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The $P2Y_1$ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells

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Abstract The human $P2Y_1$ purinoceptor has been expressed in Jurkat cells and the effects of HPLC purified nucleotides on calcium movements were measured. The most potent agonist was 2-methylthio-ADP followed by ADP. ATP, Sp-ATPαS and β,γ-methylene-ATP were competitive antagonists. Suramin and PPADS inhibited the effects of ADP. This pharmacological profile is the same as that of the so-called P2T purinoceptor responsible for platelet aggregation, which has not yet been identified. Using PCR we found the $P2Y_1$ receptor to be present in blood platelets and megakaryoblastic cell lines. These data suggest that the $P2Y_1$ receptor may be the elusive P2T receptor.

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Key words: $P2Y_1$ purinoceptor; ADP receptor; Human platelet; Megakaryoblastic cell; Heterologous expression

1. Introduction

Extracellular nucleotides exert their diverse biological effects [1] through P2 receptors, as opposed to P1 purinergic receptors which are responsive to nucleosides [2]. On the basis of differential vascular responses to ATP and its analogues, P2 receptors were first subclassified as P2X and P2Y receptors [3]. Since the first molecular characterization of a P2Y receptor in 1993 [4], there has been an explosion in the cloning of purinoceptors. These are now divided into two broad families [5]: P2X ionotropic receptors ($P2X_1$–$P2X_7$) which are ATP ligand-gated ion channel receptors, and P2Y metabotropic seven transmembrane domain receptors coupled to G-proteins ($P2Y_1$–$P2Y_8$) which are responsive to adenine or uridine nucleotides. Other P2 receptors have been described mainly from pharmacological data. This is the case of the platelet P2T receptor, responsible for platelet aggregation, where all attempts at cloning have to date been unsuccessful. The expression of P2T seems to be restricted to platelets and megakaryoblastic cell lines [6–9], ADP being its natural agonist and ATP a competitive antagonist [10,11]. Platelet activation by ADP involves not only mobilization of intracellular calcium stores but also a rapid influx of calcium [12], presumably through P2X receptors [13]. ADP also inhibits stimulated adenylate cyclase but this is not the cause of platelet aggregation [14]. On account of its coupling to a Gi protein of the α_{i2} subtype [15], the P2T receptor is thought to belong to the P2Y metabotropic receptor family.

The first metabotropic receptor to be molecularly identified was the chick $P2Y_1$ receptor [4], after which successive species homologues were cloned from turkey [16], rat and mouse [17], bovine [18] and human tissues [19]. Recently, a pharmacological characterization of the human $P2Y_1$ receptor has been reported, using measurements of inositol phosphate production in response to various ATP and ADP analogues [20,21]. Pharmacological characterization of nucleotide receptor subtypes is nevertheless hampered by the absence of selective agonists and antagonists and by the presence of synthesis by-products in commercial nucleotide sources. Furthermore, ATP and ADP analogues are more or less metabolically unstable and may be degraded by the ectonucleotidases present on cells. In the present paper, we report a new pharmacological characterization of the human $P2Y_1$ receptor using purified nucleotides. This receptor was found to be not a nucleotide triphosphate receptor but a nucleotide diphosphate receptor and was shown to strikingly resemble the platelet P2T receptor with triphosphate nucleotides antagonizing the diphosphate nucleotides effects. Moreover, this $P2Y_1$ receptor was found to be expressed both in platelets and in megakaryoblastic cell lines.

2. Materials and methods

2.1. Cell culture

Jurkat E6.1 cells (ECACC No. 88042803, Cerdic, France) and megakaryoblastic cell lines Dami, MEG-01, CHRF-288, K562 and HEL [22] were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin (media and supplements from Gibco-BRL, Life Sciences, Cergy-Pontoise, France). Cultures were kept at 37°C in a humidified atmosphere containing 5% CO_2 and cells were subcultured every 3 days so as to maintain a density of approximately 5×10^5 cells/ml.

