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The P2Y purinoceptor in rat brain microvascular endothelial cells couple to inhibition of adenylate cyclase

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1 B10 cells, a clonal line of rat brain capillary endothelial cells, exhibit a single P_2 purinoceptor, activation of which leads to increases in free intracellular calcium. In the current study the identity of this P2Y receptor was determined by its binding parameters for a range of purinoceptor ligands and by its complementary DNA (cDNA) sequence. The signal transduction mechanism activated by this receptor was also investigated.

2 The radioligand [^{35}S]-dATPαS bound with high affinity (K_d = 9.8 nM) to the P2Y purinoceptor expressed on B10 cells, which was found to be extremely abundant (B_{max} = 22.5 pmol mg^{-1} protein). The calculated K_i values of a range of P_2 purinoceptor agonists which competitively displaced binding of [^{35}S]-dATPαS led to the rank order of affinity: dATPαS (K_i 3.4 nM) > 2-chloroATP (2-ClATP) (13 nM), ATP (22 nM) > ATPγS (43 nM) > 2-methylthioATP (2-MeSATP) (88 nM) > ADP (368 nM) > > UTP, L-β,γ-methyleneATP (both > 10,000 nM). The P_2 purinoceptor antagonists, Reactive blue 2 and suramin, were also able to displace binding, with K_i values of 833 and 1358 nM respectively. In contrast pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid 4-sodium (PPADS) was able to displace only 20% of [^{35}S]-dATPαS binding at a concentration of 100 μM.

3 2-ClATP (EC_{50} = 0.22 μM), 2-MeSATP (0.54 μM), ADP (7.9 μM) and ATP (a partial agonist), but not UTP, inhibited the cyclic AMP formation stimulated by cholera toxin, in a manner that was prevented by pertussis toxin. The purinoceptor antagonist, PPADS, was found to be inactive at a concentration of 100 μM.

4 A P2Y receptor cDNA was derived from mRNA from B10 cells and from C6-2B, a rat glioma cell line known to possess a P2Y receptor that is coupled to the inhibition of adenylate cyclase. Sequence analysis of the entire coding region revealed that both were 100% identical to the rat $P2Y_1$ purinoceptor cDNA. No other P2Y-type receptor mRNA could be detected in B10 cells. Exactly the same sequence was isolated from rat brain cortical astrocytes, where 2-MeSATP has been shown to increase phospholipase C activity.

5 Since the receptor responsible for the transduction shares with the aforementioned binding site significant pharmacological features, including a strong activity of 2-MeSATP (characteristic of $P2Y_1$ receptors alone among all known P2Y purinoceptors) and an unusual insensitivity to PPADS, and since abundant mRNA is present of the $P2Y_1$ receptor but not of any other type resembling the known P2Y receptors, it is concluded that a $P2Y_1$ receptor on rat brain microvascular endothelial cells can account for all of the observations. This single $P2Y_1$ receptor, therefore, appears to couple in different native cell types to either adenylate cyclase inhibition or to phospholipase C activation.

Keywords: 2-MethylthioATP; adenylate cyclase inhibition; ATP; P2Y purinoceptors; $P2Y_1$ binding parameters; PPADS; rat brain capillary endothelium; C6-2B glioma

Introduction

Extracellular ATP mediates intercellular signalling via P_2 purinoceptors, which are of importance in both the autonomic and central nervous systems, cardiac and various types of smooth muscle, in endothelial cells, and in a variety of haematopoietic cells (Burnstock, 1990). Pharmacologically distinct receptor types have been defined on the basis of the rank order of potency of a variety of nucleotides and include the transmitter-gated ion channel P_{2X}, as well as the G protein-coupled receptors originally termed P_{2Y}, P_{2U} and P_{2T} (Dubyak & El-Moatassim, 1993). The application of molecular biology techniques in this field has revealed that more receptor types exist than were predicted by such pharmacological studies. As sequences for the G protein-coupled receptors have been derived and characterized, they have been enumerated chronologically as $P2Y_1$, $P2Y_2$, $P2Y_3$ etc. (Barnard *et al.*, 1994; Fredholm *et al.*, 1994), replacing and extending the aforementioned nomenclature.

Recombinant receptor complementary DNAs (cDNAs) have been isolated from a number of species and tissues, for both the $P2Y_1$ and $P2Y_2$ purinoceptors. Thus, $P2Y_1$ sequences have been determined from chick brain (Webb *et al.*, 1993), turkey brain (Filtz *et al.*, 1994), mouse M1N6 insulinoma cells (Tokuyama *et al.*, 1995), rat R1Nm5f insulinoma cells (Tokuyama *et al.*, 1995), bovine aortic endothelial cells (Henderson *et al.*, 1995), human erythro-leukaemia cells (Ayyanathan *et al.*, 1996) and human placenta (Léon *et al.*, 1996). $P2Y_2$ (previously P_{2U}) sequences have been isolated from the mouse component of the neuroblastoma cell line NG-108 (Lustig *et al.*, 1993), human airway epithelia (Parr *et al.*, 1994), rat alveolar type 2 cells (Rice *et al.*, 1995) and rat pituitary (Chen *et al.*, 1996).

It has previously been shown that P_2 receptors are present on rat brain capillary endothelial cells, the activation of which

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leads to an increase in the intracellular free calcium ion concentration ($[Ca^{2+}]_i$). These have been characterized as (i) a phospholipase-C-coupled $P2Y_2$ receptor and (ii) a P2Y receptor that does not achieve its rise in $[Ca^{2+}]_i$ by activation of phospholipase C (Frelin *et al.*, 1993; Vigne *et al.*, 1994; Feolde *et al.*, 1995). While in many cases activation of a tissue P2Y receptor is associated with an increase in phospholipase C activity (Boyer *et al.*, 1989), there are examples where a P2Y receptor is coupled to the inhibition of adenylate cyclase, for instance in the C6-2B glioma cell line (Boyer *et al.*, 1993). It has been suggested that distinct receptor subtypes are involved in these cases, based on the differential coupling, subtle differences in agonist activity and dissimilar antagonist effects at the turkey erythrocyte and glioma cell receptors (Boyer *et al*, 1993; 1994).

