influx. It has recently been shown that the $P2Y_1$ receptor mediates p38 MAP kinase activation in non-aspirinated platelets stimulated with ADP. The presence of

two other MAP kinases, ERK1 and ERK2, has also been reported in platelets, but the role for platelet function of these MAP kinase cascades during ADP induced activation remains to be determined. The object of the present study was to investigate whether the ERK1/ERK2 signaling pathway was activated during platelet activation with ADP and to identify the ADP receptors involved. By performing phospho-ERK1/ERK2 blotting, we found that ADP (5 μM) induced a rapid (2 min), quickly reversible, ERK2 phosphorylation in washed, aspirin-treated platelets. Interestingly, a similar activation could also be achieved by the $P2X_1$ -specific agonist, α,βMeATP (5 μM), suggesting that ADP-induced ERK2 activation was mediated through $P2X_1$. These data were confirmed in the Dami megakaryocytic cell line, endogeneously expressing the three ADP receptors, in which α,βMeATP, like ADP, induced ERK1 and ERK2 phosphorylation. In order to determine whether $P2X_1$ was able to mediate ERK2/1 activation in the absence of $P2Y_1$ and $P2T_{AC}$, two different cell lines, 1321N1 and HEK293, stably expressing the $P2X_1$ receptor were developed. In these transfected cells, ADP and α,βMeATP induced ERK2/1 activation, a phenomenon totally abolished by EGTA, indicating that the activation of ERK2/1 depends on the $P2X_1$-generated $Ca^{2+}$ influx. No such activation was observed in non-transfected cells stimulated by the same agonists. In addition, stable transfection of Dami or HEK293 cells with the dominant negative $P2X_1$ delL protein, generated cells not able to induce ERK2/1 phosphorylation in response to α,βMeATP or ADP, further demonstrating the selective involvement of $P2X_1$ in this major intracellular pathway. In conclusion, the ionotropic $P2X_1$ receptor mediates ADP-induced ERK2 activation in platelets, providing the first indication of a role of $P2X_1$ in platelet activation.

## TISSUE FACTOR-INDUCED ACUTE THROMBOEMBOLISM IS REDUCED IN $P2Y_1$-KNOCKOUT MICE

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ADP plays a key role in hemostasis as it is itself an aggregating agent and is released from dense granules during platelet activation, thus potentiating the aggregation responses induced by other agents. Two platelet ADP receptors are necessary to obtain full aggregation in response to ADP. The $P2Y_1$ receptor is responsible for shape change through intracellular calcium mobilization, while an unidentified P2 receptor ($P2_{cyc}$) coupled to adenylyl cyclase inhibition is responsible for completion and amplification of the platelet response. Recently, we showed that the $P2Y_1$ receptor plays an essential part in the thrombotic states induced by ADP or by a combination of collagen and adrenaline.[1] The aim of the present study was to assess the role of this receptor in tissue factor-induced thromboembolism. Human thromboplastin (Thromborel S®) was injected intravenously into $P2Y_1$-deficient mice or control wild-type mice and the effects on mortality and platelet count were determined and plasma thrombin-antithrombin III (TAT) complexes were quantified by ELISA. This model of acute thromboembolism leads to death of the animals within some minutes, depending on the dose of thromboplastin. After injection of 200 μL/kg thromboplastin, only 53% of the wild-type mice as compared to 73% of the $P2Y_1$ -deficient mice survived and recovered. A lower dose of thromboplastin (100 μL/kg) was used to study the effects on platelet count. In blood drawn 2 minutes after thromboplastin injection, the platelet count was strongly reduced in wild-type mice relative to control mice receiving physiologic saline (mean ±sem: 451,280±119,217 and 1168,514±57,003 platelets/μL, respectively). Surprisingly, no significant decrease in platelet count was observed in $P2Y_1$-knockout mice as compared to the corresponding control (1027,086±67,657 and 1176,000±81,302 platelets/μL, respectively). The platelet consumption in wild-type mice was most probably due to thrombin generation since this effect of thromboplastin injection was abolished by prior subcutaneous injection of 50 μg/kg hirudin. Thromboplastin injection also led to a rise in TAT complexes in plasma, again reflecting thrombin generation. TAT complexes nevertheless increased less strongly in $P2Y_1$ -knockout mice than in wild-type mice, indicating that less thrombin was generated *in vivo* in response to thromboplastin in the $P2Y_1$ -deficient mice. However, it remains to be determined whether this is the cause of the greater thromboresistance of the knockout mice, as it could also be due to a lesser procoagulant effect of $P2Y_1$ -/- platelets or to weaker interactions between platelets and the vasculature. Our results demonstrate a role of the $P2Y_1$ receptor in thrombotic states involving blood clotting and emphasize the potential relevance of this receptor as a target for antithrombotic drugs.

1. *Léon C, et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y1 receptor null mice. J Clin Invest 1999; 104:1731-7.*

## $N^6$-METHYL 2'-DEOXYADENOSINE 3'-5'-BISPHOSPHATE, A POTENT AND SELECTIVE $P2Y_1$ ANTAGONIST, INHIBITS ADP-INDUCED PLATELET AGGREGATION *IN VITRO* AND *EX VIVO* AND PROLONGS THE BLEEDING TIME

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Concomitant intracellular signaling through the $P2Y_1$ receptor coupled to phospholipase C and the $P2_{cyc}$ receptor coupled to adenylyl cyclase inhibition is essential for full ADP-induced platelet aggregation. The $P2Y_1$ receptor is necessary for ADP to trigger aggregation through an increase in intracellular calcium, since its inhibition by selective antagonists such as adenosine-2'-5' (A2P5P) and 3'-5'-bisphosphate (A3P5P) totally abolishes ADP-induced platelet aggregation, shape change and calcium mobilization. In addition, a lack of $P2Y_1$ expression confers resistance to the thromboembolism induced by intravenous injection of ADP or collagen and adrenaline (*Léon et al*

*1999*). Thus, the $P2Y_1$ receptor plays an essential role in thrombotic states and represents a potential target for antithrombotic drugs. The aim of the present study was to evaluate the effects of a potent and selective $P2Y_1$ antagonist, $N^6$-methyl 2'-deoxyadenosine-3'-5'-bisphosphate (MRS2179, *Boyer et al, 1998*), on ADP-induced human and rat platelet aggregation *in vitro*, on rat platelet aggregation *ex vivo* and on the bleeding time *in vivo*.

