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Activity of adenosine diphosphates and triphosphates on a $P2Y_T$-type receptor in brain capillary endothelial cells

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1 A P2Y (nucleotide) receptor activity in a clonal population (B10) of rat brain capillary endothelial cells is coupled to inhibition of adenylyl cyclase and has functional similarities to the $P2Y_T$ (previously designated 'P2T') receptor for ADP of blood platelets. However, the only P2Y receptor which was detectable in a previous study of B10 cells by mRNA analysis was the $P2Y_1$ receptor, which elsewhere shows no transduction *via* cyclic nucleotides. We have sought here to clarify these issues.

2 The inhibition of forskolin-stimulated adenylyl cyclase induced by purified nucleotides was measured on B10 cells. The EC_{50} value for 2-methylthioADP (2-MeSADP) was 2.2 nM and, surprisingly, 2-MeSATP was an almost equally strong agonist ($EC_{50}=3.5$ nM). ATP and 2-ClATP were weak partial agonists ($EC_{50}=26$ μM and 10 μM respectively) and under appropriate conditions could antagonise the activity on 2-MeSADP.

3 A known selective antagonist of the platelet $P2Y_T$ receptor, 2-propylthioadenosine-5′-(β,γ)-difluoromethylene) triphosphonate (AR-C 66096), was a competitive antagonist of this B10 cell receptor, with $pK_B=7.6$. That ligand is inactive at the $P2Y_1$ receptor in the same cells. Conversely, the competitive $P2Y_1$ receptor antagonists, the 3′, 5′- and 2′, 5′-adenosine *bis*-monophosphates, are, instead, weak agonists at the adenylyl cyclase-inhibitory receptor.

4 The inhibition of adenylyl cyclase by 2-MeSADP was completely abolished by pertussis toxin.

5 In summary, these brain endothelial cells possess a $P2Y_T$-type receptor in addition to the $P2Y_1$ receptor. The two have similarities in agonist profiles but are clearly distinguishable by antagonists and by their second messenger activations. The possible relationships between the B10 and platelet $P2Y_T$ receptors are discussed.

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Abbreviations: ADPβS, adenosine 5′-O-2-thiodiphosphate; A2P5P, adenosine-2′-phosphate-5′-phosphate; A3P5P, adenosine-3′-phosphate-5′-phosphate; BzATP, 3′-O-(4-benzoylbenzoyl)-adenosine 5′-triphosphate; 2-ClATP, 2-chloroATP; 2-MeSADP, 2-methylthioADP; CHA, N^6-cyclohexyladenosine; CPA, N^6-cylclopentyladenosine; CP, creatine phoshpate; CPK, creatine phosphokinase; DMEM, Dulbecco's modified Eagle's medium; IBMX, 3-isobutyl-1-methylxanthine; 2-MeSATP, 2-methylthioATP; NECA, 5′-N-ethylcarboxamidoadenosine; PKA, protein kinase A; PTX, pertussis toxin; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; PSFM-ATP/AR-C66096, 2-propylthioadenosine-5′-(β,γ-difluoromethylene)triphosphonate, RT–PCR, reverse transcriptase-polymerase chain reaction

Introduction

The cloned $P2Y_1$ receptor (Webb *et al*., 1993) is a member of a P2Y family of G-protein coupled nucleotide receptors (North & Barnard, 1997). In heterologous recombinant expression it responds to ADP to stimulate the phosphoinositide signalling pathway and intracellular Ca^{2+} mobilization (Simon *et al*., 1995; Schachter *et al*., 1996; Hechler *et al*., 1998c). It has a widespread distribution in tissues (Webb *et al*., 1993; Barnard *et al*., 1997). We are concerned here with the comparison of P2Y receptors at two important sites for them in the vascular system, namely brain microvascular endothelial cells (B10 cells) and platelets. In both B10 cells (Webb *et al*., 1996) and platelets (Leon *et al*., 1997, 1999; Jin *et al*., 1998; Fabre *et al*., 1999) molecular identification of the $P2Y_1$ receptor has been made. However, there is also evidence for a second P2Y receptor (as yet uncloned) in these cells; this receptor is also responsive to ADP, but unlike the $P2Y_1$ receptor activates a different transduction pathway i.e. causing the inhibition of adenylyl cyclase. In the platelet, this second P2Y receptor plays an essential role in platelet aggregation by ADP. There it has usually been termed the 'P2T receptor' (Gordon, 1986). In view of all of its known properties the IUPHAR rules for receptor nomenclature (Vanhoutte *et al*., 1998) would now indicate that it should be named as in a branch of the P2Y

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receptors. We therefore use the term $P2Y_T$ for the receptor(s) of this type, as a provisional term pending the numbering to be made when molecular identifications are published.

The nucleotide specificity of the cyclase inhibitory activity in the platelet is in general terms similar to that which has also been attributed to the $P2Y_1$ receptor. There has, in fact, been much debate as to whether one receptor in the platelet may operate the dual transduction pathways noted above (reviewed by Boarder & Hourani, 1998). Recent evidence has, however, strongly indicated the presence of two distinct P2Y-type receptors in platelets as stated above (Hechler *et al*., 1998c; Jin *et al*., 1998; Savi *et al*., 1998). For the $P2Y_T$ receptor, an ATP derivative, AR-C66096 (2-propylthioadenosine-5′-(β,γ-difluoromethylene)triphosphonate or PSFM-ATP) which is a selective antagonist of platelet aggregation has been obtained (Humphries *et al*., 1994) and this has been found to block the action of ADP in inhibiting platelet cyclic AMP accumulation but not in its mobilization of Ca^{2+} (Daniel *et al*., 1998). Finally, conclusive evidence on the separate identities and transductions of these two receptors in the platelet has appeared using gene targetting of the $P2Y_1$ receptor (Fabre *et al*., 1999; Leon *et al*., 1999). In those studies the platelets of $P2Y_1$ receptor-null mice are shown to lose the response to ADP of intracellular Ca^{2+} mobilization but to retain fully the adenylyl cyclase inhibition response to ADP. This evidence also confirms that these two receptor subtypes have ADP as a common agonist.

