Historical perspective on ADP-induced platelet activation

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Introduction

We have come a long way since 1960 when Hellem observed that a small molecule lost from red cells caused platelets to adhere to glass [1]. During the next year, Øllgaard [2] showed that this small molecule caused platelet aggregation, and it was identified as adenosine diphosphate (ADP) in Owren's laboratory [3]. At that time, Marjory Zucker was grading ADP-induced aggregation visually as 1–4 after shaking citrated platelet-rich plasma by hand for 1 min [4]. She had been carrying out research on platelets since 1947 and was already a major figure in the field.

The key development of the aggregometer in Gustav Born's laboratory in 1962 provided a light transmission technique for assessing and recording the rate and extent of aggregation that is still widely used today (light transmission aggregometry [LTA]) [5]. In the next few years, Born

and his colleagues used the aggregometer in detailed investigations of the changes in platelets during ADP-induced aggregation, and inhibitors of this process [6]. Much of the early work was well reviewed in the classic book *The Physiology of Blood Platelets*, subtitled *Recent Biochemical, Morphologic and Clinical Research*, that was written by Aaron Marcus and Marjorie Zucker in 1965 [7]. In 1970, another major review summarized the developments in the 1960s [6]. It had become obvious that ADP plays an important role in hemostasis and thrombosis.

The much more recent findings that platelets possess two P2Y receptors (P2Y₁ and P2Y₁₂) for ADP, and a P2X₁ receptor for ATP have made it possible to understand the reactions responsible for many of the early observations [8]. Our present knowledge about ADP-induced platelet activation is attributable to the work of thousands of investigators and this historical review can mention only some of them.

In the late 1950s and early 1960s, several groups of investigators carried out in vitro experiments showing that thrombin or collagen caused platelet aggregation and that ADP was released during this process [9–13]. In vivo, ADP and ATP do not normally circulate in the plasma, but they are stored in the dense granules of the platelets. During the formation of hemostatic plugs or arterial thrombi, platelets are stimulated by collagen and thrombin to release the contents of these platelet storage granules. In vitro, at a normal platelet count of 250,000/μl, the concentrations of ATP and ADP in plasma immediately after release of granule contents induced by collagen or thrombin have been reported in the ranges of 4–7 μM for ATP and 3–4 μM for ADP [14, 15]. The released ADP adds to the response of platelets to the other aggregating agents.

In addition to causing aggregation, the effects of ADP on platelets include shape change, refractoriness, potentiation

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of the effects of other aggregating agents, inhibition of platelet adenylyl cyclase, increase in cytosolic free calcium, and activation of specific receptors that stimulate intracellular signaling pathways that converge on the cytoplasmic domain of the integrin $\alpha\text{IIb}\beta 3$ (glycoprotein (GP) IIb–IIIa), leading to its becoming able to bind extracellular fibrinogen and von Willebrand factor [16, 17].

Platelet shape change

When ADP is added to isolated platelets in plasma or an artificial medium, a rapid change in platelet shape from discs to a rounded form with pseudopods takes place and an enormous increase in the surface area of the platelet occurs [18–20]. In an aggregometer, light transmission is seen to decrease. This alteration in morphology does not require calcium in the medium and can occur when the concentration of calcium is too low to support aggregation [18, 21]. Shape change without aggregation also occurs if ADP is added without rapid stirring [6, 22] or if the pH of the suspending medium is below 6.5 [23]. Internal changes include centralization of the granules with constriction of the marginal bundle of microtubules [24].

Later investigators have focused on the signaling pathways involved in ADP-induced shape change [25, 26]. It is now established that shape change in response to ADP involves activation of the P2Y_1 receptor which mediates a transient rise in cytoplasmic Ca^{2+} , mainly mobilized from internal stores, but partially from the external medium [8].

Refractoriness (desensitization)

Aggregation by ADP is induced by concentrations as low as 0.5 μM and can be visualized as an increase in light transmission in an aggregometer. The primary phase of ADP-induced aggregation is reversible in a medium that contains an approximately physiological concentration of calcium ions (1–2 mM) and the platelets deaggregate within a short time, becoming refractory to a further stimulation with ADP [5, 27]. Exposure to ADP without stirring for several minutes also causes this desensitization [6, 28]. The addition of apyrase to an artificial medium in which platelets have been resuspended maintains the responsiveness of platelets to ADP by degrading any of the nucleotide that may be lost from the platelets during handling or storage [29, 30].

The explanation for these early observations involves the recognition that exposure of platelets to ADP induces the internalization of both the P2Y_1 and P2Y_{12} receptors [31–34]. However, Baurand's group [32] found that “although a substantial fraction of P2Y_{12} was rapidly and reversibly

internalized”, the “ P2Y_{12} receptor-mediated inhibition of cAMP formation was not affected”. It appears that the pathways of internalization of the two receptors differ. Hardy et al. [33] reported that P2Y_{12} desensitization was mediated by G-protein-coupled receptor kinase, whereas desensitization of P2Y_1 was largely dependent on protein kinase C activity. Mundell and co-workers [34] also have shown that desensitization is rapidly reversible and they investigated the mechanism of resensitization.

Primary and secondary ADP-induced aggregation

In most of the early experiments, ADP-induced aggregation was studied in citrated platelet-rich plasma in which the concentration of ionized calcium is low enough to prevent clotting (40 μM with 10.9 mM citrate and <5 μM with 12.9 mM citrate) [35, 36]. In such a medium, ADP at concentrations above about 0.5 μM induces two phases of aggregation of human platelets, the second phase being irreversible [6, 37, 38]. Originally, this “secondary clumping” induced by ADP was attributed to the release of more ADP from the dense granules of the platelets, but it is now apparent that this is only part of the explanation and that the second phase depends mainly on the formation of thromboxane A_2 [39]. This second phase occurs with human platelets when they are brought into close contact in any way at 37°C in a medium without added calcium in which the concentration of ionized calcium is approximately 20 μM [40–42]. In platelet-rich plasma anticoagulated with heparin or hirudin, only the primary phase of ADP-induced aggregation occurs with platelets from most humans [36, 43].

In addition to human platelets, only platelets from cats, one-third of mongrel dogs, and piebald and rosette guinea pigs show this secondary response to ADP in a low calcium medium. Most other species, including rabbits, rats, mice, pigs, cows, goats, sheep, horses, mink, white guinea pigs and most dogs have platelets that undergo only a primary, reversible phase of aggregation in response to ADP in such a medium [44–46].

Aspirin irreversibly acetylates the cyclooxygenase responsible for the formation of the aggregating agent thromboxane A_2 [39, 47], so the lack of the second phase of ADP-induced aggregation of human platelets in citrated platelet-rich plasma is usually attributable to the ingestion of a drug that contains aspirin. Indeed, in 1968, the recognition by several investigators [48, 49] that only the primary phase of ADP-induced aggregation occurred when their patients had been taking aspirin added to the concurrent finding of inhibition of several other platelet functions by aspirin [50].

