0026-895X/98/040727-07$3.00/0
Copyright © by The American Society for Pharmacology and Experimental Therapeutics
All rights of reproduction in any form reserved.
MOLECULAR PHARMACOLOGY, **53:**727–733 (1998).

MOL PHARM

ATP Derivatives Are Antagonists of the $P2Y_1$ Receptor: Similarities to the Platelet ADP Receptor

BÉATRICE HECHLER, PAUL VIGNE, CATHERINE LÉON, JEAN-PHILIPPE BREITTMAYER, CHRISTIAN GACHET, and CHRISTIAN FRELIN

Institut National de la Santé et de la Recherche Médicale U311 (B.H., C.L., C.G.), Etablissment de Transfusion Sanguine de Strasbourg, BP 36, 67065 Strasbourg Cédex, France, Institut de Pharmacologie Moléculaire et Cellulaire (P.V., C.F.), Centre National de la Recherche Scientifique UPR 411, 06560 Valbonne, France, and Institut National de la Santé et de la Recherche Médicale U343 (J.-P.B.), Hôpital de l'Archet, BP 79, 06202 Nice Cédex 3, France

Received September 30, 1997; Accepted December 29, 1997

This paper is available online at http://www.molpharm.org

ABSTRACT

Pharmacological properties of the human $P2Y_1$ receptor transfected in Jurkat cells and of the endogenous receptor in rat brain capillary endothelial cells were analyzed under conditions in which the purity of adenine triphosphate nucleotides was controlled by creatine phosphate/creatine phosphokinase (CP/CPK). ATP, a partial agonist of the receptor, was inactive in the presence of CP/CPK. Results further indicated that ATP was a competitive antagonist of ADP actions. K_i values were 23.0 ± 1.5 μM in endothelial cells and 14.3 ± 0.3 μM in Jurkat cells. Solutions prepared from commercially available 2-methylthio-ATP (2-MeSATP) or 2-chloro-ATP (2-ClATP) contained ≈10% of ADP derivatives. ADP derivatives were removed from the solution by treatment with CP/CPK. Purified 2-MeSATP and 2-ClATP antagonized platelet aggregation induced by ADP. They did not activate $P2Y_1$ receptors but prevented ADP actions in a competitive manner. K_i values for 2-MeSATP were 36.5 μM in endothelial cells and 5.7 ± 0.4 μM in Jurkat cells, and K_i values for 2-ClATP were 27.5 μM in endothelial cells and 2.3 ± 0.3 μM in Jurkat cells. EDTA potentiated actions of ADP and ATP on endothelial cells by 2.4- and 3.6-fold, respectively. In conclusion, the rat and human $P2Y_1$ receptors are ADP-specific receptors that recognize ADP and 2-methylthio-ADP, whereas ATP, 2-MeSATP, and 2-ClATP are competitive antagonists. The results further point to the close pharmacological similarity of the $P2Y_1$ receptor and the platelet ADP receptor.

Purinergic responses of the P2 type are mediated by two classes of membrane receptor: ionotropic P2X receptors and metabotropic P2Y receptors (Abbracchio and Burnstock, 1994; Harden *et al*., 1995; Burnstock and King, 1996; Weissman *et al*., 1996). The structures of $P2Y_1$ receptors from chicken (Webb *et al*., 1993), turkey (Filtz *et al*., 1994), bovine (Henderson *et al*., 1995), rat, mouse (Tokuyama *et al*., 1995), and human (Ayyanathan *et al*., 1996; Léon *et al*., 1996) species are known. ATP, 2-MeSATP, 2-ClATP, 2-MeSADP, and ADP usually are considered to be potent agonists of $P2Y_1$ receptors (Boyer *et al*., 1993; Vigne *et al*., 1994; Boyer *et al*., 1996; Schachter *et al*., 1996), although ATP has been reported in some studies to be only a partial agonist (Feolde *et al*., 1995; Henderson *et al*., 1995; Léon *et al*., 1997). Characterization of the pharmacological properties of purinoceptors is, however, difficult because nucleotides from commercial sources do not have the desired purity and the purity of their aqueous solutions decreases considerably during storage. In addition, nucleotides can be degraded by cellular ectonucleotidases. These difficulties can be circumvented by using freshly purified nucleotides (Léon *et al*., 1997) or enzymatic systems that regenerate degraded nucleotides (Nicholas *et al*., 1996). In a previous article, we described the pharmacological characteristics of the human $P2Y_1$ receptor transfected into Jurkat cells. Results showed that (1) ADP was a selective agonist of this receptor, (2) freshly purified ATP and ATP derivatives were ineffective, and (3) ATP antagonized the effects of ADP (Léon *et al*., 1997). On the other hand, purified 2-MeSATP and 2-ClATP were found to be full agonists but with delayed responses compared with the corresponding diphosphate derivatives. It was suggested that the triphosphate analogues were metabolized into diphosphates by ectoenzymes, thus explaining their apparent agonistic effect. These results led us to put forward the idea that the $P2Y_1$ receptor is similar to the platelet ADP receptor, the elusive P2T receptor (Gachet *et al*., 1997).

The first aim of the current study was to determine the conditions that allow control of the purity of ATP nucleotides by means of a CP/CPK ATP-regenerating system. This tech-

This work was supported by the Centre Nationale de Recherche Scientifique and by Institut National de la Santé et de la Recherche Médicale.

ABBREVIATIONS: 2-MeSATP, 2-methylthio-ATP, CPK, creatine phosphokinase, CP, creatine phosphate, 2-MeSADP, 2-methylthio-ADP, 2-ClATP, 2-chloro-ATP; HPLC, high performance liquid chromatography; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

nique then was used to define more precisely the pharmacological properties of the human $P2Y_1$ receptor expressed in Jurkat cells and the endogenous $P2Y_1$ receptor expressed in rat brain capillary endothelial cells (Webb *et al.*, 1996). These latter cells were found previously to express specific ADP receptors at which ATP behaved as a partial agonist (Frelin *et al.*, 1993; Feolde *et al.*, 1995). Results showed the rat and human $P2Y_1$ receptors to be ADP-specific receptors antagonized by ATP and its derivatives, 2-ClATP and 2-MeSATP, thus pointing to the existence of strong pharmacological similarities between the endothelial $P2Y_1$ receptor and the platelet ADP receptor.

Experimental Procedures

Materials. ATP, ADP, iodoacetamide, creatine, CP, and CPK (Type III from bovine heart) were from Sigma Chemical (St. Quentin-Fallavier, France). Indo-1/AM was from Calbiochem (Meudon, France), whereas 2-ClATP, 2-MeSADP, and 2-MeSATP were from Research Biochemicals (Natick, MA).

