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Characteristics of nucleotide receptors that cause elevation of cytoplasmic calcium in immortalized rat brain endothelial cells (RBE4) and in primary cultures

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1 A dual-wavelength microfluorimetric method using Fura-2 as calcium indicator was applied to cells from an immortalized cell line of rat brain microvascular endothelial cells (RBE4), and to primary cultured rat brain endothelial cells.

2 In RBE4 cells, a brief (20 s) pulse of extracellular ATP (100 μM) induced a transient increase in the cytoplasmic calcium level ($[Ca^{2+}]_i$). Control responses to 100 μM ATP consisted of a ratio increase of 0.64 ± 0.03 (mean ± s.e., $n = 51$). Responses were seen at a concentration of 2.5 μM and were maximal at 100-1000 μM. When extracellular calcium was chelated with EGTA, the transient increase in $[Ca^{2+}]_i$ was not affected. The results are consistent with Ca^{2+} mobilization from intracellular stores.

3 The purinoceptor involved belongs to the P_2 subtype, since the agonist potency order among the adenine nucleotides was ATP>ADP>AMP. Moreover, the increase in $[Ca^{2+}]_i$ evoked by ATP was partially inhibited by the P_2 antagonist, suramin but was not affected by 8-phenyltheophylline, a P_1-purinoceptor antagonist. The strong desensitization observed with repeated applications of ATP is also typical of a P_2 receptor.

4 2-Methylthio-ATP (2meS-ATP 100 μM), a P_{2Y} agonist, elevated $[Ca^{2+}]_i$ in only 17% of the cells tested; however, 2meS-ATP was found to antagonize the effect of ATP in all cells tested. The increase in $[Ca^{2+}]_i$ evoked by ATP was inhibited by 500 s application of the P_{2Y} purinoceptor antagonist, Reactive Blue 2 at 10 μM, while 60 s application of 100 μM was ineffective.

5 The uracil nucleotide, UTP (100 μM) was as effective as ATP in increasing $[Ca^{2+}]_i$. The effects of ATP and UTP were not additive. Cells desensitized to the action of ATP (or UTP) were unable to respond to UTP (or ATP).

6 α,β Methylene-ATP (α,β meATP 100 μM), a P_{2x} agonist, elevated $[Ca^{2+}]_i$ in only 40% of the cells tested. In these cells it was less effective than ATP in increasing $[Ca^{2+}]_i$.

7 Cells desensitized to the action of ADP responded, to a smaller extent, to ATP. In contrast, cells desensitized to the action of ATP were unable to respond to ADP.

8 On primary cultures of brain endothelial cells the increase in $[Ca^{2+}]_i$ in response to extracellular ATP (100 μM) and UTP (100 μM) was of an equivalent amplitude, and similar to the response in RBE4 cells. The pattern of desensitization was also similar to that in RBE4 cells.

9 This comparative study indicates that in well-characterized brain microvascular endothelial cells that retain brain endothelial characteristics, the major class of nucleotide receptor is of the P_{2U} type. The implications for physiology are discussed.

Keywords: P_2 purinoceptors; P_{2U} receptor; brain endothelial cells; blood-brain barrier; RBE4 cells; calcium release; ATP; UTP; ATP analogues.

Introduction

The endothelial cells of the brain microvasculature form the blood-brain barrier. Receptors on brain endothelial cells are of interest in three main physiological contexts: control of blood-brain barrier permeability, secretion of substances to the luminal surface membrane, and control of smooth muscle tone. Our previous studies in primary cultures of rat brain microvascular endothelial cells showed that histamine, bradykinin and ATP caused an increase in cytosolic calcium (Revest *et al.*, 1991), suggesting a possible function in all three processes outlined above. The present study examines the pharmacology of the nucleotide receptors in brain endothelial cells in more detail, in order to gain insights into their physiological function.

In many cell types, extracellular adenosine triphosphate (ATP) has been shown to operate via purinoceptors located on the cell membrane. Purinoceptors are generally classified as P_1 and P_2 receptors (Burnstock, 1978): the ATP receptors are called P_2 purinoceptors, as distinct from P_1 purinoceptors which are selective for adenosine (Hoyle & Burnstock, 1991). P_1 receptors are blocked by methylxanthines (Kirkpatrick & Burnstock, 1992), while P_2 receptors are antagonized by suramin, a polyanionic detergent (Schilcker, 1989; Edwards & Gibb, 1993). Another characteristic of the P_2 purinoceptor is its desensitization by repeated applications of ATP (Burnstock, 1990).

Based on the potencies of structural analogues of ATP, P_2 purinoceptors are classified into two main subtypes, P_{2X} and P_{2Y} (Burnstock & Kennedy, 1985; Kennedy, 1990; O'Connor *et al.*, 1991). P_{2X} receptors have an agonist sensitivity order of α,β meATP>ATP>2meS-ATP. Activation of P_{2Y} purinoceptors leads to stimulation of inositol trisphosphate (InsP (1,4,5)) formation and elevation of $[Ca^{2+}]_i$ in a number of cell types (for references see O'Connor *et al.*, 1991). The order of potency of ATP analogues is 2meS-ATP>ATP>α,β meATP. Reactive Blue 2, an anthraquinone-sulphonic acid derivative

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that is a noncovalent purinoceptor antagonist, has been reported to display some selectivity for the P_{2Y} purinoceptor (Burnstock & Warland, 1987; Kennedy, 1990).

