



Regulation of cyclic AMP by extracellular ATP in cultured brain capillary endothelial cells

¹Zubeya Anwar, ¹Jennifer L. Albert, ¹Sharon E. Gubby, ¹John P. Boyle, ¹Jonathon A. Roberts, ^{2,3}Tania E. Webb & ^{*,1}Michael R. Boarder

¹Department of Cell Physiology and Pharmacology, University of Leicester, Medical Sciences Building, P.O. Box 138, University Road, Leicester LE1 9HN and ²Molecular Neurobiology Unit, Department of Anatomy, Royal Free Hospital School of Medicine, Rowland Hill Street, Hampstead, London NW3 2PF

1 In primary unpassaged rat brain capillary endothelial cell cultures (RBECs), using reverse-transcriptase PCR with primers specific for P2Y receptor subtypes, we detected mRNA for P2Y₂, P2Y₄ and P2Y₆, but not P2Y₁ receptors.

2 None of the various nucleotides tested reduced forskolin elevated cyclic AMP levels in RBECs. ATP and ATP γ S, as well as adenosine, enhanced cyclic AMP accumulation in the presence of forskolin.

3 Comparison of the concentration response curves to ATP γ S with those for ATP and adenosine, at different incubation times, indicated that the response to purine nucleotides was not wholly dependent on conversion to adenosine. Adenosine deaminase abolished the response to adenosine but only reduced the response to ATP by about 50%. These results suggest the participation of a receptor responsive to nucleotides.

4 Isobutylmethylxanthine and 8-sulphophenyltheophylline prevented the cyclic AMP response, while neither 8-cyclopentyl-1,3-dipropylxanthine nor SCH58261 were effective antagonists. 2-chloroadenosine gave a robust response, but neither 2-chloro-N⁶-cyclopentyladenosine nor CGS 21680 were agonists.

5 These results show that adenosine and ATP can elevate the cyclic AMP levels of brain endothelial cells by acting on receptors which have a pharmacology apparently distinct from known P2Y and adenosine receptors.

Keywords: P2Y receptors; purinoceptors; brain endothelial cells; cyclic AMP

Abbreviations: ATP γ S, adenosine 5'-O-(3-thiotriphosphate); [Ca²⁺]_i, cytosolic Ca²⁺; CCPA, 2-chloro-N⁶-cyclopentyladenosine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; IBMX, isobutylmethylxanthine; 2MeSATP, 2-methylthio ATP; PDE, phosphodiesterase; PLC, phospholipase C; RBECs, rat brain capillary endothelial cell cultures; RT-PCR, reverse transcriptase-polymerase chain reaction; 8-SPT, 8-sulphophenyltheophylline

Introduction

Extracellular nucleotides such as ATP, ADP, UTP and UDP exert diverse effects on cellular function by acting on two subfamilies of P2 receptors, the intrinsic ion channel P2X receptors and the G protein-coupled P2Y receptors (Fredholm *et al.*, 1997). The mammalian P2Y receptor subfamily currently comprises five cloned members (P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁). One of the challenges of this emerging pharmacology is to relate the mechanism of action of individual members to the functional responses of cells through an understanding of the second messenger systems recruited. All of these five receptors have been described as coupled to phosphoinositide-specific phospholipase C (PLC) (Harden *et al.*, 1995; Boarder & Hourani, 1998), and much of their action at different cellular sites has been interpreted as downstream of PLC. The reports of cloned receptors have revealed little evidence for direct regulation of cyclic AMP, with the exception of the P2Y₁₁ receptor which has been shown to couple to stimulation of adenylyl cyclase as well as PLC (Communi *et al.*, 1997). However, there are various reports of agonists at P2Y receptors in native systems eliciting a change in cyclic AMP. Best established is inhibition of cyclic AMP synthesis by P2 receptors in platelets (Hourani & Hall, 1994), hepatocytes

(Tomura *et al.*, 1992), C6 glioma cells (Schachter *et al.*, 1996), Schwann cells (Berti-Mattera *et al.*, 1996) and a clonal brain endothelial cell (Webb *et al.*, 1996; Hechler *et al.*, 1998a,b). The molecular nature of the negative cyclase-coupled receptors is not securely defined. In addition, native systems provide several examples of stimulation of P2Y receptors which result in sensitization of cyclic AMP responses to either other agonists acting at G_s-coupled receptors or to forskolin. Such sensitization may be direct or it may be downstream of PLC and the activation of protein kinase C (Boarder *et al.*, 1988). Following a report of sensitization by extracellular ATP in microvascular endothelial cells (Allsup & Boarder, 1991) evidence was presented which was consistent with a direct sensitization of adenylyl cyclase by P2 receptors (Johnson *et al.*, 1991). This was followed by a reported synergistic action by ATP with forskolin in aortic endothelial cells (Cote *et al.*, 1993).

Regulation of [Ca²⁺]_i and cyclic AMP are central to the control of brain microvascular permeability (Rubin *et al.*, 1991; Abbott & Revest, 1991). Brain capillary endothelial cells form a regulated blood-brain-barrier under the influence of diffusible mediators from astrocytes. The nature and mechanisms of action of these mediators are unclear, but candidates include the nucleotides. In 1991 it was shown that ATP increases [Ca²⁺]_i in rat brain endothelial cells (Revest *et al.*, 1991). Subsequent studies showed that some combination of P2X, P2Y₂ and P2Y₁-like receptors could be found on the cultured

*Author for correspondence; E-mail: mb33@le.ac.uk

³Current address: Department of Cell Physiology and Pharmacology, University of Leicester, Leicester LE1 9HN, U.K.

cells (e.g. Nobles *et al.*, 1995). The P2Y₁-like receptor was unusual in that it lead to an increase in [Ca²⁺]_i by mobilization of intracellular stores in the apparent absence of a PLC response (Vigne *et al.*, 1994; Albert *et al.*, 1997). In a clonal cell line derived from RBECs (B10) it has also been reported (Webb *et al.*, 1996) that a P2Y receptor is coupled to the inhibition of adenylyl cyclase. We found no evidence of inhibition of adenylyl cyclase by P2Y receptors, but did report a substantial increase in accumulation of cyclic AMP in response to ATP, in the presence of a concentration of forskolin which produced only a small effect on its own (Albert *et al.*, 1997). This led to the studies in the present report investigating the possibility that P2Y receptors can sensitize the adenylyl cyclase response to forskolin in native RBEC cultures.

