The P2Y1 Receptor Is Necessary for Adenosine 5'-Diphosphate–Induced Platelet Aggregation

By Béatrice Hechler, Catherine Léon, Catherine Vial, Paul Vigne, Christian Frelin, Jean-Pierre Cazenave, and Christian Gachet

The human P2Y1 receptor heterologously expressed in J urkat cells behaves as a specific adenosine 5'-diphosphate (ADP) receptor at which purified adenosine triphosphate (ATP) is an ineffective agonist, but competitively antagonizes the action of ADP. This receptor is thus a good candidate to be the elusive platelet P2T receptor for ADP. In the present work, we examined the effects on ADP-induced platelet responses of two selective and competitive P2Y1 antagonists, adenosine-2'-phosphate-5'-phosphate (A2P5P) and adenosine-3'-phosphate-5'-phosphate (A3P5P). Results were compared with those for the native P2Y1 receptor expressed on the B10 clone of rat brain capillary endothelial cells (BCEC) and for the cloned human P2Y1 receptor expressed on J urkat cells. A2P5P and A3P5P inhibited ADP-induced platelet shape change and aggregation (pA2 = 5) and competitively antagonized calcium movements in response to ADP in fura-2–loaded platelets, B10 cells, and P2Y1-J urkat cells. In contrast, these compounds had no effect on ADP-induced inhibition of adenyl cyclase in platelets or B10 cells, whereas known antagonists of platelet activation by ADP such as Sp-ATPγS were effective. These identical signaling responses and pharmacologic properties suggest that platelets and BCEC share a common P2Y1 receptor involved in ADP-induced aggregation and vasodilation, respectively. This P2Y1 receptor coupled to the mobilization of intracellular calcium stores was found to be necessary to trigger ADP-induced platelet aggregation. The present results, together with data from the literature, also point to the existence of another as yet unidentified ADP receptor, coupled to adenylyl cyclase and responsible for completion of the aggregation response. Thus, the term, P2T, should no longer be used to designate a specific molecular entity.

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receptor, the first P2 receptor subtype to be cloned,\textsuperscript{26} has a broad tissue distribution.\textsuperscript{11} Rat brain capillary endothelial cells (BCEC) have been shown to express a specific ADP receptor,\textsuperscript{31-33} which was more recently identified as a P2Y\textsubscript{1} receptor using RT-PCR in a subclone of BCEC termed B10.\textsuperscript{34} This receptor is linked to the mobilization of internal calcium stores and negatively to adenyl cyclase, thus bearing a striking resemblance to the ADP receptor of platelets.

The aim of the present study was to further address the question of the molecular identity of the platelet ADP receptor and in particular the possibility of its being of the P2Y\textsubscript{1} type. In this objective, we compared the effects of two selective P2Y\textsubscript{1} antagonists, adenosine-2'-phosphate-5'-phosphate (A2P5P) and adenosine-3'-phosphate-5'-phosphate (A3P5P),\textsuperscript{35} on ADP-induced platelet activation, on the native P2Y\textsubscript{1} receptor expressed on the B10 clone of rat BCEC and on the cloned human P2Y\textsubscript{1} receptor heterologously expressed in Jurkat cells. Platelets and BCEC are found to share a common P2Y\textsubscript{1} receptor coupled to the mobilization of intracellular calcium stores, which is necessary to allow ADP-induced platelet aggregation. These results, together with data from the literature, support the hypothesis that an ADP receptor coupled to adenyl cyclase is responsible for completion of the aggregation response.

