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$P2Y_1$-receptors in human platelets which are pharmacologically distinct from $P2Y_{ADP}$-receptors

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1 In the present study we have classified the receptor(s) mediating increases in intracellular calcium concentration ($[Ca^{2+}]_i$) in human washed platelets and compared the pharmacological profile obtained with that observed in Jurkat cells, stably transfected with a bovine $P2Y_1$-receptor.

2 The $P2Y_1$-receptor antagonist, adenosine-3′-phosphate-5′-phosphate (A3P5P), competitively antagonized agonist responses in both Jurkat cells, and in platelets with similar affinities (pK_B of 5.8 and 6.0, respectively).

3 The selective $P2Y_{ADP}$ antagonist, AR-C66096, exhibited partial agonism in the Jurkat cells with an affinity (pK_A) of 4.9. This value is consistent with its known $P2Y_1$-receptor activity. In platelets, AR-C66096 at a concentration (0.1 μM) approximately 100 fold greater than its known $P2Y_{ADP}$ receptor affinity, had no effect on ADP-induced increases in $[Ca^{2+}]_i$.

4 The ability of adenine nucleotide analogues to elevate $[Ca^{2+}]_i$ in the Jurkat cells was also determined. The rank order of agonist potency ($p[A]_{50}$) was: 2-MeSADP (8.3) > 2-ClATP (7.8) > ADP (7.5) = 2-MeSATP (7.4) > ATPγS (6.5) > ATP (6.2), with ATP appearing to be a partial agonist.

5 The same rank order of potency was observed when similar experiments were performed in platelets. However, the absolute potencies of all the agonists and the intrinsic activities of both ATPγS and ATP were lower in platelets.

6 The operational model of agonism was used to test whether the agonist concentration-effect profiles obtained in these two cell types could be explained on the basis of differences in receptor reserve. The analysis indicated that the data obtained in platelets closely resembled that predicted for a low density or poorly coupled $P2Y_1$-receptor system.

7 The hypothesis that the observed partial agonist behaviour of ATP was the result of receptor activation by contaminating ADP with concomitant receptor blockade by ATP, was tested in the platelet system. This hypothesis was supported by a theoretical analysis, which yielded an affinity value for ATP similar to that obtained previously at $P2Y_1$-receptors.

8 In summary, the results of this study indicate that human washed platelets contain $P2Y_1$-receptors which mediate increases in $[Ca^{2+}]_i$ and that this receptor population is pharmacologically distinct from $P2Y_{ADP}$-receptors.

Keywords: $P2Y_1$-receptors; Jurkat cells; human washed platelets; receptor reserve; intracellular calcium measurements; ATP; ADP; AR-C66096; adenosine-3′-phosphate-5′-phosphate

Introduction

It is well established that extracellular nucleotides are able to mediate a number of biological actions *via* P2-receptors. As a result, this receptor family has been the focus of much investigation over recent years. With advances in both molecular and pharmacological approaches, these receptors have now been divided into ionotropic ($P2X_{1\text{-}7}$) and G-protein linked receptors ($P2Y_{1\text{-}8}$) (Burnstock & King, 1996). A further G-protein linked receptor which mediates adenosine 5′-diphosphate (ADP)-induced platelet aggregation, remains elusive to cloning, with attempts being confounded by the fact that this receptor appears to be unique to the platelet which is anucleonic.

This receptor, at which ADP and its 2-substituted analogues (e.g. 2-methylthioADP (2-MeSADP)) act as agonists, whilst adenosine 5′-triphosphate (ATP) and its 2-substituted analogues (e.g. 2-methylthioATP (2-MeSATP)) act as low affinity antagonists (Hall & Hourani, 1993; Cusack & Pettey, 1996) has previously been referred to as the P_{2T} receptor. Recently, development of the potent, selective and competitive P_{2T}-receptor antagonist, AR-C66096 (formerly known as FPL 66096; Humphries *et al*., 1994) has allowed the definitive pharmacological characterization of this receptor. However, as recommended by the IUPHAR committee (Fredholm *et al*., 1997) the nomenclature used for the P_{2T}-receptor throughout the remainder of this paper is $P2Y_{ADP}$.

ADP elevates intracellular calcium concentration ($[Ca^{2+}]_i$) in platelets by mobilizing internal stores, increasing extracellular influx (Sargeant & Sage, 1994) and inhibiting adenosine 3′,5′-cyclic monophosphate cyclic AMP production (Cusack & Hourani, 1982). In terms of platelet function, ADP causes adhesion, degranulation, shape change and aggregation (Gear, 1993). The diversity of these effects, with differing agonist and antagonist potency orders, has led some workers to propose the existence of several ADP-recognizing receptors on the surface of platelets (for review see Hourani & Hall, 1994). Indeed, more recent studies have suggested that human platelets possess $P2Y_{ADP}$- (Humphries *et al*., 1994), $P2Y_1$- (Léon *et al*., 1997) and $P2X_1$-receptors (Mackenzie *et al*., 1996). However, several investigators (Nicholas *et al*., 1996; Léon *et al*., 1997) have recently highlighted the fact that the use of adenine nucleotides and their analogues in classification

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studies can be complicated by the presence of active impurities, in commercially available samples. Léon *et al*. (1997) showed that when ATP and 2-MeSATP are purified to remove contaminating diphosphates they no longer exhibited agonism at $P2Y_1$-receptors, but instead behaved as antagonists. Thus, when purified triphosphate analogues are used, the pharmacological profile observed at $P2Y_1$-receptors changes and more closely resembles that found for the $P2Y_{ADP}$-receptor (Léon *et al*., 1997). These observations have led to the suggestion that the $P2Y_1$-receptor may in fact be the $P2Y_{ADP}$-receptor.