2.2. cDNA cloning, sequencing and heterologous expression

A 2300 bp cDNA fragment was isolated from a human placental cDNA library (kind gift from Prof. P. Chambon) using a previously described procedure [19]. The clone was rescued in pMosElox (Amersham Life Science, UK) and sequenced in both directions using the Sanger dideoxy chain termination method (Sequenase kit version 2.0, Amersham). An expression vector containing the coding sequence was constructed by insertion of the *Bam*HI-*Eco*RV P2Y fragment into the *Bam*HI and *Sma*I sites of pCDNA3 (Invitrogen, Abingdon, UK). Expression vectors with and without the $P2Y_1$ coding sequence were linearized with *Pvu*I and electroporated into Jurkat E6.1 cells (Cellject, Eurogentec, Seraing, Belgium) using 250 V, 1500 μF, and infinite

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Abbreviations: ADP, adenosine 5′-diphosphate; Sp-ADPαS, adenosine 5′-*O*-(1-thiodiphosphate) (Sp isomer); ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ATP, adenosine 5′-triphosphate; 2MeSATP, 2-methylthio-adenosine 5′-triphosphate; 2MeSADP, 2-methylthio-adenosine 5′-diphosphate; 2ClATP, 2-chloro-adenosine 5′-triphosphate; 2ClADP, 2-chloro-adenosine 5′-diphosphate; αβMeATP, α,β-methylene-ATP; βγMeATP, β,γ-methylene-ATP; Sp-ATPαS, adenosine-5′-*O*-(1-thiotriphosphate); UTP, uridine 5′-triphosphate; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid

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resistance. Transfected cells were selected with geneticin (1 mg/ml; Boehringer, Mannheim, Germany) 48 h after electroporation. Cells were subsequently cloned by limiting dilution and about 20 clones were screened for expression of the P2 receptor.

2.3. Measurement of $[Ca^{2+}]_i$

Cells were washed in basal salt solution (BSS) (25 mM HEPES, pH 7.3, 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 0.1% human essentially fatty acid free serum albumin (Sigma, Saint Quentin-Fallavier, France)) supplemented with 2 mM $CaCl_2$. After centrifugation ($100\times g$, 5 min), the cells were resuspended in BSS without calcium at a concentration of 15×10^6 cells/ml and loaded with 5 μM fura-2/acetoxymethyl ester (fura-2/AM) at 37°C for 30 min in the dark. The cells were then pelleted and suspended at a density of 1×10^6 cells/ml in BSS containing calcium. Intracellular concentration of calcium ($[Ca^{2+}]_i$) was measured in a PTI Deltascan spectrofluorimeter (Photon Technology International Inc., Princetown, NJ) using 340 and 380 nm alternative excitation and 510 nm detection wavelengths. Similar $[Ca^{2+}]_i$ responses could be detected with or without pretreatment of the cells with 2 U/ml of the ATP diphosphohydrolase apyrase (EC 3.6.1.5).

2.4. Poly(A)$^+$ RNA isolation, cDNA synthesis and polymerase chain reaction (PCR) amplification

Human platelet concentrates (approximately 10^6 platelets/μl) were obtained from healthy donors. In order to eliminate white blood cells and the majority of erythrocytes, two or three successive centrifugations were performed in a Sorvall RC-3 centrifuge ($570\times g$ for 10 s, without brake), the absence of white blood cells in platelet suspensions being confirmed by counting cells with a hemacytometer (Sysmex K-1000, TOA Medical Electronics, Japan). Cell cultures in the exponential growth phase and human platelets were pelleted and total RNA was recovered using RNA NOW solution (Biogentex, Ozyme, Montigny-le Bretonneux, France). Poly(A)$^+$ RNA was obtained by oligo(dT)-cellulose chromatography (QuickPrep *Micro* mRNA Purification Kit, Pharmacia Biotech, Uppsala, Sweden) while cDNA was synthesized using a T-primed First Strand cDNA synthesizing kit (Pharmacia Biotech). The following oligonucleotides were employed to amplify the entire human $P2Y_1$ coding sequence: sense HP2YA (5′-CTCGGAGCCGCCGCCTAAGTCGAGG-3′) and antisense HP2YB (5′-CCTACCATATTACAACAGAGAGGTGT-3′). PCR reactions were carried out in a thermal cycler (GeneAmp PCR system 2400, Perkin Elmer, Roissy, France) using 35 cycles of 94°C for 30 s, 57°C for 45 s and 72°C for 45 s. Absence of exogenous or genomic DNA contamination was checked by performing experiments with no template or RNA as template instead of cDNA. The 1198 bp PCR products were purified and sequenced as described.