The evidence for the co-occurrence with the $P2Y_1$ receptor of a $P2Y_T$-type receptor activity in brain microvascular endothelial cells as well as in platelets, noted above, is now of much interest, since this, too, can be expected to be a separate entity, either similar or identical to the platelet $P2Y_T$. In a previous study (Webb *et al*., 1996) we used B10 cells, a clonal line established from a primary culture of rat brain capillary endothelial cells (Feolde *et al*., 1995) and giving a $P2Y_1$ receptor-like response to mobilize intracellular Ca^{2+}. We showed that activation by adenine (and not uridine) nucleotides led also to inhibition of adenylyl cyclase. However, the nucleotide specificity of this response was not studied in detail and specific antagonists of $P2Y_1$ and $P2Y_T$ receptors were not available to us. Moreover, analytically pure nucleotides (Hechler *et al*., 1998c) were not used, nor were endogeneous nucleotide release and degradation during the assays controlled.

The latter statements also apply for the C6-2B glioma cell line, where an inhibition of stimulated adenylyl cyclase mediated by ADP has again been observed (Valeins *et al*., 1992; Boyer *et al*., 1993), but in that case not accompanied by a $P2Y_1$ response. Only the diphosphates of adenosines have been reported to be strong agonists at the platelet $P2Y_T$ receptor, the triphosphates being antagonists (Hourani & Hall, 1996). We have now examined, with full precautions, the pharmacology of the putative $P2Y_T$ receptor of the B10 cell and its distinction from the $P2Y_1$ receptor there. The existence of an authentic $P2Y_T$ receptor on microvascular endothelial cells would be of intrinsic interest.

A preliminary report of some of this work has been presented (Simon *et al*., 1999).

Methods

Materials

All tissue culture media and components were purchased from GIBCO/BRL Life Technologies (Paisley, U.K.). ATP, ADP, adenosine 5′-O-(2-thiodiphosphate) (ADPβS), hexokinase were from Boehringer Mannheim (Lewes, U.K.). 2-MethylthioATP (2-MeSATP), 2-methylthioADP (2-MeSADP), 2-chloroATP (2-ClATP), adenosine, adenosine-2′-phosphate-5′-phosphate (A2P5P), adenosine-3′-phosphate-5′-phosphate (A3P5P), 2′- and 3′-O-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (BzATP), ATP luciferase-assay reagents, creatine, creatine phosphokinase (CPK), creatine phosphate, 3-isobutyl-1-methylxanthine (IBMX), cyclic AMP, forskolin, protein kinase A (PKA: cyclic AMP-binding regulatory subunit) and pertussis toxin, were from Sigma (Poole, U.K.). [2,8-^{3}H]-adenosine 3′5′ cyclic monophosphate ([^{3}H]-cyclic AMP: 25–40 Ci mmol^{-1}) was purchased from Amersham International (Little Chalfont, U.K.). PSFM-ATP (AR-C66096) was synthesized and characterized as described by Humphries *et al*. (1994). All other chemicals were of analytical grades from established commercial sources.

One hundred per cent purity of each of the triphosphates at the time of use in assays was assured as before by enzymic regeneration (Hechler *et al*., 1998c), using 20 u ml^{-1} creatine phosphokinase (CPK)/10 mM creatine phosphate (CP) treatment (here, 60 min for ATP, 90 min for 2-MeSATP or 2-ClATP). The same was true for the diphosphates at the time of use, but replacing the CP by 10 mM creatine, or alternatively (with the same results) using a hexokinase/glucose/$MgCl_2$ treatment (Lazarowski *et al*., 1997). The drug solutions were then used immediately in adenylyl cyclase assays. Analysis by HPLC (Hechler *et al*., 1998c) of each treated nucleotide confirmed its 100% purity. Using the regeneration system for ATP, it was also verified that no loss of purity occurred during the assay period, measured by the luciferase method as described below. The added kinase was finally inactivated where necessary by iodoacetamide (10 mM, 10 min, room temperature).

Cell culture and intracellular Ca^{2+} measurements

B10 rat brain microvascular endothelial cells (Feolde *et al*., 1995) were in two subclones, maintained separately (subclone a) at Cambridge or (subclone b) at Sophia Antipolis laboratories, respectively. They were cultured as described previously, using Dulbecco's modified Eagle's medium (DMEM)/NutMix F-12 supplemented with Glutamax-1, pyridoxine, and 10% foetal bovine serum. For the adenylyl cyclase assays, the cells were plated on flat-bottom polystyrene tissue culture plates containing (subclone a) 96 wells (at approximately 25,000 cells per well) or (subclone b) six wells (at approximately 500,000 cells per well) and were then grown to confluency. Intracellular Ca^{2+} mobilization was measured on cells, grown on 6-well plates with methods described by Hechler *et al*. (1998c). Briefly, the cells were incubated for 30 min with 5 μM indo-1/AM, diluted in Earle's solution, agonist was added and the indo-1 fluorescence ratio was measured by flow cytometry after 15 s at 37°C.

Adenylyl cyclase assay

The confluent cell layers were washed once with serum-free culture medium (with 25 mM HEPES and 5 mM glucose, pH = 7.4) at 37°C and then preincubated in medium containing 100 or 200 μM IBMX for 10–15 min at 37°C (to inhibit breakdown of cyclic AMP by phosphodiesterases). The preincubation medium was then aspirated and the incubations were initiated by addition of medium containing forskolin (10 μM, subclone a; 1 μM, subclone b), IBMX and effectors as stated. After 5 min at 37°C the incubations were terminated by aspiration of the medium and acidification to pH 5.5 or below at 4°C, 30 min. After centrifugation (in the plates, for the 96-well series), aliquots were processed for the cyclic AMP determinations, performed for subclone a as described by Carruthers *et al*. (1999) using competition for the binding of [^{3}H]-cyclic AMP to a binding fragment of protein kinase A. For the parallel analyses on subclone b (6-well plates) at Sophia Antipolis, a cyclic AMP kit (Immunotech, Marseille, France) was used according to the manufacturer's protocol. The basal level of cyclic AMP, measured in the absence of drugs and forskolin, was 46 ± 2 fmoles cyclic AMP per well (96-well plates; $n = 9$) or 2.3 ± 0.1 pmoles well^{-1} ($n = 12$) in the bulk case (6-well plate assay).