The aim of this study was to classify the receptor(s) present on human platelets responsible for increasing $[Ca^{2+}]_i$ and to determine if the $P2Y_1$-receptor is indeed the $P2Y_{ADP}$-receptor. To this end, we have generated agonist potency information for a series of adenine nucleotides and examined the effects of the $P2Y_{ADP}$-receptor antagonist AR-C66096 ($pA_2 = 9.1$; Tomlinson *et al*., 1997) and the $P2Y_1$-receptor antagonist, adenosine-3′-phosphate-5′-phosphate (A3P5P; $pK_B = 6.0$; Boyer *et al*., 1996). These studies were conducted under conditions where receptor desentitization would exclude any contribution to $[Ca^{2+}]_i$ elevation resulting from $P2X_1$-receptor activation (Mackenzie *et al*., 1996).

A preliminary account of part of this study was presented to the British Pharmacological Society (Dainty *et al*., 1997).

Methods

Cell culture

Jurkat cells transfected with a cloned bovine $P2Y_1$-receptor (Henderson *et al*., 1995) were cultured in RPMI growth media supplemented with 10% foetal calf serum, 5% l-glutamine and 10% penicillin/streptomycin. Cultures were grown at 0.2–1.2×10^6 cells ml^{-1} in a humidified atmosphere of 5% CO_2 in air and maintained at 37°C.

Loading of Jurkat cells with fura 2-AM

To remove any growth media, the Jurkat cells were centrifuged (5 min, 1000 r.p.m.) and re-suspended in basal salt solution (BSS, composition (mM): NaCl 125.0, KCl 5.0, $MgCl_2$ 1.0, $CaCl_2$ 1.5, HEPES 25.0, D-glucose 5.0 and 1 mg ml^{-1} bovine serum albumin, pH 7.3). In a loading volume of BSS (3 ml), the washed Jurkat cells were incubated with fura 2-AM (17 μM) and apyrase (2 u ml^{-1}) at 37°C with shaking for 45 min. Apyrase was included to prevent tonic desensitization of receptors by ATP or ADP (Dainty *et al*., 1997). Cells were then centrifuged (5 min, 1000 r.p.m.), the supernatant discarded and the pellet re-suspended in BSS to a final concentration of 1×10^6 cells ml^{-1}.

Preparation of human washed platelets

Suspensions of human washed platelets were prepared as previously described by Humphries *et al*. (1994). Venous blood was obtained from healthy male and female volunteers and anti-coagulated with 1/10 volume, 3.2% trisodium citrate. The blood was centrifuged (15 min, 240 *g*) to obtain platelet rich plasma (PRP) to which prostacyclin (PGI_2; 300 ng ml^{-1}) was added to stabilize platelets during the washing procedure. Red cell-free PRP was obtained by centrifugation (10 min, 125 *g*), and following further centrifugation (15 min, 640 *g*), the supernatant was discarded and the pellet re-suspended in 20 ml calcium-free Tyrode solution (CFT, composition (mM): NaCl 137.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.38, KCl 2.68, $MgCl_2$ 1.05, D-glucose 5.55, gassed with 95% O_2/5% CO_2 and maintained at 37°C) containing 300 ng ml^{-1} PGI_2 to give washed platelets.

Loading of platelets with fura 2-AM

Washed platelets (3 ml) were incubated with fura-2 AM (17 μM) and apyrase (0.06 u ml^{-1}) at 37°C for 45 min. Suspensions were then centrifuged (15 min, 640 *g*), the supernatant was discarded and the pellet re-suspended in BSS to a final platelet count of 1×10^7 cells ml^{-1}.

Experimental protocols

Fura-2 fluorescence measurements were made from stirred cell suspensions by use of a SPEX FluoroMax fluorimeter. Light emission was measured at 510 nm and the ratio of the light emissions when samples were excited at 340/380 nm was used as a measurement of $[Ca^{2+}]_i$. Aliquots (18 ml) of loaded cells (Jurkat cells or platelets) were incubated at 37°C for 15 min before the measurement of $[Ca^{2+}]_i$. Fluorescence was measured in cuvettes containing 2 ml of cells for 5 s before the addition of an agonist. Agonist concentration-effect (E/[A]) curves were constructed with each cuvette used for one agonist concentration only. When antagonists were studied, they were added to the cuvette for 15 s before the addition of 2-MeSATP (Jurkat cells) or ADP (platelets). At the end of each E/[A] curve, a maximally effective concentration of 2-MeSATP (Jurkat cells) or ADP (platelets) was tested and all responses were subsequently expressed as a percentage of these values.

Drugs

Adenosine 5′-diphosphate (sodium salt; ADP), adenosine 5′-triphosphate (disodium salt; ATP), adenosine 5′-O-(3-thiotriphosphate) (tetralithium salt; ATPγS), adenosine 3′-phosphate-5′-phosphate (monosodium salt; A3P5P), prostacyclin, apyrase (grade V) and Fura 2-AM were obtained from the Sigma Chemical Co. (Poole, U.K.). 2-pro-pylthio-D-β,γ-difluoromethylene ATP (trisodium salt AR-C66096) was synthesized in the Medicinal Chemistry Department, Astra Charnwood; 2-methylthio-adenosine 5′-di-phosphate (trisodium salt; 2-MeSADP), 2-methylthio-adenosine 5′-tri-phosphate (tetrasodium salt; 2-MeSATP) and 2-chloro-adenosine 5′-tri-phosphate (tetrasodium salt; 2-ClATP) were supplied by Research Biochemicals Inc. (St. Albans, U.K.). Fura 2-AM was dissolved in dimethylsulphoxide (DMSO); all other drugs were dissolved and diluted in distilled water.