MATERIALS AND METHODS

Materials. Adenosine 5'-O-(1-thiotriphosphate) (S-iso)mer (S-P-ATPoS) was from Boehringer (Mannheim, Germany) and 2-methylthioadenosine 5'-diphosphate (2MeSADP) from Research Biochemicals Inc (Natick, MA). ADP, ATP, A2P5P, A3P5P, isobutyl methyl xanthine (IBMX), U46619, thrombin, epinephrine, prostaglandin E1 (PGE\textsubscript{1}), adenosine-3'-5'-monophosphate (AMP) and adenosine-3'-5'-cyclic monophosphate (cAMP) dose kit was purchased from Amersham (Les Ulis, France) and apyrase was purified from potatoes as previously described.\textsuperscript{36} A2P5P and A3P5P were checked for purity by high performance liquid chromatography (HPLC) analysis on a Partisil 10 µm SAX column (Interchrom, Intermich, Monluc\textacuted, France) eluted with a linear gradient of 0 to 1 mol/L ammonium phosphate buffer, pH 3.8.

Cell cultures. Jurkat E6.1 cells (ECACC No. 88042803, Cerdic, France) stably expressing the human P2Y\textsubscript{1} receptor were grown in RPMI-1640 medium supplemented with 10% (vol/vol) heat inactivated fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 1 mg/mL genetin. B10 clone cells from rat BCEC were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) heat inactivated fetal calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cultures were kept at 37°C in a humidified atmosphere containing 5% CO\textsubscript{2} and cells were subcultured every 3 days so as to maintain a density of approximately 5 × 10\textsuperscript{5} cells/mL.

Preparation of washed human platelets. Washed human platelets were prepared as previously described.\textsuperscript{37} Briefly, fresh blood obtained from healthy donors was centrifuged at 175g for 15 minutes at 37°C and platelet-rich plasma was removed and centrifuged at 1,570g for 15 minutes at 37°C. The platelet pellet was washed twice in Tyrode’s buffer (137 mmol/L NaCl, 2 mmol/L KCl, 12 mmol/L NaHCO\textsubscript{3}, 0.3 mmol/L NaH\textsubscript{2}PO\textsubscript{4}, 5.5 mmol/L glucose, 5 mmol/L HEPES, pH 7.3) containing 0.35% human serum albumin and finally resuspended at a density of 3 × 10\textsuperscript{11} platelets/µL in the same buffer in the presence of 0.02 U/mL of the ADP scavenger apyrase (adenosine 5'-triphosphate diphosphohydrolase, EC 3.6.1.5), a concentration sufficient to prevent desensitization of platelet ADP receptors during storage. Platelets were kept at 37°C throughout all experiments.

Platelet aggregation studies. Aggregation was measured at 37°C by a turbidimetric method in a dual-channel Payton aggregometer (Payton Associates, Scarborough, Ontario, Canada). A 450-µL aliquot of platelet suspension was stirred at 1,100 rpm and activated by addition of different agonists, in the presence or absence of A2P5P or A3P5P at varying concentrations and in the presence of human fibrinogen (0.8 mg/mL), in a final volume of 500 µL. The extent of aggregation was estimated quantitatively by measuring the maximum curve height above baseline level. ADP-induced shape change was determined turbidimetrically in the presence of 5 mmol/L ethylenediaminetetraacetic acid (EDTA).

(Ca\textsuperscript{2+}), measurements. After centrifugation of human platelet-rich plasma at 1,570g for 15 minutes at 37°C, the platelet pellet was resuspended in Tyrode’s buffer containing no albumin or calcium at a density of about 6 × 10\textsuperscript{11} platelets/µL. Platelets were loaded with 2 µmol/L fura-2/AM for 45 minutes at 37°C in the dark, washed in Tyrode’s buffer containing 0.35% human serum albumin, and finally resuspended at 37°C in a density of 3 × 10\textsuperscript{11} platelets/µL in Tyrode’s buffer containing apyrase and 0.1% essentially fatty acid-free human serum albumin. Jurkat cells stably expressing the human P2Y\textsubscript{1} receptor were washed in basal salt solution (BSS: 25 mmol/L HEPES, pH 7.3, 125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl\textsubscript{2}, 5 mmol/L glucose, 0.1% human serum albumin) containing 2 mmol/L CaCl\textsubscript{2}. After centrifugation at 100g for 5 minutes, the cells were resuspended in BSS without calcium at a concentration of 15 × 10\textsuperscript{6} cells/mL and loaded with 5 µmol/L fura-2/AM in BSS containing either 2 mmol/L calcium or no calcium (0.2 mmol/L ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA)). Aliquots of fura-2–loaded platelets or cells were transferred to a 10 × 10-mm quartz cuvette maintained at 37°C and fluorescence measurements were performed under continuous stirring, using a PITI deta olm spectrophotometer (Photon Technology International Inc, South Brunswick, NJ). The excitation wavelength was alternately fixed at 340 or 380 nm and fluorescence emission was determined at 510 nm.