Four other classes of purinoceptors have been described: P_{2T}, P_{2Z}, P_{2S} and P_{2U} receptors. P_{2T} receptors have been described in platelets, and are selective for ADP and are linked to $[Ca^{2+}]_i$ mobilization (Hall & Hourani, 1993). P_{2Z} purinoceptors are responsive to UTP, ADP and ATP^{4-} and mediate permeabilization of mast cells and macrophages (Greenberg *et al.*, 1988). The existence of a P_{2S} receptor subtype was proposed by Wiklund & Gustafsson (1988) in ileal smooth muscle cells, with nucleotide potency in the order: 2meS-ATP < ATP = ADP. A further class of receptors where the pyrimidine uridine triphosphate (UTP) shows similar potency to ATP has been described recently (O'Connor *et al.*, 1991). This nucleotide receptor (ATP-UTP- or P_{2U}- receptor) is characterized by the following agonist potency order: UTP = ATP < ADP < α,β meATP < 2meS-ATP (O'Connor *et al.*, 1991; O'Connor, 1992). This class of receptor has been described in several cell types including neutrophils and monocytes (Cowen *et al.*, 1989), sheep pituitary cells (Davidson *et al.*, 1990), transformed human mandibular gland duct cells (Yu & Turner, 1991), and rat osteoblastic cells (Reimer & Dixon, 1992), and appears to correspond to the gene cloned from a neuroblastoma-glioma cell line (Lustig *et al.*, 1993; Barnard *et al.*, 1994).

In brain endothelial cells, the pharmacology of nucleotide receptors is unclear. ATP has been shown to induce an increase in cytoplasmic free calcium in primary cultures of rat brain microvascular endothelial cells, from resting levels of approximately 100 nM (Revest *et al.*, 1991). Frelin *et al.* (1993) and Vigne *et al.* (1994) reported studies on nucleotide receptors in a clonal 'capillary' endothelial cell line derived from rat brain, using dissociated cells and a fluorescence-activated cell sorter to identify distinct ATP-UTP and ADP receptors. However, as no detailed characterization of this cell line is available, it is not clear whether the cells are genuinely microvascular, and whether they retain a full brain endothelial phenotype. It is hence difficult to extrapolate from these results to the situation *in vivo*. Moreover, as the technique examined average responses of a population of cells at low time resolution (1.5–5 s), the behaviour of single cells could not be deduced. There is also some concern that trypsinisation to produce the cell suspension may have disturbed the normal receptor distribution or properties.

Recently, an immortalized cell line (RBE4) of rat brain microvascular endothelial cells has been introduced, which preserves many features of the *in situ* brain endothelium, and promises to be a useful model for *in vitro* studies of the blood-brain barrier (Durieu-Trautmann *et al.*, 1993a; Roux *et al.*, 1993; Abbott *et al.*, 1995). In this study we have used a series of ATP analogues and antagonists to investigate the pharmacological properties of the nucleotide receptors present on the RBE4 cell line. The chief findings on the RBE4 cells have been confirmed in experiments on primary cultured endothelial cells of rat brain. A preliminary account has been published (Nobles *et al.*, 1994).

Methods

Cell culture

RBE4 cells were provided by Dr P.O. Couraud. The RBE4 cell line was obtained by transfecting pure second passage primary cultures of rat brain microvascular endothelial cells with a plasmid pE1A-neo, which carries the entire E1A region of adenovirus 2 (conferring immortalization without oncogenic transformation), and the neo gene for resistance to the aminoglycoside C418, geneticin (Durieu-Trautmann *et al.*, 1993b; Roux *et al.*, 1994). RBE4 cells in culture exhibit a non-transformed phenotype showing contact inhibition, expression of endothelial differentiation markers (angiotensin-converting enzyme, factor VIII-related antigen), and non-tumourigenicity in athymic Nude mice. These cells also possess blood-brain barrier markers including expression of *p*-glycoprotein, they constitutively secrete endothelin and release nitric oxide when induced with gamma interferon, and they can be induced to express alkaline phosphatase and gamma glutamyl transpeptidase when exposed to astrocytic factors (Durieu-Trautmann *et al.*, 1993a; Roux *et al.*, 1993; 1994; Begley *et al.*, 1994; Abbott *et al.*, 1995).

Cells were stored at −70°C or in liquid nitrogen in 95% foetal calf serum (Sigma), 5% dimethylsulphoxide (DMSO) (Aldrich), at a concentration of $3-5\times10^6$ cells ml^{-1}. When needed, cells were thawed, then centrifuged at 1500 r.p.m. for 10 min. The pellet obtained was resuspended in culture medium containing alpha MEM/F10 (1:1) (Gibco), 10% foetal calf serum, 1 ng ml^{-1} basic fibroblast growth factor (Sigma), 300 $\mu g\ ml^{-1}$ geneticin (Sigma), and seeded at a concentration of 10^4 cells cm^{-2}. After thawing, cells were passaged at least once in plastic flasks before seeding onto glass coverslips, the first seeding being considered a recovery phase. Cells were studied between passage 30 and 60, and used during the two days following seeding (preconfluent cultures).