Inhibition of the second phase of ADP-induced aggregation by aspirin lasts for the lifetime of the affected

platelets and is observable for 5–8 days after ingestion. Some other non-steroidal anti-inflammatory drugs also inhibit the second phase, but their effect does not persist after the drug is cleared from the circulation. These drugs include piroxicam, naproxen, indomethacin, diclofenac, ibuprofen and diflunisal. Salicylic acid has a weak effect [51].

ADP and adenosine

Experiments by Born in 1965 with ADP labelled with ^{14}C in the adenine group seemed to indicate that ADP was taken up by platelets [52], but later experiments by Salzman et al. [53] with ^{32}P as a label of the β phosphate group showed no uptake of the label by platelets. The reason for Born's observation became evident with the demonstrations that, in plasma, ADP is hydrolysed to adenosine monophosphate (AMP) that, in turn, is dephosphorylated to adenosine [54], and that adenosine is taken up by platelets [55].

Very early, Born and Cross [37] had suggested that inhibition of ADP-induced aggregation by adenosine was attributable to its structural similarity to ADP and hence it would compete with ADP for receptor sites on the platelet membrane. Born and his co-workers [56] also found that adenosine is a strong inhibitor of ADP-induced aggregation in vivo as well as in vitro. Although this idea of adenosine as a competitive inhibitor of ADP was supported by other investigators [57], it was questionable because preincubation of platelets with adenosine increased its inhibitory effect [37]. Adenosine as a competitive inhibitor of ADP was also doubtful because it inhibited aggregation by other agonists [6, 21, 58], although inhibition of released ADP remained a possibility. Finally, in Born's laboratory in 1971, Mills and Smith [59] showed that adenosine stimulates adenylyl cyclase so that the concentration of cyclic AMP in platelets is increased, and they demonstrated that adenosine is a noncompetitive and nonselective inhibitor of ADP. In agreement with these findings, in 1973 Haslam [60] showed that "adenosine acts through an extracellular membrane receptor, which is independent of both the ADP receptor and the adenosine transport mechanism".

Role of ADP in aggregation by other agonists

The suggestion by Haslam [58] that ADP was the "final common pathway" through which other agonists induced aggregation was based on his use of enzyme systems such as phosphoenolpyruvate plus pyruvate kinase to convert extracellular ADP to ATP as ADP was released. He observed that aggregation induced by thrombin or collagen

was inhibited by this enzyme system. Studies by Macfarlane and Mills [61], however, negated the idea that ADP was the common pathway through which other agonists induced aggregation. They used freshly purified ATP and showed that (although it was a competitive inhibitor of ADP) ATP did not inhibit primary aggregation induced by thrombin and other agonists.

ADP potentiates the effects of other agonists, including weak ones such as serotonin and adrenaline [6, 62–64]. This synergistic effect of ADP can also be demonstrated with low concentrations of stronger agonists, such as collagen, thrombin, arachidonic acid and thromboxane A_2 [63, 64]. Experiments with artificially degranulated platelets, however, have shown that higher concentrations of the strong agonists can cause aggregation in the absence of releasable ADP [65].

Stabilization of platelet aggregates by released ADP [66, 67], as well as the recognition of storage pool disease (lack of dense granule contents) as a cause of some bleeding abnormalities [68], emphasize the importance of ADP in hemostasis and the contribution that ADP makes to arterial thrombosis.

Role of ADP in shear-induced aggregation

In vitro, platelets in anticoagulated plasma aggregate without the addition of a chemical agonist when they are subjected to high shear stress in devices such as a cone-and-plate viscometer [69, 70]. This aggregation was found to require von Willebrand factor multimers, extracellular calcium (Ca^{2+}), ADP released from the platelets, fibrinogen, and platelet membrane glycoproteins GPIb α and $\alpha\text{IIb}\beta 3$ [71, 72]. More recently, the P2X_1 receptor, stimulated by ATP released from platelets, has been shown to be required for shear-induced aggregation [73–75]. It has also now been found that blocking the P2Y_1 receptor for ADP prevents stabilization of aggregation [76, 77].

In vivo, shear-induced platelet aggregation is thought to occur at sites where vessels have been narrowed by atherosclerotic plaques, and during bleeding from severed arterioles [78–80].

Effects of synthetic ADP analogues

Many compounds were synthesized with structural modifications of ADP in attempts to identify the part of the molecule responsible for its ability to cause platelet aggregation. Most of these compounds had little or no biological activity. Only substitution in the 2-position of adenine produces compounds with aggregating activity similar to or greater than that of ADP [81–84].

Large lists summarizing structure–activity relationships for the inhibition of ADP-induced aggregation by putative ADP receptor antagonists have been published [81–84].

Adenylyl cyclase and cyclic AMP

An increase in the concentration of cyclic AMP in platelets inhibits aggregation and the release of granule contents induced by ADP and by all other aggregating agents. Adenosine and the prostaglandins (PG) E_1 , D_2 , and I_2 act through adenylyl cyclase to raise the concentration of cyclic AMP. Inhibitors of phosphodiesterase (methylxanthines, papaverine, pyrimidopyrimidines, dipyridamole) augment the inhibitory effect of prostaglandins by preventing the breakdown of cyclic AMP to AMP [6, 39, 59, 81].

In the early 1970s, several groups of investigators made the key observation that ADP inhibits the increase in cyclic AMP caused by PGE_1 [85–87]. Although the suggestion was made that ADP might act by decreasing the basal level of cyclic AMP in platelets [88], this idea became questionable when Haslam [60] showed that considerable ADP-induced aggregation could occur when cyclic AMP levels were well above the basal concentration. In 2002, Yang et al. [89] showed that “a decrease or increase in the basal cAMP concentration does not by itself make platelets more or less sensitive to agonists (such as ADP), and that unless PGI_2 is present, platelet agonists have little or no effect on platelet cAMP levels”.

By 1981 it was evident that inhibition of cAMP formation in platelets by ADP is a direct receptor-mediated effect of ADP on adenylyl cyclase [90]. This ADP receptor has been identified as the $P2Y_{12}$ receptor and is the target for the thienopyridine compounds ticlopidine, clopidogrel and prasugrel, and for the more recently developed inhibitors cangrelor and ticagrelor [91].

Binding sites for ADP on platelets

In the early 1990s, the search for the platelet receptor for ADP led Colman to propose that it was a 100-kDa protein (named aggregrin) that has ADP binding sites [92]. Meanwhile, Greco and his colleagues [93] suggested that the ADP receptor was αIIb (GP IIb). After a detailed review of the evidence in 1996, Mills [83] concluded that neither of these proteins had the properties of an ADP receptor. By this time, Mills and his colleagues had been investigating various aspects of the effects of ADP on platelets for 30 years and were acknowledged experts in the field.