Methods

Cell culture

The rat brain capillary endothelial cells (RBECs) were prepared and cultured as described in Albert *et al.* (1997). Briefly, the cell culture was grown out from a purified preparation of rat brain cortical capillaries plated directly into 96-well plates or T25 flasks. The culture medium was MEM-D-Val with 20% foetal calf serum, penicillin (100 iu ml⁻¹), streptomycin (100 µg ml⁻¹), glutamine (27 mg ml⁻¹) and 75 µg ml⁻¹ endothelial cell growth supplement (Sigma E-2759). The preparation was an almost completely homogenous culture of microvascular endothelial cells exhibiting a diffuse immunoreactivity for factor VIII. Cells were used at confluence in 96-well plates for the cyclic AMP assay and mRNA was extracted from confluent T25 flasks for the RT-PCR.

Cyclic AMP assay

For the cyclic AMP assay 96-well multiwells were transferred to a 37°C water bath, growth medium aspirated, and replaced with 50 µl balanced salt solution (BSS, composition in (mM): NaCl 125, KCl 5.4, NaHCO₃ 16.2, HEPES 30, NaH₂PO₄ 1, MgSO₄ 0.8, CaCl₂ 1.8, glucose 5.5, pH 7.4). After 30 min a further 50 µl of BSS was added containing, where appropriate, a 2 fold final concentration of antagonists or inhibitors, followed 10 min later with 20 µl of 6 fold final concentration of agonists and forskolin. The incubation was stopped, after 5 min except where indicated otherwise, by addition of 40 µl of 2 M trichloroacetic acid. Cyclic AMP was measured in these trichloroacetic acid extracts after neutralization with ether or freon:octylamine extraction and addition of NaHCO₃, by use of the protein binding assay (Brown *et al.*, 1971).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The RT-PCR for P2Y₁, P2Y₂, P2Y₄ and P2Y₆ mRNA were carried out exactly as described earlier (Harper *et al.*, 1998). Each run included a positive control to which was added 5 ng of plasmid DNA containing the whole coding region of the appropriate P2Y receptor. To check for contamination with genomic DNA each run also included first strand synthesis in the absence of reverse transcriptase.

Statistical analysis

Statistical analyses were with Graph-Pad Prism2 (Graphpad Software Inc., San Diego, CA, U.S.A.).

Materials

Cell culture supplies were from GIBCO (Paisley, Scotland). [³H]-Cyclic AMP was from Amersham, Bucks, U.K. 2Me-SATP, CCPA and CGS 21680 were from Research Biochemicals (Semat, Herts, U.K.). SCH 58261 was a kind gift of Scherring and Ro-20 1724 was a kind gift of Roche, Welwyn, Bucks, U.K. Other chemicals were from Fischer Scientific (Loughborough, U.K.) or Sigma (Poole, Dorset, U.K.).

Results

RT-PCR of RBECs

To understand the potential for P2 nucleotide receptor regulation of cyclic AMP in RBECs by the four P2Y receptor subtypes for which sequences were available for the rat, RNA was extracted from 25 cm² flasks of unpassaged brain endothelial cells, cDNA synthesized and used as template for PCR employing primers designed to specifically amplify regions encoding for rat P2Y₁, P2Y₂, P2Y₄ and P2Y₆ receptors. In each case amplification of the brain endothelial cell cDNA template was in parallel with amplification of the cognate receptor DNA sequence in a plasmid containing the whole coding region of each receptor. This provided a positive control for the amplification procedure. Amplification products were detected for P2Y₂, P2Y₄ and P2Y₆ (Figure 1) upon RT-PCR from RNA preparations from three separate cell preparations. These products were cloned and sequenced to confirm their identity. Amplification of the P2Y₁ receptor mRNA was not detected.

Purinergic regulation of cyclic AMP in RBECs: effect of forskolin and phosphodiesterase inhibitors

In initial experiments studying cyclic AMP responses to purinergic agonists in RBECs cells were preincubated with IBMX (10 min) followed by 5 min stimulation with nucleotides in the presence of IBMX. Under these conditions the nucleotides had no effect on the levels of cyclic AMP, although forskolin in the presence of IBMX gave a very large increase in cyclic AMP levels (Table 1A). This forskolin and IBMX elevated cyclic AMP accumulation was not affected by 300 µM

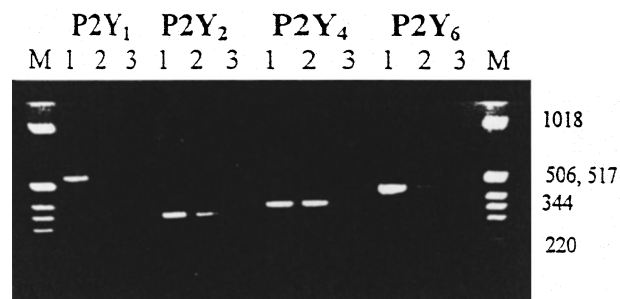


Figure 1 Agarose gel electrophoresis of RT-PCR amplification products from RBECs. Size markers (M) are 1 Kb ladder (Gibco BRL); approximate sizes are indicated. The receptors corresponding to the specific primers used in each amplification are indicated at the top. For each primer set lane 1 is a PCR from 5 ng of plasmid DNA containing the whole coding region of the appropriate P2Y receptor as a positive control, and lanes 2 and 3 used the RBEC cDNA as template in the presence (lane 2) and absence (lane 3) of reverse transcriptase respectively. The Figure is representative of three independent experiments on separate cell preparations.