Primary cultures of rat brain microvascular endothelium were prepared by a modification of the method of Abbott *et al.* (1992). The culture medium was based on standard Dulbecco medium containing 20% donor horse serum, 75 $\mu g\ ml^{-1}$ endothelial cell growth supplement, 100 $\mu g\ ml^{-1}$ penicillin/streptomycin, 2 mM glutamine, 80 $\mu g\ ml^{-1}$ heparin grade I and 4% supplement containing vitamin C, glutathione, insulin, transferrin, and selenium. Cells were used between day 5 and 7 (preconfluent cultures).

For the experiments, cells were cultured on glass coverslips treated with rat tail collagen cross-linked with carbodiimide (Aldrich), as described elsewhere (Nobles & Abbott, 1994).

Intracellular calcium measurements

Cells were loaded with Fura-2 (Molecular Probes) by incubation with the membrane permeant AM-ester (10 μM in HEPES buffer, 2% BSA, pH 7.4) for 60–80 min at 37°C. Fura-2AM was added from a 2 mM stock in DMSO (Sigma). Coverslips were mounted on a Nikon Diaphot fluorescence microscope, adapted for dual-wavelength excitation and photon-counting in single cells (Newcastle Photometric Systems). Fluorescence of Fura-2 was recorded at 200 ms intervals with alternating excitation wavelengths of 350 and 380 nm, and measurement of emitted light at 520 nm. Cells were superfused with a HEPES buffer (composition, mM: NaCl 140, $MgCl_2$ 1, $CaCl_2$ 1.5, KCl 5, D-glucose 10, HEPES 10, pH 7.4, 28–30°C) at 6 ml min^{-1}. For calcium-free experiments, $CaCl_2$ was omitted and replaced by 1.5 mM EGTA.

In view of problems in calibrating intracellular calcium measurements (for review see Williams & Fay, 1990), changes in intracellular calcium concentration are given as the ratio of fluorescence intensity measured at the peak of the response to agonists. The basal fluorescence was always subtracted.

Drugs

ATP, ADP, AMP, ATPγS, UTP, α,β meATP, histamine, phenyltheophylline were from Sigma. 2meS-ATP and Reactive Blue 2 were from Research Biochemicals International. Suramin was a gift from Dr A.J. Gibb. Agonists were routinely presented as a 20 s superfusion pulse.

Statistics

The results are given as means ± s.e.. Statistical significance was evaluated by Student's *t* test. A value of $P<0.05$ was taken as significant.

Results

RBE4 cells

Extracellular ATP-induced elevation in $[Ca^{2+}]_i$ A brief (20 s) pulse of extracellular ATP (100 μM) induced a transient increase in fluorescence ratio in Fura-2-loaded RBE4 cells (Figure 1a). Addition of ATP (100 μM) induced a rapid increase in $[Ca^{2+}]_i$, beginning within 18–21 s and lasting for 71–77 s ($n=51$). The fluorescence ratio increased by 0.64 ± 0.03 ($n=51$, peak response) for a concentration of 100 μM ATP. Longer application of ATP (150 s), caused no change in the shape or the duration of the increase in $[Ca^{2+}]_i$ (Figure 1b).

When experiments were carried out in calcium-depleted medium, with a buffer in which 1.5 mM EGTA replaced 1.5 mM $CaCl_2$, the increase in fluorescence ratio due to 100 μM ATP was slightly lower than that observed in the presence of external calcium (0.53 ± 0.09, $n=8$). However, the change in $[Ca^{2+}]_i$ was not significantly different from that in calcium-containing control solution. The shape of the response in calcium-free medium was similar to that in the presence of extracellular calcium.

Concentration-dependence of the calcium response When ATP (1 μM to 1000 μM) was added to parallel samples of Fura-2-loaded RBE4 cells, the amplitude of the calcium response was found to be dependent on ATP concentration. Consistent changes in $[Ca^{2+}]_i$ were detected at an ATP concentration as low as 2.5 μM, and the response to ATP reached a maximal amplitude at 100–1000 μM (Figure 2). These data are consistent with a saturable interaction of ATP with RBE4 cells, similar to that reported for receptor-mediated responses to ATP in other systems (Burnstock & Warland, 1987; Davidson *et al.*, 1990; Reimer & Dixon, 1992).

