Evidence for Two Distinct G-protein-coupled ADP Receptors Mediating Platelet Activation

Hans-Michael Jantzen, Laurent Gousset, Vinay Bhaskar[1], Diana Vincent, Albert Tai, Elwood E. Reynolds, Pamela B. Conley

From COR Therapeutics, Inc., South San Francisco, CA, USA

Summary

The identity of the receptors mediating platelet activation by ADP remains elusive. To distinguish between platelet ADP receptor subtypes, the effects of antagonists on platelet responses and the cloned $P2Y_1$ receptor, a putative platelet ADP receptor, have been investigated. 2-methylthio-AMP (2MeSAMP), an inhibitor of ADP-dependent platelet aggregation, antagonized ADP-mediated inhibition of adenylyl cyclase, competed with binding of [^{3}H]2-methylthio-ADP and inhibited the stimulation of [^{35}S]GTPγS binding. 2MeSAMP did not inhibit platelet shape change and was only a weak antagonist of intracellular calcium mobilization in platelets or in cells expressing the cloned human $P2Y_1$ receptor. By contrast, the $P2Y_1$ receptor antagonist adenosine 3',5'-diphosphate (A3P5P) inhibited ADP-induced platelet aggregation, completely abolished shape change, but did not antagonize ADP effects on cyclic AMP generation or [^{3}H]2-methylthio-ADP binding. However, A3P5P antagonized intracellular calcium mobilization in platelets and cells expressing the cloned $P2Y_1$ receptor. Furthermore, using a specific monoclonal antibody and flow cytometry, $P2Y_1$ receptor protein was detected on human platelets. These results support the existence of two G protein-coupled ADP receptors mediating platelet aggregation, one of which is coupled to G_i proteins and blocked by 2MeSAMP, whereas the second receptor is similar or identical to $P2Y_1$ and coupled to G_q.

Introduction

ADP induces platelet aggregation and shape change and, in concert with other agonists such as thrombin and collagen, contributes to hemostasis and to pathological thrombus formation and vascular occlusion. Inhibitors of ADP-dependent platelet activation are efficacious antithrombotic drugs (1). On the intracellular level, ADP induces various signaling events, including the activation of heterotrimeric G proteins, the inhibition of adenylyl cyclase and transient elevation of intracellular calcium. Conflicting data have been reported regarding the receptor-mediated activation of phospholipase C and A_2 (1-5).

The identity of the platelet ADP receptors mediating aggregation and other responses has been elusive. Despite some discrepancies, the pharmacological profile of platelet ADP responses and the high-affinity binding site for the potent agonist 2MeSADP were generally thought to be consistent with a unique platelet ADP receptor antagonized by ATP. The very rapid calcium influx caused by ADP in platelets has been attributed to an additional receptor with characteristics of a ligand-gated ion channel (2, 3, 5, 6). Other platelet membrane proteins that bind adenosine or ATP derivatives have been proposed to be ADP receptors but their functional relevance for platelet ADP responses remains to be defined (4, 7).

Receptors recognizing adenine and uridine nucleotides have been classified as P2 receptors, with G protein-coupled receptors being referred to as P2Y receptors and ligand-gated ion channels as P2X receptors (8-10). Since ADP activates heterotrimeric G-proteins and initiates signaling pathways typical of G-protein coupled receptors in platelets, it has been proposed to refer to the previously termed P2T receptor as $P2Y_{ADP}$, until the molecular structure has been clearly identified (10). Recently, it has been shown that in contrast to earlier observations, the previously cloned $P2Y_1$ receptor is an ADP receptor antagonized by ATP and therefore might be the elusive ADP receptor mediating platelet aggregation and typical G-protein coupled responses (11-13). In addition, $P2Y_1$ RNA has been detected in platelets and cells of megakaryoblastic origin (11). However, other investigators reported that antagonists of the cloned $P2Y_1$ receptor inhibited only some platelet responses to ADP. By contrast, ARL66096, a specific antagonist of ADP-dependent aggregation, selectively inhibited the cAMP response of ADP, contradicting the model of a single G protein-coupled ADP receptor (14, 15). The relevance for platelet function of the third ADP receptor, a ligand-gated ion channel of the P2X family, could not be determined (14-17).

In this study, three approaches have been used to help clarify the role of the $P2Y_1$-like receptor and other G protein-coupled ADP receptors on platelets. First, we have characterized previously unidentified selective properties of 2-methylthioadenosine 5'-monophosphate (2MeSAMP) and used it together with a $P2Y_1$ antagonist to discriminate between two ADP receptors on human platelets leading to aggregation, shape change, and intracellular signaling events. Second, the human $P2Y_1$ receptor has been cloned and expressed and the effect of the antagonists on the $P2Y_1$ receptor have been compared to those on platelet responses. Third, the presence on platelets of the $P2Y_1$ receptor protein was investigated with a monoclonal antibody.

Materials and Methods

Materials

Apyrase (grade V), forskolin, adenosine 3', 5' diphosphate (A3P5P), prostaglandin I_2, GDP, GTPγS, bovine serum albumin were obtained from Sigma (St. Louis, MO). 2MeSADP (2-methylthioadenosine 5'-diphosphate), 2MeSAMP (2-methylthioadenosine 5'-monophosphate, custom synthesis), IBMX (3-isobutyl-1-methylxanthine) and 8-(p-sulphophenyl)theophylline were from Research Biochemicals International (Natick, MA). ADP and protease inhibitors were purchased from Boehringer Mannheim (Indianapolis, IN). [^{3}H]2-MeS-ADP ([^{3}H]2-methylthioadenosine-5'-diphosphate, ammonium salt; specific activity 49 Ci/mmole, custom synthesis), the Biotrak cAMP[^{125}I] assay

[1] Present address: Mr. V. Bhaskar, Department of Chemistry and Biochemistry, University of California Los Angeles, Los Angeles, CA, USA

Correspondence to: H.-M. Jantzen, COR Therapeutics, Inc., 256 E. Grand Ave., South San Francisco CA 94080, USA – Tel.: +1 650 244 6831; FAX Number: +1 650 244 9208; e-mail: mjantzen@corr.com

system and [^{35}S]GTPγS were obtained from Amersham (Arlington Heights, IL). Human fibrinogen was from American Diagnostics (Greenwich, CT), Fura-2AM (fura-2 acetoxymethylester) from Molecular Probes (Eugene, OR), keyhole limpet hemocyanin from Pierce (Rockford, IL), cell culture reagents and G418 (Geneticin) from Gibco BRL (Gaithersburg, MD), the expression vector pcINeo from Promega (Madison, WI), and Jurkat cells from ATCC (Rockville, MD), F(ab')2 phycoerythrin-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Methods