Cell cultures and intracellular Ca^{2+} measurements. Rat brain capillary endothelial cells of the B10 clone (Feolde et al., 1995) and Jurkat cells stably transfected with the human $P2Y_1$ receptor (Léon *et al.*, 1997) have been described previously. The human $P2Y_1$ receptor sequence differs from that originally described (Léon *et al.*, 1996) by the presence of an additional 3 base pairs leading to an additional serine residue in transmembrane domain 3, between residues 137 and 138 (Léon *et al.*, 1997). An identical sequence was reported by Schachter *et al.* (1996).

B10 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Jurkat cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 1 mg/ml geneticin. Suspensions of Jurkat cells and B10 cells were incubated for 30 min in the presence of 5 μM indo-1/AM. The cells then were diluted in Earle's salt solution (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM glucose, 25 mM HEPES/NaOH, pH 7.4) and exposed to nucleotides, and the indo-1 fluorescence ratio was measured by flow cytometry using a FACStar Plus cytometer (Becton Dickinson, Le Pont de Claix, France) (Vigne *et al.*, 1994). Mean fluorescence ratios were determined for 5000 Jurkat cells or 1000 B10 cells sampled 15 sec after the addition of agonists and all experiments were performed at 37°. The acquisition time was 2 sec. Fluorescence ratios were calculated in arbitrary units set to a value of 100 for unstimulated cells. Triplicate determinations were performed. Each experiment was repeated at least three times. Error bars omitted on the figures were smaller than the size of the points. All experiments shown in a given figure were performed on the same batch of cells.

Purification of ATP and ATP derivative solutions. The purity of nucleotide solutions was checked by HPLC analysis (Léon *et al.*, 1997) on a Partisil 10-μm SAX column (Interchrom; Interchim, Montluçon, France) eluted with a linear gradient from 0% to 100% of 1 M ammonium phosphate buffer, pH 3.8, after 20 min with a flow rate of 1 ml/min. Absorbance was recorded at 260 nm for ATP and 274 nm for 2-ClATP and 2-MeSATP. As discussed previously (Nicholas *et al.*, 1996; Léon *et al.*, 1997), commercial nucleotide powders often are contaminated by degradation products that can be the cause of misleading results. Contamination was usually 1% for ATP and ≈10% for 2-MeSATP and 2-ClATP (see Results). Nucleotides also may be degraded by cell ectonucleotidases. Problems arising from the contamination and degradation of ATP solutions were circumvented by using an ATP-regenerating system. ATP solutions (1 mM) were treated at room temperature with 20 units/ml CPK and 10 mM CP, and the entire mixture was added to cell suspensions. The same procedure was used to purify 2-ClATP and 2-MeSATP solutions from contaminating ADP derivatives. Time course experiments indicated, however, that exposition to CPK had to be increased to remove contaminants of 2-MeSATP and 2-ClATP solutions. A 30-min incubation period was necessary to purify 2-ClATP solutions. This time was 90 min for 2-MeSATP. These differences probably reflect

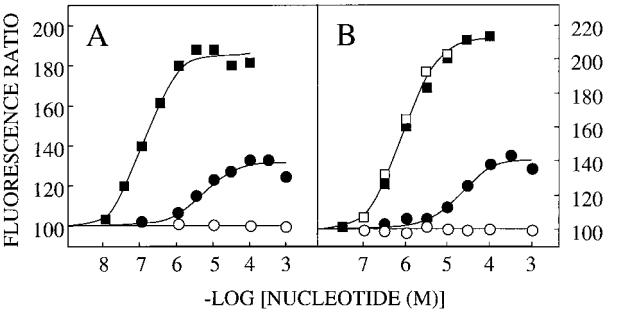

Fig. 1. An ATP-regenerating system abolishes agonistic actions of ATP in Jurkat -and B10 cells. Dose-response curves for the actions of ATP (●, ○) and ADP (■, □) in Jurkat (A) and B10 (B) cells. Actions of ATP were analyzed in the absence (●) or presence (○) of CP and CPK. Actions of ADP were analyzed in the absence (■) or presence (□) of creatine and CPK. ATP solutions (1 mM) were treated for 10 min with 10 mM CP and 20 units/ml CPK. ADP solutions (1 mM) were treated for 10 min with 10 mM creatine and 20 units/ml CPK. Appropriate dilutions were added to the cells, and $[Ca^{2+}]_i$ was measured after 15 sec. Data are from a single experiments with values determined in triplicate and are representative of the results of three similar experiments.

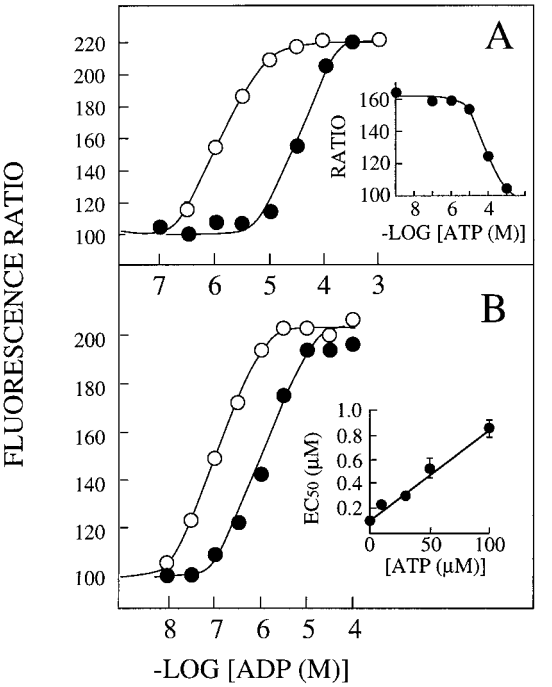

Fig. 2. ATP inhibits ADP-induced $[Ca^{2+}]_i$ responses. A, Dose-response curves for the action of ADP on B10 cells in the absence of ATP (○) and in the presence of 0.1 mM purified ATP (●). *Inset*, dose-response curve for the inhibition by purified ATP of ADP (0.3 μM) responses in B10 cells. B, Dose-response curves for the action of ADP on Jurkat cells in the absence of ATP (○) and in the presence of 0.1 mM purified ATP (●). *Inset*, plot of EC_{50} values for the action of ADP on Jurkat cells as a function of ATP concentration. EC_{50} values for ADP actions were 100 ± 20 nM (control), 230 ± 30 nM (10 μM ATP), 300 ± 40 nM (30 μM ATP), 530 ± 80 nM (50 μM ATP), and 850 ± 70 nM (100 μM ATP). The slope corresponded to a K_i value for ATP of 14 μM. ATP solutions were treated for 10 min with 10 mM CP and 20 units/ml CPK. After the addition of 10 mM iodoacetamide, the mixture was added to the cells. $[Ca^{2+}]_i$ was measured 15 sec after the addition of ADP.

MOLECULAR PHARMACOLOGY

aspet

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

different catalytic efficiencies of CPK toward ATP, 2-ClATP, and 2-MeSATP. Contaminant 2-MeSADP and 2-ClADP were identified by mass spectrometry using a Bio-Q triple quadrupole mass spectrometer (Micromass Ltd, Altrincham, UK).