ATP (not shown). The nucleotides had no effect in the absence of forskolin and IBMX (Table 1A and Figure 2).

To see whether the lack of effect of nucleotides on cyclic AMP was related to the presence of IBMX in some of the experiments described above, we sought conditions of phosphodiesterase (PDE) inhibition in the absence of a methylxanthine inhibitor. We tried the PDE 3 inhibitor siguazadon and the PDE 4 inhibitor Ro-20 1724. Siguazadon at 10 μM and Ro-20 1724 at 50 μM , either alone or together, did not lead to the substantial elevation of cyclic AMP levels by forskolin seen with IBMX (Table 1B). The combination of siguazadon and Ro-20 1724 therefore failed to provide suitable conditions for investigating regulation of cyclic AMP synthesis.

We then asked whether nucleotides could reduce forskolin elevated cyclic AMP levels in the absence of PDE inhibition. Figure 2A shows the effect of ATP on the response to increasing concentrations of forskolin. In the absence of PDE inhibition there was a modest increase in response to forskolin. Rather than attenuating this, the presence of ATP led to an unexpected and very substantial potentiation of the response to forskolin at levels of 5 μM and above. This stimulation of cyclic AMP levels by ATP did not occur at 0.1 μM forskolin, and was modest at 1 μM forskolin. Cells stimulated with 300 μM ATP in the presence of 5 μM forskolin for different periods of time showed a clear increase after 1 min, with the accumulation of cyclic AMP increasing in an approximately linear manner for up to 15 min (not shown). Subsequent experiments were with 5 min incubations except where indicated otherwise.

The effect of ATP, UTP, ATP γ S and 2MeSATP on cyclic AMP levels in the presence and absence of forskolin are shown in Figure 2B. Responses to 300 μM UTP or 300 μM 2MeSATP in the presence of 5 μM forskolin were small compared to those of 300 μM ATP or ATP γ S. All of these agonists elicit Ca²⁺ responses at these concentrations in these cells (Albert *et al.*, 1997). UDP also failed to give a substantial stimulation in the presence of 5 μM forskolin (data not shown).

Figure 3 shows the effect of increasing concentrations of ATP, UTP and α,β -methylene ATP. Typically the response to ATP shows signs of plateau between 100 and 300 μM (three out of four in this group of experiments). When the results from this series of experiments were pooled, and calculated on the assumption that the response at 300 μM was the maximum tissue response to ATP, then the apparent $-\log EC_{50}$ for ATP was 4.02 ± 0.66 ($n=4$). From Figure 3 it can also be seen that there was no substantial response to UTP or α,β -methylene ATP at any of the concentrations tested.

Adenosine gave similar responses to those of ATP and ATP γ S in the presence of 5 μM forskolin (Figure 4). Pooled

data of paired experiments showed that the responses to 5 min stimulations with 300 μM adenosine or ATP γ S were 138 ± 25 and $139 \pm 51\%$, respectively, of that of ATP (no significant difference by paired *t*-test). The apparent EC_{50} values for the three agonists were also very similar. This is shown in Figure 4 B,D,F. Apparent $-\log EC_{50}$ values pooled across experiments were 4.26 ± 0.71 ($n=4$) for ATP γ S, and 4.49 ± 0.24 ($n=4$) for adenosine.

Response to ATP is not exclusively dependent on conversion to adenosine

If the responses to ATP and ATP γ S were due solely to breakdown to adenosine we might expect that with shorter

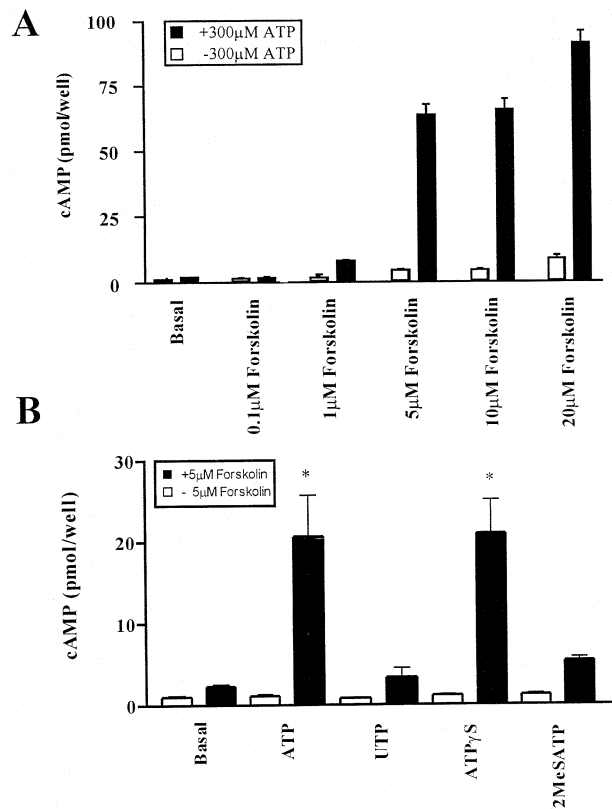


Figure 2 Cyclic AMP levels in RBECs in the presence of forskolin and nucleotide agonists. (A) The effect of increasing concentrations of forskolin in the absence and presence of 300 μM ATP with 5 min incubation times. (B) The effect of agonists at 300 μM in the presence and absence of forskolin at 5 μM with 5 min incubation times. *Significantly different from forskolin alone by ANOVA and Dunnett's post-test. Data are mean \pm s.e.mean, $n=4$, in each case.