B10 cells were loaded with 5 µmol/L indo-1/AM for 2 hours in complete culture medium at 37°C. After dissociation from the culture dishes, the cells were centrifuged at low speed and resuspended in Earle’s salt solution (25 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, 5 mmol/L KCl, 1.8 mmol/L CaCl\textsubscript{2}, 0.8 mmol/L MgSO\textsubscript{4}, 5 mmol/L glucose). Flow cytometric analysis of indo-1 fluorescence was performed as previously described\textsuperscript{37} using a FacStar Plus apparatus (Becton Dickinson, San Jose, CA). The indo-1 fluorescence ratio was measured in 5,000 single cells and collected in real time at a rate of 500 cells/second.

Measurement of adenyl cyclase activity. A 450-µL aliquot of washed platelets was stirred at 1,100 rpm in an aggregometer cuvette, and the following reagents were added at 30-second intervals: 1 µmol/L PGE\textsubscript{1}, 100 µmol/L A2P5P or A3P5P, and 5 µmol/L ADP or vehicle (Tyrode’s buffer containing no Ca\textsuperscript{2+} or Mg\textsuperscript{2+}). One minute later, the reaction was stopped by addition of 50 µL of ice-cold 6.6 N perchloric acid. B10 cells grown in 6-well tissue culture clusters were first incubated in BSS supplemented with 10^{-5} mol/L IBMX for 10 minutes at 37°C. Forskolin (1 µmol/L) and/or nucleotides were added (final volume 1 µL per well) and incubation was continued for 5 minutes at 37°C, after which the incubation solution was removed by aspiration and the cells extracted in 10% (vol/vol) ice-cold 6.6 N perchloric acid. The same procedure was applied to Jurkat cells except that the incubation solution was eliminated by centrifuging each tube at 200g for 30 seconds before extracting the cells in perchloric acid. Perchloric acid extracts were centrifuged at 11,000g for 5 minutes to eliminate
protein precipitate and cyclic AMP was isolated from the supernatants as described by Khym using a mixture of triocylamine and freon (20/22, vol/vol). The upper aqueous phase was lyophilized and the dry residue dissolved in the buffer provided with the commercial radioimmunoassay kit for cyclic AMP measurement.

Data analysis. Agonist potencies and apparent dissociation constants of inhibitors (pA2 = −log Kd) were calculated using the GraphPad software package (GraphPad, San Diego, CA).