In assays to determine the antagonistic action of ATP derivatives, CPK was inactivated with 10 mM iodoacetamide before the experiments.

Platelet aggregation. Aggregation was measured at 37° by a turbidimetric method in a dual-channel Payton aggregometer (Payton Associates, Scarborough, Ontario, Canada). A 450-μl aliquot of citrated platelet-rich plasma was stirred at 1100 rpm and activated by the addition of agonists to a final volume of 500 μl (Cazenave *et al*., 1983).

Curves were fitted with a logistic equation using SigmaPlot software (Jandel Scientific, Costa Madre, CA). Mean ± standard error values are indicated.

Results

Partial agonism of ATP is abolished in the presence of an ATP-regenerating system. Purinergic responses were analyzed in rat brain capillary endothelial cells of the B10 clone that specifically express mRNA sequences corresponding to the cloned P2Y$_1$ receptor (Webb *et al*., 1996) and on P2Y$_1$ receptor-transfected Jurkat cells. Fig. 1 shows dose-response curves for the actions of ADP on Jurkat cells and B10 cells. EC_{50} values for the actions of ADP were 0.88 ± 0.15 μM (10 experiments) in B10 cells and 0.20 ± 0.06 μM (3 experiments) in Jurkat cells. Similar results were obtained with ADPβS, which behaved as a full agonist. EC_{50} values for the action of ADPβS were 0.90 ± 0.05 μM (3 experiments) in B10 cells and 0.90 ± 0.3 μM (3 experiments) in Jurkat cells (data not shown).

Fig. 1 further shows that as reported previously (Feolde *et al*., 1995; Léon *et al*., 1997), ATP was a partial agonist compared with ADP in the two cell types. EC_{50} values for the actions of ATP were 22 ± 6 μM (10 experiments) in B10 cells and 11 ± 7 μM (3 experiments) in Jurkat cells. We noticed, however, that the maximum efficacy of ATP varied among experiments, even within the same day. This led us to postulate that actions of ATP were mediated by a contaminant that spontaneously arose during storage of the solutions. Purity of ATP solutions was assessed by HPLC. Experiments indicated that freshly prepared ATP solutions were contaminated by ≈1% ADP and that contamination of ATP solutions by ADP increased rapidly on storage of the solutions. It may reach several percentage points after a few hours. ADP also may be generated from ATP by cell ectonucleotidases. One way of avoiding spontaneous or cell-mediated degradation of ATP into ADP and to control ATP concentrations is to use an ATP-regenerating system. In the presence of CP, CPK transforms ADP into ATP and thus counteracts any degradation of ATP into ADP. Fig. 1 shows the results of experiments performed in the presence of CP and CPK. It shows that under such conditions, ATP did not raise $[Ca^{2+}]_i$ in either Jurkat or B10 cells. CP or CPK alone did not inhibit ATP responses. Fig. 1B further shows that a mixture of creatine and CPK that transformed ATP into ADP did not inhibit ADP action. Taken together, these results indicated that ATP was inactive on B10 or Jurkat cells. The actions of ATP that were observed in the absence of ATP-regenerating system were likely mediated by contaminating ADP; contamination resulted from the spontaneous degradation of ATP or from ATP hydrolysis by cellular ectonucleotidases.

ATP inhibits ADP-induced intracellular Ca^{2+} mobilization. In B10 cells, ATP (0.1 mM) inhibited the action of ADP by shifting the dose-response curve to higher concentrations (Fig. 2A). ATP did not, however, modify the maximum efficacy of ADP. EC_{50} values for ADP were 0.88 ± 0.12, 6.4 ± 0.5, and 49 ± 9 μM in the presence of no ATP, 100 μM ATP, and 1 mM ATP, respectively. The K_i value for ATP

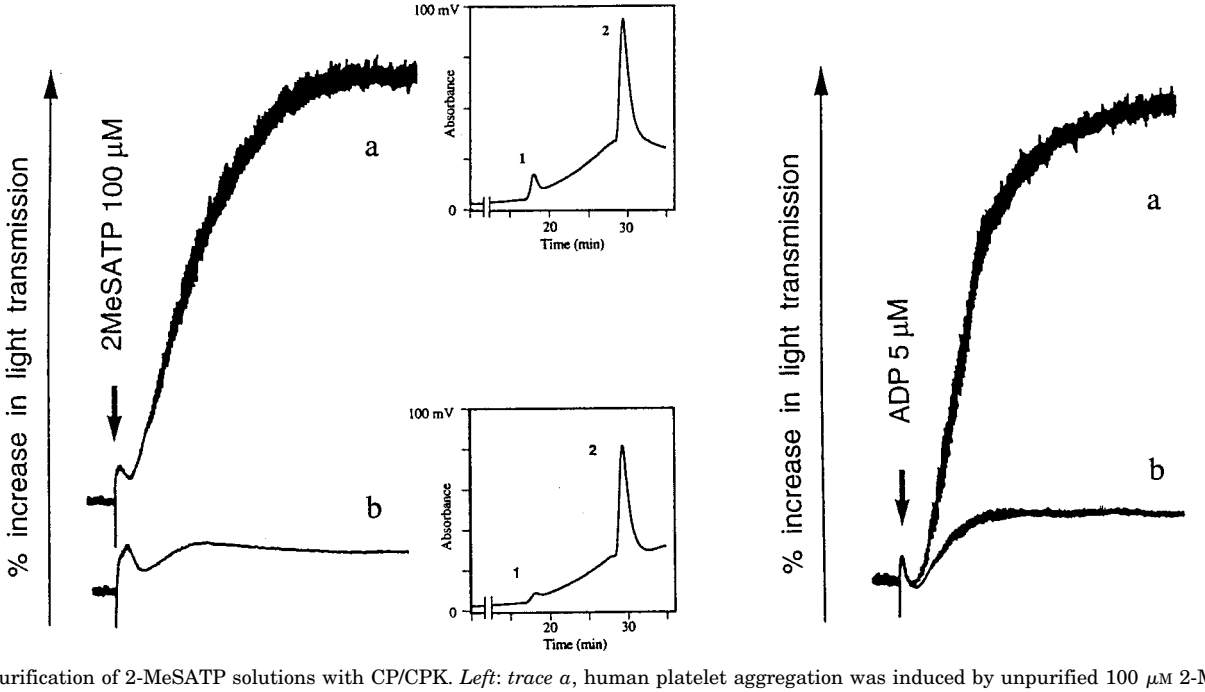

Fig. 3. Purification of 2-MeSATP solutions with CP/CPK. *Left*: *trace a*, human platelet aggregation was induced by unpurified 100 μM 2-MeSATP. *Trace b*, purified 2-MeSATP did not induce platelet aggregation. *Middle*, HPLC profiles of unpurified 2-MeSATP (*top inset*) and of 2-MeSATP that had been treated for 90 min with 20 units/ml CPK and 10 mM CP (*bottom inset*). *Peak 1*, 2-MeSADP. *Peak 2*, 2-MeSATP. *Right*, aggregation induced by 5 μM ADP (*trace a*) was prevented in the presence of 50 μM purified 2-MeSATP (*trace b*).