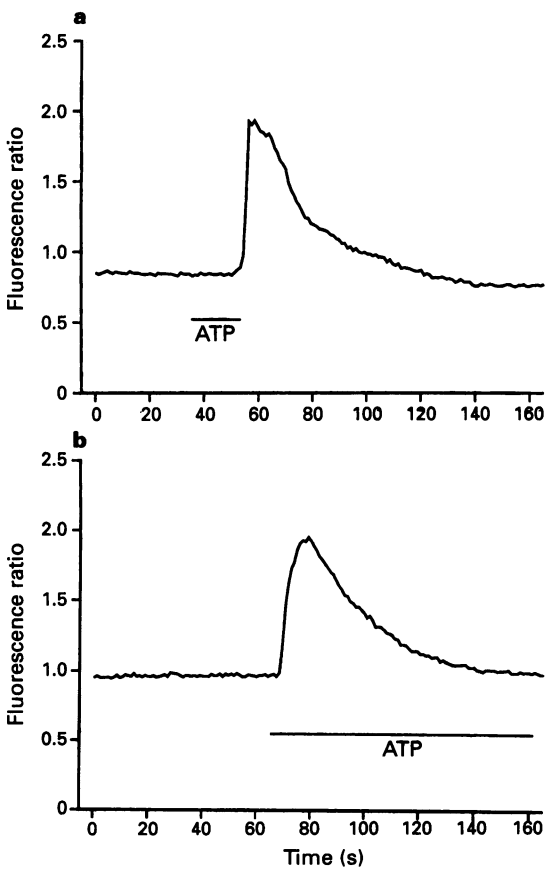

Figure 1 RBE4 cells: response to extracellular ATP. (a) Application of ATP (100 μM, 20 s) generates a transient increase in $[Ca^{2+}]_i$ in a single RBE4 cell. Intracellular calcium was monitored by microfluorimetry using the dual excitation calcium probe Fura-2. Amplitudes of the $[Ca^{2+}]_i$ responses are expressed as the increase in the fluorescence ratio after the application of ATP. (b) Transient increase in $[Ca^{2+}]_i$ due to a longer (150 s) application of extracellular ATP (100 μM).

ATP analogues We compared the potencies of several ATP structural analogues as agonists to define the P_2 purinoceptor subclass.

ADP (100 μM) induced an increase in fluorescence ratio of 0.37 ± 0.03 ($n=15$, Figure 5, $P<0.05$ compared to ATP). The response to ADP showed a concentration-dependency, reaching a plateau above 100 μM (Figure 2). The maximal increase in fluorescence ratio obtained with ADP was 58% of that with ATP.

AMP was a poor agonist on RBE4 cells, since a concentration of 100 μM led to an increase in fluorescence ratio of only 0.29 ± 0.09 ($n=7$). Moreover, not all cells responded to AMP, even for concentrations greater than 100 μM. On those cells which were not responsive to AMP, ATP always induced an increased in $[Ca^{2+}]_i$ (data not shown).

ATPγS, a poorly hydrolysable form of ATP, was slightly less effective than ATP in increasing $[Ca^{2+}]_i$. The increase in fluorescence ratio evoked by 100 μM ATPγS was 0.50 ± 0.07 ($n=7$). When tested at concentrations between 1 μM and 1000 μM, ATPγS induced increases in fluoresence ratio which were concentration dependent, with a plateau at 100–1000 μM.

α,β meATP (100 μM) caused an increase in $[Ca^{2+}]_i$ in only 40% of the cells tested. When it did so, it was less effective than ATP in raising calcium, producing an increase in fluorescence ratio of 0.43 ± 0.12 ($n=6$, $P<0.05$). Cells which did not respond to α,β meATP were tested with ATP. In all cases, ATP was able to induce an increase in $[Ca^{2+}]_i$.

Of 18 cells tested, 2meS-ATP (100 μM) elevated $[Ca^{2+}]_i$ in only three cells (17%). In these three cells the increase in fluorescence ratio was 0.48 ± 0.03. In experiments where ATP was first tested alone (increase in fluorescence ratio 0.70 ± 0.05, $n=7$), followed by a period where 100 μM 2meS-ATP was applied together with 100 μM ATP, no significant rise in $[Ca^{2+}]_i$ was obtained to the second application (change in fluorescence ratio 0.09 ± 0.06, $n=7$, $P<0.05$). 2meS-ATP appears to be a partial agonist and an antagonist of ATP on RBE4.

Desensitization of cells to ATP Sequential addition of ATP, with intervals between applications of 50 s to 300 s, led to a decrease in successive calcium signals (Figure 3a, typical trace).

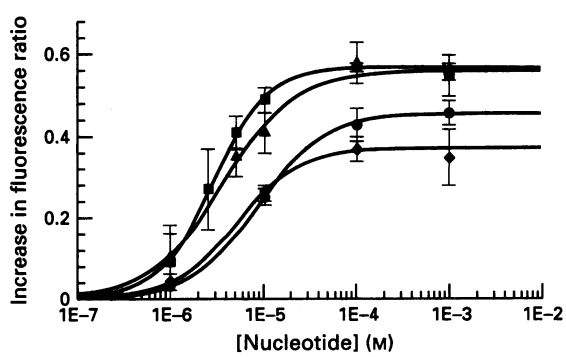

Figure 2 RBE4 cells: concentration-response curves over a concentration range of 1 μM to 1000 μM for ATP (■), UTP (▲), ATPγS (●) and ADP (◆). Curves were fitted with the Hill equation, with no parameters fixed. Points are means of at least 4 different experiments, ±s.e.. The p[A_{50}] values were for ATP (2.79 μM), UTP (3.57 μM), ATPγS (8.56 μM), and ADP (5.23 μM).