2.5. Materials

ADP, Sp-ATPαS, βγMeATP and UTP nucleotides were obtained from Boehringer (Mannheim, Germany), while ATP and αβMeATP were purchased from Sigma (Saint Quentin-Fallavier, France) and 2MeSATP, 2ClATP, 2MeSADP and PPADS from Research Biochemical Incorporation (Natick, MA, USA). Fura-2/AM and suramin were obtained from Calbiochem (Meudon, France). All nucleotides were checked for purity by high performance liquid chromatography (HPLC) analysis. When necessary, nucleotides were purified by ion exchange chromatography on a Partisil 10 μ SAX column (Interchrom, Interchim, Monluçon, France) eluted with a linear gradient of 0–500 mM triethyl ammonium bicarbonate, pH 8.4, or with 50–800 mM ammonium phosphate buffer, pH 3.8. Both buffers were previously checked for absence of effects on calcium measurements.

3. Results

Previously, we described the isolation of a human $P2Y_1$ receptor from a placental cDNA library [19]. Using exactly the same procedure, we have now isolated another 2.3 kb clone containing the same coding sequence with one exception: the presence of an additional 3 bp leading to an additional serine residue in transmembrane domain 3, between residues 137 and 138. The pCDNA3 vector alone and a vector containing the coding sequence of the serine-containing human placental $P2Y_1$ receptor were stably transfected into Jurkat E6.1 cells, a human cell line which is not naturally responsive to nucleotides [18]. Geneticin-resistant cells were cloned and screened for ATP-induced increases in $[Ca^{2+}]_i$. Three in 20 clones were found to express a functional serine-containing $P2Y_1$ receptor and the cellular clone which gave the best response to ATP was chosen for further determination of the pharmacological profile.

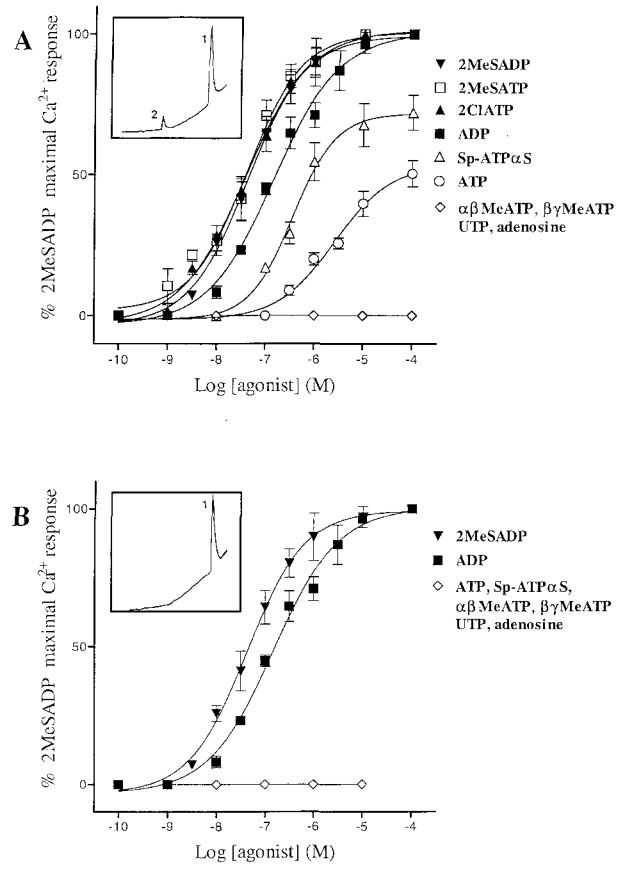

Fig. 1. Effects of nucleotides on $[Ca^{2+}]_i$ in fura-2-loaded Jurkat cells stably expressing the human $P2Y_1$ receptor. Increases in the ratio of fluorescence emission using 340 and 380 nm excitation wavelengths are expressed as a percentage of the maximal response obtained with 2MeSADP (10^{-4} M), in response to various concentrations of nucleotides, using non-purified nucleotides (A) and HPLC-purified nucleotides (B). Inset, HPLC profiles of Sp-ATPαS (1) containing 8% Sp-ADPαS (2) before (A) and after (B) purification. Curves each represent the results of five independent experiments and bars show the S.E.M.