In suspensions of washed human platelets, MRS2179 displaced the dose-response curves for ADP-induced platelet aggregation to the right in a concentration dependent manner (pA2 = 6.55± 0.05). As expected for a $P2Y_1$ antagonist, MRS2179 inhibited ADP-induced calcium mobilization in fura2 loaded washed human platelets, but had no effect on ADP-induced inhibition of adenylyl cyclase in a radioimmunoassay. The anti-aggregatory properties of MRS2179 were not influenced by the presence of apyrase (ATP-diphosphohydrolase) and it was stable in rat plasma suggesting the compound to be non-hydrolysable. A bolus i.v. injection of MRS2179 (50 mg/kg) resulted in complete inhibition of ADP-induced (1-10 μM) rat platelet aggregation in citrated platelet rich plasma prepared from blood samples drawn five minutes after injection. The bleeding time, measured from a longitudinal incision made in the rat tail one minute after MRS2179 injection, was prolonged (> 15 min) as compared to that in control rats (3 min). These results suggest this potent and selective $P2Y_1$ antagonist to be a promising tool to evaluate the *in vivo* effects of pharmacologically targeting the $P2Y_1$ receptor with a view to antithrombotic therapy.

## EFFECT OF THE NOVEL P2T RECEPTOR ANTAGONIST, AR-C69931MX, ON THROMBOSIS AND HEMOSTASIS IN THE DOG: COMPARISON WITH GPIIb/IIIa ANTAGONISTS

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*Background.* ADP-induced platelet aggregation (APA) is subserved by the $P_{2T}$-subtype of receptor which appears to be located uniquely on platelets. From a series of novel $P_{2T}$ receptor antagonists, AR-C69931MX (2-trifluoropropylthio, N-(2-(methylthio) ethyl)-β,γ-dichloromethylene ATP), a potent and selective (>1,000-fold) inhibitor of APA in human washed platelets ($IC_{50}$ 0.45 nM) and human blood ($IC_{50}$ 0.71 nM) has been selected for clinical development as an intravenous (i.v.) anti-thrombotic agent. In the present study, we compared the anti-thrombotic and anti-hemostatic effects of AR-C69931MX with those of three GPIIb/IIIa antagonists, Ro449883, GR144053 and TP9201. *Methods*. Arterial thrombosis (cyclic flow reductions (CFR) in the femoral artery), APA and bleeding time (BT) were measured in male anesthetized dogs. AR-C69931MX (n = 5), Ro449883 (n = 5), GR144053 (n = 5) and TP9201 (n = 7) were administered to separate groups of animals by stepped (30 min) i.v. infusion over dose ranges of 2.3-7,720, 47-14,000, 30-10,000 and 300-30,000 $ng.kg^{-1}.min^{-1}$, respectively. *Results.* All compounds produced dose-related inhibition of APA and CFR and prolongation of BT (Figure 1). The effective dose (CFR abolition) of

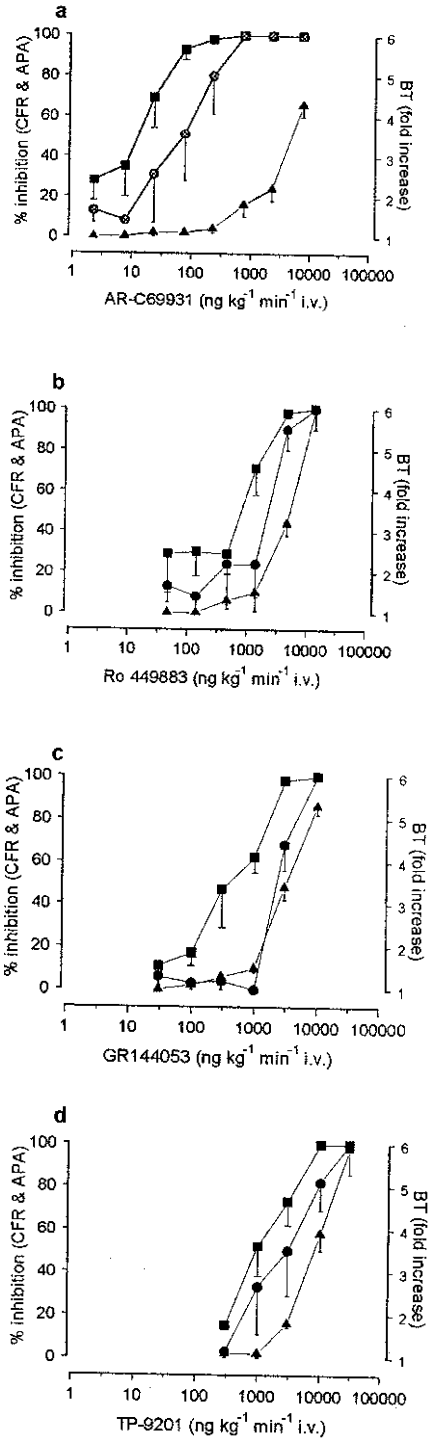


**Figure 1. Effect of AR-C69931MX (a), Ro 44-9883 (b), GR144053 (c), and TP-9201 (d) on thrombosis (λ), hemostasis (σ) and ADP-induced platelet aggregation (ν) in the anesthetized dog (n = 5-6).**

each GPIIb/IIIa antagonist produced a significant ($p < 0.05$, 2-way ANOVA) increase in BT (fold increase from baseline, mean±se: Ro449883, 4.0±0.9; GR144053, 4.3±0.5; TP9201, 3.7±0.8). In contrast, AR-C69931MX (geometric mean dose (95% confidence limits): 109 (25-479) $ng.kg^{-1}.min^{-1}$ iv) abolished CFR with minimal effect on BT (fold increase: 1.4±0.3). BT was increased significantly (4.3±0.5-fold, $p < 0.01$) at the highest dose of AR-C69931MX but, even at this substantial (71-fold) increment on the antithrombotic dose, full restoration of hemostasis was achieved within 10 min of stopping infusion. *Conclusions.* These data support a pivotal role for ADP in arterial thrombosis and indicate that, in the clinical setting, anti-thrombotic efficacy of $P_{2T}$ receptor antagonists may be associated with a reduced and, in the case of AR-C69931MX, controllable risk of bleeding compared to GPIIb/IIIa antagonists.