Platelet Preparation and Aggregation

Human venous blood was collected from healthy, drug-free volunteers into ⅛ volume of ACD containing prostaglandin I_2 (85 mM sodium citrate, 111 mM glucose, 71.4 mM citric acid, 1.6 μM PGI_2). Platelet-rich plasma (PRP) was prepared by centrifugation at 160 × g for 20 min at room temperature. PRP was centrifuged for 10 min at 730 × g and the platelet pellet resuspended in CGS (13 mM sodium citrate, 30 mM glucose, 120 mM NaCl) containing 1 U/ml apyrase. After incubation at 37° C for 15 min, the platelets were collected by centrifugation at 730 × g for 10 min and resuspended at a concentration of 3×10^8 platelets/ml in HEPES-Tyrode's buffer (10 mM HEPES, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 12 mM $NaHCO_3$, pH 7.4) containing 0.1% BSA, 1 mM $CaCl_2$ and 1 mM $MgCl_2$. This platelet suspension was kept for at least 45 min at 37° C before use in aggregation assays. Inhibition of ADP-dependent aggregation was determined in 96-well flat-bottom microtiter plates at room temperature to facilitate simultaneous processing of samples (18). This method yielded results similar to those derived from aggregometer readings. The total reaction volume of 0.2 ml/well included in HEPES-Tyrodes buffer/0.1% BSA: 4.5×10^7 apyrase-washed platelets, 0.5 mg/ml human fibrinogen, serial dilutions of inhibitors and 2 μM ADP which induces submaximal aggregation. The absorption of the samples was then determined at 490 nm using a microtiter plate reader (Softmax, Molecular Devices) resulting in the 0 minute reading. The plates were then agitated for 5 min on a microtiter plate shaker and the 5 min reading obtained in the plate reader. Aggregation was calculated from the decrease of absorption at 490 nm at 5 min compared to 0 min and expressed as % of the decrease in the ADP control samples corrected for changes in the unaggregated control samples. Dose-response curves and IC_{50}s were derived by non-linear regression analysis from three independent experiments using the Prism software (GraphPad, San Diego CA).

Platelet Shape Change

Shape change was determined with apyrase-washed human platelets prepared as described above for aggregation assays. Platelet suspensions were diluted to 5×10^7 platelets/ml. EDTA (final concentration of 5 mM) was added under stirring to 0.5 ml platelet samples in an aggregometer (Chronolog) at 37° C, followed by antagonists and 1 μM ADP. Shape change resulted in a stable decrease of transmission.

Determination of Cyclic AMP Levels

Apyrase-washed human platelets were prepared as described above and resuspended in HEPES-Tyrode's buffer/0.1% BSA/1 mM $CaCl_2$ at a concentration of 10^8/ml at room temperature. Aliquots of 0.2 ml were preincubated with 0.5 mM IBMX for 10 min at 37° C. The platelets were then incubated for an additional 10 min with 50 μM forskolin, 1 μM ADP and antagonists as indicated. The incubation was terminated by addition of 0.4 ml of ice-cold ethanol. Extracts were obtained by mixing and centrifugation. Cyclic AMP levels were determined using a radioimmunoassay (Amersham Biotrak cAMP[^{125}I] assay system).

[^{3}H]2MeSADP Binding

[^{3}H]2MeSADP binding to washed human platelets was determined using a rapid filtration assay. Apyrase-washed platelets were prepared as described above, except for a final resuspension at a concentration of 6.7×10^8 platelets/ml in HEPES-Tyrode's buffer containing 0.1% BSA without $CaCl_2$ or $MgCl_2$ to reduce ectonucleotidase activity. Platelets were allowed to recover for 45 min at room temperature and then binding experiments were performed. Apyrase-washed platelets (1×10^8) were incubated with test compounds and 1 nM [^{3}H]2MeSADP in a final volume of 0.2 ml HEPES-Tyrode's buffer/0.1% BSA. Total binding was determined in the absence of test compounds and nonspecific binding in the presence of 10 μM unlabelled 2MeSADP. After 15 min incubation at 4° C reactions were terminated by addition of ice-cold 10 mM HEPES pH 7.4, 138 mM NaCl, rapid filtration through GF/C glassfiber filters and three subsequent washes using a cell harvester (Brandel, Gaithersburg, MD). The platelet-bound radioactivity was determined in a scintillation counter. Specific binding was determined by subtraction of nonspecific binding from total binding. Competition binding data were analyzed using the Prism software (GraphPad, San Diego, CA) and K_i's were estimated according to the equation $K_i = IC_{50}/(1 + [L]/K_D)$, where [L] was the concentration of [^{3}H]2MeSADP (1 nM) and K_D the affinity constant as determined by saturation binding (0.6 nM).

Binding of [^{35}S]GTPγS to Platelet Membranes

Human platelet membranes were prepared from apheresis units obtained from blood banks. Platelets were washed with CGS and resuspended in ice-cold 10 mM HEPES pH 7.4, 5 mM EGTA, 0.1 mM PMSF at about 10^{10} platelets/ml and aliquots sonicated 2-3 × for 10 s. Lysates were centrifuged for 10 min at 550 × g and the resulting supernatant spun for 30 min at 37,500 × g to pellet crude membranes. Membranes were stored frozen in 10 mM HEPES pH 7.4, 5 mM $MgCl_2$, 0.1 mM EDTA 0.1 mM PMSF, 20 μg/ml antipain, 8 μg/ml betastatin, 10 μg/ml chymostatin, 1 μg/ml F-64, 0.5 μg/ml leupeptin, 0.66 μg/ml pepstatin, 20 μg/ml phosporamidon, and 1 μg/ml aprotinin. Before use, thawed membranes were washed twice by resuspension and centrifugation at 13,000 rpm in an Eppendorf centrifuge with 50 mM Tris 7.5, 1 mM EDTA. [^{35}S]GTPγS binding assays were performed similar to published procedures (19) in 0.1 ml containing 10 μg membrane protein, 50 mM Tris pH 7.5, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 10 μM GDP, 0.5 nM [^{35}S]GTPγS, and agonists and antagonists as indicated. Nonspecific binding was determined in the presence of 10 μM GTPγS. After 45 min at room temperature, membrane-bound [^{35}S]GTPγS was determined by rapid filtration in a cell harvester (Brandel, Gaithersburg, MD) using GF/C filters and three subsequent washes with 20 mM Tris pH 7.5 and 5 mM $MgCl_2$. Bound radioactivity was determined by liquid scintillation counting. Specific binding was determined by subtracting nonspecific from total binding.