MOLECULAR PHARMACOLOGY

aspet

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

estimated from these data was 20 μM. ATP (100 μM) also inhibited the effects of 2-MeSADP on $[Ca^{2+}]_i$ in B10 cells, raising the EC_{50} value of this agonist from 19 ± 3 to 93 ± 25 nM. This shift corresponded to a K_i value for ATP of 25 μM. Finally, Fig. 2A (*inset*) shows the dose-response curve for the inhibition by ATP of responses induced in B10 cells by 300 nM ADP. In seven independent experiments, the K_i value for ATP was estimated to be 23.8 ± 3.3 μM. The mean K_i value for ATP estimated from these three types of experiments was 23.0 ± 1.5 μM.

Similar experiments were performed on $P2Y_1$ receptor-transfected Jurkat cells (Fig. 2B). ATP (0.1 mM) shifted the dose-response curve of ADP to higher concentrations without affecting its maximum efficacy. A plot of EC_{50} values for ADP as a function of ATP concentration (Fig. 2B, *inset*) was linear, indicating a purely competitive mechanism. The slope of this representation gave a K_i value for ATP of 14 μM. Similarly, purified ATP (0.1 mM) increased the EC_{50} value for the effect of 2-MeSADP on $[Ca^{2+}]_i$ in $P2Y_1$ receptor-transfected Jurkat cells from 16 ± 3 to 131 ± 20 nM (data not shown), which corresponded to a K_i value of 14 μM. ATP likewise inhibited increases in $[Ca^{2+}]_i$ induced by 300 nM ADP with a K_i value of 16 μM (data not shown). The mean K_i value for ATP estimated from these three types of experiments was 14.3 ± 0.3 μM.

Thus, ATP behaved as a competitive antagonist of the $P2Y_1$ receptors in B10 cells and in Jurkat cells. Inhibitory constants were 23 and 14 μM, respectively.

2-MeSATP inhibits ADP-induced intracellular Ca^{2+} mobilization. Fig. 3 (*inset*) shows the HPLC profile of a freshly prepared 2-MeSATP solution that contained >10% of a major contaminant (*peak 1*). This contaminant had the same retention time as 2-MeSADP. It induced platelet aggregation and was identified as 2-MeSADP by mass spectrometry analysis. Fig. 3 further shows that a 90-min treatment of a 2-MeSATP solution with 20 units/ml CPK and 10 mM CP almost completely removed contaminating 2-MeSADP from the solution. The biological activity of purified 2-MeSATP was checked in a platelet aggregation assay. It is well known that although 2-MeSADP is a potent agonist of platelet aggregation, 2-MeSATP is an antagonist (Hall and Hourani, 1993). In Fig. 3, it can be seen that purification of 2-MeSATP solutions with CP/CPK suppressed the aggregative effect of 2-MeSATP (*traces a and b*, *left*) and further that as expected 50 μM purified 2-MeSATP abolished the action of 5 μM ADP (*traces a and b*, *right*).

Fig. 4 shows that as reported previously (Feolde et al., 1995; Léon *et al*., 1997), unpurified 2-MeSATP was a potent agonist of $P2Y_1$ receptors in Jurkat and B10 cells. EC_{50} values for the actions of 2-MeSATP were 310 ± 60 nM (five experiments) in B10 cells and 45 ± 13 nM (three experiments) in Jurkat cells. Fig. 4 further shows that a treatment of 2-MeSATP solutions with CP and CPK almost completely abolished its agonist action.

In B10 cells, purified 2-MeSATP (100 μM) competitively inhibited the action of ADP without affecting its maximum efficacy (Fig. 5A). The EC_{50} value for ADP was 0.91 ± 0.06 μM in the absence of 2-MeSATP and 3.3 ± 0.5 μM in the presence of 100 μM 2-MeSATP. The 3.6-fold shift gave an estimated K_i value of 38 μM. 2-MeSATP also inhibited responses induced by 1 μM ADP (Fig. 5A, *inset*). The concentration of 2-MeSATP producing half-maximal inhibition of the ADP signal (IC_{50}) was 77 ± 13 μM, which corresponded to a K_i value of 35 μM. The mean K_i value for 2-MeSATP estimated from these two types of experiments was 36.5 μM.

Similarly, 2-MeSATP (100 μM) antagonized ADP responses in $P2Y_1$ receptor-transfected Jurkat cells (Fig. 5B). A plot of EC_{50} values for ADP as a function of 2-MeSATP concentration was linear (Fig. 5B, *inset*), and the slope indicated a K_i

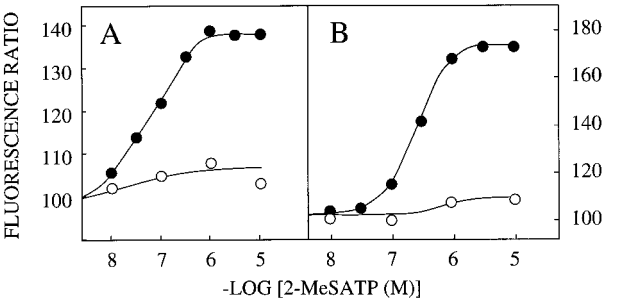

Fig. 4. 2-MeSATP does not activate $P2Y_1$ receptors in Jurkat and B10 cells. Dose-response curves for the actions of 2-MeSATP in Jurkat (A) and B10 (B) cells. Experiments were performed using unpurified 2-MeSATP (●) and purified 2-MeSATP (○). 2-MeSATP solutions (1 mM) were treated for 90 min with 10 mM CP and 20 units/ml CPK. Appropriate dilutions then were added to the cells, and $[Ca^{2+}]_i$ was measured after 15 sec. Data are from a single experiment with values determined in triplicate and are representative of the results of three similar experiments.