RESULTS

P2Yi antagonists noncompetitively inhibit ADP-induced platelet aggregation. The adenine nucleotide derivatives A2P5P and A3P5P induced no aggregation or shape change of washed human platelets, even at high concentrations (up to 100 µmol/L). On the other hand, ADP-induced platelet aggregation was inhibited by both A2P5P and A3P5P (Fig 1A). The two P2Yi receptor antagonists were also able to inhibit ADP-induced platelet shape change, as was demonstrated in the presence of 5 mmol/L EDTA, an agent that blocks aggregation by preventing the binding of fibrinogen to platelets (Fig 1B). This effect was selective, as these antagonists did not inhibit the aggregation induced by 0.1 U/mL thrombin or 2 µmol/L U46619 under conditions where the participation of ADP secreted from platelet dense granules was precluded by addition of 0.2 U/mL apyrase, a concentration sufficient to block the aggregation induced by 5 µmol/L ADP (Fig 1C and D). A3P5P produced a parallel concentration-dependent shift to the right of the dose-response curve for ADP (Fig 2). The 50% efficacy concentrations (EC50) of ADP-induced platelet aggregation were 5.2 ± 4.0 µmol/L, 8.5 ± 5.1 µmol/L, 10.2 ± 6.3 µmol/L, 14.8 ± 10.2 µmol/L, and 20.8 ± 9.7 µmol/L in the presence of 0, 3, 10, 30, and 100 µmol/L A3P5P, respectively. Schild analysis of these data led to an apparent pA2 value of 5.3 (Kd = 3 µmol/L) and a slope of 0.9, which suggests that the antagonism by A3P5P of ADP-induced platelet aggregation is noncompetitive. The isomer A2P5P produced a similar right-hand shift of the dose-response curve for ADP. EC50 of ADP-induced platelet aggregation were 4.4 ± 1.2 µmol/L, 6.2 ± 1.6 µmol/L, 8.1 ± 4.1 µmol/L, 16.7 ± 3.5 µmol/L, and 34.4 ± 14.8 µmol/L in the presence of 0, 1, 3, 30, and 100 µmol/L A2P5P, respectively. Schild analysis of the inhibition by A2P5P gave a pA2 value of 5 and a slope of 0.55, which likewise suggests that the antagonism by A2P5P of ADP-induced platelet aggregation is noncompetitive.

P2Yi antagonists competitively inhibit ADP-induced [Ca2+] increases in platelets. B10 cells, and P2Yi-transfected cells. A3P5P (100 µmol/L) had no effect on intracellular calcium levels in fura-2–loaded washed human platelets, but produced a parallel concentration-dependent shift to the right of the dose-response curve for ADP-induced [Ca2+] increases in washed platelets resuspended in Tyrode’s buffer containing 0.35% human albumin and either 2 mmol/L calcium (Fig 3A) or no calcium (0.2 mmol/L EGTA) (data not shown). EC50 values for ADP were 0.29 ± 0.1 µmol/L, 0.55 ± 0.14 µmol/L, 1.2 ± 0.4 µmol/L, 4.6 ± 0.5 µmol/L, and 12.1 ± 3 µmol/L in the presence of 0, 3, 10, 30, and 100 µmol/L A3P5P, respectively. Schild analysis of these data gave an apparent pA2 value of 5.3 (Kd, 5 µmol/L) and a slope of 1.1, which suggests competitive antagonism by A3P5P of ADP-induced [Ca2+] increases in platelets. Identical inhibition of ADP-induced [Ca2+] increases was obtained using A2P5P. EC50 values for ADP were 0.54 ± 0.4 µmol/L, 2.4 ± 1.5 µmol/L, 4.5 ± 1.8 µmol/L, and 13.3 ± 5.7 µmol/L in the presence of 0, 10, 30, and 100 µmol/L A2P5P, respectively. Schild analysis of these data led to an apparent pA2 value of 5.5 (Kd, 3 µmol/L) and a slope of 0.9, which again
suggests competitive antagonism by A2P5P of ADP-induced [Ca^{2+}]_{i} increases in platelets. The two nucleotide analogues had, on the contrary, no effect on the [Ca^{2+}]_{i} increases induced by 2 μmol/L U46619 or 0.1 U/mL thrombin in platelets (Fig 3D).