Cloning and Expression of the Human $P2Y_1$ Receptor

A genomic fragment encompassing the entire open reading frame of the human $P2Y_1$ receptor plus 220 bp of 3' untranslated region and 10 bp 5' to the ATG initiation codon was isolated from a human genomic DNA library using a human $P2Y_1$ probe derived from RT-PCR of Dami cell RNA and standard molecular biology techniques. The deduced amino acid sequence was as described (20). This fragment was cloned into the mammalian expression vector pcINeo and transfected into Jurkat cells by electroporation. G418 (Geneticin) selection and subcloning resulted in the clonal cell line hP2Y1-JA7 stably expressing the human $P2Y_1$ receptor. Transfected Jurkat cells were maintained in RPMI medium containing 10% fetal bovine serum and 0.8 mg/ml Geneticin. The pharmacological profile of the expressed receptor was verified in Ca^{++} mobilization assays with Fura-2 loaded cells.

Determination of Intracellular Ca^{++} Concentrations in Platelets and hP2Y1-JA7 Cells

Human platelet-rich plasma (PRP) was prepared as described above and incubated with 4 μM Fura-2AM in the presence of 0.008% Pluronic F-127 for 45 min at 37° C. Platelets were collected by centrifugation of PRP for 10 min at 730 × g, resuspended in CGS containing 1 U/ml apyrase and incubated at

37° C for 15 min. After centrifugation at 730 × g for 10 min platelets were resuspended at a concentration of 2×10^8 platelets/ml in HEPES-Tyrode's buffer containing 0.1% BSA and 1 mM $CaCl_2$ and kept at room temperature for calcium determinations.

Jurkat cells expressing the $hP2Y_1$ receptor (hP2Y1-JA7 cells) were washed and resuspended in HEPES-Tyrodes buffer/0.1% BSA/1 mM $CaCl_2$ at 10^7 cells/ml at 37° C. Cells were then loaded with 4 μM Fura-2AM in the presence of 0.008% Pluronic F-127 for 30 min at 37° C. After centrifugation, cells were incubated for 15 min at 37° C in buffer with 1 U apyrase/ml and after a final spin resuspended at a concentration of 2×10^6 cells/ml at 4° C.

Intracellular calcium measurements were performed with an SLM-Aminco AB2 spectrofluorimeter using the ratio method (excitation wavelengths: 340 and 380 nm; emission wavelength: 510 nm). Aliquots of platelets or cells (0.5 ml) were warmed up for 1 min at 37° C and calcium responses were determined after addition of submaximal agonist concentrations (10 μM ADP for platelets and 0.1 μM 2MeSADP for hP2Y1-JA7 cells) in the absence and presence of various concentrations of test compounds. Maximum fluorescence ratios were obtained by addition of 100 μM digitonin, minimum ratios after addition of 20 mM Tris and 10 mM EGTA. Fluorescence ratio measurements were then converted to calcium concentrations (21). Increases in intracellular calcium levels were determined by subtraction of baseline levels from peak calcium levels.

Generation of Monoclonal Antibodies to the Human $P2Y_1$ Receptor and Flow Cytometry

A peptide corresponding to the amino-terminal 16 amino acids of the deduced $P2Y_1$ protein sequence (MTEVLWPAVPNGTDAAC) was synthesized using an Applied Biosystems 431 peptide synthesizer with a carboxy-terminal cysteine residue added for conjugation. The peptide was conjugated to keyhole limpet hemocyanin according to manufacturer's specifications for use as antigen (50-100 μg/mouse) to immunize IRCF-1 mice. Positive hybridomas were detected by peptide ELISA. Supernatants from positive clones were screened by flow cytometry for activity against mammalian cells using the hP2Y1-JA7 cell line and clones with the best responses used to generate ascites. Antibodies were purified from ascites using protein A-sepharose (22). For flow cytometry, washed human platelets, Jurkat and hP2Y1-JA7 cells were resuspended in FACS Buffer (phosphate-buffered saline containing 0.1% BSA and 2% heat-inactivated fetal bovine serum). Cells (8×10^5 platelets, 5×10^5 Jurkat or hP2Y1-JA7 cells) were incubated with 5-20 μg/ml of the anti-hP2Y1 monoclonal antibody (PY1-520.1) in a total volume of 50 μl for 30 min at 4° C. In some experiments, the anti-$P2Y_1$ antibody was preincubated with a 200-fold molar excess of the amino-terminal $P2Y_1$ peptide for 30 min at room temperature. Cells were then washed with ice-cold FACS buffer and incubated with 2.5 μg/ml of F(ab')2 phycoerythrin-conjugated goat anti-mouse antibody for 30 min at 4° C. Cells and platelets were washed and resuspended in ice-cold FACS buffer and fluorescence of cell-bound phycoerythrin-conjugated secondary antibody was determined with a FACSort flow cytometer (Becton-Dickinson, San Jose, CA). Control samples contained cells without antibodies for determination of autofluorescence or cells with secondary antibody alone.

Results

Effect of P2 Receptor Antagonists on ADP-induced Platelet Aggregation

A major limitation for the investigation of the physiological role of P2 receptor subtypes on cells is the lack of a panel of selective antagonists. 2MeSAMP (2-methylthioadenosine 5'-monophosphate) has been described as an antagonist of ADP-induced platelet aggregation, but effects on other platelet responses have not been published (23, 24). A3P5P is a competitive and selective antagonist of the cloned human $P2Y_1$ receptor and has been used in this study to address the putative role of the $P2Y_1$ receptor on platelets (25). First, the effect of the antagonists on platelet aggregation was determined. ADP induced the aggregation of washed platelets with an EC_{50} of 0.76 ± 0.25 μM (mean ± s.d., n = 7). Aggregation induced by 2 μM ADP was antagonized by 2MeSAMP with an IC_{50} of 1.0 ± 0.2 μM (n = 3, Fig. 1). Interestingly, the $P2Y_1$ antagonist A3P5P inhibited aggregation only partially with 50% inhibition at 67 ± 21 μM. The inhibitory effect of 2MeSAMP on aggregation appeared to occur directly on ADP receptors and not indirectly by activation of platelet adenosine A_{2A} receptors because addition of 0.3 mM 8-(p-sulphophenyl)theophylline, an adenosine receptor antagonist, did not decrease their potency (data not shown).