For antagonism studies, either the cells were preincubated with AR-C66096 for 15 min (for full equilibration of that agent) at 37°C in medium containing 200 μM IBMX, or BzATP or A3P5P or A2P5P or purified ATP was added together with the agonist.

For pertussis toxin (PTX) treatment, the B10 cells cultured on 96-well plates to near confluency were used. Half of the wells on each plate were pretreated with 100 ng ml^{-1} pertussis toxin at 37°C for 14–16 h, followed by agonist/10 μM forskolin treatment and cyclic AMP assay as before.

ATP release and metabolism studies

B10 cells cultured to confluency on 96-well plates were washed with 200 μl serum-free DMEM and then preincubated for 15 min at 37°C in DMEM containing 200 μM IBMX. The preincubation medium was then aspirated and 150 μl of fresh media was added to each well. Aliquots (50 μl) were removed from the wells at intervals and the ATP level in each sample was then measured in a luminometer, using the luciferin-luciferase bioluminescence technique (Buell *et al*., 1996). Purified ATP standards were used in calibration of the ATP levels, prepared as above, in DMEM containing 200 μM IBMX. For the metabolism study, the cells were prepared and the medium was assayed as described above, except that ATP (to 1 μM final concentration) was added to the medium after the preincubation.

None of the adenylyl cyclase inhibition observed with the nucleotides could arise from low levels of adenosine as a breakdown product, acting at the G_i-coupled adenosine receptors on the cells, since they were blocked by the IBMX present. That was confirmed by the total lack of response to adenosine (up to 100 μM) or other adenosine receptor subtype-specific drugs, such as N^6-cyclohexyladenosine (CHA), N^6-cylclopentyladenosine (CPA), 5′-N-ethylcarboxamidoadenosine (NECA), when those were added alone up to 10 μM concentration to the assay medium (data not shown).

Data analysis

At least three independent replicate experiments, each in triplicate, were performed in all cases to give the values reported, represented as the mean ± s.e.mean. Agonist concentration-response curves (each completed on the same day and on the same batch of cells) were computer-fitted to a simple logistic equation and the pEC_{50} values calculated using GraphPad Prism (GraphPad Software) or SigmaPlot (Jandel Scientifics) programs. This also applied to the computation of pK_B values from such curves when different concentrations of antagonists were present, or otherwise these were obtained from Schild plots.

Results

Effects of adenine nucleotides on adenylyl cyclase in B10 cells

Incubation of B10 brain capillary endothelial cells with 100 nM 2-MeSADP or 100 μM ADPβS or 100 μM ADP or 100 μM ATP each had no stimulatory effect on the basal cyclic AMP level (data not shown). Rather, moderate but significant decreases were produced, e.g. $45 \pm 5\%$ with 100 μM ATP. This served to exclude the presence of the $P2Y_{11}$ (Communi *et al*., 1997) or any other adenylyl cyclase-stimulatory receptor for adenine nucleotides, which if present would have masked the true activity of the cyclase-inhibitory $P2Y_T$ receptor to be studied.

Incubation of the B10 cells with 1 or 10 μM forskolin (alone) resulted in a rapid intracellular accumulation of cyclic AMP to a stable level. Co-addition of 2-MeSADP or ADP or ADPβS with the forskolin resulted in a marked decrease in that accumulation, taken to represent the inhibition of adenylyl cyclase. The concentration-response curves fitted a simple logistic equation (Figure 1); the potencies of the latter two nucleotides were approximately 200–500 fold lower than that of the most potent agonist, 2-MeSADP (Table 1). 2-MeSATP was also a highly potent agonist, with an EC_{50} value in the low nanomolar range, almost as strong as the corresponding diphosphate (Figure 1 and Table 1).

ATP and 2-ClATP are weak partial agonists, which can act as apparent antagonists

ATP and 2-ClATP have previously been reported to be competitive antagonists at the $P2Y_T$ receptor on human platelets (reviewed by Hall & Hourani, 1993). However, in our studies on rat B10 cells we observed a partial agonist action of ATP and 2-ClATP on the forskolin-induced cyclic AMP accumulation (Figure 1b and Table 1). Their potencies are both relatively weak if compared to that of 2-MeSADP.

In contrast to the behaviour of these two agonists, the maximum decrease in the stimulated cyclic AMP level was seen with 2-MeSADP, 2-MeSATP and ADP (Figure 1a), and these were considered as full agonists in the series tested. The properties of those agonists, and the activity of ATP and of 2-ClATP as partial agonists, were consistent when observed in two subclones (a and b) of the original (Feolde *et al*., 1995) clone of B10 cells, a and b being maintained separately over several years and analysed, respectively, in our Cambridge

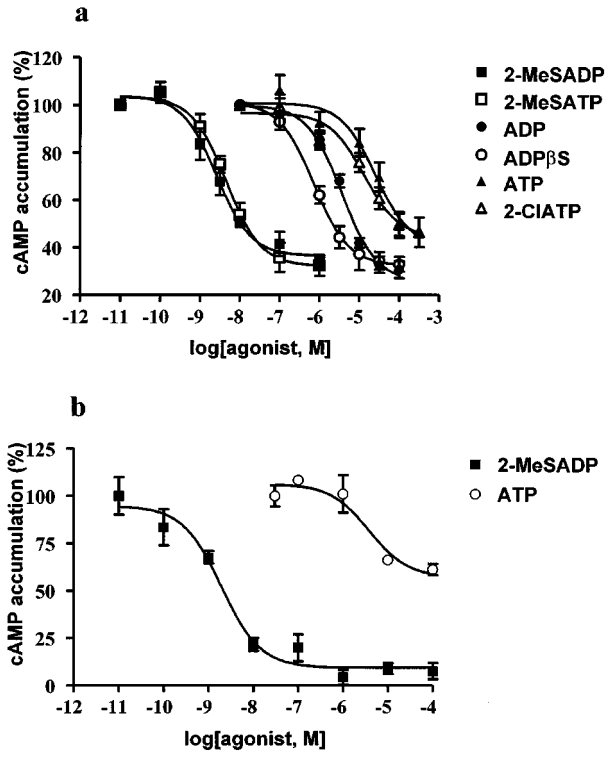

Figure 1 Concentration-dependent inhibition by nucleotides of the forskolin-induced cyclic AMP accumulation in B10 cells. The maximum stimulated activity is set at 100%, defined in the presence of forskolin and IBMX alone (see Methods). The responses were measured on (a) a subclone capable of a maximum inhibition by adenine nucleotides of 60–70%, or on a second subclone (b) capable of up to 95% inhibition (see text).The data are represented as the mean±s.e.mean from (a) 5–15 independent determinations for each agonist, each performed in triplicate on subclone a, or (b) representative experiments, performed in triplicate on subclone b, with two of the nucleotides, illustrating the partial agonist activity of ATP. The curves are theoretical fits to a logistic equation.