A3P5P also produced a parallel right-hand shift of the dose-response curve for ADP-induced [Ca^{2+}]_{i} increases in Jurkat cells stably expressing the human P2Y_{1} receptor, either in the presence of 2 mmol/L external calcium (Fig 3B) or in its absence (0.2 mmol/L EGTA) (data not shown). EC_{50} values for ADP were 0.11 ± 0.07 μmol/L, 0.13 ± 0.04 μmol/L, 0.28 ± 0.07 μmol/L, 0.6 ± 0.07 μmol/L, and 2.3 ± 0.6 μmol/L in the presence of 0, 3, 10, 30, and 100 μmol/L A3P5P, respectively. Schild analysis gave an apparent pA_{2} of 5.1 (K_{D} 8 μmol/L) and a slope of 1.1, suggesting competitive antagonism of A3P5P at the P2Y_{1} receptor. Similar results were obtained using A2P5P. EC_{50} values for ADP were 0.10 ± 0.05 μmol/L, 0.75 ± 0.03 μmol/L, 1.75 ± 0.08 μmol/L, and 7.6 ± 0.6 μmol/L in the presence of 0, 10, 30, and 100 μmol/L A2P5P, respectively. Schild analysis gave an apparent pA_{2} of 5.5 (K_{D} 3 μmol/L) and a slope of 1, likewise suggesting competitive antagonism of A2P5P at the P2Y_{1} receptor. Once again, the two nucleotide analogues had no antagonistic effect on the [Ca^{2+}]_{i} increase induced by 1 U/mL thrombin (data not shown). In the case of the B10 clone of rat BCEC, A3P5P, and A2P5P both inhibited the [Ca^{2+}]_{i} increase in response to stimulation by 1 μmol/L ADP, with 50% inhibitory concentrations (IC_{50}) of 6.6 ± 0.1 μmol/L and 10.3 ± 0.4 μmol/L, respectively (Fig 3C), corresponding to Ki values of 3.1 μmol/L and 4.8 μmol/L, respectively. At higher concentration (100 μmol/L), the two nucleotide analogues completely abolished the action of 1 μmol/L ADP. However, they once again had no antagonistic effect on the [Ca^{2+}]_{i} increase induced by 0.1 U/mL thrombin (data not shown) in B10 cells.

Lack of effect of P2Y_{1} antagonists on ADP-induced inhibition of adenylyl cyclase activity. A2P5P and A3P5P (100 μmol/L) had no impact on basal levels of cyclic AMP in human platelets (data not shown). A3P5P likewise had no influence on the increased cyclic AMP levels induced by 1 μmol/L ADP, with 50% inhibitory concentrations (IC_{50}) of 6.6 ± 0.1 μmol/L and 10.3 ± 0.4 μmol/L, respectively (Fig 3C), corresponding to Ki values of 3.1 μmol/L and 4.8 μmol/L, respectively. At higher concentration (100 μmol/L), the two nucleotide analogues completely abolished the action of 1 μmol/L ADP. However, they once again had no antagonistic effect on the [Ca^{2+}]_{i} increase induced by 0.1 U/mL thrombin (data not shown) in B10 cells.

**Fig 3.** Competitive inhibition by A3P5P of ADP-induced [Ca^{2+}]_{i} increases in washed human platelets (A) and in Jurkat cells stably expressing the human P2Y_{1} receptor (B). [Ca^{2+}]_{i} was stimulated by ADP alone or in the presence of increasing concentrations of A3P5P, in the presence of 2 μmol/L external calcium. (C) Effects of increasing concentrations of A2P5P and A3P5P on [Ca^{2+}]_{i} increases induced by 1 μmol/L ADP in B10 cells. (D) A3P5P (100 μmol/L) did not inhibit [Ca^{2+}]_{i} increases induced by 2 μmol/L U46619 (1, left) or 0.1 U/mL thrombin (1, right) in washed human platelets. Curves each represent the mean of three independent experiments and bars show the SEM.
intracellular calcium increases, and adenylyl cyclase inhibition. 8,13

In B10 cells, A3P5P (100 µmol/L) had no influence on basal levels of cyclic AMP. Cyclic AMP increased fourfold in the presence of 1 µmol/L forskolin, and A3P5P had no effect on this stimulation (data not shown). Conversely, addition of 1 µmol/L ADP to forskolin-stimulated cells resulted in a 40% reduction in cyclic AMP levels (Fig 4B). A3P5P or A2P5P (100 µmol/L) did not modify the inhibitory effect of ADP on adenylyl cyclase activity (Fig 4B), whereas under the same conditions, Sp-ATPαS (100 µmol/L) totally reversed the inhibitory action of 1 µmol/L ADP on cyclic AMP levels (Fig 4B). Finally, in Jurkat cells stably expressing the P2Y1 receptor, no positive or negative influence of A2P5P or A3P5P on adenylyl cyclase activity could be detected (data not shown).