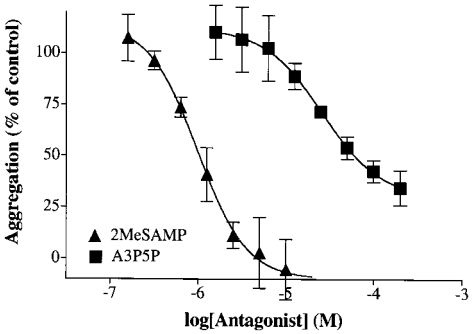

Fig. 1 Effects of P2 receptor antagonists on ADP-induced platelet aggregation. Aggregation of washed platelets was induced by 2 μM ADP in the absence and presence of the indicated concentrations of 2MeSAMP and A3P5P as described in Methods. Aggregation responses were normalized to the control without antagonists (100%). Results are the mean ± s.d. of three independent experiments each performed in duplicate

Effect of P2 Receptor Antagonists on ADP-mediated Repression of Cyclic AMP Levels

ADP represses elevated cyclic AMP levels in platelets consistent with a G_i protein-linked ADP receptor coupled to inhibition of adenylyl cyclase. Therefore we investigated whether 2MeSAMP and A3P5P inhibit ADP-induced aggregation by blocking the adenylyl cyclase-coupled receptor. We have previously established that in washed platelets, forskolin-stimulated cyclic AMP levels were repressed 90-95% by ADP with a half-maximal effect at 0.3 μM. The inhibitory effect of 1 μM ADP on stimulated cyclic AMP levels was antagonized by 2MeSAMP with an IC_{50} of 0.57 ± 0.11 μM (n = 3, Fig. 2a). The potency of 2MeSAMP in reversing the cyclic AMP effect of ADP was comparable to the potency in inhibiting ADP-induced platelet aggregation. However, the $P2Y_1$ antagonist A3P5P, which partially inhibited aggregation, had no effect even at a concentration of 200 μM (Fig. 2a). No direct effect of the antagonists on basal or forskolin-stimulated cyclic AMP levels in the absence of ADP was observed at 10 μM 2MeSAMP and 200 μM A3P5P (data not shown).

Inhibition of [^{3}H]2MeSADP Binding to Platelets

Ligand binding studies using the potent radiolabeled agonist 2MeSADP have previously demonstrated the existence of a single high affinity receptor population on human platelets (3). Consistent with these data, we have obtained a K_D of 0.6 ± 0.2 nM (n = 7) for [^{3}H]2MeSADP binding to washed platelets. In competition binding studies using 1 nM [^{3}H]2MeSADP, 2MeSAMP inhibited binding with a K_i of 0.23 ± 0.1 μM (n = 3, Fig. 2b). The $P2Y_1$ antagonist A3P5P had no effect on [^{3}H]2MeSADP binding at concentrations up to 300 μM.

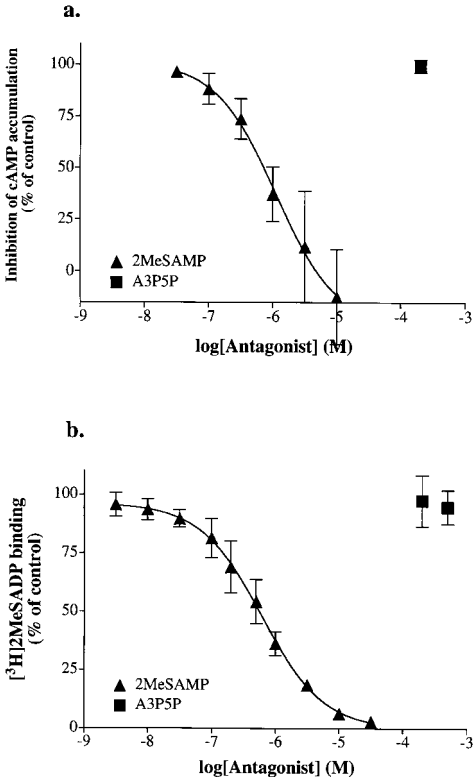

Fig. 2 Effects of P2 receptor antagonists on ADP-mediated inhibition of cyclic AMP accumulation and [^{3}H]2MeSADP binding to platelets. a) Effects of 2MeSAMP and A3P5P on ADP-mediated inhibition of cyclic AMP accumulation in platelets were determined as described in Methods. Washed platelets were preincubated with 0.5 mM IBMX followed by addition of the indicated concentrations of antagonists, 50 μM forskolin and 1 μM ADP. Cyclic AMP was extracted and quantified by radioimmunoassay. Antagonist effects were normalized to the ADP response on cyclic AMP accumulation in the absence of antagonists (100%). Results are the mean ± s.d. of three independent experiments each performed in triplicate. b) Effects of 2MeSAMP and A3P5P on [^{3}H]2MeSADP binding to platelets were determined as described in Methods. Washed platelets were incubated with 1 nM [^{3}H]2MeSADP and the indicated concentrations of antagonists and bound radioactivity determined by rapid filtration. Results are the mean ± s.d. of three independent experiments each performed in triplicate normalized to specific [^{3}H]2MeSADP binding in the absence of antagonists

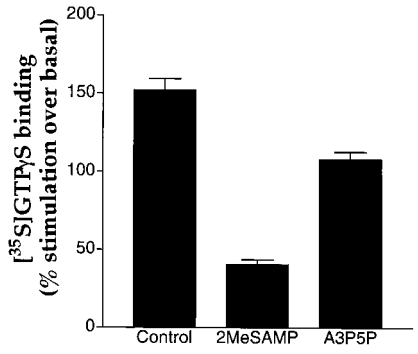

Fig. 3 Effects of P2 receptor antagonists on 2MeSADP-induced [^{35}S]GTPγS binding to platelet membranes. Specific binding of [^{35}S]GTPγS to platelet membranes was stimulated with 0.1 μM 2MeSADP in the absence and presence of 100 μM 2MeSAMP or 200 μM A3P5P. Results are the mean ± s.d. from a representative experiment performed in triplicate and were normalized to % stimulation of [^{35}S]GTPγS binding over basal binding

Effect of P2 Receptor Antagonists on 2MeSADP-induced [^{35}S]GTPγS Binding to Platelet Membranes

In platelet membranes the ADP receptor agonist 2MeSADP has been demonstrated to activate heterotrimeric G proteins, especially $G\alpha_{i2}$ (19, 26). The effect of 2MeSAMP and A3P5P on G protein activation have been determined in [^{35}S]GTPγS binding experiments with platelet membrane preparations. 2MeSADP alone at the submaximal concentration of 0.1 μM induced [^{35}S]GTPγS binding about 3-fold. This effect was inhibited by 100 μM 2MeSAMP. Interestingly, the $P2Y_1$ antagonist A3P5P was only a weak inhibitor at a concentration of 200 μM (Fig. 3).

Inhibition of Intracellular Calcium Mobilization in Platelets

ADP induces calcium mobilization from intracellular stores followed by calcium entry from the extracellular medium. Since the potency of agonists and antagonists in calcium mobilization assays correlated with their effects on platelet aggregation it has been proposed that both responses are mediated by the same ADP receptor . In contrast to this hypothesis, 2MeSAMP which antagonized platelet aggregation, the cyclic AMP response, G protein activation and [^{3}H]2MeSADP binding (Figs. 1-3) had only a weak effect on ADP-induced calcium mobilization in Fura-2 loaded platelets even at concentrations up to 300 μM (Fig. 4a). However, this response was inhibited by the $P2Y_1$ antagonist A3P5P with an IC_{50} of 45.4 ± 5.0 μM comparable to the IC_{50} for the inhibition of platelet aggregation (n = 3).