Table 1 Agonist and antagonist potencies at two nucleotide receptors in B10 cells

		EC_{50} (nM)	
Agonist		$P2Y_T$	$P2Y_1$
2-MeSADP		2.2±0.3	19*
ADP		3100±400	880*
ADPβS		623±124	900*
2-MeSATP		3.5±0.1	
ATP		26,000±2000**	
2-ClATP		13,500±770	
A2P5P		38,900±2900	[4800]†
A3P5P		39,400±1600	[3100]†
		pK_B	
Antagonist		$P2Y_T$	$P2Y_1$
AR-C66096	B10 cell	7.59±0.08	inactive (Figure 5)
	human platelet	8.6‡	inactive
BzATP	B10 cell	5.32±0.10	5.26#
	human platelet	5.20#	

The $P2Y_1$ receptor activity in each case was determined by intracellular Ca^{2+} mobilization and $P2Y_T$ activity (with the one exception noted) by adenylyl cyclase inhibition. $P2Y_T$ here denotes data on the $P2Y_T$-type receptor in B10 cells (obtained on clone a, using 10 μM forskolin: see methods) or (where stated) on the platelet $P2Y_T$ receptor. *Agonist potencies on B10 cells (Hechler *et al*., 1998a); **Estimated approximately, since the maximum inhibition could not be attained due to non-specific effects at the high nucleotide concentrations required for it; †Antagonist; the K_B values are shown for inhibition of ADP-induced $[Ca^{2+}]_i$ increase in B10 cells (Hechler *et al*., 1998b); ‡Based on the data of Daniel *et al*. (1998); #From Vigne *et al*., 1999 (for antagonism of platelet aggregation, in the $P2Y_T$ case).

and Sophia Antipolis laboratories. The observations on these were made independently and in a mutually blind fashion, furnishing a positive control for the unexpected agonism of ATP, 2-ClATP and 2-MeSATP. The two subclones gave different values for the maximum accumulation of cyclic AMP induced by forskolin above the basal level, i.e. 10 fold (in subclone a, requiring 10 μM forskolin) and 13.5 fold (in sub-clone b, but requiring only 1 μM forskolin). They were used under those optimal conditions for each. They then differed reproducibly in the maximum percentage of that cyclic AMP rise which could be inhibited by a full agonist (compare Figure 1a,b), which may indicate a higher number of receptors per cell in the Sophia Antipolis sub-clone. The high maximum value found with subclone b for the agonist inhibition of the cyclase response permitted the partial agonist character of ATP to be more clearly seen (Figure 1b). This was by comparison to 2-MeSADP as a full agonist, the latter having indistinguishable EC_{50} values of 2.2±0.3 (a) and 3.0±0.5 (b). The EC_{50} value for ATP could not be accurately determined, due to its low potency and efficacy, but could be estimated to be shifted about 8 fold leftward for subclone b relative to a, a direction of change in accord with the suggested receptor density difference. ADPβS was only examined in subclone a, and was, like the other diphosphates tested, a full agonist (Figure 1a).

We investigated the ability of ATP and 2-ClATP to oppose the action of a potent full agonist, 2-MeSADP, on the stimulated cyclic AMP accumulation. At ATP concentrations of 10–30 μM their intrinsic agonist effect could be subtracted to allow a clear rightward shift of the 2-MeSADP concentration-response curve to be observed (Figure 2). That intrinsic effect became too great for this analysis to be made above 30 μM, whereas when the agonist was at 1 μM or below its fractional occupancy became too small. The behaviour, in the effective range, is the classical type (Kenakin, 1993) of an agonist with adequate affinity but lower intrinsic efficacy denying receptor sites to a full agonist, so as to mimic a competitive antagonist, i.e. an 'apparent antagonist' action. Identical observations were made with 2-ClATP (data not shown). From individual curves ($n=3$) of the type illustrated in Figure 2, apparent pK_B values were computed of 5.1 for ATP and 5.7 for 2-ClATP.

$P2Y_1$ antagonists do not antagonize the nucleotide-induced adenylyl cyclase inhibition

The *bis*-monophosphates A2P5P and A3P5P are specific antagonists of the $P2Y_1$ receptor (Boyer *et al*., 1996). Their action on the B10 cell responses was investigated. A direct inhibition of the forskolin-induced cyclic AMP accumulation by A2P5P or A3P5P alone was found but with low potency

(Figure 3, Table 1), i.e. both compounds are weak partial agonists. It has already been shown (Hechler *et al*., 1998c) that on B10 cells these compounds competitively antagonize the $P2Y_1$ response (see Table 1), with complete abolition attained near 100 μM concentration of either. At that level, as is seen from Figure 3, they are, instead, agonists for the adenylyl cyclase inhibition.

Effects of antagonists of the platelet $P2Y_T$ receptor on the $P2Y_T$-type and $P2Y_1$ receptors in B10 cells

The ability of two compounds, known to act as antagonists at the $P2Y_T$ receptor of human platelets, to block ADP-induced adenylyl cyclase inhibition in B10 cells was examined. One of the compounds, AR-C66096 or PSFM-ATP, is an ATP derivative that has been shown on human platelets to block selectively ADP-induced aggregation or inhibition of adenylyl cyclase (Humphries *et al*., 1994; Jin *et al*., 1998). On B10 cells, we found that this compound was also a potent competitive antagonist of the 2-MeSADP-induced adenylyl cyclase inhibition, with a pK_B value of 7.6 (Figure 4a and Table 1). Alone, it had no effect (up to at least 1 μM) on the forskolin-stimulated level of cyclic AMP (data not shown). The antagonist potency, while high, is about 10 fold lower than that reported for its block of ADP-induced inhibition of adenylyl cyclase in human platelets (Table 1), but this could perhaps be accounted for by the species difference.

