ADP Receptors and Clinical Bleeding Disorders

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Abstract—ADP plays a key role in hemostasis and thrombosis. 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. The present review proposes a model of 3 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 G_{i2}, which is essential for the full aggregation response to ADP. 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. (Arterioscler Thromb Vasc Biol. 1999;19:2281-2285.)

Key Words: adenosine diphosphate ■ purino receptors ■ platelets ■ bleeding disorders ■ thrombosis

ADP, the first known low-molecular-weight, platelet-aggregating agent, was initially identified after observations that a small molecule derived from red blood cells stimulated platelet adhesion to glass and induced platelet aggregation.1,2 Adenine nucleotides, which originate from platelet dense granules and damaged cells, especially red blood cells and endothelial cells, interact with P2 receptors to regulate a broad range of physiological processes. P2 receptors are widely distributed in many different cell types. These receptors are divided into 2 main groups: the G protein–coupled, or “metabotropic,” superfamily termed P2Y and the ligand-gated ion channel, or “ionotropic,” superfamily termed P2X.3 Seven subtypes of P2X and 11 subtypes of P2Y receptor have been identified to date.

Several lines of evidence indicate that ADP plays a key role in the formation of the hemostatic plug and in the pathogenesis of arterial thrombi: (1) ADP is contained at high concentrations in the platelet dense granules and is released when platelets are stimulated by other agents, such as thrombin or collagen, thus reinforcing platelet aggregation;4 when platelets are stimulated by other activators, such as ADP receptors or those lacking ADP in platelet granules have a bleeding diathesis.4,7

ADP-Induced Platelet Activation

Addition of exogenous ADP to washed human platelets results in shape change, reversible aggregation at physiological concentrations of ionized calcium in the external medium, and finally, desensitization. In a minority of individuals, the close platelet-to-platelet contact brought about by the aggregation process triggers the formation of trace amounts of thromboxane A_2, which stimulates platelet secretion and reinforces aggregation.8 This effect is greatly enhanced9 and can be observed in most individuals8 when the concentration of ionized calcium in the extracellular medium is artifically decreased to the micromolar level, such as in citrated, platelet-rich plasma.

Transduction of the ADP-induced signal involves inhibition of adenylyl cyclase and a concomitant transient rise in free cytoplasmic calcium, due to both calcium influx and mobilization of internal calcium stores.1,2 ADP also induces a unique and extremely rapid influx of calcium from the extracellular medium, which has been attributed to ligand-gated calcium channels.1,2 In addition, it has been found to activate G_{i2}, which could explain how it inhibits adenylyl cyclase.1,2

The recent demonstration that platelets from knockout mice lacking the gene coding for the αq subunit of the Gq protein do not aggregate in response to ADP indicates that the phospholipase C pathway is necessary to raise intracellular calcium levels after ADP stimulation and that this step is essential to platelet aggregation. On the other hand, inhibition of adenylyl cyclase is a key feature of platelet activation by ADP but displays no causal relationship to aggregation. Moreover, this effect of ADP can be observed only when adenylyl cyclase has been prestimulated by prostaglandins or other activators.1,2 Thus, ADP triggers at least 2 biochemical events in platelets, a phospholipase C–mediated rise in intracellular calcium and a G_{i2}-mediated inhibition of adenylyl cyclase.
lyl cyclase. Whether 1 or more ADP receptors are responsible for these effects has been a challenging debate over the past 30 years, and the question now appears close to resolution.

**Agonists, Antagonists, and Inhibitors**

Purinoceptor pharmacology presently lacks specific and selective agonists and antagonists to discriminate clearly between receptor subtypes. The originality of platelet ADP receptors lies in the fact that while ADP is the natural agonist, ATP is a competitive antagonist of all effects of ADP on platelets except the very fast calcium entry, which seems to be triggered by both molecules.1,2 A platelet ADP receptor was previously defined on the basis of pharmacological data as the receptor responsible for ADP-induced aggregation, intracellular calcium increases, and adenylyl cyclase inhibition.1,2 This receptor was termed P2T, where T denotes thrombocytes and was presumed to be lineage specific.