Heat inactivated foetal calf serum, RPMI 1640, L-glutamine (200 mM) and penicillin/streptomycin (5000 iu ml^{-1}, 5000 ug ml^{-1}) were obtained from Gibco BRL (Paisley, U.K.).

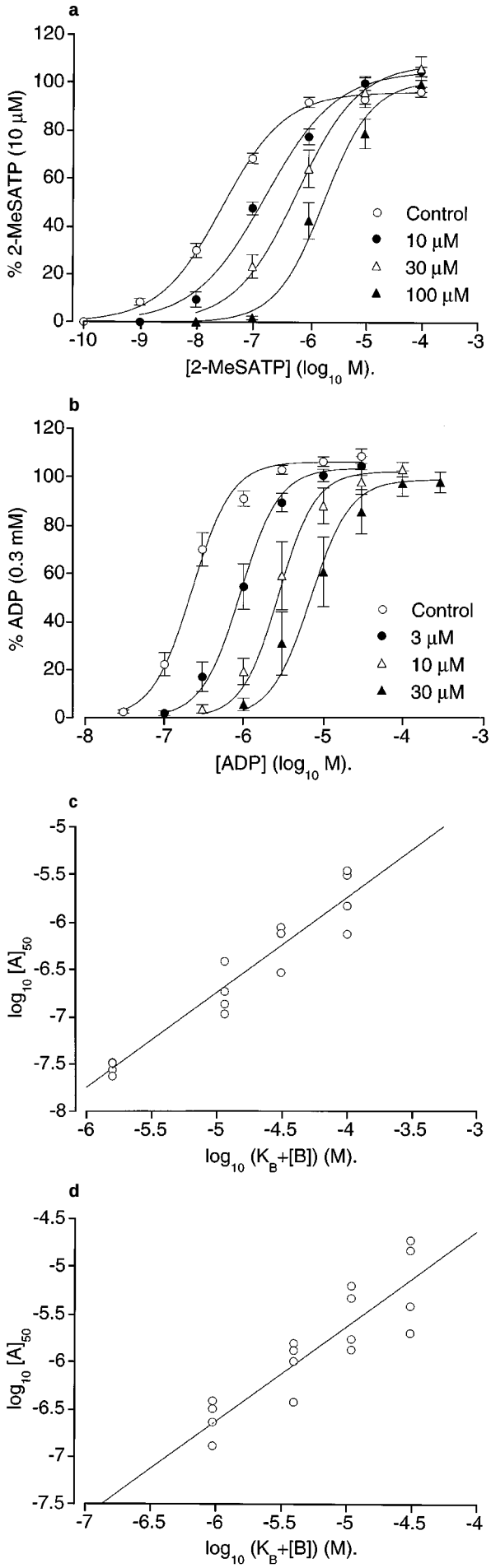
Data analysis

Logistic curve fitting Individual agonist E/[A] curve data were fitted to the following form of the Hill equation:

$$E = \frac{\alpha[A]^{n_H}}{[A]^{n_H} + [A]_{50}^{n_H}} \quad (1)$$

in which α, $[A]_{50}$ and n_H are the asymptote, location and slope parameters, respectively. $[A]_{50}$ values were assumed to be log-normally distributed and quoted as $p[A]_{50}$ ($-\log[A]_{50}$) values.

Antagonist affinity estimation $[A]_{50}$ data obtained in antagonist experiments were fitted to the following linear form of the Schild equation (Trist & Leff, 1985):



$$\log_{10}[A]_{50} = \log_{10}[A]^{c}_{50} + \log_{10}(1 + [B]^{n}/K_B) \quad (2)$$

where $[A]^{c}_{50}$ is the estimated control $[A]_{50}$ value, [B] is the concentration of the antagonist, K_B is the antagonist equilibrium constant and n is equivalent to the Schild plot slope parameter (unity for simple competition). If n was not significantly different from unity, it was constrained to unity for pK_B estimation.

Operational model-fitting The affinity and efficacy of the partial agonist, AR-C66096 (in bovine P2Y$_1$-receptor transfected Jurkat cells), was estimated by use of the operational model of agonism (Black & Leff, 1983; Black *et al*., 1985):

$$E = \frac{E_m \tau^n [A]^n}{([A]+K_A)^n + \tau^n [A]^n} \quad (3)$$

in which E and [A] are the pharmacological effect and agonist concentration respectively; E_m is the maximum possible effect; K_A is the agonist dissociation constant (estimated as the negative logarithm, i.e. pK_A); τ is the efficacy of the agonist (estimated as a logarithm) and n determines the steepness of the occupancy-effect relation.

The analysis was performed with the comparative method, in which the partial agonist (AR-C66096) E/[A] curve data were fitted to the operational model (Equation 3) simultaneously to fitting the full agonist (ADP) E/[A] curve data to a Hill equation of the form:

$$E = \frac{E_m [A]^n}{[A]^n + [A]^n_{50}} \quad (4)$$

where E_m and n are as defined above and $[A]_{50}$ is the location parameter of the full agonist curve. This allows K_A and τ for the partial agonist to be estimated as well as E_m and n from each pair of curves (see Leff *et al*., 1990 for details).