Recently, Vigne *et al*. (1999) have shown that 2′- and 3′-O-(4-benzoylbenzoyl)-ATP (BzATP) can act as an antagonist on the $P2Y_1$ receptor in B10 cells and human platelets and antagonizes platelet aggregation. They found that this agent also partially reversed the inhibition by ADP of a cholera toxin-induced increase in cyclic AMP level in the B7 cell line of Feolde *et al*. (1995). This is also an endothelial cell line, related to B10 but expressing also the $P2Y_2$ receptor. We have further found now that in B10 cells, treated with forskolin, BzATP competitively antagonises the inhibition of cyclic AMP formation evoked by 2-MeSADP (Figure 4b). The calculated pK_B value (5.3) for BzATP was identical with that found (Vigne *et al*., 1999) for its inhibition of the ADP-activated aggregation of human platelets (Table 1).

ADP and related nucleoside diphosphates exert an additional action on B10 cells, mobilizing intracellular Ca^{2+} (see Introduction). We also tested the aforementioned antagonist AR-C66096 for ability to prevent those nucleotides from activating that response. The mobilization of intracellular Ca^{2+} was produced by ADP with an EC_{50} value of 0.91 ± 0.06 μM (in agreement with that found earlier by Hechler *et al*., 1998c). This was unchanged in the presence of

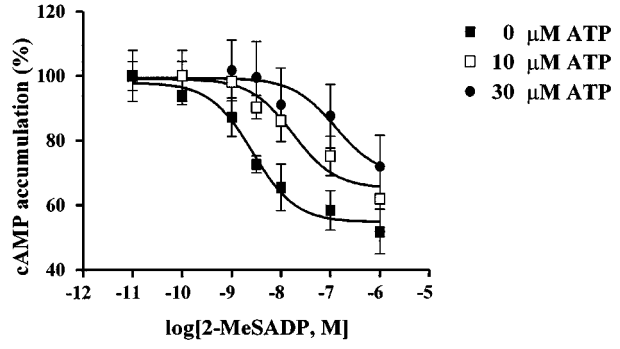

Figure 2 Apparent antagonism by ATP of the 2-MeSADP-evoked decrease in 10 μM forskolin-stimulated cyclic AMP formation in B10 cells. The cyclic AMP accumulation shown is normalized to 100% as the value prior to 2-MeSADP addition.

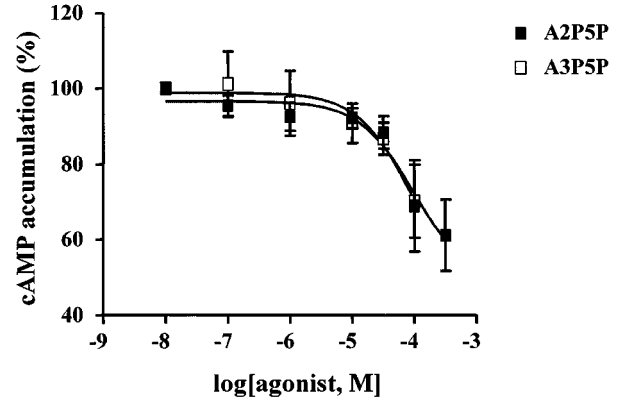

Figure 3 The weak inhibition of the 10 μM forskolin-induced cyclic AMP formation, produced by A2P5P and A3P5P in B10 cells.

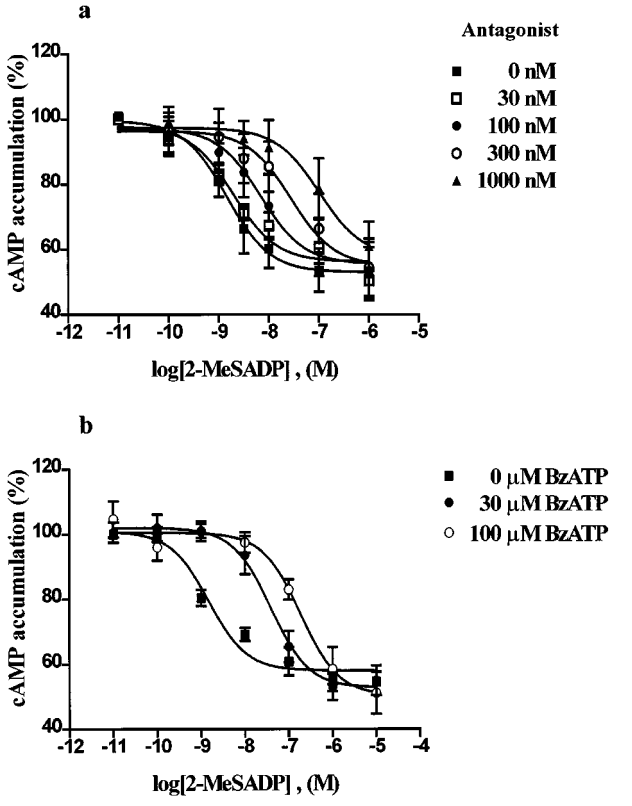

Figure 4 Concentration-dependent antagonism of the $P2Y_T$-receptor-selective antagonist PSFM-ATP (AR-C66096) (a) and BzATP (b) on the 2-MeSADP-mediated inhibition of cyclic AMP formation in B10 cells. cyclic AMP accumulation in the presence of 10 μM forskolin but in the absence of agonist and antagonist was normalized as 100%. From these and similar fitted curves (some removed for clarity from panel b) obtained at different concentrations the K_B values were computed.

AR-C66096 at 10 μM concentration (Figure 5). The latter compound also had no action alone on intracellular Ca^{2+} level (Figure 5). These results are in accord with the attribution of that Ca^{2+} response in B10 cells to the $P2Y_1$ receptor, detected therein by its mRNA (Webb *et al*., 1996). It has since been confirmed (J. Simon *et al*., unpublished data) by use of a specific antibody to the $P2Y_1$ receptor that this protein is present at the cell surface of these B10 cells.