Beginning in the 1960s, several groups of investigators had tried to determine the number of binding sites for ADP on platelets, with estimates ranging from 2,500 to 200,000

[16, 52]. In the 1980s, by the use of 2-azido-ADP that could be photoactivated [94] and also a strong aggregating ADP analogue, 2-methylthioadenosine labelled with ^{32}P (2-MeS-ADP), Macfarlane and colleagues [95] obtained values ranging from 400 to 1,200 sites per platelet for the receptor that inhibits adenylyl cyclase ($P2Y_{12}$). More recently, using the latter analogue, Gachet et al. [96] reported 600 ± 125 specific binding sites per platelet, and Nurden and co-workers [97] found 826 ± 126 per platelet. Both these latter two groups described patients with low numbers of these binding sites who exhibited a congenital impairment of ADP-induced platelet aggregation, but normal shape change. The number of binding sites was reduced by 70% on platelets from rats treated with clopidogrel [96].

In 1983, Macfarlane et al. [95] had demonstrated that the thiol complexing agent *p*-mercuribenzenesulfonate blocks inhibition by ADP of adenylyl cyclase and blocks the binding of 2-azido-ADP and 2-MeS-ADP, but does not affect the ability of ADP to induce aggregation and the shape change. In 1993, Cristalli and Mills [98] showed that a 43-kDa protein on platelets incorporated 2-(*p*-azidophenyl)-ethylthio-ADP (AzPET-ADP), a photoaffinity analogue of ADP that competitively inhibits the binding of 2-MeS-ADP. This 43-kDa protein appears to be the receptor that mediates the ADP-induced inhibition of adenylyl cyclase and is now designated as $P2Y_{12}$.

In 2001, Baurand and colleagues [99] studied the $P2Y_1$ receptor and showed 134 ± 8 binding sites per platelet. They used a potent $P2Y_1$ receptor antagonist, N^6 -methyl-2'-deoxyadenosine-3',5'-bisphosphate (MRS2179) that inhibited ADP-induced platelet shape change, aggregation and rise in Ca^{2+} , but had no effect on ADP-induced inhibition of adenylyl cyclase. MRS2179 labelled with ^{33}P was used in the binding determinations.

Cattaneo [8] has pointed out that $P2Y_1$ accounts for 20–30% of the total binding sites for ADP on the surface of human platelets.

These observations provided evidence that two different types of ADP receptors exist on platelets, one that acts on adenylyl cyclase and one that is the cause of the initial change in platelet shape. The existence of two receptors for ADP on platelets has now been confirmed by many investigators [8].

ADP-induced increases in cytosolic calcium

In the 1980s, Tsien and co-workers [100] and Rink and Sage [101] began experiments with the fluorescent calcium indicators (quin 2 and fura-2) that had been invented by Tsien et al. and could be entrapped within intact cells. They showed that ADP caused an increase in cytosolic free calcium within one second that peaked in 10 s. This ADP-induced rise

in platelets suspended in a medium with extracellular calcium was six-fold greater than that in the absence of extracellular calcium, leading the investigators to conclude that the major component of the ADP-stimulated rise was the result of an influx of calcium ions [101]. Release from internal calcium stores accounts for the elevation of internal calcium in the absence of external calcium.

It is now established that activation of the P2Y₁ receptor causes a transient increase in the concentration of intracellular ionized calcium, partially by influx of Ca²⁺ from the medium, but mainly by release from intracellular stores [102]. P2Y₁₂ amplifies the mobilization of cytoplasmic Ca²⁺ mediated by P2Y₁ and other receptors [8].

A detailed review of calcium signaling in platelets has been published recently by Varga-Szabo et al. [103].

The P2X₁ receptor

The suggestion of a P2X₁ ATP-gated, ion channel receptor on platelets was not made until 1996 when Mackenzie and his co-workers [104] showed that the nonhydrolyzable ATP analogue α,β -methylene adenosine-5-triphosphate (α,β -meATP) could induce rapid entry of calcium into platelets, but that only ADP mobilized calcium from internal stores. In the next year, Vial et al. [105] confirmed these observations and reported the finding of P2X₁ messenger RNA in platelets by the polymerase chain reaction. The suggestion that ADP also acted at the P2X₁ receptor [106] was shown to be erroneous when it was found to be based on experiments in which the commercially available ATP used was contaminated by ADP [107].

The P2X₁ receptor is desensitized by ADP [74], but the inclusion of apyrase to degrade ADP in the suspending medium of the platelets prevents this desensitization and α,β -meATP can be shown to cause a rapid, transient increase in intracellular calcium, a reversible change in platelet shape, but no aggregation [108]. Vial et al. [109] have suggested that in vivo, these ATP-induced reactions could synergize with the platelet response to ADP through the P2Y₁ receptor and amplify the increase in intracellular calcium. Oury et al. [110, 111] obtained evidence for the importance in hemostasis of a synergy between ADP and ATP released from platelets stimulated by adherence to collagen. They used transgenic mice overexpressing the P2X₁ receptor in the megakaryocyte cell lineage and found that their platelets showed enhanced aggregation and secretion in response to collagen, compared with platelets from wild-type mice. Hechler and colleagues [75] showed that platelets from mice deficient in the P2X₁ receptor had a decreased aggregation response to collagen and were protected from arterial thrombosis.

The P2Y₁ receptor

In 2000, Cusack and Hourani [84] reviewed the early studies of the P2 receptors on platelets. Studies with synthetic ADP analogues indicated that the ADP receptor had the characteristics of a P2Y receptor, and with the assumption that there was only a single such receptor, it was designated as P2_T (T for thrombocyte) [112]. A starting point for the identification of the receptors was the 1997 report of Léon and co-workers [113]. They cloned and stably expressed the P2Y₁ receptor, showed that it exhibited the pharmacological profile of the human platelet P2_T receptor, and that it was expressed in both megakaryoblastic cell lines and human platelets. They suggested that P2Y₁ was the P2_T receptor.

Although Macfarlane and his colleagues [95] had provided evidence in 1983 for two ADP receptors on platelets, one responsible for shape change and aggregation and the other for inhibition of adenylyl cyclase, it was not until 1996 that others began investigations that supported their findings [84]. Several groups used selective P2Y₁ antagonists and showed that they inhibited ADP-induced shape change, aggregation, and increases in calcium, but not the effect of ADP on adenylyl cyclase [84, 114–117]. The P2Y₁ receptor is coupled to G_q and activates phospholipase C β (PLC β) [118] that is responsible for the formation of inositol (1,4,5)-trisphosphate (IP₃) and diacylglycerol, an activator of protein kinase C. IP₃ causes calcium mobilization from internal stores [118].