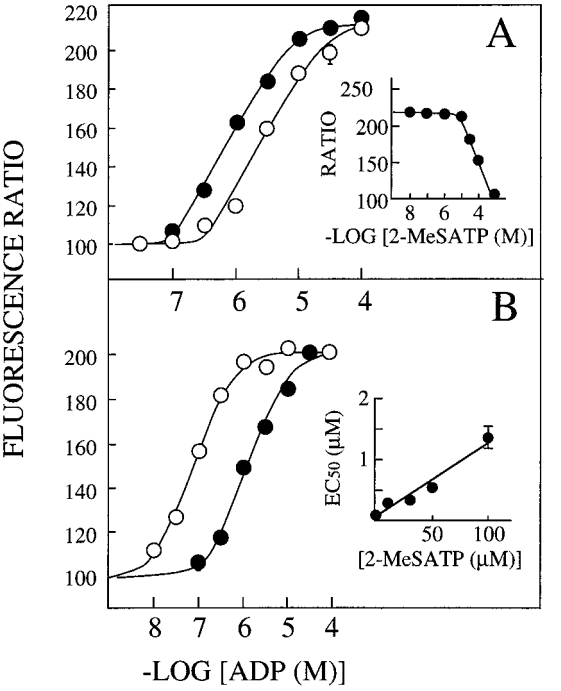

Fig. 5. 2-MeSATP inhibits ADP-induced $[Ca^{2+}]i$ responses. A, Dose-response curves for the action of ADP on B10 cells in the absence of 2-MeSATP (●) and in the presence of 0.1 mM purified 2-MeSATP (○). *Inset*, dose-response curve for the inhibition by purified 2-MeSATP of ADP (1 μM) responses in B10 cells. B, Dose-response curves for the action of ADP on Jurkat cells in the absence of 2-MeSATP (○) and in the presence of 0.1 mM purified 2-MeSATP (●). *Inset*, plot of EC_{50} values for the action of ADP on Jurkat cells as a function of 2-MeSATP concentration. EC_{50} values for the action of ADP were 98 ± 12 nM (control), 279 ± 72 nM (10 μM 2-MeSATP), 332 ± 41 nM (30 μM 2-MeSATP), 554 ± 81 nM (50 μM 2-MeSATP), and 2.1 ± 0.3 μM (100 μM 2-MeSATP). The slope corresponded to a K_i value for 2-MeSATP of 6.5 μM. 2-MeSATP solutions were treated for 90 min with 10 mM CP and 20 units/ml CPK. After the addition of 10 mM iodoacetamide, the mixture was added to the cells. $[Ca^{2+}]_i$ was measured 15 sec after the addition of ADP.

MOLECULAR PHARMACOLOGY

aspet

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

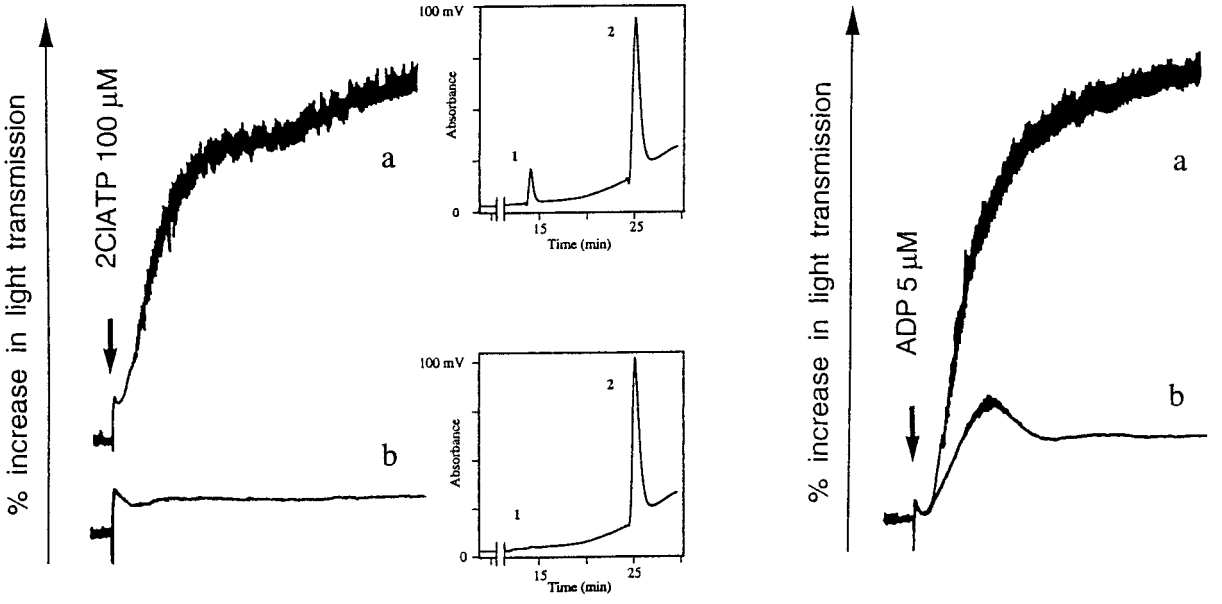

Fig. 6. Purification of 2-ClATP solutions with CP/CPK. *Left*: *trace a*, human platelet aggregation was induced by unpurified 100 μM 2-ClATP. *Trace b*, purified 2-ClATP did not induce platelet aggregation. *Middle*, HPLC profiles of unpurified 2-ClATP (*top inset*) and of 2-ClATP that had been treated for 60 min with 20 U·ml CPK and 10 mM CP (*bottom inset*). *Peak 1*, 2-ClADP. *Peak 2*, 2-ClATP. *Right*, aggregation induced by 5 μM ADP (*trace a*) was prevented in the presence of 50 μM purified 2-ClATP (*trace b*).

value of 6.5 μM. 2-MeSATP likewise shifted the dose-response curve for the action of 2-MeSADP on $[Ca^{2+}]i$. EC_{50} values for 2-MeSADP were 3.6 ± 1.3 nM in the absence of 2-MeSATP and 76 ± 12 nM in the presence of 100 μM 2-MeSATP (data not shown), giving a K_i value of 5 μM. Finally, 2-MeSATP inhibited responses to 300 nM ADP with an estimated K_i value of 5.5 μM (data not shown). The mean K_i value for 2-MeSATP estimated from these three types of experiments was 5.7 ± 0.4 μM.

Thus, 2-MeSATP behaved as a competitive antagonist of the P2Y$_1$ receptors in B10 and Jurkat cells, with inhibition constants of 36.5 and 5.7 μM, respectively.

2-ClATP inhibits ADP-induced intracellular Ca^{2+} mobilization. Fig. 6 (*left*, *inset*) shows the HPLC profile of a freshly prepared 2-ClATP solution that contained >10% of a major contaminant (*peak 1*). This contaminant induced platelet aggregation and was identified as 2-ClADP in mass spectrometry experiments. It was completely removed by 30-min treatment of the solution with 20 units/ml CPK and 10 mM CP (Fig. 6, *inset*). Purified 2-ClATP did not induce platelet aggregation (Fig. 6, *left*) and inhibited the action of ADP (Fig. 6, *right*), in accordance with the known antagonism of platelet ADP receptors by 2-ClATP (Hall and Hourani, 1993).

Fig. 7 shows that as reported previously (Feolde et al., 1995; Léon *et al.*, 1997), unpurified 2-ClATP is a potent agonist of P2Y$_1$ receptors in Jurkat and B10 cells. EC_{50} values for the actions of 2-ClATP were 200 ± 37 nM (four experiments) in B10 cells and 40 ± 4 nM nM (three experiments) in Jurkat cells. Fig. 7 further shows that a treatment of 2-ClATP solutions with CP and CPK almost completely abolished its agonist action.