In this paper we further address the question of P2Y receptor heterogeneity. We have characterized, in terms of binding properties and signal transduction, the P2Y receptor present on a clonal line of the rat brain capillary endothelial cells (B10), useful because it has lost the original $P2Y_2$ receptor of those cells (Feolde *et al.*, 1995). Further, we have isolated from these cells, by Reverse Transcriptase-Polyermase Chain Reaction (RT–PCR), a cDNA for this receptor and shown that the same sequence is also expressed in the C6-2B glioma cell line. In addition, we have isolated this sequence from rat cortical astrocytes, where application of 2-MeSATP increases phospholipase C activity (Kastritsis *et al*, 1992). Based on these data we infer that a single P2Y receptor is capable of coupling promiscuously in different cell types and have identified this receptor as $P2Y_1$.

Methods

Cell culture

B10 cells (Feolde *et al.*, 1995) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum, 100 units ml^{-1} penicillin and 100 mg ml^{-1} streptomycin. C6-2B cells were grown in Ham's F-10 medium supplemented as above. Primary astrocyte cultures were derived from the cerebral cortex of neonatal rats as described previously (Neary *et al.*, 1994).

Preparation of membranes from B10 cells

Membranes were prepared as described previously (Simon *et al.*, 1995b). Sub-confluent cells were washed twice with ice cold buffer A (Tris HCl 50 mM, EDTA 1 mM, EGTA 1 mM, pH 7.4, and harvested in buffer B [as above but also containing (as protease inhibitors) benzamidine 1 mM, phenylmethylsulphonyl fluoride 0.1 mM, bacitracin 0.01% (w/v), soybean trypsin inhibitor 0.001% (w/v) and 40 kallikrein inhibition units of aprotinin]. Cells were freeze-thawed and further disrupted by homogenization with an Ultra-Turrax J-25 homogenizer (setting 5, 15 s × 2). The membranes were collected by centrifugation at 12,000 *g*, 30 min at 4°C. Pellets were washed by resuspension and centrifugation in buffer B and incubated on ice (30 min) to chelate endogenous divalent cations, destroy labile endogenous ligands and inactivate traces of proteases. After a further two washes, the membranes were stored at −70°C. Protein concentrations throughout were determined by the dye binding method (Biorad).

Receptor binding methods

Aliquots of the membrane fraction containing 5–10 μg protein in buffer B were incubated with [^{35}S]-dATPαS, at a final concentration of 10 nM in the competition binding studies or ranging from 0.5–40 nM in the saturation experiments, in a final volume of 0.5 ml. Non-specific binding was defined in all cases with 100 μM 2-MeSATP. After a 60 min incubation at room temperature the membranes were harvested by rapid filtration through Whatman GF/C filters (pre-soaked in 20 mM sodium pyrophosphate) and the filters were immediately washed with three × 5 ml of ice 50 mM Tris HCl (pH 7.4) on a vacuum manifold (Millipore). The bound radioactivity was determined using Optiphase 'HiSafe' II (LKB) scintillant by liquid scintillation counting in a Beckman LS counter model 5000CE at a counting efficiency routinely of 95%.

All experiments were carried out in triplicate and were repeated at least three times. Data are expressed as overall mean ± s.e.mean, whereas the Figures show representative plots. Non-specific binding ranged between 10–30% of the total binding. All binding data were analysed by the EBDA-LIGAND computer programme (Biosoft). In each case alternative one-site or two-site binding models were used; the appropriate model was selected on the basis of an *F*-test on the weighted residual sums of squares and standard errors (Munson & Rodbard, 1980).

Signal transduction analysis methods

Inhibition of adenylate cyclase was assayed by first incubating cells (grown in 12-well tissue culture clusters) in a serum-free Earle's salt medium (Feolde *et al.*, 1995) buffered at pH 7.4 with 10 mM HEPES and supplemented with 100 ng ml^{-1} of cholera toxin. After 45 min of incubation at 37°C, isobutylmethylxanthine (0.1 mM) and nucleotides were added (final volume 500 μl per well) and the incubation was allowed to proceed for an additional 10 min at 37°C with constant agitation. The incubation solution was aspirated off and cells were extracted with 10% (w/v) ice-cold trichloroacetic acid. Adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in the supernatants was assayed by radioimmunoassay using the Immunotech reagents and protocol. In experiments using pertussis toxin, cells were treated with 100 ng ml^{-1} pertussis toxin for 4 h prior to the experiments. Means of triplicate assays (3 wells) were used per experiment and means of 3–7 experiments gave the data points. Dose-response curves were fitted to a logistic equation using the Sigma Plot software.

To estimate nucleotide degradation during these assays, cells were treated as above but in the presence of 0.07 μCi [α^{32}P]-ATP and 100 μM ATP. After the 10 min incubation, the incubation solution was harvested, 200 μl was diluted into 3 ml of 1 M NH_4OH/ethanol (3 vol/2 vol) and the whole mixture was applied to 2-ml columns of Dowex AG1X8 (Biorad, Boyer & Stempel, 1979). Columns were washed with 2 ml of water followed by 5 ml of 0.2 M Tris HCl, pH 8.0, AMP, ADP and ATP were then sequentially and quantitatively eluted with 2 × 3 ml 30 mM HCl, 3 × 3 ml 60 mM HCl and 3 × 3 ml 1 M HCl (analysed by the A_{260} nm profile, with standards). After 10 min, less than 2% of the initially-added ATP was degraded and ADP concentration was estimated by α^{32}P counting to be 1.2 μM. ADP was below the detection limit (0.5 μM) at times < 1 min. The separation method also showed that the ATP used here had insignificant contamination with other nucleotides.