Table 1 Cyclic AMP in RBECs: forskolin and PDE inhibitors

A. Effect of ATP and forskolin in the presence and absence of IBMX					
	Control		ATP	Forskolin	
Control	0.178 \pm 0.065		0.116 \pm 0.028	0.708 \pm 0.032	
IBMX	0.576 \pm 0.041		0.454 \pm 0.043	13.703 \pm 2.212	
B. Effect of siguazadon, Ro-20 1724 and IBMX					
	Control	Siguazadon	Ro-30 1724	Ro-20 1724 + Siguazadon	IBMX
Control	0.199 \pm 0.025	0.276 \pm 0.028	0.051 \pm 0.001	0.365 \pm 0.130	1.098 \pm 0.125
Forskolin	0.835 \pm 0.019	3.423 \pm 0.534	1.065 \pm 0.384	1.301 \pm 0.046	10.461 \pm 0.618

The PDE inhibitors IBMX (300 μM), siguazadon (10 μM) and Ro-20 1724 (50 μM) were present for 10 min prior to and during the incubation (5 min) with ATP (300 μM) or forskolin (5 μM). Data are pmols per well of cyclic AMP, mean \pm s.e.mean ($n=3$ or 4).

incubation times the response to ATP γ S would be smaller than that of ATP, and that the size of the responses to ATP and ATP γ S would be reduced compared to adenosine. Figure 4 shows a series of experiments in which the responses to the three agonists were compared with 1 min (left panels) and 5 min (right panels) incubation times. In each case the size of the response at 1 min was smaller than that at 5 min, but in each case the size of response at 1 min was the same for all three agonists. There was no change in EC₅₀ resulting from the difference in incubation times.

To further address the question of whether the response to ATP was mediated by conversion to adenosine we used adenosine deaminase to remove adenosine formed during the incubation. The response to 300 μ M adenosine was completely eliminated in the presence of 2 units ml⁻¹ of adenosine deaminase (data not shown). Over four experiments the response to 300 μ M ATP was reduced to 59.2 \pm 11.0% by adenosine deaminase. This is illustrated in Figure 5, which also shows the antagonism by 8-sulphophenyltheophylline (8-SPT) of the response to ATP and of the residual response to ATP in the presence of adenosine deaminase. Both show effectively complete antagonism by 300 μ M 8-SPT, with an IC₅₀ similar to that which we see with adenosine, of 10–30 μ M.

Effects of adenosine receptor agonists and antagonists

We undertook further studies with agonists and antagonists known to act at adenosine receptors, not only to characterize the response to adenosine but also to see if the pharmacology of the response to ATP was divergent from that of responses to adenosine. Figure 6 shows the effect of stimulation with 2-chloradenosine (agonist at A₁, A_{2A} and A_{2B} receptors), the A₁ selective agonist CCPA and the A_{2A} selective agonist CGS

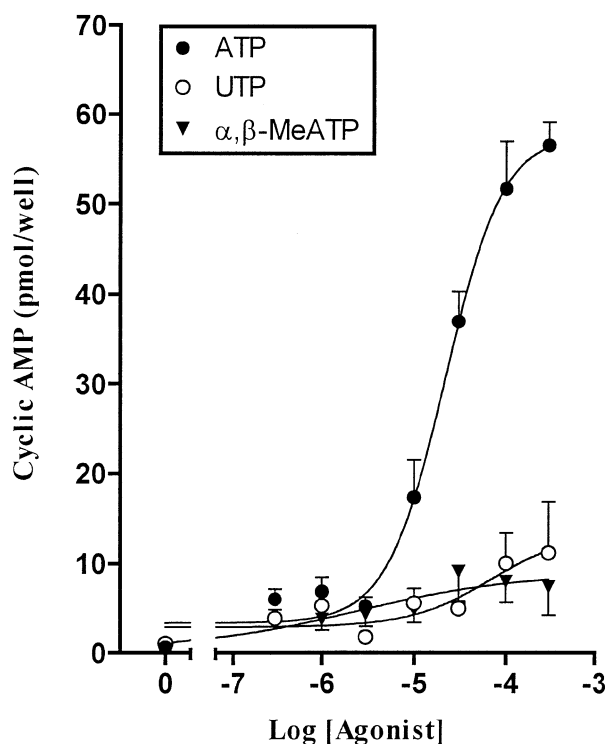
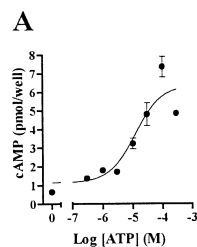


Figure 3 Cyclic AMP levels in RBECs in the presence of 5 μ M forskolin stimulated with increasing concentrations of ATP, UTP or α,β -methylene ATP. Data are mean \pm s.e. mean from a single experiment in quadruplicate representative of several similar experiments. Data pooled across experiments are presented in the text.

21680. While 2-chloradenosine gave a robust response, neither CCPA nor CGS 21680, in a range of concentrations at which they are effective agonists at A₁ and A_{2A} receptors, gave any response to all. The non-selective agonist NECA also elicited a

1 MINUTE STIMULATIONS



5 MINUTE STIMULATIONS

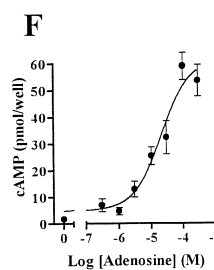
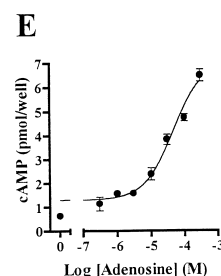
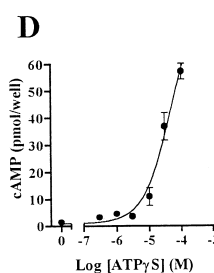
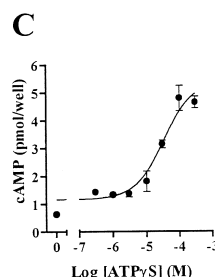
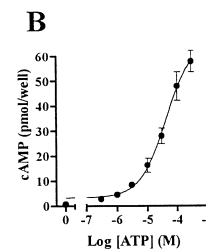


Figure 4 Accumulation of cyclic AMP in RBECs in response to 1 min or 5 min stimulation with agonists in the presence of 5 μ M forskolin. Data are mean \pm s.e. mean from a single experiment in quadruplicate. Data pooled across experiments are presented in the text.