Inhibition of ADP-induced aggregation by P2Y1 antagonists is not reversed by epinephrine. Epinephrine potentiates platelet aggregation induced by low concentrations of ADP (Fig 5A). In the presence of 100 µmol/L A3P5P, which completely inhibited aggregation and shape change, epinephrine could no longer potentiate any platelet response (Fig 5B). Cyclic AMP formation was examined under the same conditions and was found to be inhibited by both ADP and epinephrine (Fig 5C).

DISCUSSION

In a previous report, we presented the pharmacologic characteristics of the human P2Y1 receptor heterologously expressed in Jurkat cells. 25 ADP was shown to be a selective agonist of this receptor, while freshly purified ATP was an ineffective agonist, but competitively antagonized the action of ADP. Because P2Y1 receptor transcripts were found to be present in platelets and megakaryoblastic cell lines, we suggested that the P2Y1 receptor could be similar to the platelet P2T receptor for ADP. However, 2MeSATP and 2ClATP were still found to be agonistic to the P2Y1 receptor-transfected cells, contrary to their known antagonistic action on platelets. 13 It was suggested that the triphosphate analogues could have been metabolized into the corresponding diphosphates by ectoenzymes, thus explaining their apparent agonistic effect. This was later confirmed by the finding that when the purity of adenine triphosphate nucleotides was controlled with a creatine phosphokinase/creatinine phosphate ATP regenerating system, all triphosphate nucleotide derivatives were antagonists to the P2Y1 receptors on Jurkat cells and on the B10 clonal cell line of rat BCEC. 38 These data thus support the hypothesis that the P2Y1 receptor common to platelets and endothelial cells could be the P2T receptor.

In the present work, we further addressed the question as to whether the P2Y1 receptor could be the elusive P2T receptor. As probes, we chose A3P5P and A2P5P, two adenine nucleotide isomers recently demonstrated to be competitive and selective antagonists of the P2Y1 receptor. 35 The effects of these compounds on human platelets were compared with their effects on the heterologously expressed human P2Y1 receptor and on the P2Y1 receptor endogenously expressed on the B10 clonal cell line of rat BCEC. This clone is useful for studies of the P2Y1 receptor, as in contrast to other endothelial cell lines, which express both P2Y1 and P2Y2 and thus display confusing ligand structure-activity relationships, the B10 clone expresses only the P2Y1 receptor. 35,34

A3P5P and its isomer A2P5P both specifically inhibited ADP-induced platelet shape change and aggregation, demonstrating the critical role of the P2Y1 receptor in these events. Inhibition of ADP-induced aggregation was observed at relatively low concentrations of A2P5P and A3P5P and these compounds exhibited potencies (pA2 5) similar to that of the natural competitive antagonist ATP (pA2 4.6). 13 However, this effect on aggregation was found to be noncompetitive, suggesting that more than one receptor could be involved in a highly complex process. A2P5P and A3P5P were nevertheless specific and competitive antagonists of the [Ca2+]i increases induced by ADP in human platelets, in B10 cells, and in Jurkat cells stably expressing the human P2Y1 receptor, which clearly demonstrates the essential role of the calcium pathway triggered by the P2Y1 receptor in ADP-induced platelet aggregation. In further support of this view, it was recently shown that platelets from mice lacking the Gαq subunit of the phospholipase C activating G-protein Gq are unable to aggregate in response to ADP, while Inositol-1,4,5-triphosphate formation and calcium signaling are totally abolished. 39
In contrast to their inhibitory effect on calcium mobilization, A2P5P and A3P5P had no influence on the inhibition by ADP of stimulated adenylyl cyclase activity in platelets or B10 cells, even at high concentration (100 µmol/L). This indicates that under conditions where platelet aggregation was blocked, ADP could still promote the inhibition of adenylyl cyclase. It is well established that inhibition of adenylyl cyclase is not alone sufficient to support platelet aggregation.40 This is the case, for instance, when platelets are stimulated by epinephrine, which by inhibiting adenylyl cyclase in the absence of an increase in intracellular calcium, does not induce platelet aggregation, but potentiates the response to all other aggregating agents.41 Figure 5 shows that under conditions where the P2Y1 receptor was completely antagonized, epinephrine could no longer potentiate any response, even though adenylyl cyclase was still inhibited by ADP. Thus P2Y1 is absolutely necessary for ADP to induce aggregation, and inhibition of adenylyl cyclase by ADP or epinephrine is not sufficient to promote an aggregation response.