In order to characterize the pharmacology of the transfected $P2Y_1$ receptor, increases in $[Ca^{2+}]_i$ in response to various nucleotide analogues were measured and concentration-response curves were generated (Fig. 1A). In a first approach, 2MeSADP, 2MeSATP, 2ClATP and ADP were found to be full agonists of the $P2Y_1$ receptor, producing a rapid rise in $[Ca^{2+}]_i$ which peaked within 5 s and subsequently returned to basal levels in less than 3 min. 2ClATP, 2MeSATP and 2MeSADP were the most potent agonists ($EC_{50} = 40\pm4$ nM, 45 ± 13 nM and 49 ± 10 nM respectively), being four times more potent than ADP ($EC_{50} = 204\pm64$ nM). Sp-ATPαS and ATP were partial agonists ($EC_{50} = 380\pm20$ nM and 11 ± 7 μM respectively), while αβMeATP, βγMeATP, UTP and adenosine were without agonistic effect. In a medium

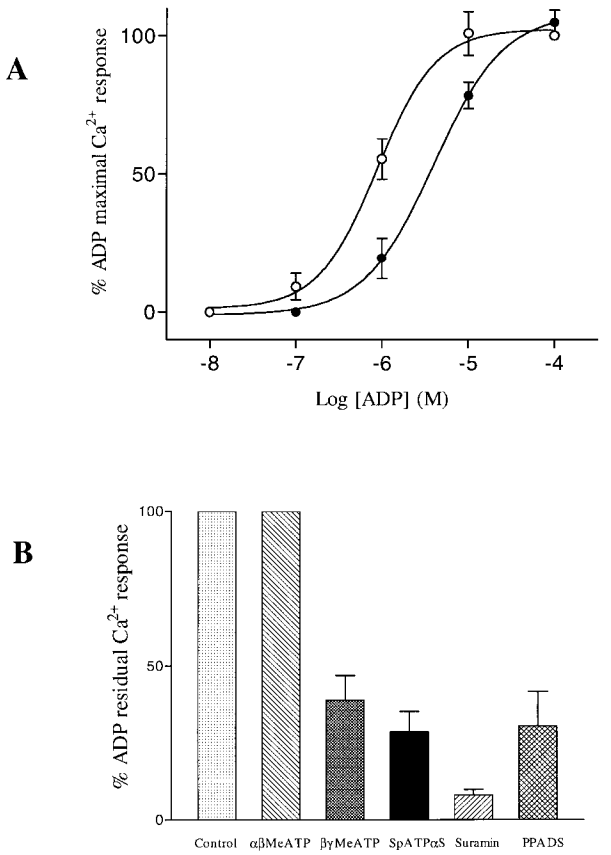

Fig. 2. Effects of triphosphate nucleotides and P2 receptor antagonists on ADP-induced $[Ca^{2+}]_i$ increase. Points are the mean of three determinations and bars show the S.E.M. A: Dose-dependent increases in $[Ca^{2+}]_i$ induced by ADP alone (○) or in the presence of ATP (100 μM) (●). B: Effects of αβMeATP (1 mM), βγMeATP (1 mM), Sp-ATPαS (10 μM), suramin (100 μM) and PPADS (10 μM) on the 1 μM ADP-induced $[Ca^{2+}]_i$ increase. Control experiment: ADP (1 μM) alone.

containing no calcium (0.3 mM EGTA), 2MeSADP still elicited a transient increase in $[Ca^{2+}]_i$, but with diminished amplitude indicating that the 2MeSADP-induced $[Ca^{2+}]_i$ rise was due both to mobilization of calcium from internal stores and to entry of external calcium (data not shown).

As already mentioned, commercial nucleotide powders are often contaminated by degradation products which can be the cause of misleading results. In order to exclude a possible contamination, and because of the observation that true antagonists of ADP-induced platelet aggregation had agonistic activities on platelets, we then systematically purified all nucleotides by HPLC immediately before use. In these conditions, we observed no further agonistic effect of ATP. Sp-ATPαS, a poorly hydrolyzable ATP analogue, contained 6–10% Sp-ADPαS; once purified, 10 μM Sp-ATPαS was ineffective, whereas the contaminating Sp-ADPαS was still agonist. Hence, the new pharmacological profile of the human $P2Y_1$ receptor was 2MeSADP > ADP with ATP, Sp-ATPαS, αβMeATP, βγMeATP, UTP and adenosine being ineffective (Fig. 1B). In addition, 100 μM ATP induced a parallel right shift in dose-dependent calcium responses to ADP, while the inhibition by ATP could be overcome at high concentrations of ADP (Fig. 2A). Hence ATP was clearly a competitive antagonist of the receptor. Purified Sp-ATPαS (10 μM) also inhibited 1 μM ADP-induced increases in $[Ca^{2+}]_i$ in a competitive manner and whereas 1 mM βγMeATP exhibited antagonistic effects, 1 mM αβMeATP was inactive (Fig. 2B; Table 1). The non-selective P2 receptors antagonists suramin (100 μM) and PPADS (10 μM) both inhibited calcium increase induced by 1 μM ADP (Fig. 2B).