$P2Y_T$-type receptor action in B10 cells depends upon a pertussis toxin-sensitive G protein

Treatment of the cells with pertussis toxin (100 ng ml^{-1}) completely and insurmountably abolished 2-MeSADP-evoked inhibition of forskolin-stimulated cyclic AMP formation (Figure 6). These results indicate that upon agonist activation this $P2Y_T$-type receptor in B10 cells couples through a G_i or G_o protein to inhibit adenylyl cyclase, as has been already established for the $P2Y_T$ receptor on platelets (Ohlmann *et al*., 1995). After that pertussis toxin treatment there was, in contrast, reproducibly no change at all in the intracellular Ca^{2+} mobilization response to ADP or 2-MeSADP, measured as in Figure 5 (data not shown).

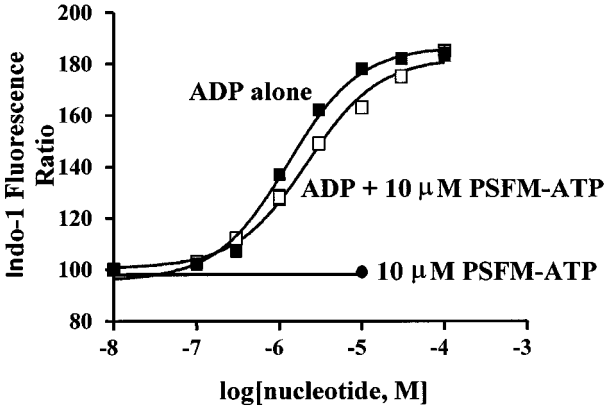

Figure 5 Lack of effect of PSFM-ATP (AR-C66096) on the response to ADP of the $P2Y_1$ receptor in B10 cells. Intracellular Ca^{2+} mobilization measurements are shown. The lowest line shows that the antagonist alone gave no response.

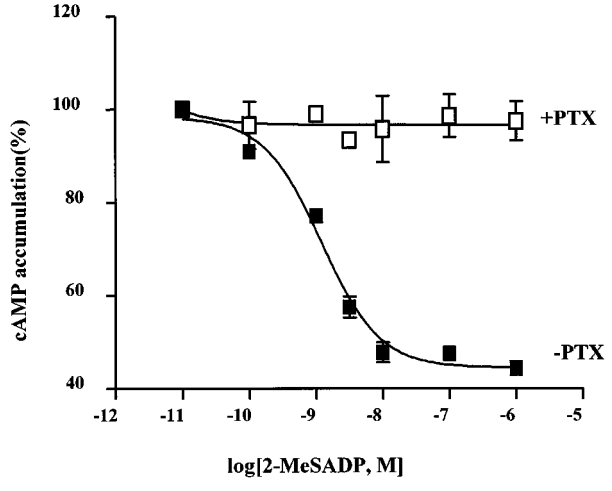

Figure 6 The 2-MeSADP-induced inhibition of 10 μM forskolin-stimulated cyclic AMP formation in B10 cells with (upper curve) or without (lower plot) pertussis toxin (100 ng ml^{-1}) pre-treatment. Basal levels of cyclic AMP were unchanged by the PTX treatment. For further details, see Methods.

ATP release and metabolism in B10 cells

Endothelial cells, like many other cell types, can release ATP and ADP into the extracellular space (for references, see Ralevic & Burnstock, 1998), especially under mechanical stimulation, which here may be produced in the course of the aspiration and addition of the media. These products could accumulate and change P2Y sensitivities, by competition or by desensitization; the latter effect has been shown with other cell types expressing a known P2Y receptor, e.g. the $P2Y_3$ receptor (Webb *et al*., 1996). Therefore, the accumulation of ATP in the bathing medium was measured from B10 cells handled and incubated as for the adenylyl cyclase assays, except that no agonist was added. The ATP content was monitored with time using a luciferin-luciferase method (Figure 7a). The concentration of ATP present due to release from the cells immediately after changing the medium (as in initiating the adenylyl cyclase assay) was close to 100 nM, and this level did not increase, but in fact decreased, during the incubation (Figure 7b). Since the adenylyl cyclase assay period was always limited to 5 min and since both ATP and ADP are very weak agonists and give no activation of the $P2Y_T$-type receptor on these cells at 100 nM (Figure 1), we could, therefore, exclude distortions of the results due to nucleotide release.

Some possible loss of triphosphates during the assays due to extracellular nucleotidase or to transfer through kinases was also considered. Even with 1 μM ATP present during the 5-min assay period the loss of ATP was only 13%, in a first-order decay (Figure 7b). For 2-MeSATP, used at low nanomolar concentrations (Figure 1), these enzymic reactions should obviously be negligible.

The experiments presented in Figure 7 were of necessity conducted without the ATP-regeneration system (see Methods) being present. However, in view of the unexpected behaviour of 2-MeSATP as a very potent agonist, and to exclude the remote possibility that this derivative but not ATP undergoes a particularly rapid conversion to the diphosphate by some enzyme activity especially pronounced on the B10 cells, we made a further test. For this, one of the 2-MeSATP plots of Figure 1 was reproduced while maintaining the full triphosphate regeneration system throughout, i.e. both up to and during the adenylyl cyclase assay. This test was also performed for ATP. In both cases no such breakdown occurred: the EC_{50} values obtained showed no significant difference (data not shown) from the values presented in Table 1.