## SUPERIOR ANTIPLATELET EFFECTS OF AR-C69931MX COMPARED TO CLOPIDOGREL IN PATIENTS WITH ISCHEMIC HEART DISEASE

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*Background.* ADP plays a major role in hemostasis and thrombosis by acting as an agonist for platelet activation via $P2X_1$, $P2Y_1$ and $P_{2T}$ receptors. The thienopyridine clopidogrel is now recognized to act at the level of the $P_{2T}$ receptor and has proven efficacy in reduction of thrombotic complications of atherosclerotic disease and, in combination with aspirin, in reducing thrombotic complications of intracoronary stent implantation. AR-C69931MX is a direct-acting $P_{2T}$ receptor antagonist that is currently being assessed as intravenous antithrombotic therapy in patients with ischemic heart disease. *Aim:* We aimed to compare the effects of clopidogrel and AR-C69931MX in ischemic heart disease patients. *Methods.* In Group 1, 13 patients with unstable angina or non-Q wave myocardial infarction received open-label intravenous AR-C69931MX infusion either 2 µg/kg/min (n=8) or 4 µg/kg/min (n=5) in addition to aspirin, with infusion duration of 24-72 hours. ADP-induced aggregation was studied using whole blood single-platelet counting and hirudin anticoagulation, before and during infusion. In Group 2, 8 patients treated with percutaneous intracoronary stent implantation were studied before and 4-7 days after this intervention. All patients received a 300 mg oral loading dose of clopidogrel at the time of stent implantation followed by 75 mg daily. At both timepoints, the effects of adding AR-C69931MX *in vitro* were studied. ADP-induced aggregation was studied using exactly the same methodology as employed in the first study. Additional measurements were performed using both whole blood single-platelet counting and $^{14}$C-5HT release in response to ADP, collagen and TRAP, and PRP turbidimetry in response to ADP (2 and 20 µM) and TRAP 20 µM. Data were analyzed using ANOVA and are expressed as mean ± standard deviation. *Results.* There was no difference between patients in groups 1 and 2 in the baseline responses to ADP in whole blood with mean $EC_{50}$ values for ADP-induced aggregation at 4 minutes in Group 1 of 2.26±1.87 mM and in group 2 of 1.39±0.57 µM ($p$=0.22). AR-C69931MX, at both infusion doses in group 1, produced substantially greater inhibition of aggregation than that achieved by clopidogrel in group 2: mean inhibition of aggregation induced by ADP 10 µM was 85±7% and 90±10% for AR-C69931MX 2 and 4 µg/kg/min, respectively, and 39±36% for clopidogrel ($p$<0.001 AR-C69931MX superior). In group 2 studies, the *in vitro* addition of AR-C69931MX 100 nM (whole blood concentration) or 150 nM (PRP concentration), approximately equivalent to the lowest plasma levels achieved in the group 1 patients, yielded superior inhibition of aggregation and $^{14}$C-5HT responses to ADP and TRAP in whole blood and PRP. This partly reflected greater uniformity of response to AR-C69931MX, with some patients responding relatively poorly to clopidogrel. Furthermore, the effects of AR-C69931MX and clopidogrel on ADP-induced aggregation were additive. For example, maximum turbidimetric responses to ADP 2 µM were 54±9% (baseline), 10±7% (baseline + AR-C69931MX), 22±12% (clopidogrel; $p$<0.05 AR-C69931MX superior) and 7±5% (clopidogrel + AR-C69931MX; $p$<0.05 additive effect); turbidimetric responses to TRAP 20 µM at 4 min, after reversal of aggregation had been allowed to occur, were 63±10% (baseline), 23±26% ($p$<0.05), 53±8% ($p$=ns) and 30±7% ($p$=ns), respectively. There was a trend towards greater inhibition of collagen-induced aggregation and $^{14}$C-5HT release by AR-C69931MX compared to clopidogrel but no significant additive effects of the combination of the two agents. *Conclusions.* Therapeutic administration of AR-C69931MX results in superior $P_{2T}$ receptor blockade, measured *ex vivo*, than clopidogrel in patients with ischemic heart disease. Direct $P_{2T}$ receptor antagonists such as AR-C69931MX therefore have the potential to achieve greater antithrombotic efficacy than clopidogrel.

## CLOPIDOGREL PRODUCES INCOMPLETE INHIBITION OF [$^{33}$P]-2MeSADP BINDING TO HUMAN PLATELETS AND LESS INHIBITION OF ADP-INDUCED PLATELET AGGREGATION THAN THE $P_{2T}$ ANTAGONIST AR-C69931MX

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*Background.* Clinical findings with clopidogrel, a pro-drug which, at the approved dose, partially inhibits ADP-induced platelet aggregation (APA), indicate a critical role for ADP in arterial thrombosis and imply that a more effective inhibitor may confer greater clinical benefit. AR-C69931MX, currently in phase II clinical trials, is a potent competitive antagonist at the platelet $P_{2T}$ receptor. *Aim*. In this study, we compared the effects of a clinically relevant concentration of AR-C69931MX added *in vitro* with the approved oral dose of clopidogrel *ex vivo* on APA. The effect of clopidogrel on binding of [$^{33}$P]-2MeSADP

to human platelets was also measured. *Methods.* Clopidogrel (75 mg po once daily) was administered to 8 healthy male volunteers for 11 days. APA concentration-response curves (0.1-300 µM) were measured on days 0 (pre-dose control), 1, 2, 3 and 11 in heparinized whole blood (hWB) using impedance aggregometry and turbidimetrically in citrated PRP (cPRP) in the absence and presence of AR-C69931MX (500 nM) added *in vitro*. Binding of [$^{33}$P]-2MeSADP (0.01-30 nM) to washed platelets was measured in subjects 5 to 8 on days 0, 2 and 11. *Results.* Figure 1 shows APA results in hWB. The anti-aggregatory effect of clopidogrel was slow to develop, incomplete and variable: inhibition of APA (day 11 cf day 0 vs 10 µM ADP) was 46±10% (mean ± s.e.). By contrast, the effect of AR-C69931MX added in vitro was complete and consistent: inhibition of APA (day 0 vs 10 µM ADP) was 97±2%. A similar pattern was observed in cPRP, with less inhibition (53 ± 5%) of the maximum extent of APA (10 µM) observed after 11 days administration of clopidogrel than with AR-C69931MX on day 0 (80±3%). Reproducible binding data were obtained for 3/4 subjects tested. In the remaining subject, control binding was substantially reduced (Figure 2). Results on day 0 were consistent with a single non-cooperative binding site (Hill coefficient 1.22±0.21) with a pK of 9.03±0.00 and a Bmax of 859±17 binding sites per platelet. Clopidogrel reduced binding (day 11 cf day 0) by approximately 70% for all concentrations of [$^{33}$P]-2MeSADP tested. *Conclusions.* Our findings confirm previous reports of slow onset partial inhibition of APA by clopidogrel and importantly, demonstrate that functional $P_{2T}$ receptors remain following clopidogrel treatment. The failure of clopidogrel to abolish binding is consistent with this view, although definitive conclusions about the pharmacological nature of the residual binding cannot be drawn in this case. In hWB, the limited effect of clopidogrel was particularly evident and contrasted markedly with that of AR-C69931MX. If anti-thrombotic efficacy is a function of inhibition of APA, these results suggest that direct $P_{2T}$ receptor antagonists such as AR-C69931MX will provide significant improvement over the modest clinical benefit demonstrated to date with clopidogrel.