RT–PCR and analysis methods

Total RNA was isolated by the method of Chomczynski & Sacchi (1987). cDNA was synthesized from 5 μg of total RNA, using an oligo$(dT)_{18}$ primer and Moloney Murine Leukaemia Virus reverse transcriptase (Gibco BRL) according to the manufacturer's recommendations. RT–PCR was performed using 1/50 volume of the reverse transcription, 200 ng of each primer, 200 μM of each dNTP and 2.5 units of Biotaq polymerase (Bioline) in a total volume of 50 μl under the following conditions: 60 s at 95°C, 30 s at 60°C, 60 s at 72°C for 30 cycles followed by 1 cycle at 72°C for 5 min. The sense primer was 5′-TGGCGTGGTGCACCCTCTCAAGTC-3′ and the antisense primer was 5′-CGGGACAGTCTCCTTCTGAATGTA-3′. Amplification products were resolved on a 1% (w/

v) agarose gel by electrophoresis. The bands were excised from the gel and after purification using Prepagene (Biorad) were cloned using the pCRIII TA cloning kit (Invitrogen). Double-stranded plasmid inserts were sequenced completely in both directions using a Sequenase kit (Amersham). Clones from three independent PCR reactions from each template source were analysed initially and additional clones were sequenced to resolve ambiguities.

Materials

[^{35}S]-dATPαS and dATPαS were purchased from NEN. 2-MethylthioATP (2-MeSATP), 2-chloroATP (2-ClATP), Reactive blue 2 (RB-2), pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid 4-sodium (PPADS) and suramin were purchased from Research Biochemicals Inc. All other nucleotides (purest grade) and other reagents were from Sigma, and tissue culture reagents were from Gibco BRL.

Results

[^{35}S]-dATPαS has previously been shown to be a suitable and convenient radioligand with very high affinity for P2Y$_1$ and related receptors (Simon *et al.*, 1995a; Webb *et al.*, 1996; Akbar *et al.*, 1996). It was applied here to membranes prepared from the B10 cells (Feolde *et al.*, 1995), where specific, high affinity, saturable binding was detected. The saturation binding isotherm was best fitted by a single site model (Figure 1). The K_d value was 9.8 ± 1.4 nM and the maximal number of binding sites was 22.5 ± 6.7 pmol [^{35}S]-dATPαS mg^{-1} protein ($n=4$). The pseudo-Hill coefficient (n_H) for this binding site was 0.98 ± 0.03.

A number of nucleotides could compete for the [^{35}S]-dATPαS binding site of these cells (Figure 2, Table 1). dATPαS, 2-ClATP, ATP, ATPγS and 2-MeSATP all displayed a high affinity for this P$_2$ receptor, with ADP displaying ~10 fold less affinity (Figure 2a). Both UTP and L-βγ-methyleneATP (L-βγ-

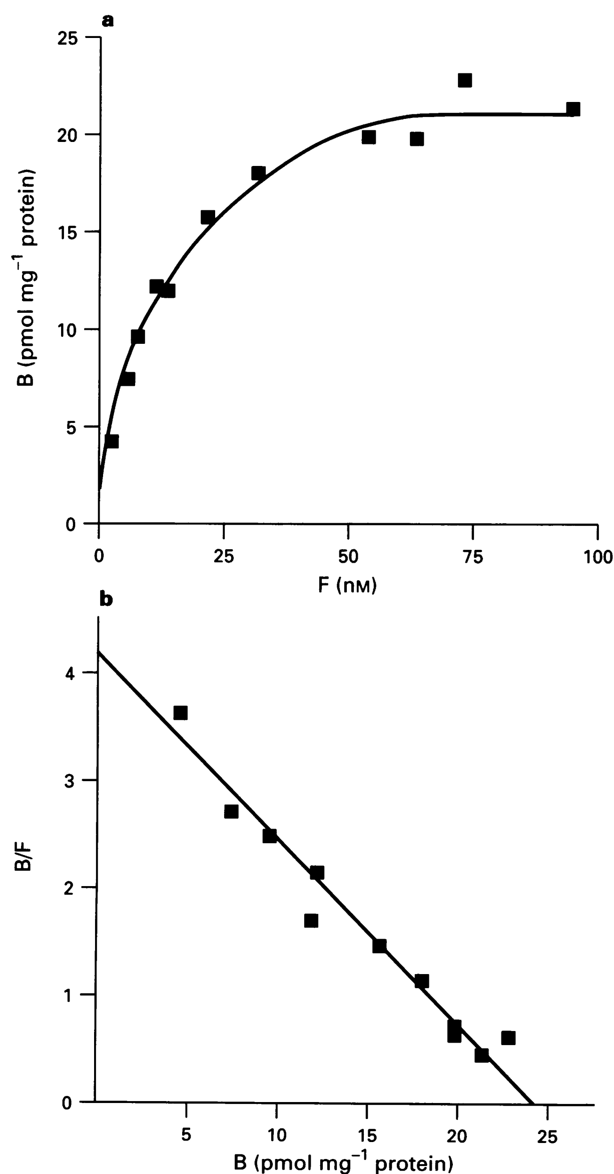

Figure 1 Equilibrium specific binding of [^{35}S]-dATPαS (0.5–40 nM) to B10 cell membranes. (a) Specific binding of [^{35}S]-dATPαS (pmol mg^{-1} protein), plotted against the free concentration (F) of radioligand (nM). (b) The specific binding data from (a) are presented as a Scatchard plot. In both (a) and (b) the lines were drawn by hand. The data were obtained from a single experiment (where each point is the mean of triplicate determinations) which is representative of the others ($n=4$).