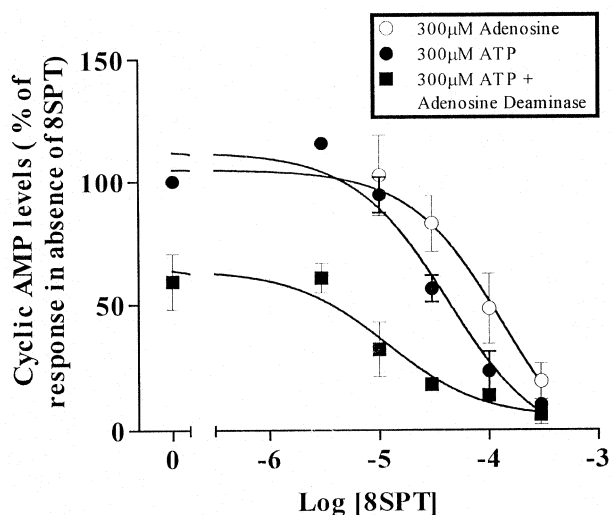


Figure 5 Effect of 8-SPT and adenosine deaminase on the stimulation of cyclic AMP by ATP (300 μ M) or adenosine (300 μ M) in the presence of 5 μ M forskolin. ATP adenosine and ATP plus 2 units ml⁻¹ adenosine deaminase. Data are mean \pm s.e. mean ($n=4$).

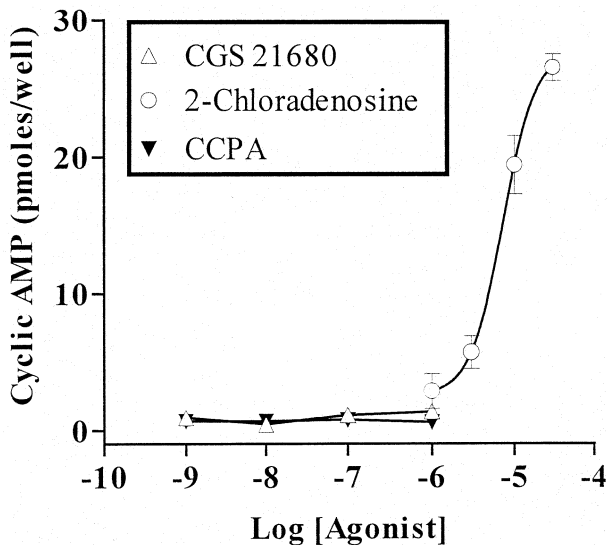


Figure 6 Effect of three adenosine receptor agonists on cyclic AMP accumulation in RBECs in the presence of $5 \mu\text{M}$ forskolin. CCPA, CGS 21680 and 2-chloradenosine. Data are mean \pm s.e. mean from a single experiment in quadruplicate (representative of several similar experiments).

response, of similar magnitude to that of ATP, which was maximal at $10 \mu\text{M}$ (not shown).

We investigated the effect of a series of antagonists present for 10 min before and during the incubation with adenosine or ATP (both at $300 \mu\text{M}$). The non-selective antagonists IBMX ($300 \mu\text{M}$) and 8-SPT (Figure 5) were effective at blocking the responses to both adenosine and ATP, as described above. However, the presence of the antagonist DPCPX and the A_{2A} selective antagonist SCH 58261, each at concentrations up to $1 \mu\text{M}$, had no effect on the responses to stimulation with $300 \mu\text{M}$ adenosine or ATP (not shown).

Effect of cyclo-oxygenase inhibition

To determine whether the stimulation of cyclic AMP levels was secondary to the formation of cyclo-oxygenase products such as prostaglandins we stimulated the cells in the presence of $1 \mu\text{M}$ indomethacin. Results from a representative experiment are shown in Table 2. ATP, $\text{ATP}\gamma\text{S}$ and adenosine gave substantial stimulations of cyclic AMP accumulation in the presence of $5 \mu\text{M}$ forskolin. In each case this was unaffected by the presence of indomethacin. Pooled over three separate experiments (each in quadruplicate) with different cell preparations the stimulation by ATP ($300 \mu\text{M}$) plus forskolin ($5 \mu\text{M}$) in the presence of $1 \mu\text{M}$ indomethacin was $96.5 \pm 8.4\%$ of the stimulation in the absence of indomethacin.

Discussion

In brain capillary endothelial cells regulation of cyclic AMP level by nucleotides is complicated by the existence of multiple P2 receptors, and by the use of different types of brain capillary cell cultures in different laboratories. Webb *et al.* (1996), using a clonal cell line (B10) of rat brain capillary endothelial cells, describe an inhibition of cyclic AMP on stimulation of a P2Y receptor. In the unpassaged brain capillary endothelial cultures (RBECs) we have previously reported that 2MeSATP does not reduce cyclic AMP levels which have been raised by forskolin (Albert *et al.*, 1997). This implies that the inhibition of cyclic AMP synthesis by P2Y₁ receptors seen in transfected 1321N1 cells (our unpublished data) does not occur in RBEC cultures. The RT-PCR work described here reinforces this view. There was no detectable expression of mRNA for P2Y₁ receptors, while sequences encoding P2Y₂, P2Y₄ and P2Y₆ receptors were easily detected. We conclude from this that P2Y₁ receptors do not regulate unpassaged cells, and in particular they do not regulate cyclic AMP in these cells, and that the $[\text{Ca}^{2+}]_i$ response to 2MeSATP and ADP seen in the absence of a detectable phosphoinositide response in these unpassaged cell cultures (Albert *et al.*, 1997) is not at a P2Y₁ receptor.