Theoretical analysis of the effect of ATP contamination by ADP Increases in $[Ca^{2+}]_i$ induced by commercially obtained 'ATP' may actually be attributed to the agonist effects of contaminating ADP. To determine whether the partial agonist effects of ATP observed in the present study were the result of receptor activation by ADP and concomitant receptor blockade by ATP, we fitted the ADP and ATP E/[A] curve data from human washed platelets simultaneously to the following equation:

$$E = \frac{E_m((1-q)X)^n}{((1-q)X)^n + \left(([A]_{50})(1 + qX/K_B)\right)^n} \quad (5)$$

in which ADP has been assumed to be a full agonist and ATP to be a competitive antagonist; X represents the total concentration of agonist (A) and antagonist (B), that is, ([A]+[B]) in the

Figure 1 Antagonist effects of A3P5P (a) on 2-MeSATP-induced increases in $[Ca^{2+}]_i$ in bovine P2Y$_1$-receptor transfected Jurkat cells and (b) on ADP-induced increases in $[Ca^{2+}]_i$ in human washed platelets: E/[A] curves to 2-MeSATP were obtained in the absence and presence of A3P5P (10–100 μM); E/[A] curves to ADP were obtained in the absence and presence of A3P5P (3–30 μM). The data are the averages of 4 replicate E/[A] curves with vertical lines indicating s.e. The lines drawn through the data are the results of curve-fitting with Equation 1 (see Methods). (c) and (d) Illustrate the corresponding $[A]_{50}$ data in Clark plot form. The adherence of the data with the unit slope line drawn through them indicates consistency with simple competition. The pK_B values estimated by fitting the $[A]_{50}$ data to Equation (2) were 5.8 ± 0.1 and 6.0 ± 0.2 for the Jurkat system and platelets, respectively.

sample; q represents the fraction of the total sample that is antagonist ($q=[B]/([A]+[B])$) and therefore $(1-q)$ represents the fraction that is agonist; E_m is the maximum possible effect; n is the slope index of the occupancy-effect function; $[A]_{50}$ is the midpoint location parameter and K_B is the antagonist equilibrium dissociation constant.

q was measured for the ATP sample by high performance liquid chromatography (h.p.l.c.) whereas q was assumed to be zero for the ADP sample. The analysis was carried out by fitting data from individual donors which allowed the estimation of K_B (as well as E_m, n and $[A]_{50}$) for each donor.

All of the data fitting procedures and simulations were carried out with Microsoft Excel. Results are expressed and plotted as mean values ± s.e. Statistical differences were assessed by the use of Student's *t* test and considered significant at the level $P<0.05$.

Results

Estimation of antagonist affinities

Exposure of $P2Y_1$-receptor transfected Jurkat cells to A3P5P (10–100 μM) caused small decreases (~15 nM) in $[Ca^{2+}]_i$. The cause of these decreases in $[Ca^{2+}]_i$ remains unclear and were not observed in platelets. A3P5P (3–100 μM) caused concentration-dependent, parallel, rightward displacements of 2-MeSATP E/[A] curves in Jurkat cells and of ADP E/[A] curves in platelets (Figure 1a and b respectively). Analysis of the computed $[A]_{50}$ values from Equation 2 for the two sets of data indicated that the interactions between A3P5P and the agonists conformed to simple competition in each case. Figure 1c and d show the $[A]_{50}$ data for Jurkat cells and platelets in Clark plot form (Stone & Angus, 1978). The Schild slope parameters were 1.1 ± 0.13 and 1.0 ± 0.10, respectively. Neither value was significantly different from unity and the resulting pK_B estimates wre 5.8 ± 0.1 ($n=4$) and 6.0 ± 0.2 ($n=4$). Statistical analysis of the resulting pK_B values showed that the affinities of A3P5P were not significantly different in the two systems.

The selective $P2Y_{ADP}$-receptor antagonist, AR-C66096, exhibited partial agonism in Jurkat cells ($n=4$, Figure 2a). Comparative analysis of these data from the operational model of agonism gave an affinity estimate (pK_A) of 4.9 ± 0.2 ($n=4$) and an efficacy estimate (τ) of 1.1 ($\log \tau = 0.04 \pm 0.05$). In contrast, AR-C66096 (100 μM) showed no agonism in platelets. Furthermore, at a concentration, approximately 100 fold greater than its published $P2Y_{ADP}$ receptor affinity (see Table 1), AR-C66096 (0.1 μM) had no effect on ADP E/[A] curves in this system ($n=4$; Figure 2b).

Agonist profiles

The ability of a series of adenine nucleotide analogues to modulate $[Ca^{2+}]_i$ in Jurkat cells and platelets was investigated. Addition of 2-MeSADP, 2-ClATP, ADP, 2-MeSATP, ATPγS or ATP induced concentration-dependent increases of $[Ca^{2+}]_i$ in both systems (Figure 3a and b). Whilst the potency order obtained was the same in both systems, the absolute potencies of all the agonists and the intrinsic activities of ATPγS and ATP were significantly lower in platelets (see Table 2). In order to test the hypothesis that platelets may contain a low reserve (low density or poorly coupled) $P2Y_1$-receptor population, we employed the operational model of agonism (see Figure 3 legend for details). The lines in Figure 3a and b are the result of this simulation and show a close correspondence to the experimental data points.