One may classify agonists and antagonists into 2 main families: *full agonists and antagonists*, which stimulate or inhibit both calcium mobilization and inhibition of stimulated adenylyl cyclase, and *partial agonists and antagonists*, which preferentially stimulate or inhibit calcium mobilization or cyclase inhibition. Some analogues of ADP are of interest on account of their preferential action on stimulated adenylyl cyclase, while others, such as adenosine 5′-O-(1-thiodiphosphate) (ADP-oS), act on platelet aggregation without affecting adenylyl cyclase. Thus, 2-methylthioadenosine 5′-diphosphate (2-MeSADP) is 200-fold more potent than ADP as an inhibitor of adenylyl cyclase, but only 5-fold more potent as an aggregating agent.1

Certain nonhydrolyzable analogues, like 2-methylthioadenosine 5′-β,γ-methyleneadenosine (2-MeSAMP) and 2-ethylthioadenosine 5′-monophosphate (2-EtSAM), competitively inhibit the action of ADP on adenylyl cyclase and inhibit platelet aggregation induced by ADP or ADP-oS (which inhibits stimulated adenylyl cyclase) but do not inhibit platelet aggregation induced by ADP-oS (which has no effect on stimulated adenylyl cyclase). Adenosine 3′-phosphate 5′-monophosphate (A3P5P), adenosine 2′,5′-diphosphate (A2P5P), and adenosine 3′,5′-diphosphate (A3P5P) inhibit platelet aggregation to ADP by competitively antagonizing the intracellular calcium rise induced by this agonist.1,14,15 These AMP analogues, known to be selective P2Y1 receptor antagonists,1 had, on the contrary, no effect on adenylyl cyclase inhibition.

The thienopyridine compounds ticlopidine and clopidogrel, 2 specific and potent inhibitors of ADP-induced platelet aggregation, are used clinically as antithrombotic drugs.3 These drugs, which are inactive in vitro and must be metabolized in the liver to acquire their antiaggregating properties, selectively antagonize the ADP-induced inhibition of prostaglandin E2-activated adenylyl cyclase but do not modify other effects of ADP on platelets, including shape change and calcium movements. They cause a dose-dependent reduction in the number of 2-MeSADP binding sites on platelets.1,15

Under conditions where the 2-MeSADP binding sites are maximally reduced (up to 70% reduction) and ADP-induced aggregation and inhibition of adenylyl cyclase are completely blocked by thienopyridine treatment, low concentrations of ADP can still promote platelet shape change and a rise in intracellular calcium. Such findings point to the existence of a platelet ADP receptor insensitive to thienopyridines and responsible for shape change and calcium signaling.

In contrast to thienopyridines, ATP analogues like AR-C66096 are direct inhibitors of ADP-induced platelet activation in vitro. Although no binding data are as yet available, the molecular target of AR-C66096 seems to be very similar to that of the thienopyridine compounds.11

Measurements of the binding of [33P]2-MeSADP to the platelets of a patient (V.R.) with a congenital deficiency of ADP-induced aggregation thought to be related to a receptor defect16 revealed a reduction of up to 70% in the number of binding sites compared with control platelets, without modification of the binding affinity (see below).15 Interestingly, the clinical profile and platelet functions of this patient are the same as when thienopyridines are administered to humans or animals. The main feature is a strong and selective inhibition of the aggregation response to ADP, despite conserved shape change. At the intracellular level, ADP-induced responses are blocked, with the exception of the intracellular calcium rise, as after thienopyridine treatment.

The above findings may be interpreted on the basis of 2 principal ADP receptors on blood platelets: 1 coupled to calcium mobilization and necessary to initiate ADP-induced aggregation and 1 coupled to adenylyl cyclase, responsible for amplification of the response. Probably, this latter receptor is defective in patients with congenital impairment of platelet responses to ADP and is the molecular target of thienopyridines.

**Molecular Identity of the Platelet ADP Receptors**

Attempts to clone a platelet-specific ADP receptor by using megakaryoblastic cell lines as sources for a cDNA library have to date been unsuccessful. However, several P2 purinoceptors have been cloned in human erythroleukemia (HEL) cells, in particular the P2Y1 receptor, which was the first P2 purinoceptor to be cloned, originally from a chick brain cDNA library.17 After the human endothelial P2Y1 receptor was cloned and sequenced, P2Y1 mRNA was found to be present in megakaryoblastic cell lines (HEL, MEG-01, Dam, CHRF-288, and K562) and in human platelets.18 After its stable expression in Jurkat cells, the human P2Y1 receptor proved to be an ADP receptor at which Sp-ATP-oS and ATP were competitive antagonists, and partial agonistic responses to triphosphate nucleotides were found to be due to degradation of the commercial reagents into diphosphate nucleotides.18 These studies were extended to platelets and to brain capillary endothelial cells expressing the P2Y1 receptor, and it was shown that the agonistic responses of purified triphosphate nucleotides were due to enzymatic transformation into diphosphate analogues by ectonucleotidases present at the surface of the cells.19