Platelets from mice deficient in the P2Y₁ receptor did not aggregate in response to concentrations of ADP up to 10 μ M, whereas 100 μ M caused only a slow formation of small aggregates with an atypical shape change and no calcium movement [119, 120]. The observation that platelets from these mice still showed ADP-induced inhibition of adenylyl cyclase, provided more evidence for two ADP receptors [119]. Injection of ADP into these mice did not cause thromboembolism, in contrast to its effect in wild type mice. This finding raised the possibility that the P2Y₁ receptor might be a target for antithrombotic drugs [121, 122].

The P2Y₁₂ receptor

The ADP receptor that is negatively coupled to adenylyl cyclase has had several names. First P2_T [112], followed by P2Y_{cyc} [123], P2Y_{AC} [106], P2Y_{ADP} [124], and finally, in 2001, P2Y₁₂ after it was cloned and analyzed by Hollopeter et al. [125]. The latter investigators showed that the receptor has four extracellular cysteines and that the thiol reagent *p*-chloromercuriphenylsulfonic acid (pCMBS) inhibits ADP-induced P2Y₁₂ activation [125]. Twenty years earlier, Macfarlane and Mills [90] had already used a

similar reagent to prevent ADP-induced inhibition of cyclic AMP formation in platelets.

In the early 1990s, the first patient with a mutation of the P2Y₁₂ receptor was discovered on the basis of a lifelong history of excessive bleeding and defective ADP-induced aggregation [126]. A few other patients of this sort have been identified and Cattaneo [8] has described the molecular defects in the P2Y₁₂ receptor in these patients. In 2001, mice that are deficient in the P2Y₁₂ receptor were produced and found to have a prolonged bleeding time [127]. Their platelets aggregate weakly in response to ADP, but shape change and calcium mobilization are normal. In the presence of PGE₁, adenylyl cyclase is not inhibited by ADP. It is evident that for full platelet activation by ADP, both P2Y receptors are necessary.

The P2Y₁₂ receptor is negatively coupled to adenylyl cyclase activity through activation of a G protein, as shown by experiments with a radiolabelled GTP photoaffinity ligand [128], receptor antagonists [129], and genetically engineered mice [89, 130]. This G protein was identified as G_{i2} by Ohlmann et al. in 1995 [128].

Although ADP, acting through its P2Y₁₂ receptor, inhibits cyclic AMP formation when adenylyl cyclase has been stimulated, when it has not been stimulated the need for this G protein-dependent signaling during platelet activation by ADP points to an essential role for other effectors [8, 89]. Several groups of investigators have been exploring G_i signaling pathways that involve other such effectors. G_{i2} couples P2Y₁₂ to pathways that include phosphoinositol 3-kinase (PI3K) and Rap1B [131]. G_i-dependent activation of PI3K also promotes activation of Akt, a serine–threonine kinase [132]. Schoenwaelder and coworkers have identified a co-operative PI3K signaling mechanism that regulates the adhesive function of αIIbβ3 in platelets [133].

Drugs that act on the P2Y₁₂ receptor

In 1974, Maffrand and Eloy [134] synthesized the thienopyridine, ticlopidine, the first drug specifically developed to inhibit platelet reactions that was not directed at cyclooxygenase products. Ticlopidine's inhibitory effect was only demonstrable after oral administration, pointing to the production of an active metabolite *in vivo*. There was extensive research on ticlopidine in experimental models of thrombosis and in clinical trials in humans [135] before it was recognized in the early 1990s as a specific inhibitor of ADP-induced aggregation [136, 137]. Concern about the toxic effects of ticlopidine (neutropenia, thrombotic thrombocytopenic purpura) led to the development of a similar prodrug, clopidogrel, that did not have these adverse effects and has become widely used. Both ticlopidine and

clopidogrel exert their inhibitory effect through an active metabolite that has a thiol group that reacts irreversibly with a cysteine residue of P2Y₁₂ [138].

Cattaneo [91] has discussed the problem of the high inter-individual variability in platelet inhibition by clopidogrel, that is attributed to differences in the extent of metabolism of the prodrug. A newer prodrug of this class, prasugrel, is more efficiently converted to its active metabolite, displays much faster and uniform inhibition of platelet functions, and lower inter-individual variability [91]. All three of these thienopyridine drugs, however, share the problem of a slow offset of action because they irreversibly inhibit P2Y₁₂. Consequently, new, short-acting direct P2Y₁₂ antagonists, cangrelor, ticagrelor and elinogrel, have been developed and are being tested in clinical trials with patients at risk of major cardiac events [91, 139].

The effects of drugs that act on the P2Y₁₂ receptor illustrate the key role that ADP, a so-called “weak” agonist, plays in hemostasis and thrombosis.

Conclusion

The 50 years of investigation of the role of ADP in platelet aggregation have contributed to major increases in our understanding of many of the processes involved in hemostasis and thrombosis. Early years saw the recognition of the importance of the release of the contents of the platelet storage granules by collagen and thrombin, and the recognition that the lack of their contents, particularly ADP in the dense granules, led to bleeding. Later findings included the role of thromboxane A₂ and inhibition by aspirin of its formation, the stimulation of integrin αIIbβ3 to become able to bind fibrinogen, inhibition of aggregation by prostaglandins, particularly PGI₂, the finding that ADP inhibits the increase in cyclic AMP caused by these prostaglandins, the increases in cytosolic calcium that occur in response to aggregating agents, and the roles played by ADP and von Willebrand factor in shear-induced aggregation. More recent investigations have focused on the receptors for ADP and ATP on platelets, the discovery of patients lacking the P2Y₁₂ receptor, and the development of drugs that inhibit this receptor. In the future, the P2Y₁ and the P2X₁ receptors may become targets for antithrombotic drugs.

References

1. Hellem AJ (1960) The adhesiveness of human blood platelets *in vitro*. *Scand J Clin Lab Invest* 12(Suppl):1–117
2. Øllgaard E (1961) Macroscopic studies of platelet aggregation. Nature of an aggregating factor in red blood cells and platelets. *Thromb Diath Haemorrh* 6:86–97