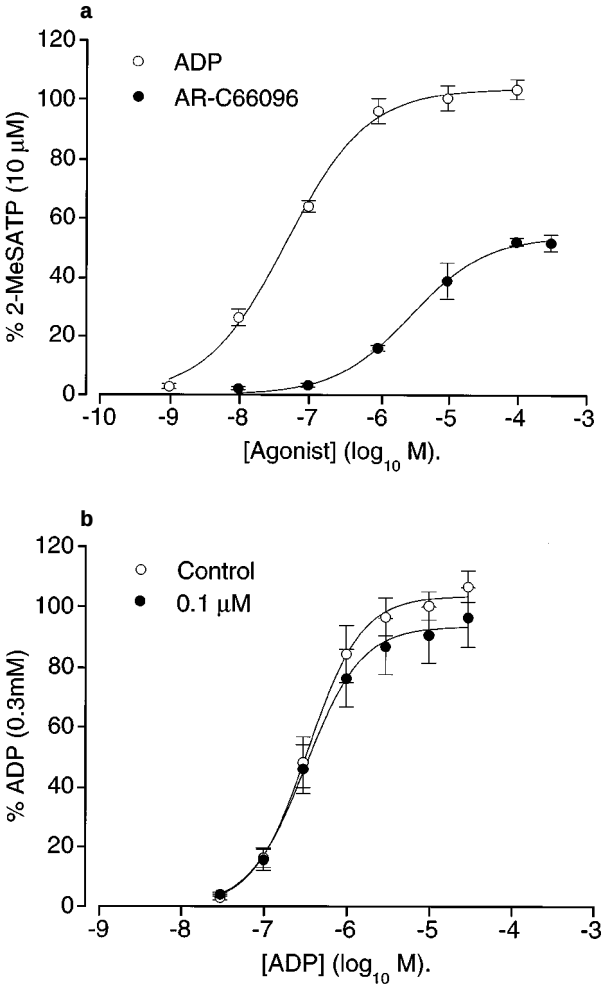


Figure 2 Effects of AR-C66096 on (a) E/[A] curves for agonist-induced increases in $[Ca^{2+}]_i$ to ADP and AR-C66096 in bovine $P2Y_1$-receptor transfected Jurkat cells. (b) E/[A] curves to ADP were obtained in the absence and presence of AR-C66096 (0.1 μM) in human washed platelets. The data are the mean of 4 experiments with vertical lines indicating s.e. The lines drawn through the data are the results of operational model-fitting in (a) and curve-fitting with Equation 1 (see Methods) in (b).

Table 1 Comparison of the affinity estimations for A3P5P and AR-C66096 in bovine $P2Y_1$-receptor transfected Jurkat cells and human washed platelets with published values at $P2Y_1$- and $P2Y_{ADP}$- receptors.

	Jurkat cells	*Platelets*	*$P2Y_{ADP}$-receptor*	*$P2Y_1$-receptor*
A3P5P	$pK_B = 5.8 \pm 0.1^{\#}$	$pK_B = 6.0 \pm 0.2^{\#}$	–	$pK_B = 6.0^{a}$
AR-C66096	$pK_A = 4.9 \pm 0.2$	No effect at 0.1 μM	$pA_2 = 9.1^{b}$	$pA_2 = 4.7^{c}$

Results are presented as mean ± s.e. of 4 experiments. #Affinity estimations were not significantly different between systems. Published values taken from [a]Boyer *et al.* (1996), [b]Tomlinson *et al.* (1997) and [c]Humphries *et al.* (1994).

Theoretical analysis of the effect of ATP contamination by ADP

H.p.l.c. analysis of the commercially obtained 'ATP' sample used in this study showed it to be approximately 99% pure with ADP being the major impurity (data not shown). Computer fitting of the experimentally obtained data for 'ATP' in human washed platelets to Equation 5, with a q value of 0.99, gave a close fit to this model (Figure 4) and yielded an average pK_B value for ATP of 5.2 ± 0.04 ($n=4$).

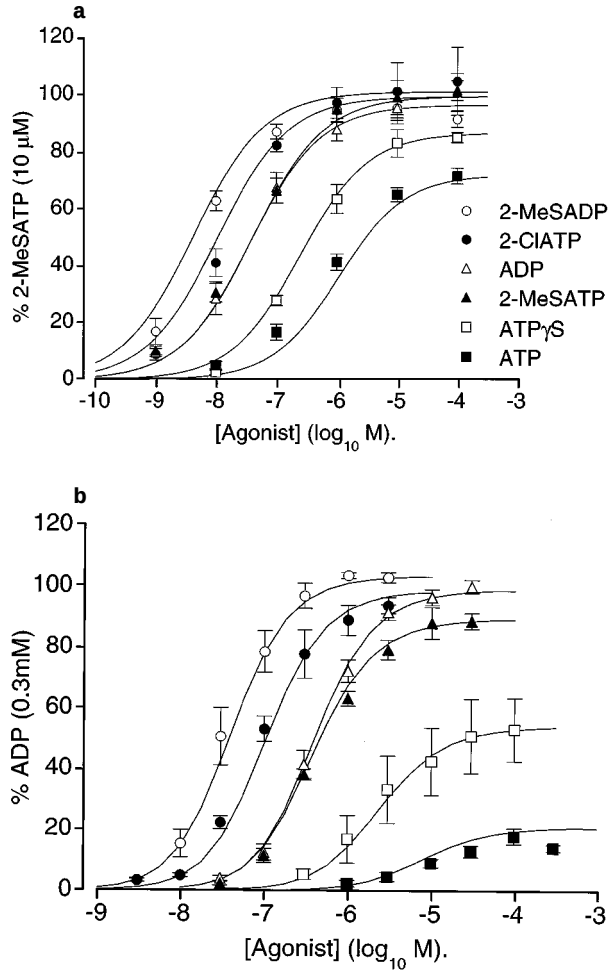


Figure 3 E/[A] curves for agonist-induced increases in $[Ca^{2+}]_i$ to 2-MeSADP, 2-ClATP, ADP, 2-MeSATP, ATPγS and ATP in (a) bovine $P2Y_1$-receptor transfected Jurkat cells and (b) human washed platelets. The data are the mean of 4–10 experiments with vertical lines indicating the s.e. Lines drawn through the data are the result of simultaneously fitting all the experimental data to the operational model of agonism (see Equation 3, Methods). This procedure assumed that responses were mediated by the same receptor in the two systems but that the systems have different receptor reserves. Accordingly, the affinities (K_A) of the agonists were kept constant but the efficacies (τ) were allowed to differ by a fixed value (estimated by the fitting-procedure). The parameters used for the fitting-procedure were as follows:

	2-MeSADP	*2-ClATP*	*ADP*	*2-MeSATP*	*ATPγS*	*ATP*
Affinity (pK_A)						
Jurkat/ platelets	6.5	6.3	5.7	6.0	5.5	5.2
Efficacy (τ)						
Jurkats	58.5	38.8	39.0	22.9	7.6	2.7
Platelets*	7.5	5.0	5.0	2.9	1.0	0.3

Jurkats: $E_m = 106.4$, $n=0.7$ for all agonists; platelets: $E_m = 110.2$, $n=1.4$ for all agonists. *Receptor reserve approximately 8 fold lower in platelets.

Discussion

The objective of this study was to classify the receptor(s) mediating increases in $[Ca^{2+}]_i$ in human washed platelets and to compare the pharmacological profile obtained with that observed in Jurkat cells stably transfected with the bovine $P2Y_1$-receptor.

Competitive antagonist studies

Recently, Boyer *et al.* (1996) have shown that A3P5P is a competitive antagonist in human $P2Y_1$-receptor transfected 1321N1 cells, with a pK_B value of 6.0. This value is similar to the pK_B estimate of 5.8 obtained in our study with bovine $P2Y_1$-receptor transfected Jurkat cells. The similarity of this value to the antagonist affinity obtained in human washed platelets (Table 1) suggests that ADP-induced increases in $[Ca^{2+}]_i$ in platelets are elicited by $P2Y_1$-receptor activation.

The affinity of A3P5P for $P2Y_{ADP}$-receptors, mediating platelet aggregation has not been demonstrated. Therefore, it could be argued that the above results do not definitively ascribe the observed ADP-induced increases in $[Ca^{2+}]_i$ in platelets to $P2Y_1$-receptor activation. To address this concern, we determined the affinity of the potent and selective $P2Y_{ADP}$-receptor antagonist, AR-C66096 ($pA_2 = 9.1 \pm 0.1$; Tomlinson *et al.*, 1997) in both Jurkat cells and platelets. In the Jurkat cells, AR-C66096 exhibited partial agonism with an affinity estimate (pK_A) of 4.9, which is consistent with its $P2Y_1$-receptor affinity in the guinea-pig isolated aorta ($pA_2 = 4.7 \pm 0.2$; Humphries *et al.*, 1994). The agonist effect of AR-C66096 in Jurkat cells cannot be explained by its metabolism to an active compound since pre-incubation of this compound with Jurkat cells did not affect its subsequent affinity determined by a platelet aggregation bio-assay (data not shown). In platelets, AR-C66096 (0.1 μM; a concentration approximately 100 fold greater than its $P2Y_{ADP}$-receptor affinity), had no effect on ADP-induced increases in $[Ca^{2+}]_i$ (Table 1). The data obtained with this selective $P2Y_{ADP}$-receptor antagonist provides compelling evidence that, under the experimental conditions used, ADP-induced $[Ca^{2+}]_i$ increases in platelets are not mediated by $P2Y_{ADP}$-receptors.

Agonist profiles

Our hypothesis that ADP-induced increases in $[Ca^{2+}]_i$ in platelets, are mediated by $P2Y_1$-receptors is further strengthened by the observation that agonist profiles obtained in both the transfected Jurkat system and human washed platelets were consistent with those found previously in other $P2Y_1$-receptor assays (Filtz *et al.*, 1994; Boyer *et al.*, 1995). In all these systems the most potent compounds were the 2-substituted analogues, with the diphosphates being more potent than their triphosphate equivalents (Table 2). However, although the rank order of potencies were identical in both systems, the absolute potencies of the agonists were lower in platelets. These results might indicate that platelets possess a low reserve $P2Y_1$-receptor population. To test this hypothesis, we fitted the data obtained in platelets and Jurkat cells to the operational model of agonism, assuming that the same receptor was activated in both systems but the receptor reserve differed (see Figure 3 legend). This analysis led us to conclude

Table 2 Summary of E/[A] curve parameters for adenine nucleotide analogues in bovine $P2Y_1$-transfected Jurkat cells and human washed platelets

	2-MeSADP	*2-ClATP*	*ADP*	*2-MeSATP*	*ATPγS*	*ATP*
$P2Y_1$-receptor transfected Jurkat cells						
α	95.0±3.5	99.4±6.6	97.1±5.3	103.7±5.0	91.2±3.0	75.5±3.1
n_H	1.1±0.3	1.0±0.04	0.9±0.1	0.7±0.1	0.8±0.1	0.7±0.04
$p[A]_{50}$	8.3±0.1	7.8±0.1	7.5±0.2	7.4±0.1	6.5±0.1	6.2±0.1
Human washed platelets						
α	103.6±1.1	96.0±2.9	100.6±2.1	88.3±3.2	54.4±10.5	17.4±1.2
n_H	1.5±0.1	1.2±0.1	1.3±0.1	1.2±0.1	1.1±0.1	1.1±0.3
$p[A]_{50}$	7.5±0.1	7.1±0.1	6.4±0.1	6.4±0.04	5.6±0.2	5.0±0.1

Results are presented as mean±s.e. of 4–10 experiments.