These results supported the hypothesis that the P2Y1 receptor could be the elusive P2T receptor. Nevertheless, the fact that the selective P2Y1 antagonists A2P5P and A3P5P inhibited ADP-induced platelet aggregation but had no effect on ADP-induced adenylyl cyclase inhibition led to the suggestion that another as-yet-unidentified receptor must mediate this effect of ADP on platelets.11,14,20,21 The suggestion was later strongly supported by the demonstration, at both genetic and pharmacological levels, that the P2Y1 receptor was normal in a patient (V.R.) with congenital impairment of
platelet responses to ADP. This receptor should be of the P2Y type, since ADP is known to activate the G<sub>a2</sub> heterotrimeric G protein in human platelet membranes. It also should exhibit a pharmacological profile identical to that of the P2Y<sub>1</sub> receptor but with subtle differences in the selectivity of certain ligands and should be the molecular target of thienopyridines and the direct antagonist AR-C66096. Unfortunately to date, no such receptor appears to have been cloned.

Apart from the now-well-characterized P2Y<sub>1</sub> receptor and the putative P2Y receptor coupled to G<sub>a2</sub>, a P2X<sub>1</sub> receptor could be responsible for the fast calcium entry induced by ADP in human platelets. Polymerase chain reaction amplification of human cDNA with oligonucleotides specific for the ADP in human platelets. The functional P2X<sub>1</sub> purinoceptors on blood platelets. However, the putative P2Y receptor coupled to G<sub>a2</sub>, a P2X<sub>1</sub> receptor could be responsible for the fast calcium entry induced by ADP in human platelets. Polymerase chain reaction amplification of human cDNA with oligonucleotides specific for the human P2X<sub>1</sub> purinoceptor subtype demonstrated the presence of P2X<sub>1</sub> transcripts in platelets and megakaryoblastic cell lines, whereas the selective P2X<sub>1</sub> agonist αβ-MeATP was found to trigger a calcium influx into fura 2-loaded human platelets. These findings confirmed the existence of functional P2X<sub>1</sub> purinoceptors on blood platelets. However, because αβ-MeATP does not induce platelet shape change, aggregation, or phospholipase C activation and because desensitization of the P2X<sub>1</sub> receptor is without effect on ADP-induced aggregation, the physiological role of P2X<sub>1</sub> receptors on platelets remains unclear and would in fact seem to be discrete.

In conclusion, at least 3 distinct ADP receptors seem to be involved in the complex process of platelet activation and aggregation: the P2X<sub>1</sub> ionotropic receptor, the role of which remains to be established; the P2Y<sub>1</sub> receptor, which is necessary to initiate ADP-induced shape change and aggregation through calcium mobilization but not sufficient to support a full platelet response; and a P2Y receptor coupled to G<sub>a2</sub>, which is essential for the full aggregation response of platelets to ADP.