3. Gaarder A, Jonsen J, Laland S, Hellem A, Owren PA (1961) Adenosine diphosphate in red cells as a factor in the adhesiveness of human blood platelets. *Nature* 192:531–532
4. Jerushalmy Z, Skoza L, Zucker MB, Grant R (1966) Inhibition by guanidine compounds of platelet aggregation induced by adenosine diphosphate. *Biochem Pharmacol* 15:1791–1803
5. Born GVR (1962) Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature* 194:927–929
6. Mustard JF, Packham MA (1970) Factors influencing platelet function: adhesion, release, and aggregation. *Pharmacol Rev* 22:97–187
7. Marcus AJ, Zucker MB (1965) The physiology of blood platelets: recent biochemical, morphologic and clinical research. Grune and Stratton, New York, pp 1–162
8. Cattaneo M (2007) The platelet P2 receptors. In: Michelson AD (ed) *Platelets*, 2nd edn. Elsevier, New York, pp 201–210
9. Grette K (1962) Studies on the mechanism of thrombin-catalyzed hemostatic reactions in blood platelets. *Acta Physiol Scand Suppl* 195:1–93
10. Zucker MB, Borrelli J (1962) Platelet clumping produced by connective tissue suspensions and by collagen. *Proc Soc Exp Biol Med* 109:779–787
11. Hovig T (1963) Release of a platelet-aggregating substance (adenosine diphosphate) from rabbit blood platelets induced by saline “extract” of tendons. *Thromb Diath Haemorrh* 9:264–278
12. Hugues J, Lapière CM (1964) Nouvelles recherches sur l’accolement des plaquettes aux fibres de collagène. *Thromb Diath Haemorrh* 11:327–354
13. Mills DCB, Robb IA, Roberts GCK (1968) The release of nucleotides, 5-hydroxytryptamine and enzymes from human blood platelets during aggregation. *J Physiol* 195:715–729
14. Holmsen H, Weiss HJ (1970) Hereditary defect in the platelet release reaction caused by a deficiency in the storage pool of platelet adenine nucleotides. *Br J Haematol* 19:643–649
15. D’Souza L, Glueck HI (1977) Measurement of nucleotide pools in platelets using high pressure liquid chromatography. *Thromb Haemost* 38:990–1001
16. Gachet C, Cazenave JP (1991) ADP induced blood platelet activation: a review. *Nouv Rev Fr Hematol* 33:347–358
17. Plow EF, Ginsberg MH, Marguerie GA (1986) Expression and function of adhesive proteins on the platelet surface. In: Phillips DR, Shuman MA (eds) *Biochemistry of platelets*. Academic Press, Orlando, pp 225–256
18. Born GVR (1970) Observations on the change in shape of blood platelets brought about by adenosine diphosphate. *J Physiol* 209:487–511
19. Mills DCB (1973) Changes in adenylate energy charge in human blood platelets induced by adenosine diphosphate. *Nature, New Biol* 243:220–222
20. Deranleau DA, Dubler D, Rothen C, Lüscher EF (1982) Transient kinetics of the rapid shape change of unstirred human blood platelets stimulated with ADP. *Proc Natl Acad Sci USA* 79:7297–7301
21. Zucker MB, Jerushalmy Z (1967) Studies on platelet shape and aggregation: effect of inhibitors on these and other platelet characteristics. In: Johnson SH, Seegers WH (eds) *Physiology of hemostasis and thrombosis*. Charles C. Thomas, Springfield, pp 249–265
22. Skjorten F (1968) Studies on the ultrastructure of pseudopod formation in human blood platelets: 1. Effect of temperature, period of incubation, anticoagulants and mechanical forces. *Scand J Haematol* 5:401–414
23. McLean JR, Veloso H (1967) Change of shape without aggregation caused by ADP in rabbit platelets at low pH. *Life Sci* 6:1983–1986
24. White JG (1968) Fine structural alterations induced in platelets by adenosine diphosphate. *Blood* 31:604–622
25. Paul BZS, Daniel JL, Kunapuli SP (1999) Platelet shape change is mediated by both calcium-dependent and -independent signalling pathways. *J Biol Chem* 274:28293–28300
26. Wilde JJ, Retzer M, Seiss W, Watson SP (2000) ADP-induced platelet shape change: an investigation of the signalling pathways involved and their dependence on the method of platelet preparation. *Platelets* 11:286–295
27. Gachet C (2006) Regulation of platelet functions by P2 receptors. *Annu Rev Pharmacol Toxicol* 46:277–300
28. O’Brien JR (1962) Platelet aggregation: Part II. Some results from a new method of study. *J Clin Pathol* 15:452–455
29. Ardlie NG, Perry DW, Packham MA, Mustard JF (1971) Influence of apyrase on stability of suspensions of washed rabbit platelets. *Proc Soc Exp Biol Med* 136:1021–1023
30. Kinlough-Rathbone RL, Perry DW, Rand ML, Packham MA (1999) Most apyrase preparations are impure and contain inhibitors of cathepsin G: suggestions for use of apyrase in preparation and stabilization of platelet suspensions. *Thromb Haemost* 81:849–850
31. Baurand A, Eckly A, Bari N, Léon C, Hechler B, Cazenave J-P, Gachet C (2000) Desensitization of the platelet aggregation response to ADP: differential down-regulation of the P2Y₁ and P2_{cyt} receptors. *Thromb Haemost* 84:484–491
32. Baurand A, Eckly A, Hechler B, Kauffenstein G, Galzi J-L, Cazenave J-P, Léon C, Gachet C (2010) Differential regulation and relocalization of the platelet P2Y receptors after activation: a way to avoid loss of hemostatic properties? *Mol Pharmacol* 67:721–733
33. Hardy AR, Conley PB, Luo J, Benovic JL, Poole AW, Mundell SJ (2005) P2Y₁ and P2Y₁₂ receptors for ADP desensitize by distinct kinase-dependent mechanisms. *Blood* 105:3552–3560
34. Mundell SJ, Barton JF, Mayo-Martin MB, Hardy AR, Poole AW (2008) Rapid resensitization of purinergic receptor function in human platelets. *J Thromb Haemost* 6:1393–1404
35. Herrmann RG, Laceyfield WB, Crowe VG (1970) Effect of ionic calcium and magnesium on human platelet aggregation. *Proc Soc Exp Biol Med* 135:100–103
36. Lages B, Weiss HJ (1981) Dependence of human platelet functional responses on divalent cations: aggregation and secretion in heparin- and hirudin-anticoagulated platelet-rich plasma and the effects of chelating agents. *Thromb Haemostas* 45:173–179
37. Born GVR, Cross MJ (1963) The aggregation of blood platelets. *J Physiol* 168:178–195
38. Packham MA, Rand ML, Kinlough-Rathbone RL (2002) Aggregation. In: Gesele P, Page CP, Fuster V, Vermylen J (eds) *Platelets in thrombotic and non-thrombotic disorders*. Cambridge University Press, Cambridge, pp 338–356
39. Smith JB (1987) Prostaglandins in platelet aggregation and haemostasis. In: Bloom AL, Thomas DP (eds) *Haemostasis and thrombosis*, 2nd edn. Churchill Livingstone, Edinburgh, pp 78–89
40. Mustard JF, Perry DW, Kinlough-Rathbone RL, Packham MA (1975) Factors responsible for ADP-induced release reaction of human platelets. *Am J Physiol* 228:1757–1765
41. Packham MA, Bryant NL, Guccione MA, Kinlough-Rathbone RL, Mustard JF (1989) Effect of the concentration of Ca²⁺ in the suspending medium on the responses of human and rabbit platelets to aggregating agents. *Thromb Haemost* 62:968–976
42. Cattaneo M, Gachet C, Cazenave J-P, Packham MA (2002) Adenosine diphosphate (ADP) does not induce thromboxane A₂ generation in human platelets. *Blood* 99:3868–3869
43. Kinlough-Rathbone RL, Packham MA, Mustard JF (1983) Platelet aggregation. In: Harker LA, Zimmerman TS (eds)