In Jurkat cells stably expressing the human P2Y1 receptor, it was not possible to detect any positive or negative coupling of the receptor to adenylyl cyclase. Although this might be due to weak expression of the P2Y1 receptor in these cells, it more probably reflects the fact that the P2Y1 receptor is selectively coupled to calcium mobilization rather than to adenylyl cyclase inhibition.42 The specific inhibition by A2P5P and A3P5P of the intracellular calcium increases induced by ADP in platelets and endothelial cells, in the absence of any inhibition of the effects of ADP on adenylyl cyclase in these cells, points to the existence of two ADP receptors, the P2Y1 receptor coupled to calcium movements and an as yet unidentified receptor coupled to adenylyl cyclase inhibition. Other lines of evidence reinforce this hypothesis. Thus, thienopyridines inhibit ADP-induced platelet aggregation and inhibition of adenylyl cyclase without affecting the concomitant ADP-induced [Ca2+]i increase.14,15 Clopidogrel, in particular, inhibits only 70% of the binding of radiolabeled 2MeSADP, leaving residual binding sites even at the highest doses giving maximal blockade of platelet aggregation.18,19 The compound, ARL66096, an ATP analogue that has been proposed as a selective P2T antagonist on the basis of its inhibitory effect on ADP-induced platelet aggregation,43 has been recently reported to block ADP-induced inhibition of adenylyl cyclase without affecting ADP-mediated [Ca2+]i increases or shape change.44 Overall, these data strongly support the view that a full aggregation response when platelets are stimulated with ADP involves the P2Y1 receptor, which triggers calcium signaling, shape change, and initial aggregation, while another ADP receptor coupled to the inhibition of adenylyl cyclase potentiates and completes the initial response. This amplification pathway would also be involved in aggregation induced by other agonists when ADP is released from platelet dense granules. One can then speculate that the antithrombotic properties of clopidogrel and ARL66096 are due to blockade of this ADP pathway whatever the original stimulus. In earlier work, we reported that a full aggregation response could be restored in the platelets of ticlopidine-treated humans by inhibiting adenylyl cyclase with epinephrine.15 Similarly, in the case of the specific defect of ADP-induced platelet aggregation described in two patients5,6 who display a “ticlopidine-like syndrome”,19 one may anticipate that the defect should lie on the receptor coupled to adenylyl cyclase. Indeed, the calcium
response is almost normal in these patients and shape change is not abolished, whereas 2MeSADP binding sites are reduced and adenylyl cyclase inhibition is blocked. Sequencing of the P2Y₁ receptor gene and platelet mRNA along with functional studies using selective P2Y₁ antagonists will be required to resolve this question.