**Figure 1**

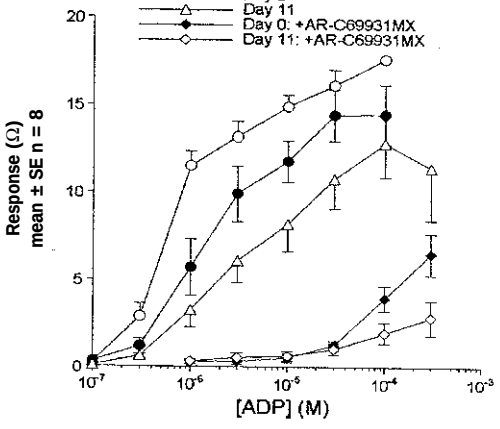
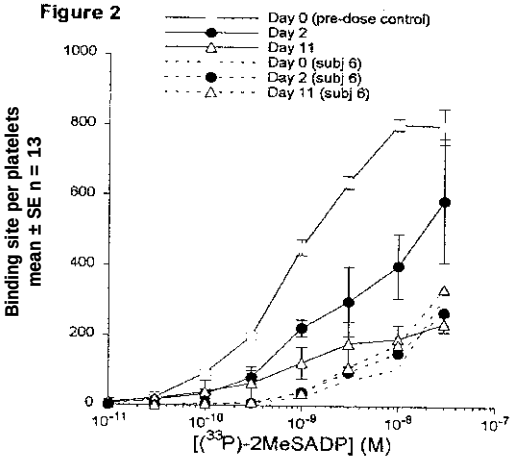
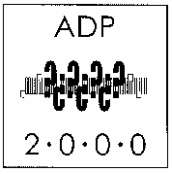



**Figure 2**






# POSTERS

## INVOLVEMENT OF THE $P2_{cyc}$ BUT NOT THE $P2Y_1$ ADP RECEPTOR IN THE PHOSPHATIDYLSERINE EXPOSURE OF ACTIVATED PLATELETS

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The activation of platelets and coagulation are related events. Platelet activation leads to secretion and aggregation and simultaneously to the exposure of negatively charged phospholipids which provide a catalytic surface for the generation of thrombin. ADP is itself a key agonist and enhances the platelet response to strong agonists such as thrombin and collagen, thereby acting as an essential cofactor in many platelet functions. However, little is known about the role of ADP in the exposure of procoagulant phospholipids by platelets.

The aim of this study was to examine *in vitro* the role of ADP and its receptors in the exposure of phosphatidylserine at the surface of activated platelets. Two ADP receptors are potentially involved: $P2Y_1$, the metabotropic receptor responsible for the mobilization of ionized calcium from internal stores, which initiates aggregation, and/or the unidentified $P2_{cyc}$ receptor, coupled to adenylyl cyclase inhibition, which is essential for the full aggregation response to ADP. FITC-annexin V was used to detect exposure of phosphatidylserine at the surface of washed platelets activated with thrombin or a mixture of thrombin and collagen. Adenosine-3'-phosphate-5'-phosphate (A3P5P), a selective $P2Y_1$ antagonist, and AR-C69931MX, a selective $P2_{cyc}$ antagonist, were employed to distinguish between the two pathways. Experiments were performed using normal human platelets and wild type and $P2Y_1$ deficient ($P2Y_1$-/-) mouse plalelets. Washed human or mouse platelets ($1.5x10^5$ platelets/µL) resuspended in Tyrode's buffer containing 0.1% (w/v) fatty acid free albumin were activated with thrombin (0, 0.05, 0.1, 1 U/mL), alone or in the presence of collagen (25 µg/mL), for 10 min at 37°C. Activation was stopped by 10 fold dilution with the same buffer containing 50 U/mL hirudin. Aliquots (20 µL) were incubated with FITC-annexin V (10 µg/mL) for 10 min at room temperature and annexin V binding was detected by flow cytometry. Quadrant analyses were performed by plotting FSC against FL1 and determining the percentage of annexin V labeled platelets.

*In vitro* treatment of human platelets with collagen and increasing concentrations of thrombin raised the percentage of annexin V labeled cells by 4 to 15 fold as compared to resting platelets. The proportion of annexin V labeled platelets decreased by 30 to 60% in the presence of AR-C69931MX (10 µM), whereas A3P5P (400 µM) had no significant effect. When wild type or $P2Y_1$ -/- mouse platelets were treated with thrombin and/or collagen, FITC-annexin V labeled $P2Y_1$-/- and wild type platelets to a comparable extent. AR-C69931MX (10 µM) reduced the percentage of annexin V labeled $P2Y_1$-/- or wild type platelets by 30 to 60% depending on the dose of thrombin, while A3P5P (200 µM) again had no significant effect. Thus, when the $P2Y_1$ receptor was blocked either with a specific antagonist or by gene knock-out in mice, human and mouse platelets were still able to expose procoagulant phospholipids at their membrane surface. In contrast, phospholipid exposure was inhibited when the $P2_{cyc}$ receptor was blocked with a specific antagonist. These results suggest that ADP is involved in the exposure of negatively charged phospholipids at the surface of activated platelets and that this function of ADP is dependent on the $P2_{cyc}$ pathway. Our data are consistent with the observation of Hérault *et al.* (*Thromb Haemost 1999*) that clopidogrel inhibits thrombin generation on rat platelets and support the idea that antiplatelet agents affecting the $P2_{cyc}$ pathway may also act as antithrombotic agents by reducing thrombin generation.

## THROMBOPOIETIN UPREGULATES $P2Y_1$ RECEPTOR GENE EXPRESSION AND $P2Y_1$ mRNA LEVELS IN MEGAKARYOCYTES

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Pharmacologic data and studies on $P2Y_1$ knockout mice have clearly established that two independent receptors contribute to the platelet aggregation induced by adenosine 5'-diphosphate (ADP): the $P2Y_1$ metabotropic receptor responsible for the mobilization of ionized calcium from internal stores which initiates aggregation and as yet unidendified P2Y receptor coupled to adenylyl cyclase inhibition, termed $P2Y_{ADP}$, $P2T_{AC}$ or $P2_{CYC}$, which is essential for a full aggregation response to ADP (*Hechler et al., Blood 1998; Léon et al., J Clin Invest 1999*). Although it has been shown that the $P2Y_1$ receptor is expressed early in the megakaryocytic lineage, the regulation of its expression in the process of megakaryocyte maturation is unknown. Thrombopoietin (TPO) is the pivotal physiologic regulator of megakaryocytopoiesis and platelet production. It stimulates megakaryocyte progenitor cell proliferation, induces the expression of platelet specific proteins and increases endomitosis. We wondered if TPO affected the expression of the $P2Y_1$ receptor during differentiation of megakaryocytes. In this issue, we took advantage of the Y10/L8057 mouse megakaryocytic cell line, a subclone of the megakaryocytic cell line L8057, which has been shown to respond to recombinant TPO (PEG-rHuMGDF, generous gift of Amgen, Inc. CA,