- Methods in hematology. Measurements of platelet function. Churchill Livingstone, New York, pp 64–91
44. Macmillan DC, Sim AK (1970) A comparative study of platelet aggregation in man and laboratory animals. *Thromb Diath Haemorrh* 24:385–389
 45. Meyers KM, Katz JB, Clemmons RM, Smith JB, Holmsen H (1980) An evaluation of the arachidonate pathway of platelets from companion and food-producing animals, mink, and man. *Thromb Res* 20:13–24
 46. Nunn B (1981) Some characteristics of mouse platelet aggregation and a comparison of the activity of a range of compounds in mouse and human platelet-rich plasma in vitro. *Thromb Haemost* 45:1–5
 47. Roth GJ, Majerus PW (1975) The mechanism of the effect of aspirin on human platelets: I. Acetylation of a particulate fraction protein. *J Clin Invest* 56:624–632
 48. Zucker MB, Peterson J (1968) Inhibition of adenosine diphosphate-induced secondary aggregation and other platelet functions by acetylsalicylic acid ingestion. *Proc Soc Exp Biol Med* 127:148–158
 49. Weiss HJ, Aledort LM, Kochwa S (1968) The effect of salicylates on the hemostatic properties of platelets in man. *J Clin Invest* 47:2169–2180
 50. Evans G, Packham MA, Nishizawa EE, Mustard JF, Murphy EA (1968) The effect of acetylsalicylic acid on platelet function. *J Exp Med* 128:877–894
 51. Cronberg S, Wallmark E, Söderberg I (1984) Effect on platelet aggregation of oral administration of 10 non-steroidal analgesics to humans. *Scand J Haematol* 33:155–159
 52. Born GVR (1965) Uptake of adenosine and of adenosine diphosphate by human blood platelets. *Nature* 206:1121–1122
 53. Salzman EW, Ashford TP, Chambers DA, Neri LL (1969) Platelet incorporation of labelled adenosine and adenosine diphosphate. *Thromb Diath Haemorrh* 22:304–315
 54. Holmsen H, Day HJ (1971) Adenine nucleotides and platelet function. *Ser Haemat* 4:28–58
 55. Rozenberg MC, Holmsen H (1968) Adenine nucleotide metabolism of blood platelets: II. Uptake of adenosine and inhibition of ADP-induced platelet aggregation. *Biochim Biophys Acta* 155:342–352
 56. Born GVR, Honour AJ, Mitchell JR (1964) Inhibition by adenosine and by 2-chloroadenosine of the formation and embolization of platelet thrombi. *Nature* 202:761–765
 57. Skoza L, Zucker MB, Jerushalmy Z, Grant R (1967) Kinetic studies of platelet aggregation induced by adenosine diphosphate and its inhibition by chelating agents, guanidino compounds, and adenosine. *Thromb Diath Haemorrh* 18:713–725
 58. Haslam RJ (1967) Mechanisms of blood platelet aggregation. In: Johnson SH, Seegers WH (eds) *Physiology of hemostasis and thrombosis*. Charles C. Thomas, Springfield, pp 88–112
 59. Mills DCB, Smith JB (1971) The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3':5'-cyclic monophosphate in platelets. *Biochem J* 121:185–196
 60. Haslam RJ (1973) Interactions of the pharmacological receptors of blood platelets with adenylate cyclase. *Ser Haematol* 6:333–350
 61. Macfarlane DE, Mills DCB (1975) The effect of ATP on platelets: evidence against the central role of released ADP in primary aggregation. *Blood* 46:309–320
 62. Ardlie NG, Glew G, Schwartz CJ (1966) Influence of catecholamines on nucleotide-induced platelet aggregation. *Nature* 212:414–417
 63. Kinlough-Rathbone RL, Mustard JF (1986) Synergism of agonists. In: Holmsen H (ed) *Platelet responses and metabolism*, Vol 1. CRC Press, Boca Raton, pp 193–207
 64. Ware JA, Smith M, Salzman EW (1987) Synergism of platelet-aggregating agents. Role of elevation of cytoplasmic calcium. *J Clin Invest* 80:267–271
 65. Kinlough-Rathbone RL, Perry DW, Guccione MA, Rand ML, Packham MA (1993) Degranulation of human platelets by the thrombin receptor peptide SFLLRN: comparison with degranulation by thrombin. *Thromb Haemost* 70:1019–1023
 66. Cattaneo M, Canciani MT, Lecchi A, Kinlough-Rathbone RL, Packham MA, Mannucci PM, Mustard JF (1990) Released adenosine diphosphate stabilizes thrombin-induced human platelet aggregates. *Blood* 75:1081–1086
 67. Trumel C, Payrastre B, Plantavid M, Hechler B, Viala C, Presek P, Martinson EA, Cazenave J-P, Chap H, Gachet C (1999) A key role of adenosine diphosphate in the irreversible aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. *Blood* 94:4156–4165
 68. Holmsen H, Weiss HJ (1972) Further evidence for a deficient storage pool of adenine nucleotides in platelets from some patients with thrombocytopathia — “storage pool disease”. *Blood* 39:197–209
 69. Hardwick RA, Hellums JD, Peterson DM, Moake JL, Olson JD (1981) The effect of PGI₂ and theophylline on the response of platelets subjected to shear stress. *Blood* 56:678–681
 70. Moake JL, Turner NA, Stathopoulos NA, Nolasco L, Hellums JD (1988) Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. *Blood* 71:1366–1374
 71. Chow TW, Hellums JD, Moake JL, Kroll MH (1992) Shear stress-induced von Willebrand factor binding to platelet glycoprotein Ib initiates calcium influx associated with aggregation. *Blood* 80:113–120
 72. Ruggeri ZM, Dent JA, Saldívar E (1999) Contribution of distinct adhesive interactions to platelet aggregation in flowing blood. *Blood* 94:172–178
 73. Cattaneo M, Marchese P, Jacobson KA, Ruggeri Z (2002) New insights into the role of P2X₁ in platelet function. *Haematologica* 87:13–14
 74. Oury C, Toth-Zsomboki E, Thys C, Tytgat J, Vermylen J, Hoylaerts MF (2001) The ATP-gated P2X₁ ion channel acts as a positive regulator of platelet responses to collagen. *Thromb Haemost* 86:1264–1271
 75. Hechler B, Lenain N, Marchese P, Vial C, Heim V, Freund M, Cazenave J-P, Cattaneo M, Ruggeri ZM, Evans R, Gachet C (2003) A role of the fast ATP-gated P2X₁ cation channel in thrombosis of small arteries in vivo. *J Exp Med* 198:661–667
 76. Mazzucato M, Cozzi MR, Pradella P, Ruggeri ZM, De Marco L (2004) Distinct roles of ADP receptors in von Willebrand factor-mediated platelet signaling and activation under high flow. *Blood* 104:3221–3227
 77. Maxwell MJ, Westein E, Nesbitt WS, Giuliano S, Doppeide SM, Jackson SP (2007) Identification of a 2-stage platelet aggregation process mediating shear-dependent thrombus formation. *Blood* 109:566–576
 78. Fuster V, Badimon L, Badimon JJ, Chesebro JH (1992) The pathogenesis of coronary artery disease and the acute coronary syndromes (1). *New Engl J Med* 326:242–250
 79. Fuster V, Badimon L, Badimon JJ, Chesebro JH (1992) The pathogenesis of coronary artery disease and the acute coronary syndromes (2). *N Engl J Med* 326:310–318
 80. Tangelder GJ, Slaaf DW, Arts T, Reneman RS (1988) Wall shear rate in arterioles in vivo: least estimates from platelet velocity profiles. *Am J Physiol* 254:H1059–H1064
 81. Haslam RJ, Cusack NJ (1981) Blood platelet receptors for ADP and for adenosine. In: Burnstock G (ed) *Purinergic receptors*.