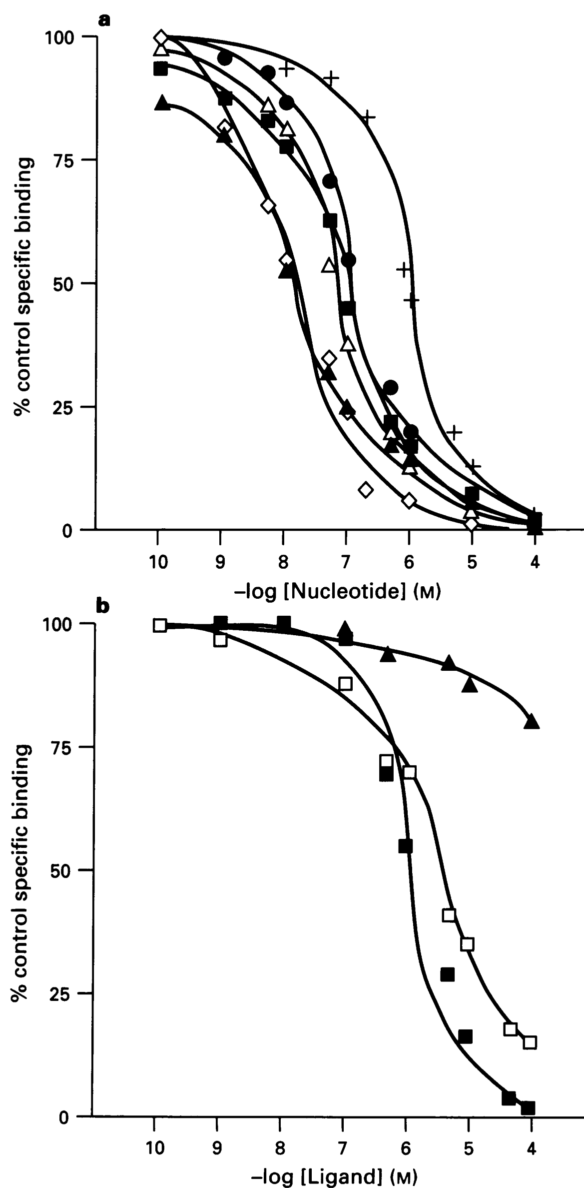

Figure 2 Competition curves for [^{35}S]-dATPαS binding. (a) (▲) dATPαS, (◇) 2-ClATP, (△) ATP, (■) ATPγS, (●) 2-MeSATP and (+)ADP. (b) (■) RB-2, (□) suramin and (▲) PPADS. Points represent the mean values of triplicate determinations, in one of three or more experiments used together to obtain the K_i values. The curves are theoretical ($n_H=1$) for a one-site binding model (which always gave the best fit).

Table 1 Affinities of purinoceptor-active ligands at the B10P2Y and comparison with the recombinant and native chick brain P2Y$_1$ purinoceptors

Ligand	*B10 P2Y*	K_i (nM) *Recombinant chick brain P2Y$_1$*[a]	*Native chick brain P2Y$_1$*[a]
dATPαS	3.6±1.4	23±7	17±2
2-ClATP	13±5	–	–
ATP	22±3	48±13	48±4
ATPγS	43±16	52±17	–
2-MeSATP	88±22	69±23	32±8
ADP	368±63	171±19	530±183
RB-2	833±111	944±201	1472±278
Suramin	1358±404	1592±206	1052±244
UTP	>10,000	>10,000	>10,000
L-β,γ-meATP	>10,000	>10,000	>10,000
PPADS	>100,000	–	–

K_i values were computed from the competition data (Figure 2) using the observed K_d value for [^{35}S]-dATPαS of 9.8 nM. All values are expressed as the mean±s.e.mean of at least three independent determinations. [a]Values obtained on the recombinant chick brain P2Y$_1$ receptor expressed in COS-7 cells and on chick brain membranes (Simon *et al.*, 1995a,b) are shown for comparison.

meATP) had K_i values in excess of 10 μM. P$_2$ purinoceptor antagonists were also tested, RB-2, a P2Y receptor antagonist (Rice & Singleton, 1989) and suramin, a non-selective antagonist of P$_2$ purinoceptors (Dunn & Blakely, 1988), competed with the [^{35}S]-dATPαS binding with K_i values of ~8.0 and 1.4 μM respectively. However, PPADS, an antagonist at P2X responses in rabbit urinary bladder and at the P2Y$_1$ receptor of the turkey erythrocyte (Ziganshin *et al.*, 1993; Boyer *et al.*, 1994) had little effect here, being able to displace only 20% of the [^{35}S]-dATPαS binding at a high concentration, 100 μM (Figure 2b). These data establish a rank order of affinity for the P2Y purinoceptor of rat brain capillary endothelial cells as: dATPαS>2-ClATP, ATP, ATPγS≥2-MeSATP>ADP>RB-2>suramin>>UTP, L-β,γ-meATP, PPADS. This binding profile is in good agreement (where tested) with that found for both the native and the recombinant chick brain P2Y$_1$ purinoceptors (Webb *et al.*, 1994; Simon *et al.*, 1995a,b).

Previously it has been shown that nucleotides are able to raise [Ca^{2+}]$_i$ in B10 cells in the absence of phospholipase C activation (Feolde *et al.*, 1995). Therefore, involvement of adenylate cyclase was investigated. 2-ClATP, ATP, 2-MeSATP, ADP and UTP each had no effect on basal cyclic AMP levels. Addition of 100 ng ml^{-1} of cholera toxin increased cyclic AMP levels from 0.33±0.03 nmol mg^{-1} of protein to 2.36±0.46 nmol mg^{-1} of protein (n=10). 2-ClATP, 2-MeSATP, ATP and ADP were each able to decrease this stimulated cyclic AMP level. The maximum inhibition induced by ADP, 2-ClATP or 2-MeSATP was 50 to 60%, whereas that observed with ATP was ~35% and UTP was inactive (Figure 3a,b; Table 2). Dose-response curves indicated a rank order of activity 2-ClATP, 2-MeSATP>ADP>ATP for the inhibition of adenylate cyclase (Figure 3b). Calculated EC$_{50}$ values are shown in Table 2. An accurate EC$_{50}$ value for ATP could not be determined due to its weak partial agonist activity (Figure 3b); its EC$_{50}$ was estimated to be of the order of 200 μM. PPADS at a concentration of 100 μM was unable to antagonize the inhibitory effect of ADP on adenylate cyclase activity (Table 3). The actions of the adenine nucleotides were completely preventable by pertussis toxin pre-treatment (Table 4).