The change in fluorescence ratio in response to ATP was 0.59 ± 0.02 ($n=17$) during the first application. The second application caused a smaller increase in fluorescence ratio (0.30 ± 0.04, $n=17$, 51% of the initial response, $P<0.05$), while the third application caused a further reduction to 0.11 ± 0.03 ($n=17$, 19% of the initial response, $P<0.05$).

A typical trace showing desensitization in calcium-free medium is shown in Figure 3b. Averaging 8 experiments, the first ATP application gave 0.53 ± 0.09, the second application 0.26 ± 0.07 (49% of the initial response, $P<0.05$), and the third application 0.06 ± 0.03 (11% of the initial response, $P<0.05$). There was no significant difference in the degree of progressive desensitization in control and calcium-free conditions.

Receptor pharmacology It is well established that P_1 purinoceptors are inhibited by low doses of methylxanthines. We have tested the effect of phenyltheophylline on the response to ATP. The ATP-induced Ca^{2+} transient was first measured in control medium, then the cells were exposed to phenyltheophylline (10 μM) for 150–200 s before a second application of ATP (100 μM) in the continued presence of phenyltheophylline. Phenyltheophylline did not reduce the calcium signal evoked by ATP ($n=8$) (Figure 4a, typical trace).

The trypanocide compound suramin, described as a specific P_2 purinoceptor antagonist, was tested (Figure 4b). A first ATP-induced Ca^{2+} increase was measured in control solution, then cells were superfused with suramin for 100 s. ATP was tested again in the presence of suramin. Finally cells were superfused with the control buffer and ATP was tested again after 200 s. Suramin caused a 30% inhibition of the response to ATP, when used at a concentration of 20 μM (change in fluorescence ratio 0.14 ± 0.04, $n=8$). At a concentration of 300 μM, suramin caused 100% inhibition of the response induced by 100 μM ATP (no change in fluorescence ratio, $n=6$, $P<0.05$). The effect of suramin on the ATP response was reversible. The percentage inhibition induced by suramin was calculated relative to the decrease expected from desensitization. On the third application of ATP, the response was larger than in the presence of suramin, but still lower compared to the first application, as expected from the progressive desensitization shown in Figure 3.

Reactive Blue 2, an anthraquinone sulphonic acid derivative, which is a reported antagonist of the P_{2Y} receptor, was

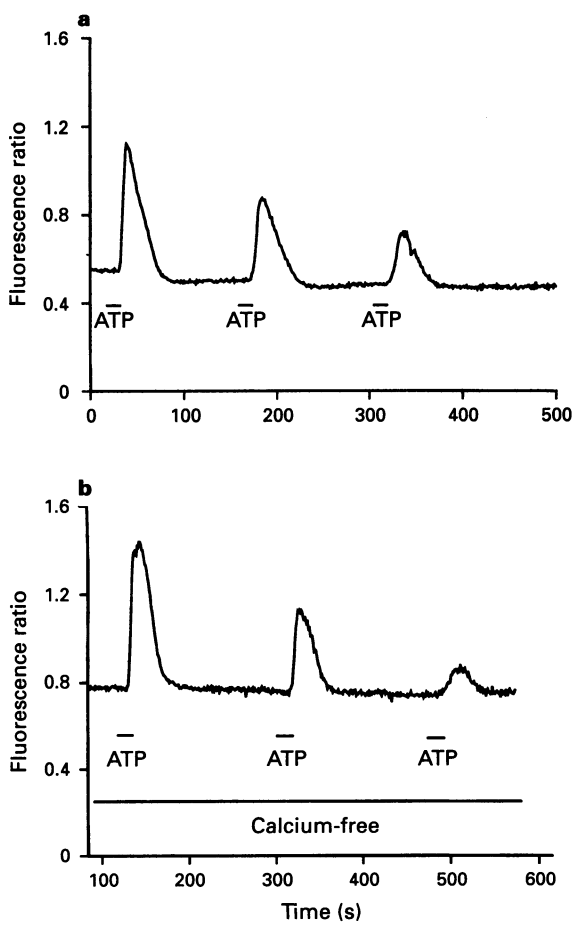

Figure 3 Desensitization in RBE4 cells: changes in $[Ca^{2+}]_i$ elicited by successive additions of extracellular ATP (100 μM, 20 s), (a) in the presence of 1.5 mM $CaCl_2$ in the bathing solution and (b): in the absence of added $CaCl_2$ and in the presence of 1.5 mM EGTA (calcium-free).