The presence of mRNA encoding P2Y₄ and P2Y₆ receptors indicate a potential for P2Y mediated, forskolin-dependent, stimulations of cyclic AMP. However, the elevations of cyclic AMP by ATP seen here do not have the characteristics consistent with the currently cloned P2Y receptors. Firstly, stimulation cannot be seen in the presence of IBMX. Secondly, 8-SPT is an effective antagonist. This excludes, not only the P2Y₁, P2Y₂, P2Y₄ and P2Y₆ receptors described above, but also the P2Y₁₁ receptor, which appears to be a candidate for an ATP receptor coupled to adenylyl cyclase, since 8-SPT is not an antagonist at P2Y₁₁ receptors (Communi *et al.*, 1997). The failure of either UTP or UDP to stimulate the cyclic AMP response seen with ATP is another indication that neither P2Y₂, P2Y₄ or P2Y₆ are involved. For this reason no further work with antagonists directed at P2 receptors was undertaken.

The observation that both ATP and adenosine are able to stimulate cyclic AMP accumulation raises the possibility that ATP is acting by conversion to adenosine and subsequent stimulation of G_s-coupled A₂ receptors. We report here various observations which make this unlikely. Both adenosine and $\text{ATP}\gamma\text{S}$ had the same potency as ATP. This was true for both 1 min and 5 min stimulations. If the rate of conversion of ATP to adenosine was sufficiently rapid as to be complete in less than 1 min of incubation, then ATP could have the same potency even if it had no action of its own. However, this cannot be true of the ectonucleotide resistant analogue $\text{ATP}\gamma\text{S}$, even taking into account that it is not completely resistant to breakdown and that it is not completely pure. Impurities (e.g. ATP/ADP) would have to account for the majority of the compound if this were the explanation. The comparisons of

Table 2 Effect of indomethacin on ATP, $\text{ATP}\gamma\text{S}$ and adenosine stimulation of cyclic AMP accumulation

	Control	Forskolin	+ ATP	+ $\text{ATP}\gamma\text{S}$	+ Adenosine
Control	0.97 ± 0.11	3.21 ± 0.20	21.85 ± 0.96	15.51 ± 2.41	25.61 ± 4.34
Indomethacin	—	—	22.91 ± 2.80	18.90 ± 2.90	18.34 ± 2.59

Indomethacin ($100 \mu\text{M}$) and was present for 10 min before and during the 5 min incubation with agonists (all at $300 \mu\text{M}$). Forskolin ($5 \mu\text{M}$) was present with each of the agonists. Data are mean \pm s.e. mean of cyclic AMP content (pmole per well), $n=3$ or 4. There were no significant differences between the presence and absence of indomethacin.

concentration response curve for these three agonists at 1 min and 5 min (Figure 4) are inconsistent with the hypothesis that stimulation by ATP γ S is dependent on breakdown to adenosine. The data indicate the presence of a receptor which responds to ATP γ S itself, and not just one which responds to adenosine.

The hypothesis that the action of ATP is dependent on conversion to adenosine was further explored with the adenosine deaminase experiments. Removal of adenosine in the bulk phase was complete, as indicated by the elimination of responses to added adenosine, but there was only a partial loss of the response to added ATP. Two hypotheses must be considered. Firstly, that there is a biophase at the cell surface where adenosine formed from ATP reaches a sufficient concentration locally to stimulate adenosine receptors before it is metabolized by adenosine deaminase. This is possible, even though we are working with cell monolayers. Secondly, that ATP is partially converted to adenosine, and that both ATP and adenosine act with forskolin to stimulate the accumulation of cyclic AMP. The conversion of ATP to adenosine then only results in a loss of response when the adenosine is removed by adenosine deaminase.

If both adenosine and ATP act at cell surface receptors to stimulate cyclic AMP then the pharmacology of the adenosine portion of the stimulation does not clearly indicate one of the described adenosine receptors (Alexander & Peters, 1998). A₁ receptors are described as G_{i/o} coupled to inhibition of adenylyl cyclase, and are also excluded because CCPA does not act as an agonist and DPCPX does not act as an antagonist. A₂ receptors are described as coupled to the stimulation of adenylyl cyclase *via* G_s. A_{2A} receptors are excluded because the selective agonist CGS 21680 does not elicit a response and the responses to ATP and adenosine are not antagonized by SCH58261. Neither is the response blocked by DPCPX. This is an A_{2B} antagonist with a pA₂ of around seven at cloned and transfected A_{2B} receptors (Alexander *et al.*, 1996), so under conditions used in these studies DPCPX would be expected to be an effective antagonist at A_{2B} receptors. However, these data do not completely exclude a contribution from A_{2B} receptors.

We conclude that the stimulation by ATP of cyclic AMP levels in RBECs is not at known P2Y receptors, and neither is it likely to be completely dependent on conversion to adenosine and action at known P1 receptors. The answer could lie in the form of separate receptors for adenosine and ATP, although the results here are inconsistent with a contribution from known P2 receptors. If two receptors are responsible, there is the potential for complex interactions between the receptors and their signalling pathways in the generation of the data reported here. An alternative explanation is that the response is at a common receptor for ATP and adenosine, as proposed for presynaptic regulation of noradrenaline release from peripheral nerve terminals (Shinozuka *et al.*, 1988; Kurz *et al.*, 1993; Smith *et al.*, 1997) and stimulation of adenylyl cyclase in NG108-15 cells (Matsuoka *et al.*, 1995).