- Receptors and recognition. Series B, Vol. 12. Chapman & Hall, London, pp 221–285
82. Hourani SMO, Cusack NJ (1991) Pharmacological receptors on blood platelets. *Pharmacol Rev* 43:243–298
 83. Mills DCB (1996) ADP receptors on platelets. *Thromb Haemost* 76:835–856
 84. Cusack NJ, Hourani SMO (2000) Platelet P2 receptors: from curiosity to clinical targets. *J Auton Nerv Syst* 81:37–43
 85. Cole B, Robison GA, Hartmann RC (1971) Studies on the role of cyclic AMP in platelet function. *Ann New York Acad Sci* 185:477–487
 86. Mills DCB, Smith JB (1972) The control of platelet responsiveness by agents that influence cyclic AMP metabolism. *Ann NY Acad Sci* 201:391–399
 87. Haslam RJ, Taylor A (1971) Role of cyclic 3',5'-adenosine monophosphate in platelet aggregation. In: Caen J (ed) platelet aggregation. Masson et Cie, Paris, pp 85–93
 88. Salzman EW, Levine L (1971) Cyclic 3',5'-adenosine monophosphate in human blood platelets. *J Clin Invest* 50:131–141
 89. Yang J, Wu J, Jiang H, Mortensen R, Austin S, Manning DR, Woulfe D, Brass LF (2002) Signaling through G_i family members in platelets. Redundancy and specificity in the regulation of adenylyl cyclase and other effectors. *J Biol Chem* 277:46035–46042
 90. Macfarlane DE, Mills DCB (1981) Inhibition by ADP of prostaglandin induced accumulation of cyclic AMP in intact human platelets. *J Cyclic Nucleotide Res* 7:1–11
 91. Cattaneo M (2010) State of the art of new P2Y₁₂ antagonists. *Intern Emerg Med* 5:385–391
 92. Colman RW (1990) Aggregin: a platelet ADP receptor that mediates activation. *FASEB J* 4:1425–1435
 93. Greco NJ, Yamamoto N, Jackson BW, Tandon NN, Moos M Jr, Jamieson GA (1991) Identification of a nucleotide-binding site on glycoprotein IIb. Relationship to ADP-induced platelet activation. *J Biol Chem* 266:13627–13633
 94. Macfarlane DE, Mills DCB, Srivastava PC (1982) Binding of 2-azidoadenosine [β -³²P]diphosphate to the receptor on intact human blood platelets which inhibits adenylylase. *Biochemistry* 21:544–549
 95. Macfarlane DE, Srivastava PC, Mills DCB (1983) 2-Methylthioadenosine [β -³²P]diphosphate. An agonist and radioligand for the receptor that inhibits the accumulation of cyclic AMP in intact blood platelets. *J Clin Invest* 71:420–428
 96. Gachet C, Cattaneo M, Ohlmann P, Hechler B, Lecchi A, Chevalier J, Cassel D, Mannucci PM, Cazenave J-P (1995) Purinoceptors on blood platelets: further pharmacological and clinical evidence to suggest the presence of two ADP receptors. *Br J Haematol* 91:434–444
 97. Nurden P, Savi P, Heilmann E, Bihour C, Herbert JM, Maffrand JP, Nurden A (1995) 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* 95:1612–1622
 98. Cristalli G, Mills DCB (1993) Identification of a receptor for ADP on blood platelets by photoaffinity labelling. *Biochem J* 291:875–881
 99. Baurand A, Raboisson P, Freund M, Léon C, Cazenave J-P, Bourguignon J-J, Gachet C (2001) Inhibition of platelet function by administration of MRS2179, a P2Y₁ receptor antagonist. *Eur J Pharmacol* 412:213–221
 100. Tsien RY, Pozzan T, Rink TJ (1984) Measuring and manipulating cytosolic Ca²⁺ with trapped indicators. *TIBS* 9:263–266
 101. Rink TJ, Sage SO (1990) Calcium signaling in human platelets. *Annu Rev Physiol* 52:431–449
 102. Sage SO, Reast R, Rink TJ (1990) ADP evokes biphasic Ca²⁺ influx in fura-2-loaded human platelets. Evidence for Ca²⁺ entry regulated by the intracellular Ca²⁺ store. *Biochem J* 265:675–680
 103. Varga-Szabo D, Braun A, Nieswandt B (2009) Calcium signaling in platelets. *J Thromb Haemost* 7:1057–1066
 104. MacKenzie AB, Mahaut-Smith MP, Sage SO (1996) Activation of receptor-operated cation channels via P_{2X1} not P_{2T} purinoceptors in human platelets. *J Biol Chem* 271:2879–2881
 105. Vial C, Hechler B, Léon C, Cazenave J-P, Gachet C (1997) Presence of P2X₁ purinoceptors in human platelets and megakaryoblastic cell lines. *Thromb Haemost* 78:1500–1504
 106. Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP (1998) Molecular basis for ADP-induced platelet activation: I. Evidence for three distinct ADP receptors on human platelets. *J Biol Chem* 273:2024–2029
 107. Mahaut-Smith MP, Ennion SJ, Rolf MG, Evans RJ (2000) ADP is not an agonist at P2X₁ receptors: evidence for separate receptors stimulated by ATP and ADP on human platelets. *Br J Pharmacol* 131:108–114
 108. Rolf MG, Brearley CA, Mahaut-Smith MP (2001) Platelet shape change evoked by selective activation of P2X₁ purinoceptors with α,β -methylene ATP. *Thromb Haemost* 85:303–308
 109. Vial C, Pitt SJ, Roberts J, Rolf MG, Mahaut-Smith MP, Evans RJ (2003) Lack of evidence for functional ADP-activated human P2X₁ receptors supports a role for ATP during hemostasis and thrombosis. *Blood* 102:3646–3651
 110. Oury C, Toth-Zsomboki E, Vermeylen J, Hoylaerts MF (2002) P2X₁-mediated activation of extracellular signal-regulated kinase 2 contributes to platelet secretion and aggregation induced by collagen. *Blood* 100:2499–2505
 111. Oury C, Kuijpers MJE, Toth-Zsomboki E, Bonnefoy A, Danloy S, Vreys I, Feijge MAH, De Vos R, Vermeylen J, Heemskerk JWM, Hoylaerts MF (2003) Overexpression of the platelet P2X₁ ion channel in transgenic mice generates a novel prothrombotic phenotype. *Blood* 101:3969–3976
 112. Gordon JL (1986) Extracellular ATP: effects, sources and fate. *Biochem J* 233:309–319
 113. Léon C, Hechler B, Vial C, Leray C, Cazenave J-P, Gachet C (1997) The P2Y₁ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Letters* 403:26–30
 114. Boyer JL, Romero-Avila T, Schachter JB, Harden TK (1996) Identification of competitive antagonists of the P2Y₁ receptor. *Mol Pharmacol* 50:1323–1329
 115. Jin J, Daniel JL, Kunapuli SP (1998) 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* 273:2030–2034
 116. Hechler B, Léon C, Vial C, Vigne P, Frelin C, Cazenave J-P, Gachet C (1998) The P2Y₁ receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. *Blood* 92:152–159
 117. Savi P, Beauverger P, Labouret C, Delfaud M, Salel V, Kaghad M, Herbert JM (1998) The role of P2Y₁ purinoceptor in ADP-induced platelet activation. *FEBS Lett* 422:291–295
 118. Torti M, Lapetina EG (2002) Roles of phospholipase C and phospholipase D in receptor mediated platelet activation. In: Gesele P, Page CP, Fuster V, Vermeylen J (eds) Thrombotic and non-thrombotic disorders. Cambridge University Press, Cambridge, pp 238–259
 119. Léon C, Hechler B, Freund M, Eckly A, Vial C, Ohlmann P, Dierich A, LeMeur M, Cazenave J-P, Gachet C (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y₁ receptor-null mice. *J Clin Invest* 104:1731–1737
 120. Fabre J-E, Nguyen M, Latour A, Keifer JA, Audoly LP, Coffman TM, Koller BH (1999) Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y₁-deficient mice. *Nat Med* 5:1199–1202
 121. Cattaneo M (2003) The platelet P2Y receptors as targets for new antithrombotic drugs. *J Thromb Haemost* 1:1133–1135