USA) (*Zhang et al., J Biol Chem 1998*). Northern blot analysis indicated that PEG-rHuMGDF (25 ng/mL) increased $P2Y_1$ mRNA levels by two fold in Y10/L8057 cells. The enhancement of $P2Y_1$ receptor mRNA by TPO was associated with the upregulation of platelet factor 4 (PF4) and glycoprotein IIb (GPIIb) mRNA. The $P2Y_1$ receptor mRNA upregulation appeared to be selective, since PEG-rHuMGDF did not induce an increase of the adenosine A2a receptor mRNA in Y10/L8057 cells. The half-life of the $P2Y_1$ receptor mRNA in megakaryocytes was determined to be 2.5 hours and this was not affected by PEG-rHuMGDF treatment. In contrast, PEG-rHuMGDF increased the transcription of the $P2Y_1$ gene in Y10/L8057 cells, as assessed by nuclear run-on experiments. Moreover, *in vivo* studies consisting of injection of 50 µg/kg PEG-rmMGDF in FVB mice induced an increase in the $P2Y_1$ receptor mRNA level in megakaryocytes from spleen, as shown by *in situ* hybridization analysis. These results show that TPO upregulates $P2Y_1$ receptor mRNA expression in the process of megakaryocyte maturation, both *in vivo* and *in vitro*. Whether the TPO-induced increase in $P2Y_1$ receptor mRNA level correlates with an increase in $P2Y_1$ receptor protein and whether it affects platelet functions remains to be determined.

## DIFFERENTIAL INVOLVEMENT OF THE $P2Y_1$ AND $P2_{cyc}$ RECEPTORS IN MORPHOLOGICAL CHANGES DURING PLATELET AGGREGATION

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Platelets are discoid in their resting state but upon activation by various stimuli rapidly change their morphology to become spherical and form two distinct types of surface protusion, lamellipodia and filopodia. This shape change precedes platelet aggregation and secretion. ADP is stored at high concentrations in the dense granules of platelets, from which it is released during platelet activation, thus potentiating aggregation in response to other agonists. ADP-induced platelet aggregation results from simultaneous activation of the $P2Y_1$ and $P2_{cyc}$ receptors, coupled to calcium mobilization and adenylyl cyclase inhibition, respectively. The aim of the present work was to assess the relative contributions of these two receptors to the morphologic changes induced by ADP itself and by ADP releasing agonists such as thrombin and U46619. The effects of selective antagonists of $P2Y_1$ (A2P5P) and $P2_{cyc}$ (AR-C67085) on the ultrastructure of platelet aggregates were examined by scanning and transmission electron microscopy.

A2P5P (1 mM) totally blocked platelets stimulated with ADP (5 µM) in the discoid shape typical of resting cells. When platelets were activated with a low concentration of thrombin (0.02 U/mL), A2P5P prevented aggregation and markedly affected shape change, as demonstrated by the presence of a large proportion of discoid cells (75%) and only some isolated platelets extruding lamellipodia and filopodia (25%). At a higher concentration of thrombin (1 U/mL) A2P5P had no effect on the ultrastructure of platelet aggregates. When platelets were stimulated with a low concentration of U46619 (0.25 µM), A2P5P inhibited the formation of filopodia without significantly affecting lamellipodia, while at a higher concentration of U46619 (10 µM) A2P5P was again ineffective. These results point to a role of the $P2Y_1$ receptor in the formation of filopodia in weakly activated platelets. In contrast, AR-C67085 (1 µM), which blocks the action of ADP on adenylyl cyclase, did not affect platelet shape change but led to a decrease in the size of the aggregates induced by ADP (5 µM), thrombin (0.02 U/mL) or U46619 (0.25 µM). These aggregates were composed of loosely packed platelets with few contact points, as compared to the tight macroaggregates formed under control conditions. In the presence of a higher concentration of thrombin (1 U/mL) or U46619 (10 µM), AR-C67085 did not significantly modify the morphologic changes of platelet aggregation.

It is concluded that the $P2Y_1$ and $P2_{cyc}$ receptors are differently involved in the morphologic changes of the platelet aggregation induced by ADP or low concentrations of thrombin or U46619, conditions under which aggregation is dependent on released ADP. Activation of the P2cyc receptor appears to be essential for the formation of stable macroaggregates, whereas $P2Y_1$ seems to be involved in shape change and to play a role in the extrusion of filopodia. Studies are currently underway to elucidate the molecular mechanisms through which the $P2Y_1$ receptor regulates the cytoskeletal reorganization induced by ADP.

## CHARACTERIZATION OF THE $P_{2T}$ RECEPTOR ANTAGONIST PROPERTIES OF AR-C69931MX IN HUMAN WASHED PLATELETS *IN VITRO*

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*Background.* The $P_{2T}$ antagonists, AR-C66096 and AR-C67085 inhibit ADP-induced platelet aggregation in a competitive manner when tested under equilibrium conditions. However, because they dissociate relatively slowly from the receptor, the antagonism can be insurmountable under conditions of hemi-equilibrium. *Aim*. The aim of this study was to investigate the pharmacology of the structural analog, AR-C69931MX, which is currently in phase II clinical development. *Methods.* Aggregation of human washed platelets (WP) was assessed turbidimetrically as a decrease in absorbance (650 nm), 5, 10 and 60 min after the addition of ADP to aliquots (150 µL) of platelet suspension in 96-well microtiter plates. Concentration/effect (E/[A]) curves to ADP (0.03-1,000 µM) were constructed in the absence and presence of AR-C69931MX (3-100 nM) added either 5 min before or simultaneously with ADP and, in the latter case, left for 15 min before the response was initiated by shaking. Protection experiments were also performed in the presence of the non-depressing,

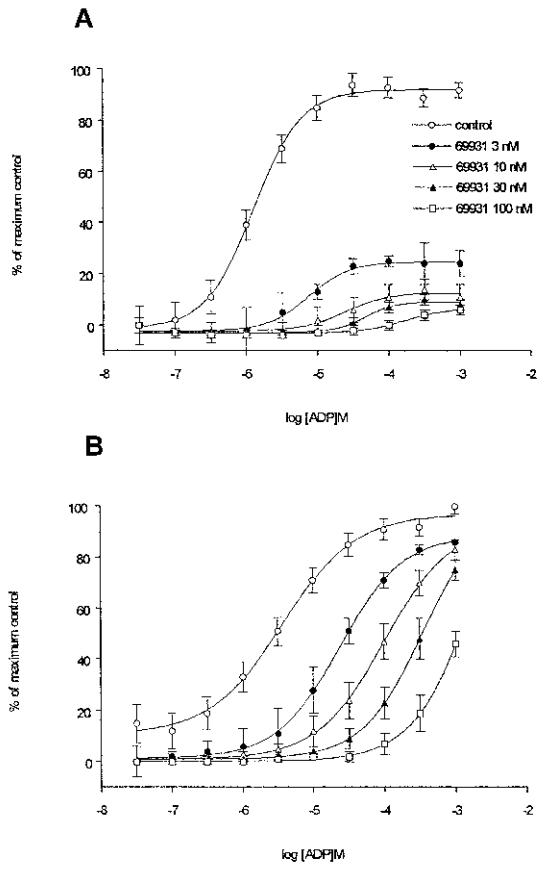


**Figure 1. The effect of AR-C69931MX pre-incubated for 5 min on ADP-induced platelet aggregation read after a) 5 min, b) 60 min. Values are means ± s.e. (n = 4).**