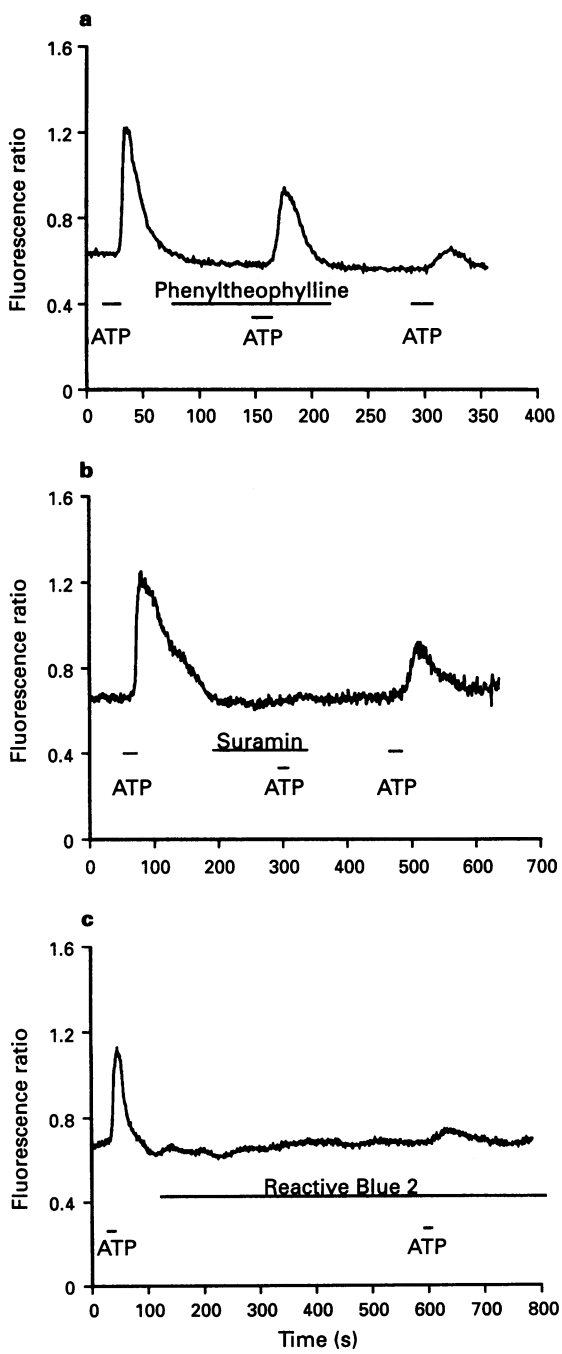

Figure 4 Pharmacology of the RBE4 cell ATP receptor: ATP (100 μM, 20 s) was first tested alone as a control, then ATP was tested again in the continued presence of (a) phenyltheophylline (10 μM, 150–200 s), (b) suramin (300 μM, 100 s), (c) Reactive Blue 2 (10 μM, 500 s). Each trace is representative of 6 to 8 experiments.

tested (Figure 4c). When cells were incubated for 60 s with 100 μM Reactive Blue 2, the response to ATP had the same amplitude as the second response to ATP in controls (change in fluorescence ratio 0.26 ± 0.04, $n=5$). When the duration of incubation was increased to 500 s, 10 μM Reactive Blue 2 induced a complete inhibition of the ATP effect on $[Ca^{2+}]_i$ (change in fluorescence ratio 0.03 ± 0.02, $n=6$, $P<0.05$). The effect of Reactive Blue 2 was not reversible within the time period tested (150 s).

The specificity of Reactive Blue 2 towards the purinoceptor was checked. For this purpose the action of this compound was tested on the cellular response to histamine. Cells were first challenged with histamine (100 μM, 20 s), and an increase in fluorescence ratio of 0.23 ± 0.03 ($n=4$) was obtained. After incubation of cells for 500 s with 10 μM Reactive Blue 2, no significant decrease of the response to histamine was observed (increase in fluorescence ratio 0.21 ± 0.06, $n=4$).

Pyrimidine nucleotide The pyrimidine nucleotide, UTP (100 μM) caused a transient rise in fluorescence ratio of 0.59 ± 0.04 (Figure 5, $n=14$), which was not significantly different from the effect of ATP at the same dose.

For a range of concentrations from 1 μM to 1000 μM, the response to UTP showed the same concentration-dependency as for ATP. The responses to UTP and ATP reached the same plateau levels above 100 μM (Figure 2).

When ATP was applied together with UTP (100 μM), an increase in fluorescence ratio of 0.63 ± 0.08 was obtained (Figure 5, $n=7$). This increase in $[Ca^{2+}]_i$ was not significantly different from that caused by ATP alone.

When UTP was applied three times, with a delay between successive applications of 150–200 s, a desensitization was observed (Figure 6a, typical trace). The first application gave an increase in fluorescence ratio of 0.59 ± 0.06 ($n=7$), the second application 0.30 ± 0.04 ($n=7$, 50% of the initial response, $P<0.05$), and the third application 0.08 ± 0.04 ($n=7$, 13% of the initial response, $P<0.05$). There was no significant difference in the degree of progressive desensitization with ATP or UTP.

When applied after the third UTP application, ATP was unable to induce a significant increase in $[Ca^{2+}]_i$ (Figure 6a, $n=7$). On cells desensitized to the action of ATP, UTP had no effect (data not shown).