The possible partial degradation of ATP by ectonucleotidase (Kennedy & Leff, 1995), if this enzyme were to be present on these cells in sufficient amount, was considered as a possible cause of its reduced efficacy. Cells were incubated under the conditions used in the cyclic AMP experiments, with 100 μM ATP trace-labelled with [^{32}P]-ATP. In the

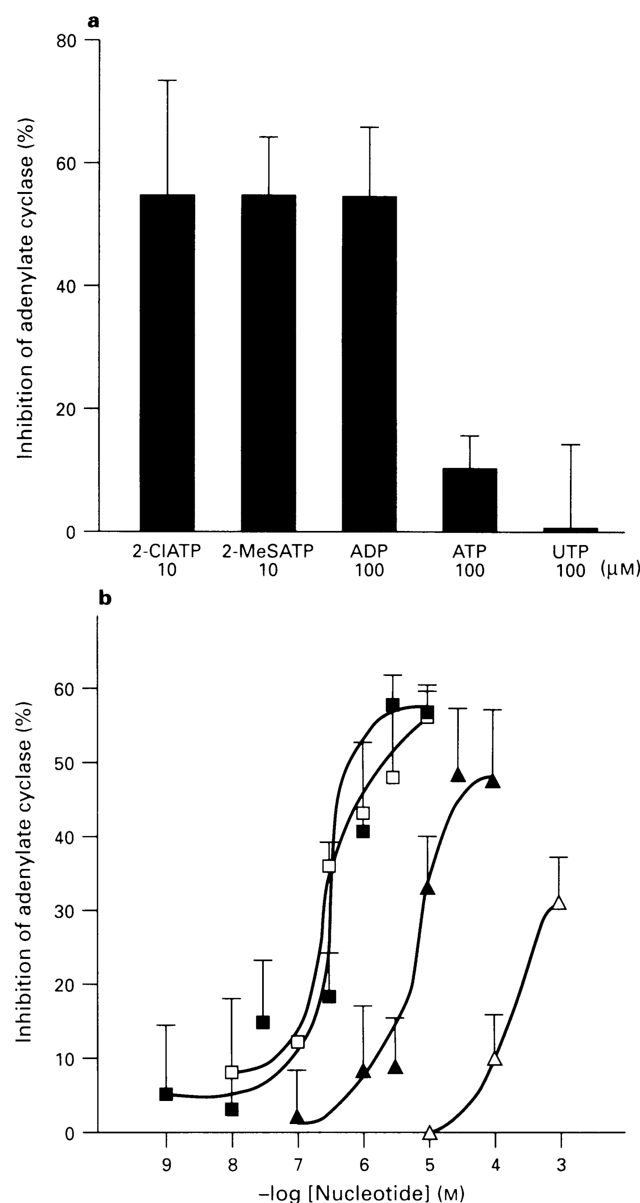

Figure 3 Inhibition by nucleotides of cholera toxin-induced cyclic AMP formation in B10 cells. (a) Cells were incubated in the presence of 100 ng ml^{-1} cholera toxin, 0.1 mM isobutylmethylxanthine; 10 μM 2-ClATP, 10 μM 2-MeSATP, 100 μM ADP or 100 μM ATP were present as indicated and cyclic AMP levels were measured. Basal activity (see Table 3) was defined in the presence of isobutylmethylxanthine alone and has been subtracted. Maximum activity (100%) was defined in the presence of isobutylmethylxanthine and cholera toxin alone. Means and s.e.mean values for 3–7 experiments are indicated. (b) Concentration-dependent effect of (□) 2-ClATP, (■) 2-MeSATP, (▲) ADP and ATP (△) on cyclic AMP accumulation. Means and s.e.mean (n=6) are shown (separate experiments from those of panel a).

10 min incubation period, <2% of the ATP present was found to be converted to ADP and other products (data not shown). Therefore, the low activity of ATP at the receptor on these cells is unlikely to be an artefact due to a preferential degradation of that nucleotide (as discussed further, below), and with these cells inhibition of ectonucleotidase is not required for the determinations made here. The plausible possibility that ATP is only an apparent weak agonist, with its activity being due to an ADP impurity in it, could be rejected because we used an ATP sample where ADP was not significantly detectable (see Methods), and also because a treatment with apyrase (1 unit ml^{-1}, 1 h, 25°C) of the ATP solution to be used, *increased* the activity in a manner

Table 2 Parameters for adenylate cyclase inhibition by nucleotides

Ligand	EC_{50} (μM)	*Maximum inhibition (%)*
2-ClATP	0.22±0.07	54.6±3.8
2-MeSATP	0.54±0.23	62.9±6.3
ADP	7.90±2.70	55.1±5.3

For details see Methods. Values were derived from curve fitting of the dose-response data; all values are expressed as the mean±s.e.mean of six independent determinations.

Table 3 PPADS sensitivity of adenylate cyclase inhibition

Experimental condition	*Cyclic AMP production (nmol cyclicAMP mg^{-1} protein)*
Control	0.30±0.02
Cholera toxin	3.89±0.22
Cholera toxin+ADP 10 μM	1.40±0.10
Cholera toxin+ADP 10 μM+ PPADS 10 μM	1.21±0.21
Cholera toxin+ADP 10 μM+ PPADS 100 μM	1.38±0.12

For details see Methods. All values are expressed as the mean±s.e.mean of three independent determinations carried out in triplicate.