Discussion

This study describes the pharmacological characterization of a P2Y receptor present in B10 rat brain capillary endothelial cells, which is coupled to the inhibition of adenylyl cyclase. The pharmacological properties and signal transduction of

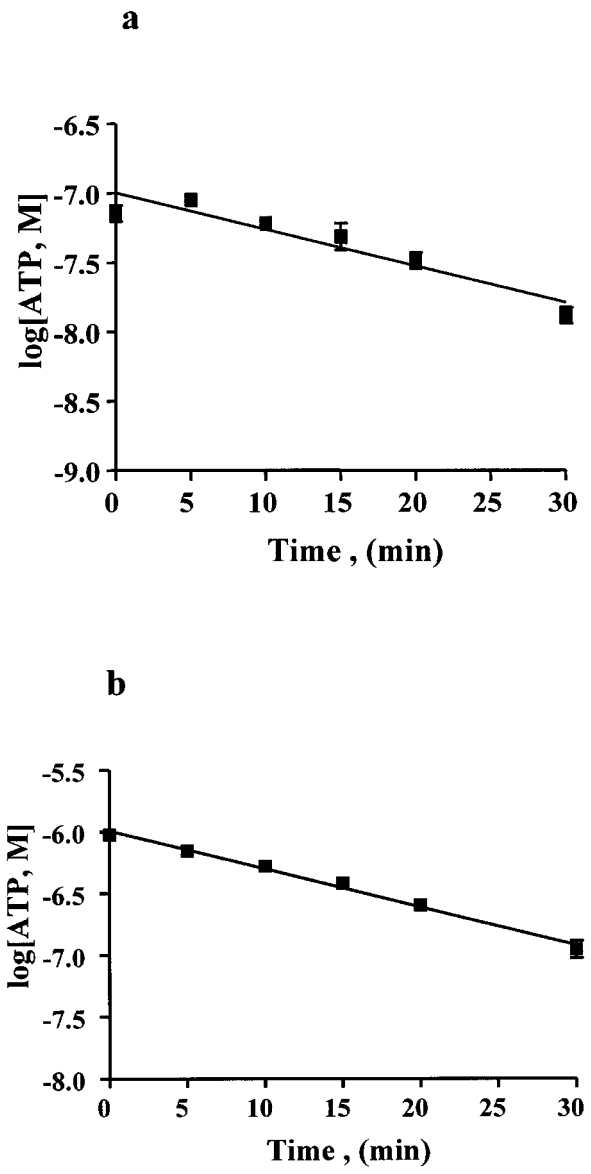

Figure 7 The rate of appearance (a) and metabolism (b) of ATP in the incubation medium of B10 cells. Confluent cultures were pre-washed and the medium replaced at zero time, with the conditions as in an adenylyl cyclase assay (without agonist), and the ATP content of the medium was monitored (see Methods). The stress due to the change of medium results in a release to give ~100 nM ATP in the assay volume (a), but no further net release is seen. In another set of experiments (b), ATP was added initially to 1000 nM, and its disappearance with time was determined. Note that during the standard adenylyl cyclase assay period of 5 min, only a relatively small conversion of ATP to other products occurs, even in the absence (as here) of the ATP regeneration system.

this receptor are in most respects very similar to those of the $P2Y_T$ receptor present on platelets, which plays an essential role in platelet aggregation (Hourani & Hall, 1994; Kunapuli, 1998). Our earlier studies have already shown that adenine nucleotides induce inhibition of adenylyl cyclase through a P2Y-type receptor in B10 cells (Webb *et al*., 1996). Activation of those cells by the same agonists failed to lead to a measurable increase in inositol phosphate levels (Feolde *et al*., 1995). RT–PCR and DNA cloning studies identified P2Y-type transcripts only for the $P2Y_1$ subtype in B10 cells and hence it was suggested (Webb *et al*., 1996) that the $P2Y_1$ receptor might couple in different native cell types to either adenylyl cyclase inhibition (e.g. in B10 cells) or to the more commonly observed phospholipase C activation. However, selective antagonists have since become available and were applied in the present study to examine this further.

We had previously noted (Webb *et al*., 1996) that alternatively a second, unknown P2Y receptor could be a possible origin of the adenylyl cyclase inhibition in B10 cells. That is now seen to be the case, since the adenylyl cyclase inhibition is sensitive to a specific antagonist of the platelet $P2Y_T$ receptor, AR-C66096 (Figure 4a), which does not affect the activity of the $P2Y_1$ receptor (Figure 5) in the B10 cells. The two receptors are also totally distinguished by the *bis*-monophosphate $P2Y_1$ antagonists (Figure 3).

In platelets, the ADP-mediated adenylyl cyclase inhibition is due to the $P2Y_T$ receptor (see Introduction) and we can conclude that this inhibition in the B10 cells is produced by an endothelial cell receptor which is functionally very similar, if not identical, to that platelet receptor. Thus, (i) both of those receptors respond to ADP with adenylyl cyclase inhibition and without intracellular Ca^{2+} mobilization (as just discussed), (ii) both are competitively antagonized by AR-C66096 (Daniel *et al*., 1998 (platelets) and Figure 4a) and also (iii) by BzATP (Vigne *et al*., 1999 (platelets) and Figure 4b), (iv) neither are blocked by the *bis*-phosphate antagonists of the $P2Y_1$ receptor (Fagura *et al*., 1998; Hechler *et al*., 1998b; Jin & Kunapuli, 1998 (platelets) and Figure 3), (v) both involve a G-protein, sensitive to pertussis toxin (Ohlmann *et al*., 1995 (platelets) and Figure 6), unlike the $P2Y_1$ receptor in the B10 cells, and (vi) both are (again unlike the $P2Y_1$ receptor) not antagonized by 100 μM PPADS (Geiger *et al*., 1998 (platelets); Webb *et al*., 1996 (B10)). Further, in agonist potencies for adenylyl cyclase inhibition, ADP is 400 to 500 fold weaker than 2-MeSADP in both the rat platelet (Savi *et al*., 1994a) and the rat B10 cell (Table 1).

There are, however, differences in the activity of some adenosine triphosphate derivatives between the platelet $P2Y_T$ receptor and the similar receptor in B10 cells. 2-ClATP, 2-MeSATP and ATP itself are clearly agonists for the adenylyl cyclase inhibition in rat B10 cells (Figure 1), but are well established to be antagonists of the ADP-induced adenylyl cyclase inhibition (as well as aggregation) in human platelets (Cusack & Hourani, 1982; Park & Hourani, 1999). There is no evidence as yet to indicate that the species difference here is likely to cause this major change. The $P2Y_T$ receptor of platelets of another rodent, the mouse, behaves identically to that of man in its ADP responses and their AR-C66096 sensitivity (Kim *et al*., 1999). ADP-mediated inhibition of adenylyl cyclase shows only a 4 fold greater potency on the rat platelet (Savi *et al*., 1994a) than on the human platelet (with pure nucleotide: Geiger *et al*., 1998).