The molecular identity of the ADP receptor coupled to inhibition of adenylyl cyclase should be of the P2Y type, as we have previously shown that ADP activates the Gₛ₃₂ heterotrimeric G-protein in human platelet membranes.⁴⁵ Such a receptor should exhibit a pharmacologic profile identical to that of the P2Y₁ receptor. ADP being an agonist and triphosphate nucleotides competitive antagonists, but with subtle differences in the selectivity of a number of ligands. A₂P₅P and A₃P₅P, in particular, do not appear to interact with this receptor. Conversely, two other adenine nucleotide derivatives, 2-methylthioadenosine 5′-β, γ-methylentetraphosphonate (2MeSATMPCP) and 2-ethylthioadenosine 5′-monophosphonate (2EsAMP), reported to selectively and competitively inhibit the effects of ADP on adenylyl cyclase in platelets while only partially inhibiting ADP-induced platelet aggregation,⁴⁶ should interact with this receptor. Other compounds have long been known to display specificity for shape change and aggregation or for inhibition of adenylyl cyclase. Thus, ADP/xS induces platelet aggregation without affecting adenylyl cyclase, whereas the thiol reagent p-mercurybenzoysulfate (pCMBS) blocks inhibition of adenylyl cyclase, but not shape change (extensively reviewed in Mills⁸). Altogether, the currently available data best fit a model of two P2Y receptors mediating the effects of ADP on platelet aggregation.

Because of the key pharmacologic feature of agonism by ADP and antagonism by ATP, an adenylyl cyclase-coupled P2Y receptor (P2Ycyc) should display high molecular identity with the P2Y₁ receptor and be detectable by RT-PCR using wide receptor (P2Ycyc) should display high molecular identity with ADP and antagonism by ATP, an adenylyl cyclase-coupled P2Y receptor, ADP being an agonist and triphosphate nucleotides competitive antagonists, but with subtle differences in the selectivity of a number of ligands. A₂P₅P and A₃P₅P, in particular, do not appear to interact with this receptor. Conversely, two other adenine nucleotide derivatives, 2-methylthioadenosine 5′-β, γ-methylentetraphosphonate (2MeSATMPCP) and 2-ethylthioadenosine 5′-monophosphonate (2EsAMP), reported to selectively and competitively inhibit the effects of ADP on adenylyl cyclase in platelets while only partially inhibiting ADP-induced platelet aggregation,⁴⁶ should interact with this receptor. Other compounds have long been known to display specificity for shape change and aggregation or for inhibition of adenylyl cyclase. Thus, ADP/xS induces platelet aggregation without affecting adenylyl cyclase, whereas the thiol reagent p-mercurybenzoysulfate (pCMBS) blocks inhibition of adenylyl cyclase, but not shape change (extensively reviewed in Mills⁸). Altogether, the currently available data best fit a model of two P2Y receptors mediating the effects of ADP on platelet aggregation.

Because of the key pharmacologic feature of agonism by ADP and antagonism by ATP, an adenylyl cyclase-coupled P2Y receptor (P2Ycyc) should display high molecular identity with the P2Y₁ receptor and be detectable by RT-PCR using wide range primer sets covering the known P2Y receptors. However, such experiments only allowed detection of the P2Y₁ receptor of B₁0 cells,⁴⁶ leaving open the search for P2Ycyc. A P2Y receptor of this type has been reported to be present in a subclone of glioma cells termed C₆-2B,⁴⁷ which has the advantage of expressing no other P2 receptors, but whether this receptor is the same as that of platelets remains to be established. One would wish to know the effects of compounds like ARL66096 on both C₆-2B and B₁0 cells to clarify this point and such compounds are unfortunately not yet commercially available.

In conclusion, our results demonstrate that platelets and endothelial cells share a common P2Y₁ receptor involved in ADP-induced vasodilation and platelet shape change and aggregation and that this receptor is necessary to trigger ADP-induced platelet aggregation. Our findings and other data from the literature also strongly suggest the existence of another as yet unidentified P2Y receptor coupled to the inhibition of adenylyl cyclase. This means that the receptor previously known as “P₂T” is probably composed of three distinct receptors, the P₂Y₁ receptor, the P₂Ycyc receptor, and the P₂X₁ receptor, the role of which appears to be discrete. Thus, the term “P₂T” should no longer be used to designate a specific molecular entity. In the near future, it should be possible to establish the respective contributions of each of these receptors not only to platelet aggregation induced by ADP itself, but also to the potentiation of platelet activation by ADP released in different physiologic situations such as adhesion or aggregation in response to thrombin or other strong platelet agonists.

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