Table 4 Pertussis toxin sensitivity of adenylate cyclase inhibition

Experimental condition	*CyclicAMP production (nmol cyclicAMP mg^{-1} protein)*
Control	0.29±0.03
Cholera toxin	2.01±0.36
pertussis toxin	0.32±0.04
Cholera toxin+pertussis toxin	2.32±0.10
Cholera toxin+2-MeSATP 10 μM	1.26±0.10
Cholera toxin+2-MeSATP 10 μM+pertussis toxin	2.73±0.03

For details see Methods. All values are expressed as the means±s.e.mean of three independent determinations carried out in triplicate.

consistent with a high degree of conversion to ADP providing a more potent agonist activity with higher efficacy (data not shown). Further, an apyrase pretreatment of the B10 cells (as in Henderson *et al.*, 1995) did not change the effects of ADP or of ATP (data not shown), indicating that prior desensitization by any released nucleotide does not compromise these results.

Based upon the chick $P2Y_1$ receptor cDNA sequence, we had previously isolated a partial cDNA sequence encoding the region from transmembrane domain-3 to transmembrane domain-7 of a rat brain P2Y purinoceptor (Webb, T.E., unpublished). This sequence has 87% and 96% amino acid sequence identity to the same regions of the chick and bovine $P2Y_1$ purinoceptors respectively (Webb *et al.*, 1993; Henderson *et al.*, 1995) and was therefore considered as encoding part of the rat $P2Y_1$ receptor. This was subsequently found to be the case, by the report of Tokuyama *et al.* (1995) of a rat $P2Y_1$ receptor cDNA (cloned from an insulinoma cDNA library), which is completely identical (across the region covered) with the rat partial cDNA which we had isolated. Given the highly similar profiles of the receptor on B10 cells compared with the chick $P2Y_1$ receptor, primers based on this rat partial cDNA sequence were used in RT-PCR using B10 RNA as a template source. A single product of 540 base pairs was generated (Figure 4), which upon complete sequencing was shown to be identical to the sequence previously isolated from rat brain.

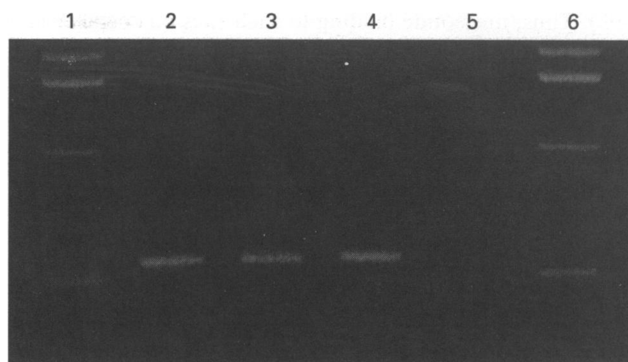

Figure 4 Agarose gel electrophoresis of RT-PCR products. Lane 1 and 6 marker lanes of 1 Kbp ladder (Gibco BRL). Lanes 2, 3 and 4, amplification products of 540 base pairs were produced using RNA from B10 cells, C6-2B cells and cortical astrocytes, respectively, as a template source. Lane 5: control RT-PCR without template.

Since a P2Y receptor coupled to adenylate cyclase inhibition has also been identified on the C6-2B glioma cell line (Boyer *et al.*, 1993), RT-PCR was also carried out on RNA from that cell line using the same primers. Again a product of 540 base pairs was generated which was found to be identical in sequence to that isolated from rat brain and the B10 line. It was also possible to amplify the same sequence from rat cortical astrocytes (Figure 4); sequence analysis of this partial cDNA confirmed that it, too, shared 100% sequence identity with the B10 and C6-2B sequences. In confirmation, specific primers designed to amplify the whole coding region of the rat $P2Y_1$ receptor were used in PCR with template cDNA derived from B10, C6-2B and astrocyte RNA and again, upon complete sequencing, the amplified cDNAs were found to be 100% identical to each other and to the published rat $P2Y_1$ sequence (data not shown).

Discussion

Previous pharmacological and cross-desensitization studies have indicated that a single P2Y receptor is expressed by B10 microvascular endothelial cells (Feolde *et al.*, 1995). Here we have identified this purinoceptor as the $P2Y_1$ subtype by its sequence and by the lack of any other receptor mRNAs having any similarity in their transmembrane regions to the known P2Y receptors. We have further shown that this receptor couples negatively to adenylate cyclase in these cells. In this identification, comparison of its binding properties with those of the recombinant chick $P2Y_1$ and the native chick brain $P2Y_1$ purinoceptors was highly supportive of the concept that the B10 cell receptor is the rat equivalent of the avian $P2Y_1$; the same rank order of affinity was found for all three cases (Webb *et al.*, 1994; Simon *et al.*, 1995a,b and our results).

As found previously for chick and rat brain membranes, the [^{35}S]-dATPαS binding site on B10 cell membranes is highly abundant (22 pmol mg^{-1} protein). A B_{max} value of 14 pmol mg^{-1} for a high affinity binding site has been reported previously for a different radioligand, [^{35}S]-ADPβS, binding to primary cultures of bovine aortic endothelial cells (Wilkinson & Boarder, 1995). However, this is not the site involved here, since under the conditions used the rank order of ligand affinity did not resemble that of a P2Y receptor (2-MeSATP having extremely low affinity) and β,γ-methyleneATP at 10^{-4} M abolished the high-affinity binding sites; the authors concluded that non-receptor sites were contributing to the high number of binding sites found. A major difference in the binding protocol which we have used is the complete absence here of magnesium and calcium, which are necessary for binding and catalysis at many ecto-enzymes using nucleotides such as ecto-ATPases (Culic *et al.*, 1990; Ziganshin *et al.*,

1995). Thus, nucleotide binding to such sites on cochlear hair cells has been shown to be decreased by 74% by the removal of divalent cations (Mockett *et al.*, 1994). Furthermore, Filtz *et al.* (1994) found that astrocytoma cells expressing transfected $P2Y_1$ receptors gave no associated binding of [^{35}S]-ADPBβS. Most of the sites labelled by Wilkinson & Boarder (1995) using [^{35}S]-ADPβS, Ca^{2+} and high Na^+ medium are, therefore, likely to be high affinity non-receptor nucleotide-binding sites, very numerous on primary cultures of bovine aortic cells. In contrast, on membranes of the same cell type but using medium free of divalent cations and [^{35}S]-ATPγS instead of [^{35}S]-ADPβS, Motte *et al.* (1996) more recently found a set of high affinity sites with a pharmacology like ours which appeared to represent P2Y receptors. For all of these reasons, we discount a significant contribution of non-receptor binding sites to the B_{max} value obtained here and conclude that the binding site characterized in this study is that of a $P2Y_1$ receptor.