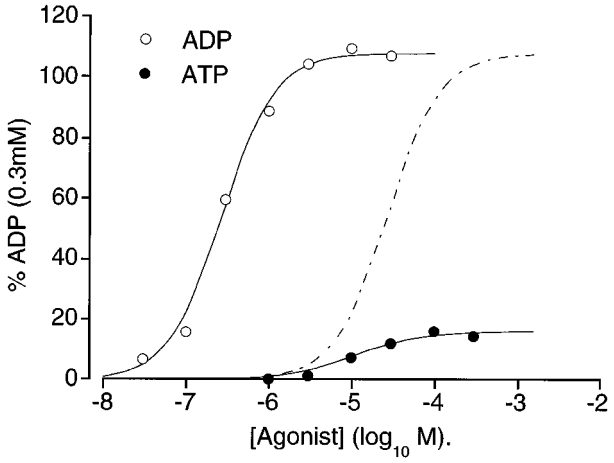


Figure 4 Theoretical analysis of the effect of ATP contamination by ADP in human washed platelets. Shown are representative E/[A] curve data observed for ADP and ATP in a single donor. The solid lines drawn through the data are the result of simultaneously fitting the ADP and ATP E/[A] curves to Equation 5 respectively (see Methods). The fitting procedure for ATP assumed: the partial agonist effects of commercially obtained 'ATP' were solely due to a 1% contamination with ADP and that ATP acted as a competitive antagonist of these responses. The dashed line indicates the expected position of the 'underlying' ADP E/[A] curve resulting from this 1% contamination of ATP (100 fold to the right of the observed ADP E/[A] curve). The estimated affinity (pK_B) of ATP from this set of data was 5.1.

Table 3 Comparison of agonist potencies ($p[A]_{50}$) in bovine $P2Y_1$-receptor transfected Jurkat cells and human washed platelets with published values in megakaryocyte-like cell lines

	Jurkat cells	*Platelets*	*Megakaryocyte-like cells**
2-MeSADP	8.3	7.5	7.4
2-ClATP	7.8	7.1	7.0
ADP	7.5	6.4	6.4
2-MeSATP	7.4	6.4	6.4

*Potency estimations in Dami, and K562 megakaryocyte-like cell lines (from Table 2 of Murgo *et al*., 1994). A similar agonist profile has been obtained in UMR106 cells (Sistare *et al*., 1994).

that the agonist data observed in this study could be accommodated by platelets having an 8 fold lower receptor reserve than the $P2Y_1$-receptor transfected Jurkat cells (Figure 3a and b).

One criticism of this conclusion could be that some of the ligands used are broken down by ecto-ATPases and the effects seen are not those of the parent compound. This is unlikely to be the case since transfected Jurkat cells have been shown to have minimal ecto-ATPase activity (Dainty *et al*., 1997) and in washed platelets, nucleoside diphosphokinase activity predominates over ecto-ATPases activity (Hourani *et al*., 1991). Moreover, the kinetics of the responses to equi-effective concentrations of di- and triphosphates were identical (data not shown), which would not be expected if the observed responses were due to the breakdown of tri- to diphosphates (Léon *et al*., 1997).

A further criticism of this conclusion could be that the agonists used in this study were from commercial sources and unlikely to be chemically pure. Others have shown that when purified ligands are used, triphosphates appear to be devoid of agonist activity at $P2Y_1$-receptors (Léon *et al*., 1997) or even act as antagonists of ADP-induced $[Ca^{2+}]_i$ increases in platelets (Hall & Hourani, 1993). In theory, the similarity of the agonist profiles in the Jurkat system and platelets may be the fortuitous result of activation of different receptors in these two systems, by the parent compounds and/or their active impurities. However, if this was the case, it seems highly unlikely that our data could be described so accurately by the fitting procedure adopted. Furthermore, even if the data obtained with the triphosphates were discounted, the lower potencies of ADP and 2-MeSADP in human washed platelets, compared to the transfected Jurkat cells, still indicate that platelets have a lower $P2Y_1$-receptor reserve.

The possibility of a complicating influence by the stimulation of $P2X_1$-receptors on agonist potencies in platelets (Mackenzie *et al*., 1996) can also be excluded; firstly, the diphosphates analogues used are known to be highly selective for $P2Y_1$- and $P2Y_{ADP}$-receptors in comparison to $P2X_1$-receptors; secondly, $P2X_1$-receptor-mediated $[Ca^{2+}]_i$ increases in platelets are only observed under conditions where receptor desensitization, by release of endogenous adenine nucleotides, is prevented by apyrase treatment during the measurement of $[Ca^{2+}]_i$ (Mackenzie *et al*., 1996).

A number of haematopoietic cell lines exhibiting megakaryocytic characteristics have been described in the literature and possess similar agonist profiles to that obtained in the present study (Murgo & Sistare, 1992; Sistare *et al*., 1994; Murgo *et al*., 1994; see Table 3). However, in contrast to the results observed in our study, ATP acted as an antagonist in these cell lines. This, together with the observation that the diphosphates were agonists led the authors to conclude that they had identified a novel ADP-recognizing receptor.

However, we suggest that these haematopoietic cell lines contain a low reserve P2Y$_1$-receptor population and the ATP activity observed in this study may be due to other phenomena discussed below.