competitive $P_{2T}$ antagonist, AR-C67938MX. Binding data were obtained in WP in 96-well plates, with each well containing [$^{33}$P]-2MeSADP (0.36 nM), putative displacers and WP. After a 30 min incubation, the reaction was terminated by filtration. Specificity of the antiplatelet effect was tested against responses to a combination of the $TxA_2$ mimetic, U46619 (3 µM) and adrenaline (0.3 µM), rendered ADP-independent by addition of the standard $P_{2T}$ antagonist, AR-C67085 (1 µM). Selectivity data were obtained against other P2 receptor subtypes found on platelets by measuring ADP- ($P2Y_1$) or (in the presence of apyrase) ATP-induced ($P2Y_1$) calcium increases. *Results.* When pre-incubated for 5 min before the addition of ADP, with the response measured at 5 min after this addition, AR-C69931MX caused a concentration-dependent rightward displacement of the E/[A] relationship and depressed the asymptote (Figure 1a). Less depression was observed when responses were measured following a longer co-incubation period (60 min reading, Figure 1b). Under conditions in which equilibrium was believed to be achieved, AR-C69931MX caused parallel rightward displacement consistent with simple competition. The Clark slope parameter was 1.08±0.13 and, with this parameter constrained to unity, the $pK_B$ value was 9.35±0.14 (mean±s.e., n = 4). This was consistent with the $pK_i$ value for AR-C69931MX of 9.5± 0.1 (mean±s.e., n = 6) for the displacement of [$^{33}$P]-2MeSADP binding from human washed platelets. In addition, the asymptote at 10 min in the presence of AR-C69931MX (10 nM) was increased from 43% to 69% of maximum when the $P_{2T}$ antagonist AR-C67938MX was used to protect the receptor. At a concentration of up to 100 µM, AR-C69931MX had no effect on an ADP independent aggregation response and showed no agonist or antagonist activity at $P2Y_1$ or $P2X_1$ receptors. *Conclusions.* AR-C69931MX is a potent, selective, competitive P2T receptor antagonist which, under non-equilibrium conditions, can exhibit non-competitive properties.

## INHIBITION OF ADP-INDUCED PLATELET AGGREGATION BY AR-C69931MX: COMPARISON OF EFFECTS IN HUMAN WHOLE BLOOD AND PLATELET RICH PLASMA *IN VITRO*

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*Background.* AR-C69931MX, currently in phase II clinical development for the treatment of acute coronary syndromes, is a potent, selective and competitive antagonist at the platelet $P_{2T}$ receptor. Platelet aggregation can be measured using a variety of techniques, each capable of producing different results which require specific interpretation. For example, the response is commonly measured in citrated platelet rich plasma (cPRP) using optical aggregometry, but an alternative is impedance aggregometry using the more physiologic milieu of whole blood. *Aim.* The aim of this study was to characterize the inhibition of ADP-induced platelet aggregation (APA) by AR-C69931MX in cPRP using optical aggregometry, and in heparinized whole blood (hWB) using impedance aggregometry. *Methods.* Impedance aggregometry was performed in hWB (heparin, 10 U/mL) from 5 subjects and optical aggregometry in cPRP (citrate, 0.11M, 3.2%) from 4 subjects. Concentration-response curves to ADP were generated in the absence (control) and presence of increasing concentrations of AR-C69931MX (3-1,000 nM). The response in hWB was measured in ohms 5 min after the addition of ADP. In cPRP, 3 indices of aggregation were measured: final extent, maximum extent and rate. Data were fitted to a 4 parameter logistic model incorporating a model of competitive antagonism. Statistical tests were carried out to assess whether any inhibition conformed to standard conditions of competitive antagonism. *p* values of >0.10 were considered non-significant; *p* values of <0.01 were considered significant. Intermediate values were considered of borderline significance. Data are presented as mean ± s.e. *Results.* AR-C69931MX inhibited APA in both hWB and cPRP. In 2/5 subjects in hWB, there was no significant deviation from parallelism and, in the remaining 3 subjects, there was a borderline deviation. In 3/5 subjects, the Schild slope

did not differ significantly from unity and in the remaining 2 there was a borderline deviation. The pKB of AR-C69931MX in hWB was 9.11±0.22 and the Schild slope was 1.06±0.07. The effect of AR-C69931MX on the maximum extent of aggregation in cPRP was characterized by a concentration-related reduction in the Hill coefficient, caused by the persistence of a transient $P2Y_1$-mediated aggregation. The effect on rate of aggregation was a concentration-related depression of the agonist curve. The effect on the final extent differed from that observed previously in heparinized PRP, in that the inhibition did not conform strictly to the criteria of competitive antagonism, but manifest a concentration-related reduction in the Hill coefficient. In particular, the control curves were markedly steeper than those previously observed (7.1±3.2). The extent of rightward shift of the curves however was consistent with competitive antagonism (Schild slope = 1.04±0.02). *Conclusions.* These data demonstrate that AR-C69931MX is a potent inhibitor of APA in both hWB and cPRP. The competitive nature of the antagonism in hWB illustrates that the extent of the response is determined by the occupancy of the $P_{2T}$ receptor while the $P2Y_1$ receptor remains activated. The steepness of the control agonist curve for the final extent of aggregation in cPRP may have been due to $TxA_2$ generation and release of dense granule contents, a phenomenon which is not observed in heparinized PRP, under which circumstances, antagonism by $P_{2T}$ antagonists is competitive. It is concluded that while both methods can clearly demonstrate the anti-aggregatory properties of AR-C69931MX, the impedance technique is a more appropriate method for monitoring and quantifying this effect owing to its direct dependence on activation of the $P_{2T}$ receptor.

## EFFICIENCY OF PLATELET ADHESION TO FIBRINOGEN DEPENDS ON BOTH CELL ACTIVATION AND FLOW

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The kinetics of adhesion of platelets to fibrinogen (Fg) immobilized on polystyrene latex beads (Fg-beads) was determined in suspensions undergoing Couette flow at well-defined homogeneous shear rates. The efficiency of platelets adhesion to Fg-beads was compared for ADP-activated versus *resting* platelets.