This conclusion was further strengthened by the high degree of sequence identity of the cDNA isolated from B10 cell RNA to both the chick $P2Y_1$ cDNA sequence (Webb *et al.*, 1993) and the $P2Y_1$ cDNA sequence obtained from bovine aortic endothelial cells (Henderson *et al.*, 1995). Finally, when the rat $P2Y_1$ cDNA was isolated from an insulinoma cell line and functionally characterized as such in *Xenopus* oocyte expression (Tokuyama *et al.*, 1995), we confirmed that the entire coding region of the B10 receptor is identical to the rat $P2Y_1$ receptor DNA sequence. The B10 cell line is therefore of particular interest in P2Y receptor expression comparisons, because the $P2Y_1$ receptor activity here is not due to an artificially integrated exogenous receptor but to a native receptor gene expressing in its original genomic environment. Thus, in the B10 cells a native $P2Y_1$ receptor can be studied in a microvascular endothelial cell.

We have found that 2-ClATP, 2-MeSATP, ADP and ATP inhibit cholera toxin stimulated adenylate cyclase activity in B10 cells. The rank order of potency (2-ClATP, 2-MeSATP > ADP > > ATP) is similar to that found for the adenine nucleotide-induced $[Ca^{2+}]_i$ elevation in these cells (Feolde *et al.*, 1995). The same rank order of potency was seen in Jurkat cells expressing the recombinant bovine $P2Y_1$ receptor (Henderson *et al.*, 1995). A slightly different potency order was seen (Filtz *et al.*, 1994; Schachter *et al.*, 1996) for the accumulation of inositol phosphates in 1321N1 astrocytoma cells expressing either the human or turkey $P2Y_1$ receptors (2-MeSATP > ADP > 2-ClATP > ATP). However, ATP displayed significantly reduced potency and was a partial agonist in all of these cases.

While the rank order was the same for cyclase inhibition and for $[Ca^{2+}]_i$ increases, there are some differences in potency in the two responses of the B10 cells, with ADP and ATP showing a reduced potency for cyclase inhibition. One possibility was that this could arise from some preferential degradation of these ligands during the longer incubation time in the adenylate cyclase assay (10 min) compared with that in the previous calcium measurements (15 s). This is not considered likely, however, because the discrepancy occurs with ATP and ADP, but not with the other ligands tested: 2-MeSATP and 2-ClATP show (within experimental error limits) the same potency in the two systems in the B10 cells. Where ecto-enzymes which hydrolyse ATP have been studied, they destroy 2-MeSATP as rapidly as ATP (Ziganshin *et al.*, 1995) and, further, on endothelial cells of small aortic arteries, ADP is resistant (Juul *et al.*, 1991). Hence, at least a major element here must be an intrinsically lower potency of ATP. To test this further, we measured the ability of the cell samples (in the conditions of the adenylate cyclase assay) to hydrolyse ATP and found this to be negligible (see Results). With the 10 min period used and the small volume of incubation medium (500 μl) employed and agitated throughout, an equilibrium state must be established and the concentration of ATP at the receptors should approximate that in the medium.

We should also note that in the rat C6-2B glioma cells (Boyer *et al*, 1993) a low potency of ATP, acting (as here) as a partial agonist in adenylate cyclase inhibition, was also seen. While in similar measurements of degradation made on primary cultures of endothelial cells taken from native tissues such as bovine aorta (Motte *et al*, 1996) considerable hydrolysis of applied ATP is found if (and only if) divalent cations are present at millimolar levels, the ecto-nucleotidase activity is obviously much lower on the cultured permanent lines of a single cell type considered here.

The binding affinities for the nucleotides at the B10 purinoceptor are considerably greater than their EC_{50} values for cyclase inhibition. Such differences shown by a G protein-coupled receptor for a given agonist between its affinity in binding and in second messenger response represent a not uncommon situation, since the EC_{50} value for agonist activity in a transduction is a composite quantity based on a number of parameters and need not approach the equilibrium binding constant. However, for other receptors the rank order of agonist potency is usually maintained in that comparison, whereas here there is a discrepancy between the rank order of the affinities determined from the binding data (2-ClATP, ATP > 2-MeSATP > ADP) and that of the activities in cyclase inhibition (2-ClATP, 2-MeSATP > ADP > ATP). While this requires further investigation, it is not without precedent in the G-protein coupled receptors, since in some opioid receptors different ligand profiles have been attributed to the G-protein coupled and uncoupled states (Richardson *et al.*, 1992), and here the high density suggests that most of the receptors may be unable to couple.

The use of selective antagonists is preferable to the use of agonist potency orders for the pharmacological definition of a receptor type (Kenakin *et al.*, 1992). It has previously been shown that suramin, RB-2 and PPADS are all antagonists at the turkey $P2Y_1$ purinoceptor (Boyer *et al.*, 1994). PPADS has also been found to be active as an antagonist at the recombinant human $P2Y_1$ receptor (Schachter *et al.*, 1996) and at the $P2Y_1$ receptor of bovine aortic endothelial cells (Brown *et al.*, 1995). In contrast to the $P2Y_1$ purinoceptors mentioned above we find that while suramin and RB-2 have ~ μM affinity at the B10 cell P2Y receptor, PPADS has very low affinity in competitive binding at the latter receptor and has no effect on nucleotide-induced adenylate cyclase inhibition. This raises the question of whether there is also a rat $P2Y_1$ purinoceptor that, as in the cases of the avian, bovine and human $P2Y_1$ receptors, is PPADS-sensitive. Alignment of the amino acid sequence of the rat $P2Y_1$ receptor with those of the avian, bovine and human receptors reveals a high degree of sequence identity, as would be expected of species homologues. However, a lysine residue at position 41 (numbered as in the human sequence), conserved in the avian, bovine and human sequence, is replaced by arginine in the rat receptor sequence. As PPADS is predicted to form a Schiff's base with lysine, its potential inability to do so at this position in the rat $P2Y_1$ receptor may account for the PPADS insensitivity noted there. This possible explanation of the difference could be tested by mutation in that region.