In B10 cells, purified 2-ClATP (100 μM) increased the EC_{50} value for the action of ADP on $[Ca^{2+}]_i$ from 0.98 ± 0.09 to 3.7 ± 0.8 μM without modifying its maximum efficacy (Fig. 8A). This 3.8-fold shift indicated a K_i value of 36 μM. 2-ClATP further inhibited responses to 1 μM ADP (Fig. 8A, *inset*), with an IC_{50} value of 41 ± 5 μM corresponding to a K_i value of 19 μM. The mean K_i value for 2-ClATP estimated from these two types of experiments was 27.5 μM.

2-ClATP antagonized ADP responses in P2Y$_1$ receptor-transfected Jurkat cells in a similar manner (Fig. 8B). A plot of EC_{50} values for ADP as a function of 2-ClATP concentration was linear (Fig. 8B, *inset*) and gave a slope indicating a K_i value of 2.5 μM. 2-ClATP also shifted the dose-response curve for the action of 2-MeSADP on $[Ca^{2+}]_i$. EC_{50} values for 2-MeSADP were 3.6 ± 1.3 nM in the absence of 2-ClATP and 213 ± 46 nM in the presence of 100 μM 2-ClATP (data not shown), which corresponded to a K_i value of 1.7 μM. Last, 2-ClATP inhibited responses to 300 nM ADP with an estimated K_i value of 2.7 μM (data not shown). The mean K_i value for 2-ClATP estimated from these three types of experiments was 2.3 ± 0.3 μM.

Thus, 2-ClATP behaved as a competitive antagonist of the P2Y$_1$ receptors in B10 and Jurkat cells, with inhibition constants of 27 and 2.3 μM, respectively.

EDTA potentiates the effects of ADP and ATP. In physiological buffers, ATP and ADP exist as mixtures of several species, including forms that are complexed with monovalent and divalent cations and free ADP^{3-} and ATP^{4-} forms. The species constituting the preferential ligand of a receptor can be determined by investigating the effects of divalent cations on the action of ADP or ATP. Fig. 9A compares dose-response curves for the action of ADP on $[Ca^{2+}]_i$ in B10 cells in experiments performed in the presence or absence of 5 mM EDTA. The addition of EDTA decreased the EC_{50} value for ADP action from 0.86 ± 0.08 μM (7 experiments) to 0.35 ± 0.03 μM (4 experiments). Thus, chelation of extracellular divalent cations with EDTA increased by 2.4-fold the potency of ADP. EDTA also potentiated the inhibitory action of ATP on ADP-induced $[Ca^{2+}]_i$ increase in B10 cells (Fig. 9B). The mean K_i value for ATP action decreased from 23.8 ± 3.3 μM (7 experiments) in the absence of EDTA to 6.5 ± 1.3 μM (13 experiments) in the presence of 5 mM EDTA. These values were calculated by using EC_{50} values for

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

ADP actions measured in the presence of EDTA. EDTA therefore increased 3.6-fold the apparent affinity of ATP for the $P2Y_1$ receptor of B10 cells.

Discussion

ATP, 2-ClATP, and 2-MeSATP usually are considered to be agonists of $P2Y_1$ receptors (Boyer *et al.*, 1993; Vigne *et al.*, 1994; Boyer *et al.*, 1996; Burnstock and King, 1996; Schachter *et al.*, 1996). In the current study, the pharmacological properties of rat and human $P2Y_1$ receptors were studied under conditions in which care was taken to purify the agonist solutions of contaminating ADP or ADP derivatives by treatment with CP/CPK. The agonistic action of ATP, 2-MeSATP, and 2-ClATP was suppressed in the presence of this ATP-regenerating system. Any possibility that CP/CPK inhibited purinergic responses through a nonspecific mechanism was ruled out by the following observations: (1) CPK or CP alone was inactive, (2) creatine/CPK did not modify ADP responses, and (3) iodoacetamide-inactivated CPK and CP did not alter ADP responses. These findings suggested that the previously reported agonistic effects of ATP and its derivatives were most likely due to contaminating ADP or ADP derivatives, present in the unpurified agonist solutions or produced at the cell surface by ectoenzymes, or both. Thus, the human $P2Y_1$ receptor expressed in Jurkat cells and the endogenous $P2Y_1$ receptor of B10 cells appear to be ADP-specific receptors. This, together with a previous report (Nicholas *et al.*, 1996), points to a remarkable specificity of P2Y receptors. Mammalian P2Y receptors can be classified as an ADP-specific receptor ($P2Y_1$ receptor), a UDP-specific receptor ($P2Y_6$ receptor), a UTP-selective receptor ($P2Y_4$ receptor), or a mixed ATP/UTP receptor ($P2Y_2$ receptor) (Nicholas *et al.*, 1996).

This study also showed that purified solutions of ATP, 2-ClATP, or 2-MeSATP shifted ADP dose-response curves to higher concentrations in both B10 cells and $P2Y_1$ receptor-transfected Jurkat cells. The maximum efficacy of ADP was not modified, indicating that ATP and its derivatives are competitive antagonists of ADP at $P2Y_1$ receptors. K_i values were estimated as 23 μM for ATP, 31 μM for 2-MeSATP, and 27 μM for 2-ClATP for the rat $P2Y_1$ receptor and as 5.6 μM for 2-MeSATP, 2.3 μM for 2-ClATP, and 15 μM for ATP for the human receptor. Hence, ATP, 2-ClATP, and 2-MeSATP are weak antagonists of $P2Y_1$ receptors, whereas ADP (K_{app} = 0.2 μM in Jurkat cells and 0.9 μM in B10 cells) and 2-MeSADP (K_{app} = 49 nM in Jurkat cells and 19 nM in B10 cells) are potent agonists.

Different results were reported by Schachter *et al.*(1996), who reported full agonistic activity of 2-MeSADP, 2-MeSATP, ADP, and ATP. This pharmacological profile resulted from measurement of inositol phosphate production after 10-min incubation of the human $P2Y_1$ receptor-transfected 1321N1 cells with nonpurified nucleotides. These experimental conditions, under which metabolic transformation of the nucle-

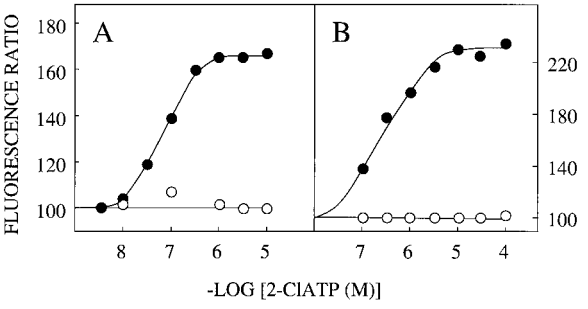

Fig. 7. 2-ClATP does not activate $P2Y_1$ receptors in Jurkat and B10 cells. Dose-response curves for the actions of 2-ClATP in Jurkat (A) and B10 (B) cells. Experiments were performed using unpurified 2-ClATP (●) and purified 2-ClATP (○). 2-ClATP solutions (1 mM) were treated for 30 min with 10 mM CP and 20 units/ml CPK. Appropriate dilutions were added to the cells, and $[Ca^{2+}]_i$ was measured after 15 sec. Data are from a single experiment with values measured in triplicate and are representative of the results of three similar experiments.