The effects of the shear rate (100 to 2000 $s^{-1}$), Fg-density on the beads (24 to 2,882 Fg/$mm^2$), the concentration of ADP used to activate the platelets, and the presence of soluble fibrinogen were assessed. *Resting* platelets did not specifically adhere to Fg-beads at levels detectable with our methodology. The apparent efficiency of platelet adhesion to Fg-beads readily correlated with the proportion of platelets quantally activated by doses of ADP, i.e. only ADP-activated platelets appeared to adhere to Fg-beads, with a maximal adhesion efficiency of 6-10% at shear rates of 100-300 $s^{-1}$, decreasing with increasing shear rates up to 2000 $s^{-1}$. The adhesion efficiency was found to decrease by only threefold when decreasing the density of Fg at the surface of the beads by 100-fold, with only moderate decreases in the presence of physiologic concentrations of soluble Fg. These adhesive interactions were also compared using activated GPIIbIIIa-coated beads. Our studies provide novel model particles for studying platelet adhesion relevant to hemostasis and thrombosis, and show how the state of activation of the platelet and the local flow conditions regulate Fg-dependent adhesion.

## CONTROL OF ADP-EVOKED CALCIUM RELEASE BY THE CELL POTENTIAL IN THE RAT MEGAKARYOCYTE

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Platelets and megakaryocytes lack voltage-dependent calcium channels, therefore the cell potential (Em) is believed to control $[Ca^{2+}]_i$ only by altering the driving force for $Ca^{2+}$ entry. However we now show that Em can modulate $Ca^{2+}$ release from intracellular stores in a more direct manner during stimulation of metabotropic purinoceptors in the rat megakaryocyte. Whole-cell patch clamp recordings and simultaneous measurements of intracellular $Ca^{2+}$ (fluo-3 or fura-2) demonstrated that voltage steps from –75mV to either 0mV or –115mV had no effect on $[Ca^{2+}]_i$ in unstimulated cells. During exposure to 1 µM ADP, depolarization evoked an increase, and hyperpolarization a decrease in $[[Ca^{2+}]_i$. The control of $[Ca^{2+}]_i$ by Em was observed in $Ca^{2+}$-free medium and was blocked by several treatments known to inhibit endogenous IP3 receptors: dialysis with heparin (10 mg $mL^{-1}$ in the pipette), cyclic AMP elevation with carbacyclin or flash photolysis, and exposure to caffeine. A physiologically relevant oscillating Em command paradigm (range -75 to -45mV) induced synchronous $[Ca^{2+}]_i$ oscillations. These experiments suggest that membrane potential changes may have an active role in the control of $Ca^{2+}$ release during purinoceptor signaling in hematopoietic cells.

*Funded by the British Heart Foundation (BS/10 and PG/94151 & 95005)*

## REVERSAL OF ADP-INDUCED AGGREGATION BY APYRASE MODULATED BY WORTMANNIN, A NEW METHOD TO ASSESS PLATELET ACTIVATION *IN VIVO*

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*Background.* Addition of apyrase (AP) to platelet suspensions to protect platelets against unwanted exposure to ADP was introduced by Mustard *et al*. in 1972. Recently Marcus *et al.* have shown that a similar mechanism exists in circulation, where the scavenger is the ecto-ADPases of endothelial cells. The introduction of ADP to platelets in the presence of apyrase results in a *spike* of platelet aggregation/deaggregation when observed in an aggregometer. *Aim*. We propose that

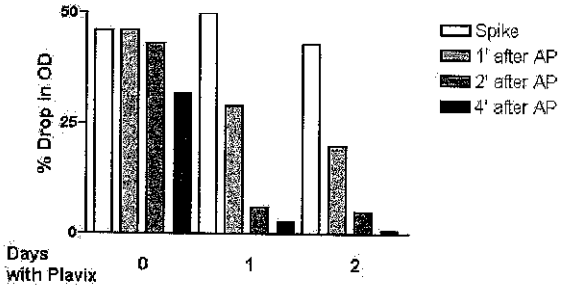
when apyrase is added after the addition of ADP, the speed and completeness of the return of the aggregation tracing to baseline indicates the extent to which platelets have undergone activation in circulation. *Methods*. Blood was collected in green-top vacutainers. Ten min before centrifugation to prepare platelet-rich plasma 0 or 1 µL Wortmannin (W) was added (5 mM in DMSO). An additional 2 µM W was added in plasma. Aggregation was initiated in a ChronoLog aggregometer with 10 or 25 µM ADP, and at least 1 unit/mL apyrase added 3 min later. Percent drop in OD was noted at the peak of aggregation and 1, 2 and 4 min after apyrase addition.

*Results.*

| | [W], µM | Spike | 1' after AP | 2' after AP | 4' after AP | |
|---|---|---|---|---|---|---|
| Low activity | 0 | 43.8 | 42.5 | 24.3 | 13.8 | |
| platelets | 0.5 | 41.5 | 22.0 | 2.3 | -0.3 | n=4 |
| | 2.5 | 31.0 | 1.5 | -0.8 | 0.3 | |
| Hyperactive | 0.5 | 45.0 | 42.7 | 17.0 | 3.0 | |
| platelets | 2.5 | 41.7 | 19.0 | 2.7 | 1.3 | n=3 |

The figure presents results obtained when a donor with hyperactive platelets was given a 75 mg tablet of Plavix™ (clopidogrel) per day for 2 days. Column 1-4, spike result before Plavix; 5-8, day 1 after Plavix; 9-12, day 2 after Plavix.

*Conclusion*. Using the *spike* modulated by W we can quantify the degree of activation of platelets *in vivo*. The *in vivo* effect of Plavix indicates that in the present case platelet hyperactivity is linked to activation of the $P_{2Tac}$ receptor.




## *IN VITRO* EVALUATION OF BLOOD PLATELET FUNCTION DURING THE ACUTE PHASE OF CEREBROVASCULAR ISCHEMIC DISEASE

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A new *in vitro* system for the detection of platelet dyfunction PFA100™, has been developed. PFA100™ is a system for *in vitro* measurement of platelet function in anticoagulated whole blood. The measurement scheme of the system is based on the original principle described by Kratzer and Born. The instrument aspirates a blood sample under constant vacuum form the sample reservoir through a capillary and a microscopic hole cut into a membrane. The membrane is coated with collagen and ephinephrine or adenosine 5'-diphosphate. The instrument is useful as a screening test for hemorragic disease.

The aim of our study is to evaluate this type of technology also in thrombophilic diseases such as the ischemic cerebrovascular events. Herein, we report the results obtained from the general population (n=100, 70 males and 30 females, aged 37±11.5 yrs) referred to our laboratory of Haemostasis and Thrombosis and those found in cerebrovascular ischemic patients (n= 60, 41 males and 19 females) suffering from ischemic stroke (aged 59±17 yrs). Forty patients have a documented diagnosis of atherothrombotic stroke (lacunar and large vessell disease) and 20 a diagnosis of cardioembolic stroke. Samples from cerebrovascular patients were obtained within 24 hours the onset of ischemic event.

The results obtained in our general population were 136±13 seconds for collagen and ephinephrine membrane and 125±40 seconds for adenosine 5'-diphosphate membrane, respectively. In the group of patients we found significant lower results compared to those found in controls (59±35 seconds for collagene and ephinephrine membrane and 75±28 seconds for adenosine 5'-diphosphate membrane, respectively). Notably the lowest levels of PFA100™ tests were found in patients with a cardioembolic stroke (45±20.5 sec. and 60.5±17.5 sec., respectively; $p<.0001$, Fisher exact test).