Effect of P2 Receptor Antagonists on the Cloned Human $P2Y_1$ Receptor

To address the role of the $P2Y_1$ receptor in platelet function, the human $P2Y_1$ receptor has been cloned and expressed in mammalian cells and the effects of 2MeSAMP and A3P5P were evaluated. In Jurkat cells, as reported for other expression systems, the cloned $hP2Y_1$ receptor couples to phospholipase C but not to adenylyl cyclase. When intracellular calcium mobilization was analyzed with freshly HPLC-purified nucleotides in $hP2Y_1$-expressing cells loaded with Fura-2, the $hP2Y_1$ receptor behaved like an ATP-antagonized ADP receptor similar to platelet ADP receptors (H.-M. Jantzen, data not shown), confirming recent reports (11, 13). 2MeSAMP at a concentration of 100 μM had only a weak effect on $hP2Y_1$-dependent calcium mobilization (Fig. 4b). The $P2Y_1$ antagonist A3P5P inhibited the $hP2Y_1$ response with an IC_{50} of 9.7 ± 1.2 μM (n = 3), similar to the inhibition of intracellular calcium mobilization in platelets (Fig. 4a).

Inhibition of ADP-mediated Platelet Shape Change

Upon exposure to ADP platelets rapidly change their shape from discoid to spherical and spiny which results in a decrease of light transmission in an aggregometer. 2MeSAMP, an antagonist of aggregation, cyclic AMP response and G_i-protein activation, had no effect on ADP-induced platelet shape change at concentrations up to 200 μM. By contrast, the $P2Y_1$ receptor antagonist A3P5P partially inhibited shape change at 100 μM and abolished it at 200 μM (Fig. 5).

Detection of the Human $P2Y_1$ Receptor Protein on Platelets

The presence of $P2Y_1$ receptor RNA in platelets has been previously detected by RT-PCR and $P2Y_1$ clones have been isolated from plate-

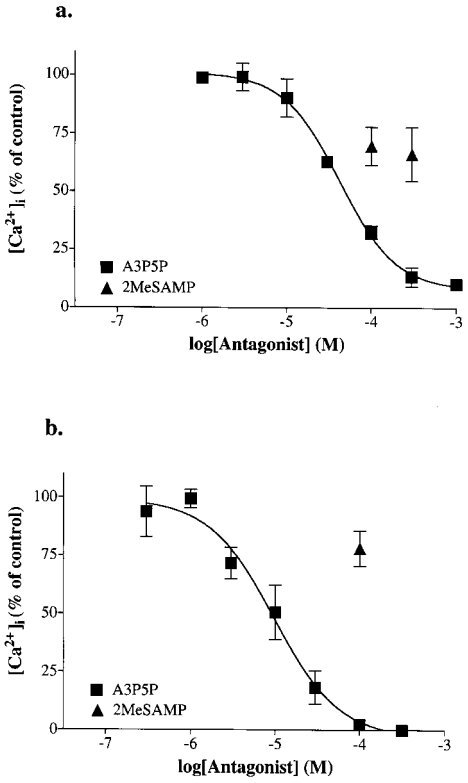

Fig. 4 Effects of P2 receptor antagonists on intracellular calcium mobilization in platelets and cells expressing the cloned human $P2Y_1$ receptor. a) Peak elevations of intracellular calcium were determined in Fura-2 loaded washed platelets with 10 μM ADP and the indicated concentrations of 2MeSAMP and A3P5P. Results are the mean ± s.d. of three independent experiments each performed in duplicate normalized to the calcium response in the absence of antagonists. b) Jurkat cells expressing the cloned $hP2Y_1$ receptor were loaded with Fura-2 and peak elevations of intracellular calcium determined with 0.1 μM 2MeSADP and the indicated concentrations of 2MeSAMP and A3P5P. Results are the mean ± s.d. of three independent experiments each performed in duplicate normalized to the calcium response in the absence of antagonists

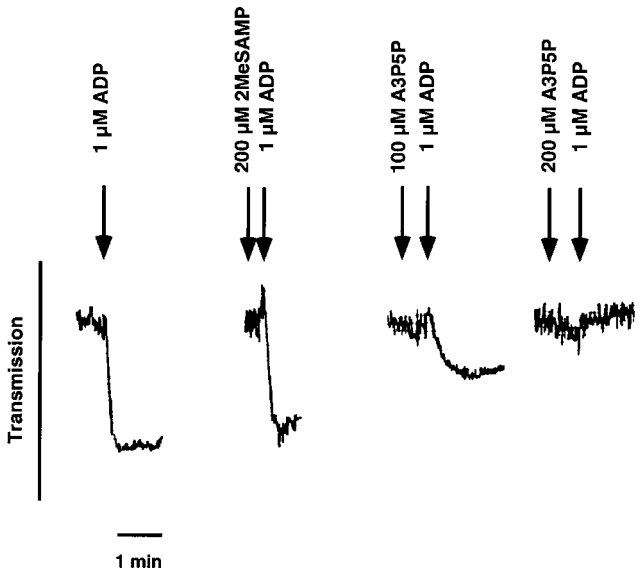

Fig. 5 Effect of P2 receptor antagonists on ADP-mediated platelet shape change. Shape change was determined in washed platelets as described in Methods. Representative aggregometer tracings are shown with arrows indicating addition of 1 μM ADP or antagonists