For the agonist action of 2-MeSATP and other ATP derivatives in cyclase inhibition in B10 cells, we can eliminate as an explanation potential artefacts due to metabolism (Figure 7) of a triphosphate to produce the agonist diphosphate, a problem recognized and removed previously in $P2Y_1$ receptor studies (Hechler *et al*., 1998c). All the triphosphate compounds were 100% pure, using the CPK/CK regeneration system as before, and it was shown (Figure 7b) that enzymic conversions of ATP on the B10 cells in the

assay conditions were negligible. Furthermore, 2-MeSATP appears as almost as strong an agonist as 2-MeSADP (Table 1), so that the latter product formed in the 5-min assay period could not possibly account for the activity that is observed of that triphosphate derivative.

Therefore, ATP and at least two of its derivatives behave at this receptor in B10 cells in an opposite manner to the otherwise similar receptor in platelets. We consider here two main possibilities to explain this phenomenon. The first is that two different $P2Y_T$ receptor subtypes exist, one seen in the platelet and one in the B10 endothelial cell, which are similar in all of the other properties enumerated above, but differ in their interactions with triphosphates. These could be from two genes or from splice variants (although no introns are reported in the coding regions of the known genes of the P2Y receptor family).

The second explanation is that the antagonist action of ATP derivatives in platelets is apparent only, due, for example, to a particularly low receptor reserve there or to differences in interacting proteins. As analysed by Hoyer & Boddeke (1993) and Kenakin (1993; 1997), a change from a very low to a high receptor reserve could give a great increase in agonist potency, and even convert a ligand of lower intrinsic efficacy from acting as an apparent antagonist or inverse agonist to an agonist. That effect has been invoked recently (Palmer *et al*., 1998; Filippov *et al*., 2000) to interpret a similar agonist/antagonist variation observed for these same adenosine triphosphates acting at the $P2Y_1$ receptor when it is expressed in different cell types. That receptor is similar in almost all of its agonist selectivities to the $P2Y_T$ receptor (Hechler *et al*., 1998c), suggesting much similarity in their binding sites, although their transductions are entirely different. The $P2Y_1$ receptor can exhibit two alternative activity series for the same set of ligands when expressed in different cells at what appear to be very different receptor densities. In these two series ADP derivatives are always agonists but (as here) ATP derivatives appear either as antagonists (Leon *et al*., 1997; Hechler *et al*., 1998c) or agonists (Palmer *et al*., 1998; Filippov *et al*., 2000). These are cases where unambiguously a single P2Y receptor is studied (since one recombinant human receptor subtype was expressed in different cells), where ligand impurity or metabolism was rigorously excluded in each study, and where entirely different stages in the transduction pathway (Filippov *et al*., 2000) can give the same result. Overall, this is a close parallel to the comparisons of $P2Y_T$ receptors made here.

For the receptor density factor, we can note that a total of only $\sim$500 $P2Y_T$ receptors per platelet has been found by saturation binding of [^{33}P]2-MeSADP (in the presence of excess A3P5P to block $P2Y_1$ receptors) or by such binding sensitive to prior clopidogrel administration (Savi *et al*., 1994b; 1998; Hechler *et al*.,1998a). This corresponds to a mean surface density of the order of 60 μm^{-2}, an extremely low level. We do not know the density of the $P2Y_T$-type receptor on the B10 cell, but from the relatively high level of forskolin-induced cyclic AMP per cell and its ADP-evoked decrease (up to 90%, Figure 1b) it appears likely that a much higher receptor density is present on those cells.

We have shown (Figure 2) that ATP and 2-ClATP can each act as an apparent antagonist of the action of the very potent agonist 2-MeSADP on the B10 cell, which can be explained because each is a partial and much less potent agonist than 2-MeSADP (Figure 1). Hence, at the ratio of $P2Y_T$-type receptors to G-protein/effectors existing in the B10 cells, ATP already has mixed agonist/antagonist character, unlike ADP. If that partial agonist character derives from a low enough intrinsic efficacy, then at a much lower receptor density the antagonist behaviour could predominate. This may occur in the platelet.

A more extreme change in this $P2Y_T$-type response between the B10 cell and the platelet is exhibited by 2-MeSATP. This is an agonist with an EC_{50} value of 5.7 nM at the B10 cells and it is as full an agonist there as the most potent diphosphate derivative, 2-MeSADP (Figure 1), while pure 2-MeSATP becomes a very potent antagonist at the platelet. Thus, a K_B value of 65 nM has been found for inhibition of ADP-induced cyclic AMP increase in human platelets by pure 2-MeSATP (Park & Hourani, 1999) and somewhat lower antagonist potencies in various earlier studies in the literature (the latter all using unpurified nucleotide, which generally contains agonistic diphosphate contamination). 2-MeSATP, therefore, presents an exceptional difference in the $P2Y_T$ pharmacology between the B10 cell and the platelet. With ATP and 2-ClATP that difference is of the same type but distinctly lesser in extent, those being agonists on B10 cells but far less potent than 2-MeSATP (Table 1) and weak antagonists ($pK_B \sim 4-5$) of the adenylyl cyclase inhibition in platelets (Hourani & Hall, 1996; Geiger *et al*., 1998).

However, precedents with other receptors for such a large change in a ligand activity within the same transduction response, although uncommon, are known with a single receptor protein responsible. Such effects have been found, for example, on several 5-HT1 receptor subtypes (Hoyer & Boddeke, 1993; Schoeffter *et al*., 1997). In the maximum change reported there, at the transfected 5-$HT1_A$ receptor several specific ligands acted at relatively high receptor density as partial agonists, with EC_{50} values down to 250 nM, whereas at a receptor density only 7 fold lower they were antagonists, with K_B of 6.3 nM. That the major role there is played by receptor density and not by different effector interactions is underlined by the fact that all of those data were recorded in the same cell type (a HeLa cell line) and for the same transduction step (Ca^{2+} mobilization).

In summary, further studies are now needed to distinguish between possible causes for the quantitative differences discussed here, but we conclude that in any case there is a $P2Y_T$-type receptor present in the rat brain capillary endothelial cells. Whether it is identical to, or another subtype of, that in the blood platelets is an open question, brought into focus by the functional similarities and differences described here.

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