ADP response When ADP was applied sequentially (Figure 6b, typical trace), decrease in the response was observed, as for ATP and UTP. The increase in fluorescence ratio was 0.57 ± 0.04 ($n=6$) during the first application. The second application caused an increase in fluorescence ratio of 0.46 ± 0.03 ($n=6$, 86% of the initial response, $P<0.05$), the third application 0.33 ± 0.03 ($n=6$, 57% of the initial response, $P<0.05$), and the fourth 0.18 ± 0.02 ($n=6$, 31% of the initial response, $P<0.05$). The range of desensitization obtained for ATP and ADP was statistically different ($n=6$, $P<0.05$).

When ADP was tested together with ATP (100 μM for each nucleotide), the increase in fluorescence ratio was 0.56 ± 0.06 (Figure 5, $n=7$), not significantly different from that to ATP alone (100 μM).

On cells desensitized to the action of ADP, ATP was still able to induce an increase in $[Ca^{2+}]_i$ (Figure 6b, typical trace). However, the response to ATP in these conditions was 0.38 ± 0.006 ($n=5$, 59% of the response to ATP in control conditions $P<0.05$). In cells desensitized to the action of ATP, ADP was unable to induce an increase in $[Ca^{2+}]_i$ ($n=6$)

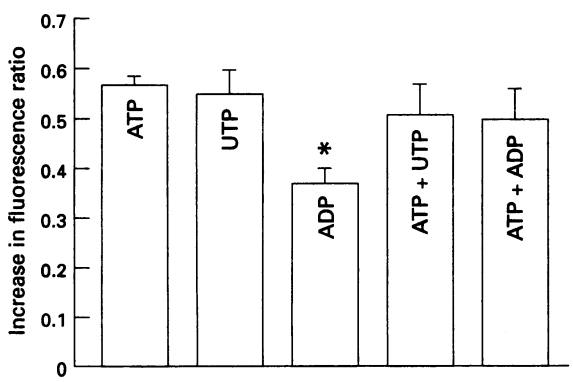

Figure 5 Non additivity of ATP/UTP and ATP/ADP actions on RBE4 cells. Responses to UTP (100 μM, 20 s, $n=14$), ADP (100 μM, 20 s, $n=15$) and to a mixture of ATP/UTP ($n=7$) or ATP/ADP ($n=7$) (100 μM for each nucleotide) are compared to the effect of ATP alone (100 μM, 20 s, $n=51$). Means ± s.e. are shown, $^*P<0.05$.

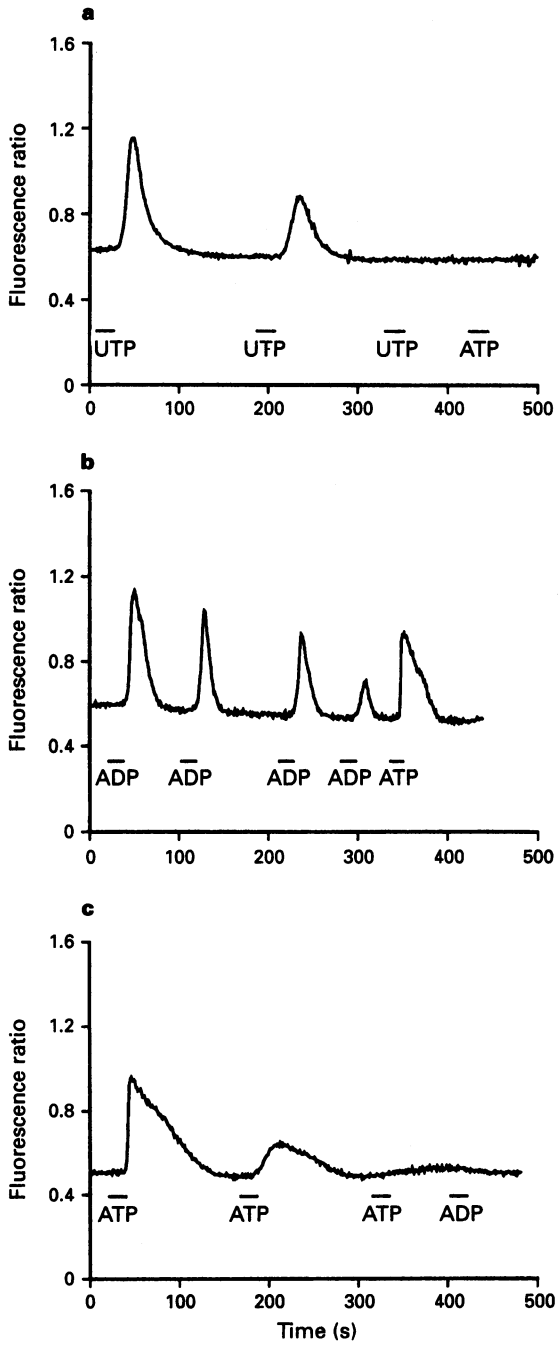

Figure 6 Cross-desensitization in RBE4 cells. Sequential additions of nucleotides (100 μM) were made where indicated for 20 s. Each trace is representative of from 6 to 7 cells.