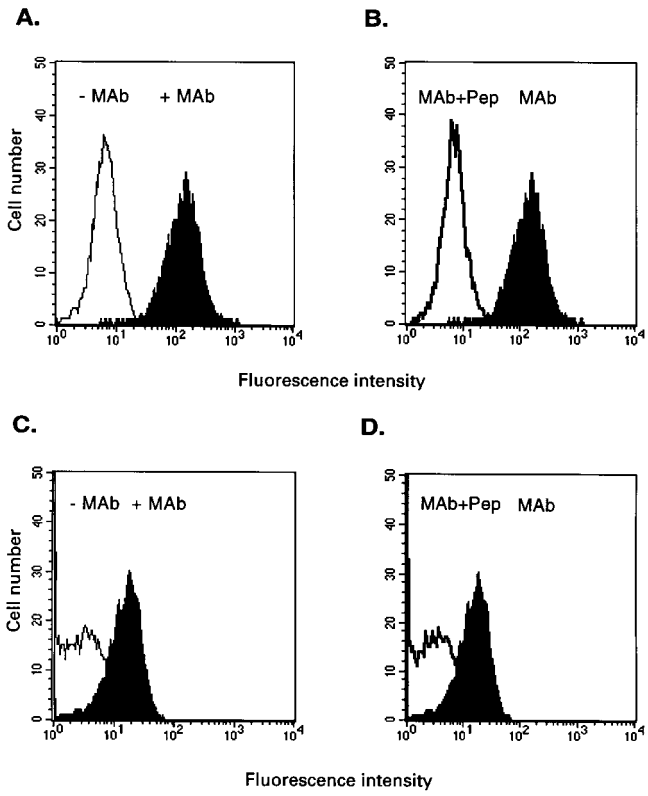

Fig. 6 Binding of anti-$P2Y_1$ monoclonal antibody to platelets and Jurkat cells expressing the $P2Y_1$ receptor. a) Jurkat cells expressing the cloned human $P2Y_1$ receptor (hP2Y1-JA7) were incubated with or without 10 μg/ml anti-$P2Y_1$ monoclonal antibody followed by phycoerythrin-coupled secondary antibody. Fluorescence intensities of bound antibody were determined by flow cytometry. b) hP2Y1-JA7 cells were incubated with 10 μg/ml anti-$P2Y_1$ monoclonal antibody in the absence or presence of a 200-fold molar excess of $P2Y_1$-peptide followed by phycoerythrin-coupled secondary antibody. c, d) Flow cytometry experiments with washed human platelets. Incubations were the same as in (a) and (b). Data shown are from one representative experiment out of three performed in duplicate or triplicate

let cDNA libraries (11, 15, P. Conley, unpublished observations). However, the presence of $P2Y_1$ protein on the platelet surface has not yet been demonstrated. To investigate the $P2Y_1$ receptor protein expression, mouse monoclonal antibodies have been generated against a peptide comprising the 16 amino-terminal amino acids of the human $P2Y_1$ receptor. Binding of purified antibody to hP2Y1-JA7 cells expressing the cloned $P2Y_1$ receptor was determined by flow cytometry. At an antibody concentration of 10 μg/ml, binding resulted in a 19-fold shift of the mean fluorescence intensity compared to controls incubated with the secondary antibody alone (Fig. 6a). When the antibody was preincubated with a 200-fold molar excess of the amino-terminal $P2Y_1$ peptide, binding was reduced to control values (Fig. 6b). Furthermore, no anti-$P2Y_1$ antibody binding to untransfected Jurkat cells was detected (data not shown). Importantly, the same anti-$P2Y_1$ antibody bound to washed human platelets resulting in a 6-fold shift of the fluorescence when compared with the control (Fig. 6c) and this binding was eliminated by preincubation with the amino-terminal $P2Y_1$ peptide (Fig. 6d).

Discussion

The identity of the platelet receptors mediating ADP responses is still unclear. Here we report new evidence for the presence of two G

protein-coupled ADP receptors on platelets, one of them likely to be $P2Y_1$. Selective antagonists, such as ARL66096, which distinguish between the two receptors (14, 15, 27) are not generally available. We have identified 2MeSAMP, previously described only as an antagonist of ADP-dependent platelet aggregation (23, 24) as a preferential antagonist of the ADP receptor coupled to the inhibition of adenylyl cyclase, and as a competitor of 2MeSADP binding and G protein activation as determined by [^{35}S]GTPγS binding. 2MeSAMP does not affect platelet shape change and has only small effects on intracellular calcium mobilization. Using the cloned human $P2Y_1$ receptor expressed in mammalian cells, we have determined that 2MeSAMP affects the $P2Y_1$ receptor only at high concentrations, and therefore is a preferential antagonist of a G_i-linked ADP receptor distinct from $P2Y_1$, validating the model of two G protein-coupled ADP receptors on human platelets (14, 27). The IUPHAR Committee on Receptor Nomenclature has proposed to name G-protein coupled platelet ADP receptors $P2Y_{ADP}$ instead of P2T until they have been definitively cloned (10). We therefore refer to the G_i-linked receptor as $P2Y_{ADP}$.

The results with the $P2Y_1$ antagonist A3P5P confirm that a $P2Y_1$-like receptor is not only mediating intracellular Ca^{++} mobilization and shape change, but also aggregation of human platelets. Furthermore, we demonstrate the presence of $P2Y_1$ receptor protein on the surface of platelets using a monoclonal antibody. Based on the pharmacological data and the detection of $P2Y_1$ protein on human platelets, it is intriguing to speculate that the receptor mediating Ca^{++} mobilization is in fact $P2Y_1$. However, until definitive proof is available, e.g. from loss of function experiments using blocking antibodies or gene knockouts, this receptor might be better referred to as a $P2Y_1$-like receptor.

The data in this manuscript strongly support the hypothesis of two different G-protein coupled receptors initiating signaling pathways leading to an aggregation response, whereas only one pathway mediates platelet shape change, confirming and extending the model proposed by Daniel et al. (14) (Fig. 7). However, both pathways are not redundant, since antagonism of either receptor is sufficient to strongly inhibit aggregation. It is possible that the amount of signal generated by each receptor pathway is insufficient for aggregation, but both together, either additively or synergistically, can lead to a full response (Fig. 7). In this context, the observation that platelet aggregation by ADP and other agonists was strongly inhibited in mice lacking $G\alpha_q$, further supports the role of a G_q-coupled receptor like $P2Y_1$ in aggregation (28).

Whereas one of the ADP receptors on platelets might be $P2Y_1$, the molecular identity of the G_i-linked ADP receptor is unknown. None of the cloned P2Y receptors have been demonstrated to couple to the inhibition of adenylyl cyclase. $P2Y_1$ does not seem to be linked to this pathway even in rat glioma C6-2B cells, which express an endogenous G_i-linked ADP receptor (29). It is possible that this C6-2B receptor might be similar or identical to the elusive ADP receptor.