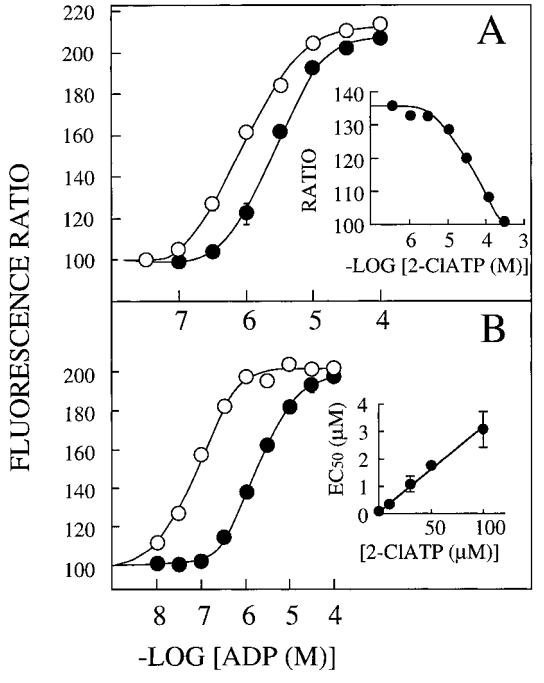

Fig. 8. 2-ClATP inhibits ADP-induced $[Ca^{2+}]_i$ responses. A, Dose-response curves for the action of ADP on B10 cells in the absence of 2-ClATP (○) and in the presence of 0.1 mM purified 2-ClATP (●). *Inset*, dose-response curve for the inhibition by purified 2-ClATP of ADP (1 μM) responses in B10 cells. B, Dose-response curves for the action of ADP on Jurkat cells in the absence of 2-ClATP (○) and in the presence of 0.1 mM purified 2-ClATP (●). *Inset*, plot of EC_{50} values for the action of ADP on Jurkat cells as a function of 2-ClATP concentration. EC_{50} values for ADP actions were 139 ± 60 nM (control), 376 ± 37 nM (10 μM 2-ClATP), 1.09 ± 0.27 μM (30 μM 2-ClATP), 1.78 ± 0.16 μM (50 μM 2-ClATP), and 3.08 ± 0.64 μM (100 μM 2-ClATP). The slope corresponded to a K_i value for 2-ClATP of 2.5 μM. 2-ClATP solutions were treated for 30 min with 10 mM CP and 20 units/ml CPK. After the addition of 10 mM iodoacetamide, the mixture was added to the cells. $[Ca^{2+}]_i$ was measured 15 sec after the addition of ADP.

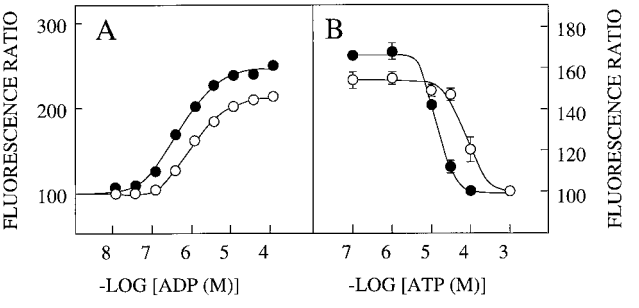

Fig. 9. EDTA potentiates the effects of ADP and ATP on B10 cells. A, Dose-response curves for the action of ADP on B10 cells in the absence of EDTA (○) and in the presence of 5 mM EDTA (●). B, Dose-response curves for the inhibition by ATP of ADP (0.3 μM) responses in B10 cells. Experiments were performed in the absence of EDTA (○) or in the presence (●) of 5 mM EDTA. Experiments were performed as described in the legends of Figs. 1 and 2.

MOLECULAR PHARMACOLOGY

aspet

Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012

MOL PHARM

otides could occur, may explain the discrepancies with our results. More recently, at the "Purine and Their Receptors" meeting in New Orleans, Harden *et al.* reported a new pharmacological profile, with 2-MeSADP and ADP being the strongest agonists and 2-MeSATP and ATP being the weakest. In their study, $[Ca^{2+}]_i$ increase was measured on few cells adhering to a coverslip perfused with HPLC-purified nucleotides but in the absence of an ATP-regenerating system. These new data are fully consistent with the results presented in the current report.

The potencies of ADP and ATP at the rat $P2Y_1$ receptor were increased 2.4- and 3.6-fold, respectively, when extracellular divalent cations were chelated with EDTA (Fig. 6). This could imply that the $P2Y_1$ receptor is more responsive to charged forms of nucleotides than to forms that are complexed with divalent cations. However, the situation probably is not so simple because a larger shift in affinity for ATP would be expected if only ATP^{4-} were acting on receptors; although ATP^{4-} is the preferred ligand of the $P2Y_1$ receptor, it also may be recognized by other forms of ATP.

Despite spectacular advances in recent years in the fields of ionotropic P2X and metabotropic P2Y receptors, the putative platelet-specific ADP receptor (P2T), which was the first purinoceptor to be defined pharmacologically, has not yet been cloned (Hourani and Hall, 1996; Mills, 1996; Gachet *et al.*, 1997). In a previous publication, we suggested that the $P2Y_1$ receptor could be this elusive ADP receptor of platelets (Léon *et al.*, 1997). The results presented here provide further evidence of close similarities in the pharmacological profiles of the $P2Y_1$ receptor and the platelet ADP receptor.

First, ATP, 2-ClATP, and 2-MeSATP are antagonists of the ADP receptor of platelets (Hall and Hourani, 1993) and of the rat and human $P2Y_1$ receptors. The K_i value of ATP for the human $P2Y_1$ receptor in Jurkat cells (16 μM) is similar to that reported for the ADP receptor of platelets (10 μM) (Hall and Hourani, 1993). The K_i value of 2-ClATP in Jurkat cells (2.3 μM) likewise is close to that reported for the platelet ADP receptor (2.5 μM) (Hall and Hourani, 1993). The only difference between the human $P2Y_1$ receptor in Jurkat cells and the platelet ADP receptor relates to 2-MeSATP. 2-MeSATP is a competitive antagonist of ADP actions in Jurkat cells (Fig. 4B), whereas it was found to be an insurmountable antagonist of ADP actions in platelets (Hall and Hourani, 1993).

Second, ADP^{3-} is a 2.4 times more potent activator of the rat $P2Y_1$ receptor than ADP (Fig. 6A) and a 1.9 times more potent activator of the ADP receptor of platelets (Hall *et al.*, 1994). Conversely, chelation of divalent cations with EDTA increased by 3.6-fold the capacity of ATP to inhibit the rat $P2Y_1$ receptor, whereas the ability of ATP to inhibit the platelet ADP receptor increased ≈10-fold when experiments were performed in the absence of divalent cations (Hall *et al.*, 1994).