122. Baurand A, Gachet C (2003) The P2Y₁ receptor as a target for new antithrombotic drugs: a review of the P2Y₁ antagonist MRS-2179. *Cardiovascular Drug Reviews* 21:67–76
123. Hechler B, Eckly A, Ohlmann P, Cazenave J-P (1998) 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* 103:858–866
124. Fagura MS, Dainty IA, McKay GD, Kirk IP, Humphries RG, Robertson MJ, Dougall IG, Leff P (1998) P2Y₁-receptors in human platelets which are pharmacologically distinct from P2Y_{ADP}-receptors. *Br J Pharmacol* 124:157–164
125. Hollopeter G, Jantzen H-M, Vincent D, Li G, England L, Ramakrishnan V, Yang R-B, Nurden P, Nurden A, Julius D, Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 409:202–207
126. Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM (1992) Identification of a new congenital defect of platelet function characterized by severe impairment of platelet responses to adenosine diphosphate. *Blood* 80:2787–2796
127. Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, Zhang FL, Gustafson E, Monsma FJ Jr, Wiekowski MT, Abbondanzo SJ, Cook DN, Bayne ML, Lira SA, Chintala MS (2001) Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. *J Clin Invest* 107:1591–1598
128. Ohlmann P, Laugwitz K-L, Nürnberg B, Spicher K, Schultz G, Cazenave J-P, Gachet C (1995) The human platelet ADP receptor activates G_{i2} proteins. *Biochem J* 312:775–779
129. Woulfe D, Yang J, Brass L (2001) ADP and platelets: the end of the beginning. *J Clin Invest* 107:1503–1505
130. Jantzen H-M, Milstone DS, Gousset L, Conley PB, Mortensen RM (2001) Impaired activation of murine platelets lacking G_{αi2}. *J Clin Invest* 108:477–483
131. Brass LF (2005) The molecular basis of platelet activation. In: Hoffman R, Benz EJ Jr, Shattil SJ, Furie B, Cohen HJ, Silberstein LE, McGlave P (eds) *Hematology basic principles and practice*, 4th edn. Elsevier, Philadelphia, pp 1899–1914
132. Woulfe DS (2010) Akt signaling in platelets and thrombosis. *Expert Rev Hematol* 3:81–91
133. Schoenwaelder SM, Ono A, Sturgeon S, Chan SM, Mangin P, Maxwell MJ, Turnbull S, Mulchandani M, Anderson K, Kauffenstein G, Rewcastle GW, Kendall J, Gachet C, Jackson SP (2007) Identification of a unique co-operative phosphoinositide 3-kinase signaling mechanism regulating integrin $\alpha_{IIb}\beta_3$ adhesive function in platelets. *J Biol Chem* 282:28648–28658
134. Maffrand JP, Eloy F (1974) Synthesis of the thienopyridines and furopyridines of therapeutic interest. *Eur J Med Chem* 9:483–486
135. Bruno JJ, Molony BA (1983) Ticlopidine. In: Scriabine A (ed) *New drugs annual: cardiovascular drugs*. Raven, New York, pp 295–316
136. Gachet C, Cazenave J-P, Ohlmann P, Bouloux C, Defreyne G, Droit F, Maffrand J-P (1990) 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* 40:2683–2687
137. Defreyne G, Gachet C, Savi P, Droit F, Cazenave JP, Maffrand JP (1991) Ticlopidine and clopidogrel (SR 25990C) selectively neutralize ADP inhibition of PGE₁-activated platelet adenylate cyclase in rats and rabbits. *Thromb Haemost* 65:186–190
138. Savi P, Pereillo JM, Uzabiaga MF, Combalbert J, Picard C, Maffrand JP, Pascal M, Herbert JM (2000) Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost* 84:891–896
139. Gurbel PA, Bliden KP, Antonino MJ, Stephens G, Gretler DD, Jurek MM, Pakyz RE, Shuldiner AR, Conley PB, Tantry US (2009) The effect of elinogrel on high platelet reactivity during dual antiplatelet therapy and the relation to cyp 2c19*2 genotype: first experience in patients. *J Thromb Haemost* 8:43–53