It is intriguing that [^{3}H]2MeSADP binding sites are not displaced by A3P5P and therefore do not represent the $P2Y_1$-like receptor, although 2MeSADP is a potent agonist of intracellular calcium mobilization in platelets and the cloned $P2Y_1$ receptor (20). These data are consistent with previous observations that the potency of compounds in competing with [^{3}H]2MeSADP binding correlates much better with their potency in affecting ADP-mediated inhibition of adenylyl cyclase than Ca^{++} mobilization (2, 3). Recently, it has been shown that A3P5P can displace a small subpopulation of [^{3}H]2MeSADP binding sites on rabbit platelets (30). Although we have been unable to detect this subpopulation in human platelets, potentially due to lower $P2Y_1$ receptor number on human vs. rabbit platelets or because of different experimental

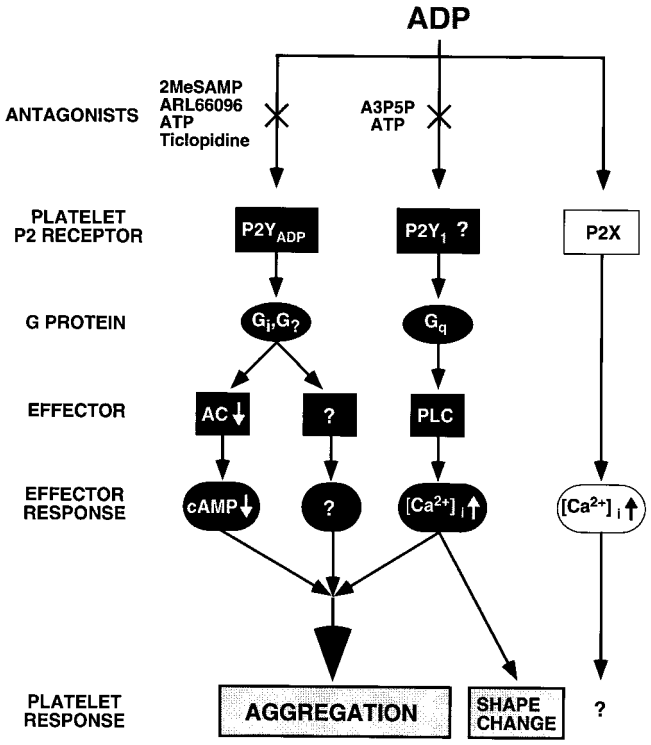

Fig. 7 ADP receptors on platelets. This diagram shows the two proposed G protein-coupled ADP receptors on human platelets mediating aggregation or shape change: A G_i-linked P2Y receptor mediates inhibition of adenylyl cyclase, leads to aggregation and is the target of antithrombotic drugs. The second receptor is similar or identical to the cloned G_q-coupled $P2Y_1$ receptor and mediates elevation of intracellular calcium, aggregation and shape change. The physiological role of the $P2X_1$-like ligand-gated ion channel mediating rapid calcium entry is still unknown (see discussion)

procedures, the data are consistent with the majority of the binding sites representing the G_i-linked ADP receptor.

Previously, platelet ADP receptors have been shown to couple to G proteins in membrane preparations, but only activation of $G\alpha_{i2}$ could be clearly identified (19, 26). We have shown here that 2MeSAMP, the antagonist of the adenylyl cyclase-coupled receptor inhibits stimulation of [^{35}S]GTPγS binding in platelet membranes whereas the antagonist of the G_q-coupled $P2Y_1$ receptor, A3P5P, has much less effect. Apparently, G_q activation by the $P2Y_1$-like receptor cannot be well detected because [^{35}S]GTPγS binding assays reflect predominantly activation of G_i and G_o proteins (31). The signaling pathways leading from activation of G_i proteins to aggregation are presently unknown. It is generally assumed that repression of elevated cyclic AMP levels is necessary to allow for aggregation but by itself is not sufficient to induce it (32). It is possible, that βγ subunits released from activated $G\alpha_i$ will initiate other pathways leading to GPIIb-IIIa activation and aggregation. Alternatively, this receptor might couple to G proteins different from G_i, the activation of which has not yet been detected.

In addition to the two G protein-coupled ADP receptors, a third ADP receptor, similar or identical to the ligand-gated ion channel $P2X_1$ appears to be expressed on platelets and to mediate a very rapid Ca^{++} influx (Fig. 7) (16, 17, 33, 34). However, unlike for aggregation and other platelet responses described here, α,β-methylene-ATP and ATP are agonists of this receptor. Therefore, the relevance of this receptor for platelet activation remains unclear. The antagonist effects described here do not involve P2X receptors, since P2X receptors are desensitized in the absence of apyrase in our platelet suspensions as well as in standard platelet preparations.

Interestingly, the antithrombotic drugs ticlopidine and clopidogrel which appear to act through an as yet unidentified metabolite target the G_i-linked platelet ADP receptor and do not inhibit the Ca^{++} mobilization in platelets (1). Similarily, ARL66096 which also has antithrombotic activity (35), is selective for this receptor (14, 15, 27). Furthermore, patients with mild bleeding disorders and impaired ADP-dependent aggregation had defects associated with the same pathway (36, 37). Together, these findings not only support the critical role of the G_i-linked ADP receptor in thrombosis and hemostasis but also as a target for the development of novel antithrombotic drugs.

Acknowledgements

The authors wish to thank Shirlee Yonkovich and Merlyn Alphonso for generation and screening of monoclonal antibodies, Stan Hollenbach and Kathleen Ministri for assistance in the animal facility, Arnie Martelli and Robert Scarborough for peptide synthesis, Raymond Kong for DNA sequencing, Vanitha Ramakrishnan for help with flow cytometry, and Richard Wong for help with preparing figures. We thank David R. Phillips, Donna Oksenberg and Debbie Law for stimulating discussions and critical reading of the manuscript.

Note added in proof. After submission of this manuscript, Jin and Kunapuli (38) and Hechler et al. (39) reported evidence for the critical requirement of the $P2Y_1$-like platelet ADP receptor in addition to the adenylyl cyclase-coupled receptor for human platelet aggregation.