**Congenital Defects of Platelet ADP Receptor(s)**

In 1992, the first patient (V.R.) with a selective defect of platelet responses to ADP was described. He is a man of white origin, aged 49 years at the time of diagnosis, who had a lifelong history of excessive bleeding, prolonged bleeding time, and an abnormality of platelet aggregation that was similar to that observed in patients with defects of platelet secretion (reversible aggregation in response to weak agonists and impaired aggregation in response to low concentrations of collagen or thrombin), except that the aggregation response to ADP was severely impaired. This platelet aggregation profile is the same one that occurs when thienopyridines are administered to humans and is compatible with a selective impairment of responses to ADP, which also affects the full aggregation response to release-inducing agonists, as a consequence of the loss of the potentiating effect of released ADP on platelet aggregation. Other abnormalities of platelet function found in this patient were common to those induced by thienopyridine compounds, including (1) no inhibition by ADP of prostaglandin E<sub>1</sub>–stimulated platelet adenyl cyclase but normal inhibition by epinephrine; (2) normal shape change and normal (or only mildly reduced) mobilization of cytoplasmic ionized calcium induced by ADP; and (3) the presence of 30% of the normal number of platelet binding sites for [35S]labeled ADP (see above). Three additional patients, 1 male and 2 females, with very similar characteristics were subsequently described; in 1 of them, it was shown that the ADP receptor pathway that is defective in this condition is linked to a selective tyrosine phosphorylation response. The many similarities among the patients suggest that they are affected by the same type of disorder of platelet function, probably associated with defective interaction between ADP and the P2Y receptor that is coupled to G<sub>a2</sub>. The functional defects of these patients could result from (1) a quantitative or qualitative defect of the receptor or (2) a permanently downregulated, nonfunctional protein. Similarities between these patients and humans or animals that were treated with clopidogrel have been reported in terms of the morphology of ADP-induced platelet aggregates as well as in signal transduction studies. The defect is probably inherited as an autosomal recessive trait, because all patients so far described were born of consanguineous parents. The son of 1 of them, who is considered to be an obligate heterozygote, bound intermediate levels of 2-MeSADP. His bleeding time was mildly prolonged (13 minutes), and his platelets underwent a normal first wave of aggregation after stimulation with ADP but did not secrete normal amounts of ATP after stimulation with different agonists. The secretion defect of his platelets could not be ascribed to impaired production of thromboxane A<sub>2</sub> or low concentrations of platelet granule contents, which were normal, and are therefore similar to that of platelets with the so-called primary secretion defect (PSD). PSD is the most common congenital defect of platelet secretion and is characterized by a normal primary wave of aggregation induced by ADP and other agonists, a normal concentration of platelet granule contents, and normal production of thromboxane A<sub>2</sub>. Because PSD patients also have a moderately decreased number of platelet-binding sites for 2-MeSADP, it is likely that they are heterozygous for the severe defect of the platelet ADP receptor that is coupled to Goi<sub>2</sub> and that the full complement of platelet-binding sites for ADP is necessary for full platelet secretion. Studies of normal platelets that had been pretreated with acetylsalicylic acid and stimulated under nonstirring conditions to avoid the formation of large aggregates indicated that ADP potentiates platelet secretion directly and independently of platelet aggregation and the production of thromboxane A<sub>2</sub>. The role of ADP in potentiating platelet secretion has been recently confirmed in studies of platelet activation/aggregation induced by sera from heparin-induced thrombocytopenia patients.

**The Congenital Defect in Platelet ADP Receptor(s) as a Model to Study the Role of Released ADP in Platelet Function**

With the use of platelets from patient V.R., it was possible to demonstrate that ADP plays a role in the stabilization of thrombin-induced platelet aggregates and contributes to shear-induced platelet aggregation but that it does not play an essential role in platelet aggregation and fibrinogen binding induced by the prostaglandin endoperoxide-thromboxane A<sub>2</sub> mimetic U46619.

**Therapy**

The intravenous infusion of the vasopressin analogue DDAVP (0.3 μg/kg body weight) shortened the prolonged
bleeding time of patient V.R. from 20 minutes to 8.5 minutes.\(^{34}\) After the infusion of DDAVP, which was repeated twice at 24-hour intervals, the patient underwent a surgical intervention for herniated disc repair, which was not complicated by excessive bleeding. Although the efficacy of DDAVP in reducing bleeding complications of patients with defects of primary hemostasis is anecdotal, its administration is generally without serious side effects and can therefore be recommended for the prophylaxis and treatment of bleeding episodes in these patients.

**Conclusions**

Although itself a weak platelet-aggregating agent, ADP is a key platelet agonist. At least 3 distinct ADP receptors are involved in the complex process of platelet activation and aggregation: the P2X\(_1\) ionotropic receptor, the role of which remains to be established; the P2Y\(_1\) receptor, which is necessary to initiate ADP-induced aggregation through calcium mobilization but not sufficient to support a full platelet response; and a P2Y receptor coupled to G\(_{\text{flight}}\), which is essential for the full aggregation response of platelets to ADP. The P2Y receptor is necessary for ADP-induced platelet aggregation.\(^{35}\) The P2Y\(_1\) receptor is required for ADP-induced platelet aggregation, \(^{35}\) and inhibition of adenyl cyclase by ADP is preserved.\(^{36}\) The demonstration that P2Y\(_1\) receptor knockout mice are resistant to the thromboembolic induction by the intravenous injection of ADP or collagen suggests that P2Y\(_1\) represents a potential target for antithrombotic drugs.

**Note Added in Proof**

It was recently shown that platelets from P2Y1 receptor knockout mice do not aggregate in response to ADP and aggregate poorly in response to collagen; in contrast, inhibition of adenyl cyclase by ADP is preserved.\(^{36}\) The P2Y1 receptor knockout mice do not aggregate in response to ADP.\(^{35}\)

**References**


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