On the other hand, the only form of P2Y receptor detected to date in platelets is the $P2Y_1$ receptor (Léon *et al.*, 1997), as also is true in B10 cells (Webb *et al.*, 1996). Another striking similarity is the fact that although ADP induces the mobilization of intracellular Ca^{2+} stores in platelets and B10 cells, in both cases inositol-1,4,5-trisphosphate seems to be only poorly involved in this response (Feolde *et al.*, 1995; Hourani and Hall, 1996; Mills, 1996; Gachet *et al.*, 1997).

In summary, our results and data from the literature strongly suggest that platelets and endothelial cells share a common $P2Y_1$ receptor that mediates platelet aggregation and vasodilation. Whether P2T receptors and $P2Y_1$ receptors are encoded by the same gene remains to be established through knock-out experiments.

Acknowledgments

We thank J. N. Mulvihill for reviewing the use of English in the manuscript, D. Loew and A. Van Dorsselaer for the mass spectrometry analysis, and Dr. R. Ventura Clapier for advice.

References

Abbracchio MP and Burnstock G (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? *Pharmacol Ther* **64**:445–475.
Ayyanathan K, Webb TE, Sandhu AK, Athwal RS, Barnard EA, and Kanapuli SP (1996) Cloning and chromosomal localization of the human $P2Y_1$ purinoceptor. *Biochem Biophys Res Commun* **218**:783–788.
Boyer JL, Lazarowski R, Chen X-H, and Harden TK (1993) Identification of a P2Y purinergic receptor that inhibits adenylyl cyclase. *J Pharmacol Exp Ther* **267**: 1140–1146.
Boyer JL, Romero-Avila T, Schachter JB, and Harden TK (1996) Identification of competitive antagonists of the $P2Y_1$ receptor. *Mol Pharmacol* **50**:1323–1329.
Burnstock G and King BF (1996) The numbering of cloned P2 purinoceptors. *Drug Dev Res* **38**:67–71.
Cazenave JP, Hemmendinger A, Beretz A, Sutter-Bay A, and Launay J (1983) Platelet aggregation: a tool for clinical investigation and pharmacological study: methodology. *Ann Biol Clin (Paris)* **41**:167–179.
Feolde E, Vigne P, Breittmayer JP, and Frelin C (1995) ATP, a partial agonist of atypical P2Y purinoceptors in rat brain microvascular endothelial cells. *Br J Pharmacol* **115**:1199–1203.
Filtz TM, Li Q, Boyer JL, Nicholas RA, and Harden TK (1994) Expression of a cloned P2Y purinoceptor that couples to phospholipase C. *Mol Pharmacol* **46**:8–14.
Frelin C, Breittmayer JP, and Vigne P (1993) ADP induces inositol phosphate independent intracellular Ca^{2+} mobilization in brain capillary endothelial cells. *J Biol Chem* **268**:8787–8792.
Gachet C, Hechler B, Léon C, Vial C, Leray C, Ohlmann P, and Cazenave JP (1997) Activation of ADP receptors and platelet function. *Thromb Haemost* **78**:271–275.
Hall DA, Frost V, and Hourani SMO (1994) Effects of extracellular divalent cations on responses of human blood platelets to adenosine 5′diphosphate. *Biochem Pharmacol* **48**:1319–1326.
Hall DA and Hourani SMO (1993) Effects of analogues of adenine nucleotides on increases in intracellular calcium mediated by P2T-purinoceptors on human platelets. *Br J Pharmacol* **108**:728–733.
Harden TK (1998) *Purine and Their Receptors*, New Orleans, LA. In press.
Harden TK, Boyer JL, and Nicholas RA (1995) P2-purinergic receptors: subtype-associated signaling responses and structure. *Annu Rev Pharmacol Toxicol* **35**: 541–579.
Henderson DJ, Elliot DG, Smith GM, Webb TE, and Dainty IA (1995) Cloning and characterization of a bovine P2Y receptor. *Biochem Biophys Res Commun* **212**: 648–656.
Hourani SMO and Hall DA (1996) P2T purinoceptors: ADP receptors on platelets, in *P2 Purinoceptors: Localization, Function and Transduction Mechanisms*. Chichester, UK, Wiley, Ciba Foundation Symposium 198, pp 53–70.
Léon C, Hechler B, Vial C, Leray C, Cazenave JP, and Gachet C (1997) The $P2Y_1$ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryocytic cells. *FEBS Lett* **403**:26–30.
Léon C., Vial C, Cazenave JP, and Gachet C (1996) Cloning and sequencing of a human cDNA encoding endothelial $P2Y_1$ purinoceptor. *Gene* **171**:295–297.
Mills DC (1996) ADP receptors on platelets. *Thromb Haemost* **76**:835–856.
Nicholas RA, Watt W, Lazarowski ER, Li Q, and Harden TK (1996) Uridine nucleotide selectivity of three phospholipase C-activating P2 receptors: identification of a UDP-selective, UTP-selective, and a ATP- and UTP-specific receptor. *Mol Pharmacol* **50**:224–229.
Schachter JB, Li Q, Boyer JL, Nicholas RA, and Harden TK (1996) Second messenger cascade specificity and pharmacological selectivity of the human $P2Y_1$-purinoceptor. *Br J Pharmacol* **118**:167–173.
Tokuyama Y, Hara M, Jones EMC, Fan Z, and Bell GI (1995) Cloning of rat and mouse P2Y purinoceptors. *Biochem Biophys Res Commun* **211**:211–218.
Vigne P, Feolde E, Breittmayer JP, and Frelin C (1994) Characterization of the effects of 2-methylthio-ATP and 2-chloro-ATP on brain capillary endothelial cells: similarities to ADP and differences from ATP. *Br J Pharmacol* **112**:775–780.
Webb TE, Feolde E, Vigne P, Neary JT, Runberg A, Frelin C, and Barnard E (1996) The P2Y purinoceptor in rat brain microvascular endothelial cells couples to inhibition of adenylate cyclase. *Br J Pharmacol* **119**:1385–1392.
Webb TE, Simon J, Krishek BJ, Bateson AN, Smart TG, King BF, Burnstock G, and Barnard EA (1993) Cloning and functional expression of a brain G-protein coupled ATP receptor. *FEBS Lett* **324**:219–225.
Weissman GA, Turner JT, and Fedan JS (1996) Structure and function of P_2 purinoceptors *J Pharmacol Exp Ther* **277**:1–9.

Send reprint requests to: Dr. Christian Frelin, Institut de Pharmacologie Moléculaire et Cellulaire, CNRS UPR 411, 660 route des Lucioles, 06560 Valbonne, France, E-mail: frelin@ipmc.cnrs.fr
or
Dr. Christian Gachet, INSERM U311, ETSS, 10 rue Spielmann, BP 36, 67065 Strasbourg Cédex, France, E-mail: christian.gachet@etss.u-strasbg.fr

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org by guest on December 1, 2012