2MeSATP and 2ClATP, once purified, still appeared to be agonists but elicited a different calcium response, characterized by a lag time (15–20 s) followed by a gradual increase (40 s) leading to a sustained calcium signal which returned progressively to basal levels (Fig. 3A). In order to assess the action of 2MeSATP and 2ClATP on the $P2Y_1$ receptor, we examined their susceptibility to enzyme degradation. Classical methods of nucleotide analysis using perchloric acid for protein precipitation could not be employed for 2MeSATP and 2ClATP due to their instability at acidic pH. To circumvent this problem, we used a direct bioassay in which 100 μM purified 2MeSATP or 2ClATP was incubated with mock-transfected Jurkat cells (2×10^6 cells) at 37°C under slow agitation for 5 min. These cells were then centrifuged and an aliquot of the supernatant was added to fura-2-loaded $P2Y_1$-transfected Jurkat cells. The resulting 2MeSATP or 2ClATP calcium signals were similar to that obtained after addition of non-purified 2MeSATP or 2ClATP (Fig. 3B). Equivalent results were observed when 2MeSATP and 2ClATP were incubated with the ATP diphosphohydrolase apyrase (EC 3.6.1.5), which converted 2MeSATP to 2MeSADP and 2ClATP to 2ClADP (data not shown). Control supernatants from mock-transfected Jurkat cells incubated with buffer alone had no effect.

In terms of $[Ca^{2+}]_i$ responses, the pharmacological properties of the $P2Y_1$ receptor are strikingly similar to those of the ADP receptor present on human platelets [23] (Table 1). Thus, we employed PCR amplification to look for the presence of $P2Y_1$ receptor mRNA in megakaryoblastic cell lines (MEG-01, Dami, CHRF-288, HEL and K562) and in human platelets. As shown in Fig. 4, a 1198 bp amplification product corresponding to the entire coding sequence of $P2Y_1$ was

Table 1
Comparison of the pharmacological characteristics, in terms of $[Ca^{2+}]_i$ movements, both of the human $P2Y_1$ receptor heterologously expressed and of the human P2T receptor

	'P2T' receptor[a]	$P2Y_1$ receptor
Agonists		
2MeSADP	+ (full) EC_{50} = 41 nM	+ (full) EC_{50} = 49 nM
ADP	+ (full) EC_{50} = 800 nM	+ (full) EC_{50} = 204 nM
ADPαS	+ (partial)	+ (nd)
ADPβS	+ (partial)	+ (nd)
Antagonists		
Sp-ATPαS	+ (competitive)	+ (competitive)
ATP	+ (competitive)	+ (competitive)
βγMeATP	+ (competitive)	+ (competitive)
2ClATP	+ (competitive)	?
2MeSATP	+ (non-competitive)	?
Suramin	+	+
PPADS	+	+
Without effect		
αβMeATP	+	+
UTP	+	+
Adenosine	+	+

[a]According to [23].
nd: not determined; ?: inconclusive (delayed effect due to enzymatic degradation and chemical instability in solution).

detected in all the megakaryoblastic cell lines tested and in platelets. U373 cells and native Jurkat cells were found to be negative for $P2Y_1$ mRNA (data not shown). When this amplification product was purified and sequenced, its nucleic acid sequence showed 100% identity with the human $P2Y_1$ receptor.