Theoretical analysis of ATP contamination

ATP has been widely used as a tool for assigning numerous platelet functions to the P2Y$_{ADP}$-receptor. Apparently without antagonist effects at other P2-receptors, ATP was shown to antagonize ADP-induced effects on platelet aggregation, $[Ca^{2+}]_i$ and adenylyl cyclase activity with similar affinity (pA_2 values of 4.6–5.2; Cusack & Hourani, 1982; Hall & Hourani, 1993). These observations led the authors to conclude that the same ADP-recognizing receptor was responsible for all of these platelet functions. However, Léon *et al.* (1997) have recently shown that purified ATP is a competitive antagonist at human P2Y$_1$-receptors. This brings into question the use of ATP as a selective antagonist of P2Y$_{ADP}$-receptors.

In the present study, ATP exhibited an apparent partial agonism in both the Jurkat system and in platelets (Figure 3a and b). However, using computer fitting of the data in human washed platelets, we have demonstrated that these results are consistent with the supposition that ATP acts as a P2Y$_1$-receptor antagonist, blocking the effects of ADP which is an impurity of commercially available 'ATP'. Whilst we have not directly shown that ATP is devoid of agonist activity at P2Y$_1$-receptors, the estimated pK_B value of 5.2 obtained from this analysis is similar to the pA_2 value of 4.5 which can be derived from the data of Léon *et al.* (1997). In light of these observations, studies showing ATP exhibiting partial agonism at P2Y$_1$-receptors may now need to be re-evaluated (Feolde *et al.*, 1995; Dainty *et al.*, 1997). Furthermore, these studies serve to highlight the limited use of ATP as a pharmacological tool for the characterization of ADP-recognizing receptors.

Is the P2Y$_1$-receptor the elusive P2Y$_{ADP}$-receptor?

Whilst the successful cloning of P2Y$_{ADP}$-receptor remains unaccomplished, mRNA for P2Y$_1$-receptors have been found in both human platelets and megakaryoblastic cell lines (Conley *et al.*, 1996; Léon *et al.*, 1997). When purified ligands are used, the agonist profile at human P2Y$_1$-receptors is similar to that expected at P2Y$_{ADP}$-receptors. These findings have led Léon *et al.* (1997) to propose that the P2Y$_1$-receptor may in fact be the P2Y$_{ADP}$-receptor.

The results of our study provide evidence that human platelets possess P2Y$_1$-receptors mediating increases in $[Ca^{2+}]_i$. Furthermore, using the selective P2Y$_{ADP}$-receptor antagonist, AR-C66096, and the P2Y$_1$-receptor antagonist, A3P5P, we have conclusively demonstrated that these ADP recognizing P2Y$_1$-receptors are pharmacologically distinct from the P2Y$_{ADP}$-receptors which mediate platelet aggregation (Humphries *et al.*, 1994).

Other lines of evidence also suggest that there is more than one ADP-recognizing receptor on human platelets. The antithrombotic agents clopidogrel and ticlopidine inhibit ADP-induced aggregation but do not prevent ADP-induced $[Ca^{2+}]_i$ increase or shape change (Gachet *et al.*, 1990). Additionally, in patients with a congenital deficiency of ADP-induced platelet aggregation, the shape change response to ADP remains intact (Cattaneo *et al.*, 1992). These observations led Gachet and colleagues (1995) to propose a model for the existence of two distinct ADP receptor subtypes on platelets: one receptor mediating aggregation and the other mediating shape change and calcium influx. More recently, Gachet and colleagues (1997) have incorporated the work of Léon *et al.* (1997) into this model. They now propose aggregation is mediated by P2Y$_1$-receptors and shape change/calcium influx is mediated by P2X$_1$-receptors.

In the present study, we have shown that ADP-induced $[Ca^{2+}]_i$ increases are mediated by P2Y$_1$-receptors which are pharmacologically distinct from P2Y$_{ADP}$-receptors. P2Y$_{ADP}$-receptors have been shown to mediate ADP-induced platelet aggregation, defined pharmacologically by AR-C66096 (Humphries *et al.*, 1994). However, the full functional role of P2Y$_1$-receptors and P2X$_1$-receptors (which also cause $[Ca^{2+}]_i$ elevation in platelets) remains to be defined. Sanderson *et al.* (1996) have shown that AR-C66096 antagonizes ADP-induced platelet aggregation but not shape change in whole blood. Furthermore, P2Y$_1$-receptors have been found to mobilize $[Ca^{2+}]_i$ from intracellular stores by activation of phospholipase C and formation of inositol trisphosphate, possibly via coupling to Gαq (Schachter *et al.*, 1997). Studies showing deficiencies in Gαq have shown impaired shape change and aggregation responses in platelets (Gabberta *et al.*, 1997; Offermanns *et al.*, 1997). On this basis, it is likely that the ADP-recognizing receptor mediating shape change in the platelet is the P2Y$_1$-receptor. However, these studies also suggest P2Y$_{ADP}$-receptors and P2Y$_1$-receptors may both play a role in ADP-induced platelet aggregation. The precise mechanism of ADP-induced platelet aggregation therefore requires further study.

Conclusions

The results of the present study demonstrate that human washed platelets contain a low reserve P2Y$_1$-receptor population that mediates ADP-induced increases in $[Ca^{2+}]_i$. This receptor population is pharmacologically distinct from the P2Y$_{ADP}$-receptor. Furthermore, the endogenous activator of platelets, ADP, appears to be non-selective and is capable of activating several receptors on the surface of platelets, namely P2Y$_{ADP}$, P2Y$_1$ and P2X$_1$.

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