Other P2Y purinoceptors have, where transduction evidence is available, generally been found to increase phospholipase C activity, for example the P2Y receptor of bovine aortic (Motte *et al.*, 1993) or adrenal micro-vasculature endothelial cells (Puskiss *et al.*, 1993) and of turkey erythrocytes (Boyer *et al.*, 1989; Filtz *et al.*, 1994). However, a well-characterized case of adenylate cyclase inhibition exists for a P2Y purinoceptor present in both the C6 rat glioma cell line and its subclone C6-2B (Boyer *et al.*, 1993; 1994). The rank order of potency of inhibition of adenylate cyclase by nucleotides in B10 cells is quite similar to that found in C6-2B rat glioma cells (Boyer *et al.*, 1993). The only difference, for the agonists studied here, is that 2-MeSATP was much more potent than 2-ClATP in the Boyer *et al.* (1993) study while the potencies of these two ligands were not significantly different here. Furthermore, as in B10 cells, in the C6 line PPADS has no antagonistic effect on nucleotide inhibition of adenylate cyclase (Boyer *et al.*, 1994). In addition in both cases, 2-MeSATP has no effect on phos-

pholipase C activity (Boyer *et al*., 1993; Feolde *et al*., 1995) and we show that the same P2Y$_1$ receptor sequence can be amplified from both cell types. This is of most significance in the B10 cells, as this is the only P$_2$ receptor sequence that we have been able to detect by PCR with wide-range primers for P2Y receptors, while in C6-2B cells we have been able to amplify cDNAs of other known subtypes of P2Y purinoceptors (Webb T.E., unpublished observations); it is noteworthy that of all of the known series of P2Y purinoceptors it is the P2Y$_1$ subtype (from any species studied) which is highly sensitive to 2-MeSATP as an agonist (Burnstock & King, 1996). However, the detection of P2Y$_1$ receptor RNA in C6-2B cells may also be significant in its own right, as the only effect of 2-MeSATP on these cells is through the inhibition of adenylate cyclase activity rather than the activation of phospholipase C (Boyer *et al*., 1993). It has been suggested that different P2Y receptor subtypes were involved in these couplings to phospholipase C and to adenylate cyclase (Boyer *et al*., 1993; 1994). This conclusion was based on the comparison of an avian and human receptor with that from a rodent, thus complicating the analysis. However, since the present paper was submitted it has been reported by Schachter *et al*. (1996) that transfection of the human P2Y$_1$ receptor into the rat C6 line confers upon these cells the ability to activate phospholipase C in response to 2-MeSATP and other agonists active at the human P2Y$_1$ receptor. Therefore, if it is the rat P2Y$_1$ receptor that is coupling to adenylate cyclase in this cell line it could be a consequence of the rat receptor sequence difference but not of high receptor density.

We cannot exclude the possibility that a further P2Y receptor subtype RNA is expressed on B10 cells, or indeed that a further 2-MeSATP-activated P2Y receptor is expressed in the C6 glioma cell line. However, any such receptor is not detectable with the wide-range primer sets which we have used, which will recognise the diagnostic features of certain transmembrane domains of all the P2Y receptors known to date. Given also that the previous cross-desensitization and other studies carried out on B10 cells indicated that a single P2Y receptor is present (Feolde *et al*, 1995), we believe that we have identified this receptor as P2Y$_1$ by its sequence and by its pharmacological profile. Furthermore, while there is no overall concordance of the binding and adenylate cyclase data there are some striking correlations between them. In both assays, firstly 2-ClATP and 2-MeSATP are equi-active; secondly, ADP shows lower activity than those agonists and finally, in both PPADS is inactive. We must also note that despite the abundant mRNA present for the rat P2Y$_1$ receptor there is no nucleotide activation at all of phospholipase C. Therefore we conclude that in B10 cells the identified rat P2Y$_1$ purinoceptor can indeed be naturally coupled to adenylate cyclase. We have also isolated the same P2Y$_1$ purinoceptor sequence from rat brain cortical astrocytes, where 2-MeSATP has been shown to increase phospholipase C activity (Kastritsis *et al*., 1992). The present case involves a native P2Y$_1$ purinoceptor and not one produced by DNA transfection into a host cell, where the native access to particular G proteins may be altered. In the capillary endothelial cells used here, the cyclase inhibition and the pertussis toxin-sensitivity (Table 2) show that the P2Y$_1$ receptor is coupling to a G$_i$ type of G protein, whereas in other cells such as the turkey erythrocyte (Boyer *et al*., 1989), where phospholipase C activation is the pathway, a G protein such as G$_q$ may be involved. Such a dichotomy of the pathways for a single receptor has been shown by Kenakin (1995a,b), citing several other cases in the G protein-coupled receptor superfamily, to be explicable by a detailed analysis of agonist efficacy and G protein trafficking selection. One of the variables noted by Kenakin to affect the trafficking is the ratio between the concentrations of the receptor and a G protein in the cell; this is pertinent in our case because the P2Y$_1$ receptor concentration is high at 22 pmol mg^{-1} protein. The findings reported or reviewed here have led us to surmise that the rat P2Y$_1$ receptor is capable of coupling in different cell types to either the adenylate cyclase inhibition of the phospholipase C activation pathways.

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