4. Discussion

Previously, we reported the cloning of a human $P2Y_1$ receptor highly homologous to the chick, rodent and bovine $P2Y_1$ receptor [19]. In the present paper, we report the pharmacological characteristics of another cloned $P2Y_1$ receptor, differing from the first by the presence of an additional serine residue in transmembrane domain 3. This serine residue is present in all the P2Y receptors sequenced so far and is highly conserved in most of the G-protein-coupled receptors. Whether the absence of this serine in our first clone represents a point mutation or a polymorphism is not clear at the moment. Whatever it is, we only got responsive cellular clones expressing the serine residue-containing receptor. The pharmacological profile first obtained was consistent with observations for the other heterologously expressed $P2Y_1$ receptor [4,16–18,20,21]. We found 2MeSATP, 2ClATP and 2MeSADP to be more potent agonists than ADP, Sp-ATPαS and ATP being partial agonists. αβMeATP and βγMeATP were without agonistic effect. A partial agonistic effect of ATP has been observed for the bovine $P2Y_1$ receptor [18] and also for the chick $P2Y_1$ receptor for which several related ATP derivatives were partial agonists [24,25]. This partial agonistic feature was remarkable since $P2Y_1$ receptors have long been described as adenine nucleotide triphosphate receptors.

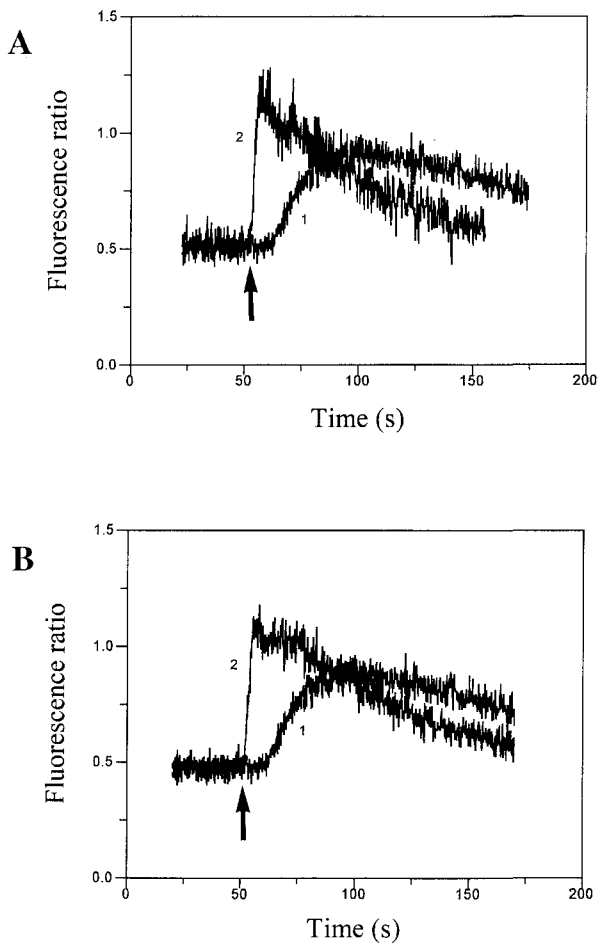

Fig. 3. Variations in fluorescence ratio in fura-2-loaded Jurkat cells expressing the human $P2Y_1$ receptor induced by purified 2MeSATP (10 μM) (1) and by non-purified 2MeSATP (10 μM) (2) A: Demonstration of 2MeSATP catabolism at the surface of human Jurkat cells. Comparison between purified 2MeSATP (10 μM) (1) or purified 2MeSATP (10 μM) previously incubated for 5 min with mock-transfected Jurkat cells (2) B: Arrows indicate the time of nucleotide addition.

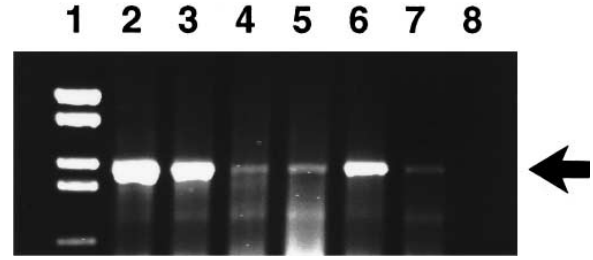

Fig. 4. Detection of $P2Y_1$ mRNA by PCR amplification. A 1198 bp amplification product was analyzed on 1% agarose gel and visualized by ethidium bromide staining (arrow). Lane 1, molecular weight marker VI (Boehringer); lane 2, MEG-01 cells; lane 3, DAMI cells; lane 4, K562 cells; lane 5, CHRF-288 cells; lane 6, HEL cells; lane 7, human platelets; lane 8, control reaction in the absence of cDNA template.