Our findings demonstrate that the PFA100™ may have potential applications in routine evaluation of platelet function in the clinical setting because of its accurancy, easy of operation and rapid turnaround of results. Therefore, it may be of great interest also for studying patients not only with hemorrhagic phenomena but with cerebrovascular ischemic episodes under high shear stress rates of flow such as the brain territory.

## THE INFLUENCE OF DEMOGRAPHY ON THE VARIABILITY OF ADP-INDUCED PLATELET AGGREGATION IN HUMAN BLOOD *IN VITRO*

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*Background.* Aggregation of blood platelets is believed to be a critical process in hemostasis and the pathogenesis of arterial thrombosis. Moreover, ADP is considered to be an important mediator of the aggregation response *in vivo*. Optical techniques are used commonly to measure ADP-induced platelet aggregation (APA) although other methods exist, including impedance aggregometry. Although this latter technique has been criticized for its variability, it has not been considered that this variability may

reflect actual differences between subjects. *Aim:* The aim of this study was to investigate whether demographic or clinical differences between subjects have any influence upon APA measured using impedance aggregometry. *Methods:* The concentration-response profile of APA was measured in heparinized whole blood from 100 subjects using impedance aggregometry. Demographic data (age, sex, height, weight, smoking status) and coagulation parameters (including von Willebrand factor (vWF) and fibrinogen) were collected for each individual. The structural model used to fit the data was a three parameter logistic equation which generated mean and variance (inter and intra individual) estimates of the following parameters: Maximum response (*Max*); -log concentration giving a 50% response ($pA_{50}$); Hill coefficient (*nH*). By using non-linear mixed effects modeling demographic and clinical data were incorporated into the model to explain differences observed between subjects response profiles. *Results*. 48 males and 52 females were recruited into the study. The average age was 36 years (s.d. = 10) and ranged from 21 to 57. Ten subjects were current smokers. The analysis showed that *Max* differed significantly ($p<0.001$) between males and females [male: 21.7± 0.4 ohms; female: 23.5±0.4 ohms (mean ± s.e.)]. The $pA_{50}$ was significantly affected by sex ($p<0.001$) and age ($p<0.001$), being greater in females than males, and increasing with age, as illustrated by the predicted values in Table 1. Fibrinogen concentration, but not vWF, positively correlated with *Max* ($p<0.01$) and was significantly higher in females than males ($p = 0.027$); however, fibrinogen concentration did not contribute to *Max* independently of its association with sex which was the dominant contributor in the model. *Conclusions*. It is concluded that demographic factors have a significant influence on APA. The dominant demographic factor is sex, with females showing both increased sensitivity to ADP and a greater maximum response compared to males. Increasing age increased sensitivity alone. It is likely that the higher levels of fibrinogen in females contributed to the increase in *Max* seen in this sex. However, the fact that the effect of sex was not wholly explained by fibrinogen, suggests that other unidentified gender specific factors also contributed to this phenomenon. This study also shows that whole blood impedance aggregometry and non-linear mixed effects modeling are valuable methods for investigating population-based variability in platelet responsiveness.

Table 1. Predicted $pA_{50}$ values as a function of age and sex.

| | *Age (years)* | | | |
|---|---|---|---|---|
| *Sex* | *25* | *35* | *45* | *55* |
| Male | 6.00 | 6.03 | 6.06 | 6.09 |
| Female | 6.12 | 6.20 | 6.28 | 6.35 |

## MECHANISM OF ATP-MEDIATED PLATELET AGGREGATION

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ATP and ADP are both released from activated platelets during thrombosis, and are thought to have absolutely different actions on platelet reactivity. According to the two-receptor model for ADP-induced platelet aggregation, simultaneous activation of $P_{2Y1}$ and $P_{2T}$ receptors by ADP, followed by coupling to Gq and Gi, respectively, are required to cause platelet aggregation. In this model, the inhibitory effects of ATP were explained by its antagonistic action at the $P_{2T}$ receptor. However, ATP, as an agonist at $P_{2Y1}$ receptors, should also promote platelet aggregation. Using optical measurement of platelet aggregation, we have demonstrated that, although ATP by itself is unlikely to induce platelet aggregation, it can substantially stimulate platelets in the presence of epinephrine or norepinephrine. We have evidence that ATP induces platelet aggregation via $P_{2Y1}$ receptors. Since both catecholamines act at $\alpha$2A receptors coupled to Gi in platelets, these results are consistent with the proposal that concomitant activation of Gi and Gq are necessary and sufficient for platelet aggregation. In addition, these data suggest a possible prothrombotic role of extracellular ATP during ischemia and stress when concentrations of both catecholamines are significantly increased in blood.

## SYNERGY BETWEEN CONVULXIN AND ADP IN PLATELET AGGREGATION

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Convulxin is a specific agonist for the platelet receptor GPVI, which signals through a tyrosine kinase-dependent pathway leading to platelet aggregation. The G protein-coupled receptor agonist ADP induces platelet aggregation via two receptors, $P2T_{AC}$ and $P2Y_1$. It has been suggested that co-activation of these two receptors is critical for aggregation by ADP although the underlying mechanism is not understood. In cases in which aggregation is induced by a Gq-coupled receptor, e.g. thromboxane receptor, the Gi component is supplied through transmitter release, which can be blocked by a PKC inhibitor. In the present study we investigate whether activation of PLC$\gamma$2 by GPVI requires co-activation of a Gi-regulated pathway to cause platelet aggregation. The PKC inhibitor, Ro 31-8220 was used to investigate the involvement of transmitter release dependent on PKC in aggregation by convulxin. We have also investigated whether there is synergy between convulxin and, $P2T_{AC}$ and/or $P2Y_1$ ADP receptors. Ro 31-8220 causes a 95.6±2.1% (n=4) decrease in the aggregation response to convulxin measured in a Born-aggre-

gometer, whereas it only causes a 15.1±3.6% (n=3) decrease in response to ADP. We found that the ability of Ro 31-8220 treated platelets to respond to convulxin was restored by the addition of adrenaline (a Gi-coupled receptor agonist). We also found that the addition of ADP just prior to addition of convulxin causes a 3-fold (n=4) leftward shift in the dose response curve to convulxin. The largest effect was seen at low concentrations of convulxin where the platelet response can be shifted from minimal aggregation up to 50% aggregation in the presence of ADP. We can conclude that aggregation to convulxin is largely dependent on the activity of PKC and this may be an indirect effect due to release of a Gi-coupled receptor agonist. From the observed shift in the dose response curve we can conclude that ADP does potentiate platelet aggregation to convulxin. Since activation of platelets by convulxin/collagen causes ADP release, this synergy may be an important physiologic mechanism for the amplification of the aggregation response in hemostasis.

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