References

1. Savi P, Herbert JM. ADP Receptors on Platelets and ADP-Selective Antiaggregating Agents. Med Res Rev 1996; 16: 159-79.
2. Hourani SMO, Hall DA. Receptors for ADP on Human Blood Platelets. Trends Pharmacol Sci 1994; 15: 103-8.
3. Mills DCB. ADP Receptors on Platelets. Thromb Haemost 1996; 76: 835-6.
4. Puri RN, Colman RW. ADP-Induced Platelet Activation. Crit Rev Biochem Mol Biol 1997; 3: 437-502.
5. Boarder MR, Hourani SMO. The regulation of vascular function by P2 receptors: multiple sites and multiple receptors. Trends Pharmacol Sci 1998; 19: 99-107.
6. Sage SO. Calcium Entry Mechanisms in Human Platelets. Exp Physiol 1997; 82: 807-23.
7. Greco NJ, Yamamoto N, Jackson BW, Tandon NN, Moos M, Jamieson GA. Identification of a nucleotide-binding site on glycoprotein IIb. J Biol Chem 1991; 266: 13627-33.
8. Harden TK, Boyer JL, Nicholas RA. P2 Purinergic Receptors: Subtype-Associated Signaling Responses and Structure. Annu Rev Pharmacol Toxicol 1995; 35: 541-79.
9. North RA, Barnard EA. Nucleotide receptors. Curr Opin Neurobiol 1997; 7: 346-57.
10. Fredholm BB, Abbracchio MP, Burnstock G, Dubyak GR, Harden TK, Jacobson KA, Schwabe U, Williams M. Towards a revised nomenclature for P1 and P2 receptors. Trends Pharmacol Sci 1997; 18: 79-82.
11. Leon C, Hechler B, Vial C, Leray C, Cazenave JP, Gachet C. The P2Y1 Receptor is an ADP Receptor Antagonized by ATP and Expressed in Platelets and Megakaryoblastic Cells. FEBS Lett 1997; 403: 26-30.
12. Gachet C, Hechler B, Leon C, Vial C, Leray C, Ohlmann P, Cazenave JP. Activation of ADP Receptors and Platelet Function. Thromb Haemost 1997; 78: 271-5.
13. Hechler B, Vigne P, Leon C, Breittmeyer J-P, Gachet C, Frelin C. ATP Derivatives Are Antagonists of the $P2Y_1$ Receptor: Similarities to the Platelet ADP Receptor. Mol Pharmacol 1998; 53: 727-33.
14. Daniel JL, Dangelmaier C, Jin J, Ashby B, Smith JB, Kunapuli SP. Molecular Basis for ADP-induced Platelet Activation. J Biol Chem 1998; 273: 2024-9.
15. Jin J, Daniel JL, Kunapuli SP. Molecular Basis for ADP-induced Platelet Activation. J Biol Chem 1998; 273: 2030-4.
16. Savi P, Bornia J, Sabel V, Delfaud M, Herbert JM. Characterization of P2x1 Purinoceptors on Rat Platelets: Effect of Clopidogrel. Br J Haematol 1997; 98: 880-6.
17. Vial C, Hechler B, Leon C, Cazenave JP, Gachet C. Presence of P2X1 Purinoceptors in Human Platelets and Megakaryoblastic Cell Lines. Thromb Haemost 1997; 78: 1500-4.
18. Fratantoni JC, Poindexter BJ. Measuring Platelet Aggregation with Microplate Reader. Am J Clin Pathol 1990; 94: 613-7.
19. Gachet C, Cazenave JP, Ohlmann P, Hilf G, Wieland T, Jakobs KH. ADP receptor-induced activation of guanine-nucleotide binding proteins in human platelet membranes. Eur J Biochem 1992; 207: 259-63.
20. Schachter JB, Li Q, Boyer JL, Nicholas RA, Harden TK. Second Messenger Cascade Specificity and Pharmacological Selectivity of the Human P2Y1-Purinoceptor. Br J Pharmacol 1996; 118: 167-73.
21. Grynkiewicz G, Poenie M, Tsien RY. A New Generation of Ca^{2+} Indicators with Greatly Improved Fluorescence Properties. J Biol Chem 1985; 260: 3440-50.
22. Harlow E, Lane D, eds. Antibodies: a laboratory manual. Cold Spring Harbor NY: Cold Spring Harbor Laboratory, 1988.
23. Michal F, Maguire MH, Gough G. 2-Methylthioadenosine-5'-phosphate: a Specific Inhibitor of Platelet Aggregation. Nature 1969; 222: 1073-4.
24. Cusack NJ, Hourani SMO. Specific but noncompetitive inhibition by 2-alkylthio analogues of adenosine 5'-monophosphate and adenosine 5'-triphosphate of human platelet aggregation induced by adenosine 5'-diphosphate. Br J Pharmacol 1982; 75: 397-400.
25. Boyer JL, Romero-Avila T, Schachter JB, Harden TK. Identification of Competitive Antagonists of the P2Y1 Receptor. Mol Pharmacol 1996; 50: 1323-9.
26. Ohlmann P, Laugwitz KL, Nurnberg B, et al. The human platelet ADP receptor activates G_{i2} proteins. Biochem J 1995; 312: 775-9.
27. Fagura MS, Dainty IA, McKay GD, Kirk IP, Humphries RG, Robertson MJ, Dougall IG, Leff P. $P2Y_1$-receptors in human platelets which are pharmacologically distinct from $P2Y_{ADP}$-receptors. Br J Pharmacol 1998; 124: 157-64.
28. Offermanns S, Toombs CF, Hu YH, Simon MI. Defective Platelet Activation in $G\alpha_q$-deficient Mice. Nature 1997; 389: 183-6.
29. Schachter JB, Boyer JL, Li Q, Nicholas RA, Harden TK. Fidelity in Functional Coupling of the Rat P2Y1 Receptor to Phospholipase C. Br J Pharmacol 1997; 122: 1021-4.
30. Savi P, Beauverger P, Labouret C, et al. Role of P2Y1 purinoceptor in ADP-induced platelet activation. FEBS Lett 1998; 422: 291-5.
31. McKenzie F. Basic techniques to study G-protein function. In: Signal Transduction: A Practical Approach. Milligan G, ed. Oxford: IRL Press 1992; 31-56.
32. Savi P, Pflieger AM, Herbert JM. cAMP is not an Important Messenger for ADP-induced Platelet Aggregation. Blood Coagul Fibrinol 1996; 7: 249-52.
33. MacKenzie AB, Mahaut-Smith MP, Sage SO. Activation of Receptor-Operated Cation Channels via P2x1 Not P2T Purinoceptors in Human Platelets. J Biol Chem 1996; 271: 2879-81.
34. Scase TJ, Heath MF, Allen JM, Sage SO. Identification of a P2X1 Purinoceptor Expressed on Human Platelets. Biochem. Biophys Res Commun 1998; 242: 525-8.
35. Humphries RG, Robertson MJ, Leff P. A Novel Series of P2T Purinoceptor Antagonists: Definition of the Role of ADP in Arterial Thrombosis. Trends Pharmacol Sci 1995; 16: 179-81.
36. Cattaneo M, Lecchi A, Randi AM, McGregor JL, Mannucci PM. Identification of a New Congenital Defect of Platelet Function Characterized by Severe Impairment of Platelet Responses to Adenosine Diphosphate. Blood 1992; 80: 2787-96.
37. Nurden P, Savi P, Heilmann E, et al. An Inherited Bleeding Disorder Linked to a Defective Interaction between ADP and Its Receptor on Platelets. J Clin Invest 1995; 95: 1612-22.
38. Jin J, Kunapuli SP. Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. Proc Natl Acad Sci USA 1998; 95: 8070-4.
39. Hechler B, Leon C, Vial C, Vigne P, Frelin C, Cazenave JP, Gachet C. The $P2Y_1$ Receptor Is Necessary for Adenosine 5'-Diphosphate-Induced Platelet Aggregation. Blood 1998; 92: 152-9.

Received June 10, 1998 Accepted after revision September 9, 1998