Since a partial agonistic action could arise from degradation of the nucleotide triphosphates, ATP and ATP analogues were checked for purity by HPLC and repurified when necessary. Purification changed the pharmacological profile of the human $P2Y_1$ receptor. In this report we show that contrary to common knowledge, the human $P2Y_1$ receptor is a nucleotide diphosphate receptor for which nucleotide triphosphates cannot be considered to be agonists. 2MeSADP is a more potent agonist than ADP, while ATP, Sp-ATPαS and βγMeATP are competitive antagonists of the ADP-induced $[Ca^{2+}]_i$ rise, αβMeATP, adenosine and UTP being without effect. This discrepancy with previous studies is mainly due to the fact that commercial nucleotide preparations are not pure. Such preparations require further purification, as even small amounts of contaminant products can lead to an artefactual agonistic effect. Erroneous pharmacological profiles have already been observed for other P2 receptors, due to contamination of nucleotide stock solutions and enzymatic metabolization [26,27]. Although purified, 2MeSATP and 2ClATP still elicited a calcium signal. This signal was delayed probably due to the metabolization by ectoenzymes and to the relatively high chemical instability of these compounds in solution, although we cannot absolutely rule out the possibility that 2MeSATP and 2ClATP exert a real agonistic effect. Therefore this is also a possible explanation why ATP was found to be a full agonist when IP_3 production was measured at 10 min stimulation [20]. It would be useful to have selective inhibitors of the various ectonucleotidases in order to distinguish between these different phenomena.

Since we show that the human $P2Y_1$ receptor is in fact an ADP receptor for which ATP is an antagonist, the question arises as to whether the $P2Y_1$ receptor might be the ADP receptor expressed by platelets and commonly referred to as the P2T receptor. The P2T receptor is indeed activated by ADP, 2-substituted ADP analogues being the most potent

agonists [14]. ATP, Sp-ATPαS, βγMeATP and 2ClATP are competitive antagonists, whereas 2MeSATP is a non-competitive antagonist and αβMeATP and adenosine are inactive [11,14,23]. The pharmacological profile determined in the present work for the human $P2Y_1$ receptor is in agreement with that of the P2T receptor (Table 1). In view of these results, it appears that the human $P2Y_1$ and P2T receptors display very similar if not identical pharmacological properties, although on account of the delayed action of 2MeSATP and 2ClATP, we were unable to demonstrate an antagonistic effect of these 2-substituted nucleotides at the $P2Y_1$ receptor. Moreover, the $P2Y_1$ mRNA was found to be present both in human platelets and in megakaryoblastic cells. The cloning of human $P2Y_1$ receptor from HEL cells was also reported during the course of our study [28].

Pharmacological studies and the evidence for the presence of $P2Y_1$ mRNA in platelets and megakaryoblastic cells strongly support that the $P2Y_1$ receptor is the platelet P2T receptor. In platelets, ADP induces $[Ca^{2+}]_i$ increase and inhibits adenylate cyclase [10,11] whereas most $P2Y_1$ receptors described to date are coupled to phospholipase C. However, transduction mechanisms may vary according to the cell type expressing the receptor and some $P2Y_1$ receptors have been shown to be coupled to adenylate cyclase in some native cells, for example in the case of the rat $P2Y_1$ receptor [24,29]. When endogenously expressed by B10 cells, a brain capillary endothelial cell line, this receptor is indeed coupled to adenylate cyclase, and likewise in the rat glioma cell line C6-2B, whereas in cultured brain astrocytes it is associated with inositol phosphate production [24]. However, whether the $P2Y_1$ receptor is the receptor involved in platelet aggregation remains to be demonstrated. It would be of interest to check whether so-called selective P2T antagonists such as ARL66096 and ARL67085 (previously known as FPL) [30] have an effect on this receptor. Unfortunately, these compounds are not commercially available. ARL66096 has been shown to antagonize ADP-induced platelet aggregation with a pK_B of 8.66 while it inhibited 2MeSATP-induced guinea pig aorta relaxation with a pK_B of 4.71 [31]. This discrepancy in potency might be due firstly to the fact that aorta contains more than one subtype of P2 receptors [1]. Secondly, the antagonistic potency of ARL66096 has not been determined toward 2MeSADP which is 20-fold more potent than ADP as an agonist of platelet aggregation.

In summary, we have cloned and stably expressed the human $P2Y_1$ receptor and report that: (i) this receptor exhibits the pharmacological profile of the human platelet P2T receptor and (ii) it is expressed by both megakaryoblastic cell lines and human platelets. These data strongly suggest that the $P2Y_1$ receptor may be the elusive P2T receptor.

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