References

- ABBOTT, N.J. & REVEST, P.A. (1991). Control of brain endothelial permeability. *Cerebrovasc. Brain. Metab. Rev.*, **31**, 1–34.
- ALBERT, J.L., BOYLE, J.P., ROBERTS, J.A., CHALLISS, R.A.J.C., GUBBY, S.E. & BOARDER, M.R. (1997). Regulation of brain capillary endothelial cells by P2Y receptors coupled to Ca²⁺, phospholipase C and mitogen activated protein kinase. *Br. J. Pharmacol.*, **122**, 935–941.
- ALEXANDER, S.P.H., COOPER, J., SHINE, J. & HILL, S. (1996). Characterisation of the human brain putative A_{2B} adenosine receptor expressed in Chinese hamster ovary (CHO.A_{2B4}) cells. *Br. J. Pharmacol.*, **119**, 1286–1290.
- ALEXANDER, S.P.H. & PETERS, J.A. (1998). 1998 Receptor & Ion Channel Nomenclature Supplement. *Trends Pharmacol. Sci.*, (Suppl): 1–98.
- Conigrave *et al.* (1998) have recently reported evidence for a novel cyclic AMP-linked P2 receptor which responds to ATP but not UTP. However, this response appears distinct from that described in the present paper in several respects. For example, adenosine is a weak agonist, and the response is enhanced by IBMX and insensitive to 8-SPT. A better fit with the present series of observations is the forskolin enhanced cyclic AMP response to ATP of bovine aortic endothelial cells (Cote *et al.*, 1993), where adenosine is an effective agonist, 2MeSATP and UTP are ineffective, and ATP γ S gives the same response as ATP. This implies that an atypical cyclic AMP elevating response to ATP may occur in diverse endothelial cells.
- With respect to the mechanisms by which cyclic AMP is elevated in RBECs on stimulation with ATP, the most likely explanation is augmentation of a G_s coupled response by forskolin similar to that reported earlier (Cote *et al.*, 1993, and references therein). An effect *via* elevated [Ca²⁺]_i is unlikely, since both UTP and 2MeSATP elevate [Ca²⁺]_i but do not effect cyclic AMP. A further possibility is that phospholipase A₂ is activated by these agonists, leading to the prostaglandin-dependent stimulation of cyclic AMP. Such a mechanism for nucleotide receptor regulation of cyclic AMP has been described by Post *et al.* (1996), who show that indomethacin can block this paracrine pathway for stimulation of adenylyl cyclase. The responses to ATP and adenosine in the brain capillary endothelial cells are seen in the presence of indomethacin, indicating that this is not the pathway for stimulation of cyclic AMP accumulation in these experiments.
- In this paper we have shown that the cyclic AMP levels of rat brain capillary endothelial cells, maintained in culture without passage, are regulated by extracellular adenosine and ATP. ATP probably acts in part by conversion to adenosine, but experiments with ATP γ S provide evidence that a receptor for nucleotides also contributes to these results. Both agonists act on receptors which have a pharmacology which is distinct from those previously cloned and characterized. The nature of the receptors responsible will be the subject of further investigations. *In situ* these cells form the blood-brain-barrier by a process maintained by adjacent astrocytes and involving the regulation of cyclic AMP levels. It has previously been shown that the 1321N1 astrocytoma cell line releases ATP in a regulated manner into the medium (Lazarowski *et al.*, 1995). The results presented here provide further information on the way in which nucleotides released by astrocytes could contribute to the regulation of the blood-brain-barrier. A further understanding of these events will require clarification of the receptors involved and study of how the cyclic AMP cascade interacts with other cell signalling pathways, such as the p42 and p44 mitogen activated protein kinases, which are also regulated by P2Y receptors in these endothelial cells (Albert *et al.*, 1997).

We thank The Wellcome Trust and The Medical Research Council for financial support.

- ALLSUP, D.J. & BOARDER, M.R. (1991). Comparison of P2 purinergic receptors of aortic endothelial cells with those of adrenal medulla: evidence for heterogeneity of receptor subtype and of inositol phosphate response. *Mol. Pharmacol.*, **38**, 84–91.
- BERTI-MATTERA, L.N., WILKINS, P.L., MADHUM, Z. & SUCHOVSKY, D. (1996). P2-purinergic receptors regulate phospholipase C and adenylyl cyclase activities in immortalised Schwann cells. *Biochem. J.*, **314**, 555–561.
- BOARDER, M.R. & HOURANI, S.M.O. (1998). The regulation of vascular function by P2 receptors: multiple sites and multiple receptors. *Trends Pharmacol. Sci.*, **19**, 99–107.
- BOARDER, M.R., PLEVIN, R. & MARRIOTT, D.B. (1988). Angiotensin II potentiates prostaglandin stimulation of cyclic AMP levels in intact bovine adrenal medulla cells but not adenylyl cyclase in permeabilised cells. *J. Biol. Chem.*, **263**, 11273–11284.
- BROWN, B.L., ALBANO, J.D., EKINS, R.P., SGHERZI, A.M. & TAMPION, J.W. (1971). A simple and sensitive saturation assay method for the measurement of adenosine 3',5'-cyclic monophosphate. *Biochem. J.*, **121**, 561–562.
- COMMUNI, D., GOVAERTS, C., PARMENTIER, M. & BOYNAEMS, J. (1997). Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J. Biol. Chem.*, **272**, 31969–31973.
- CONIGRAVE, A.D., LEE, J.Y., VAN DER WEYDEN, L., JIANG, L., WARD, P., TASEVSKI, V., LUTTRELL, B.M. & MORRIS, M.B. (1998). Pharmacological profile of a novel cyclic AMP-linked P2 receptor on undifferentiated HL-60 leukemia cells. *Br. J. Pharmacol.*, **124**, 1580–1585.
- COTE, S., VAN SANDE, J. & BOEYNAEMS, J.-M. (1993). Enhancement of endothelial cAMP accumulation by adenine nucleotides: role of methylxanthine-sensitive sites. *Am. J. Physiol.*, **264**, H1498–H1503.
- FREDHOLM, B.B., ABBRACCHIO, M.P., BURNSTOCK, G., DUBYAK, G.R., HARDEN, T.K., JACOBSEN, K.A., SCHWABE, U. & WILLIAMS, M. (1997). Towards a revised nomenclature of P1 and P2 receptors. *Trends Pharmacol. Sci.*, **18**, 79–82.
- HARDEN, T.K., BOYER, J.L. & NICHOLAS, R.A. (1995). P2-purinergic receptors; subtype associated signalling responses and structure. *Annu. Rev. Pharmacol. Toxicol.*, **35**, 541–579.
- HARPER, S., WEBB, T.E., CHARLTON, S.J., NG, L.L. & BOARDER, M.R. (1998). Evidence that P2Y₄ nucleotide receptors are involved in the regulation of rat aortic smooth muscle cells by UTP and ATP. *Br. J. Pharmacol.*, **124**, 703–710.
- HECHLER, B., LEON, C., VIAL, C., VIGNE, P., FRELIN, C., CAZENAVE, J.P. & GACHET, C. (1998a). The P2Y₁ receptor is necessary for adenosine 5'-diphosphate-induced platelet aggregation. *Blood*, **92**, 152–159.
- HECHLER, B., VIGNE, P., LEON, C., BREITTMAYER, J.-P., GACHET, C. & FRELIN, C. (1998b). ATP derivatives are antagonists of the P2Y₁ receptor: similarities to the platelet ADP receptor. *Mol. Pharmacol.*, **53**, 727–733.
- HOURANI, S.M.O. & HALL, D.A. (1994). Receptors for ADP on human blood platelets. *Trends Pharmacol. Sci.*, **15**, 103–108.
- JOHNSON, J.A., FRIEDMAN, J., HALLIGAN, R.D., BIRNMAUMER, M. & CLARK, R.B. (1991). Sensitization of adenylyl cyclase by P2 purinergic and M5 muscarinic receptor agonists in L cells. *Mol. Pharmacol.*, **39**, 539–546.
- KURZ, K., VON KUGELGEN, I. & STARKE, K. (1993). Prejunctional modulation of release in mouse and rat vas deferens: contribution of P1 and P2 receptors. *Br. J. Pharmacol.*, **110**, 1465–1472.
- LAZAROWSKI, E.R., WATT, W.C., STUTTS, M.J., BOUCHER, R.C. & HARDEN, T.K. (1995). Pharmacological selectivity of the cloned human P_{2U}-purinoceptor: potent activation by diadenosine tetraphosphate. *Br. J. Pharmacol.*, **116**, 1619–1627.
- MATSUOKA, I., ZHOU, Q., ISHIMOTO, H. & NAKINISHI, H. (1995). Extracellular ATP stimulates adenylyl cyclases and phospholipase C through distinct purinoceptors in NG108-15 cells. *Mol. Pharmacol.*, **47**, 855–862.
- NOBLES, M., REVEST, P.A., COURAUD, P.-O. & ABBOTT, N.J. (1995). Characterisation of nucleotide receptors that cause elevation of cytoplasmic calcium in immortalised rat brain endothelial cells (RBE4) and in primary cultures. *Br. J. Pharmacol.*, **115**, 1245–1252.
- POST, S.R., JACOBSEN, J.P. & INSEL, P.A. (1996). P2 purinergic agonists enhance cyclic AMP production in Madin-Darby canine kidney epithelial cells via an autocrine/paracrine mechanism. *J. Biol. Chem.*, **271**, 2029–2032.
- REVEST, P.A., ABBOTT, N.J. & GILLESPIE, J.I. (1991). Receptor mediated changes in intracellular Ca²⁺ in cultured rat brain endothelial cells. *Brain Res.*, **549**, 159–161.
- RUBIN, L.L., HALL, D.E., PORTER, S., BARBU, K., CANNON, C., HORNER, H.C., JANATPOUR, M., LIAW, C.W., MANNING, K., MORALES, J., TANNER, L.I., TOMASELLI, K.J. & BARD, F. (1991). A cell culture model of the blood-brain-barrier. *J. Cell Biol.*, **115**, 1725–1735.
- SCHACHTER, J.B., LI, Q., BOYER, J.L., NICHOLAS, R.A. & HARDEN, T.K. (1996). Second messenger cascade specificity and pharmacological selectivity of the human P2Y₁ purinoceptor. *Br. J. Pharmacol.*, **118**, 167–173.
- SHINOZUKA, K., BJUR, R.A. & WESTFALL, D.P. (1988). Characterisation of prejunctional purinoceptors on adrenergic nerves of the rat caudal artery. *N.S. Archives Pharmacol.*, **338**, 221–227.
- SMITH, A.D., CHEEK, D.J., BUXTON, I.L.O. & WESTFALL, D.P. (1997). Competition of adenine nucleotides for a 1,3-[³H]-dipropyl-8-cyclopentylxanthine binding site in rat vas deferens. *Clin. Exper. Pharm. Physiol.*, **B24B**, 492–497.
- TOMURA, H., OKAJIMA, F. & KONDO, Y. (1992). Discrimination between two types of P2 purinoceptors by suramin in rat hepatocytes. *Eur. J. Pharmacol.*, **226**, 363–365.
- VIGNE, P., FEOLDE, E., BREITTMAYER, J.P. & FRELIN, C. (1994). Characterisation of the effects of 2-methylthio-ATP and 2-chloro-ATP on brain capillary endothelial cells. Similarities to ADP and differences from ATP. *Br. J. Pharmacol.*, **112**, 775–780.
- WEBB, T.E., FEOLDE, E., VIGNE, P., NEARY, J.T., RUNBERG, A., FRELIN, C. & BARNARD, E.A. (1996). The P2Y purinoceptor in rat brain microvascular endothelial cells couple to inhibition of adenylyl cyclase. *Br. J. Pharmacol.*, **119**, 1385–1392.

(Received June 4, 1999

Revised June 16, 1999

Accepted June 22, 1999)