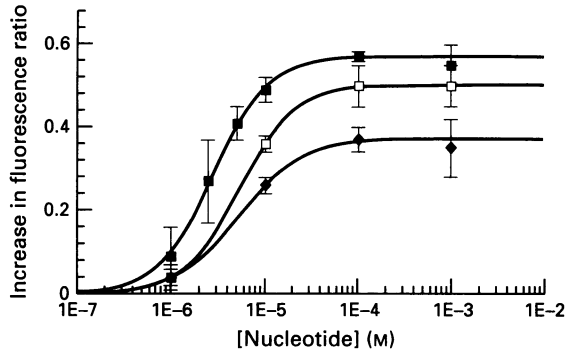

Figure 7 RBE4 cells: concentration-response curves over a concentration-range of 1 μM to 1000 μM for ATP (■), ADP (◆), and for a co-application of ATP and ADP (□, both at the concentration indicated on the abscissa scale). Curves were fitted with the Hill equation, with no parameters fixed. Points are means of at least 4 different experiments, ±s.e.. The p[A_{50}] values were for ATP (2.79 μM), ADP (5.23 μM), and ATP+ADP (5.33 μM).

(Figure 6c, typical trace). A similar result was obtained with UTP: ADP was unable to induce a significant increase in $[Ca^{2+}]_i$ in cells desensitized to the action of UTP (change in fluorescence ratio 0.026±0.004, $n=5$).

A full dose-response curve for the co-application of ADP and ATP was obtained (Figure 7). It remains below the ATP dose-response curve at all concentrations tested, which is consistent with competition between ATP and the less efficient ADP for a single receptor.

Primary cultures

In order to test the validity of our results on the ATP-receptor of RBE4 cells, we have conducted a parallel study with primary rat brain microvascular endothelial cell cultures.

We found that ATP at a concentration of 100 μM induced an increase in the fluorescence ratio of the brain endothelial cells of 0.62±0.05 ($n=10$, Figure 8), that lasted for 69±8 s. UTP (100 μM), gave an increase in fluorescence ratio of 0.55±0.11 ($n=4$, Figure 8), not significantly different from the response to ATP. Repeated applications of ATP or UTP led to desensitization of the receptor, while cells desensitized to the action of ATP (or UTP) no longer responded to UTP (or ATP) (data not shown).

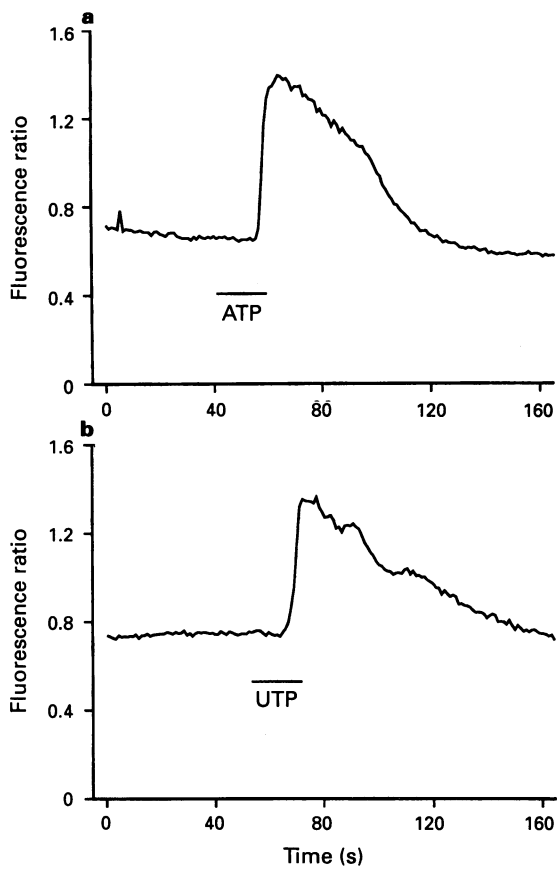

Figure 8 Primary cultures of brain microvascular endothelial cells. Action of extracellular ATP (100 μM, representative of $n=10$) and UTP (100 μM, representative of $n=4$).

Discussion

The purpose of this study was to investigate the pharmacology of the nucleotide receptors present on immortalized rat brain endothelial cells and to check the findings on cells in primary culture.

RBE4 cells

We found that the natural nucleotides ATP, ADP, AMP and UTP all elicited a transient elevation of $[Ca^{2+}]_i$ in RBE4 cells. The calcium transient due to ATP occurred as a single peak and lasted around 70 s. In contrast to observations made on endothelial cells from large peripheral blood vessels (Carter *et al.*, 1988) and on a clonal brain endothelial cell line (Frelin *et al.*, 1993; Vigne *et al.*, 1994), no plateau in the calcium signal was observed, even in the sustained presence of ATP. The persistence of the response and its similarity in shape in calcium-depleted medium argues in favour of an ATP-induced release of calcium from intracellular stores.

The agonist potency order AMP<ADP<ATP, together with the lack of inhibition by phenyltheophylline (a P_1 purinoceptor antagonist), the inhibitory effect of suramin (a P_2 purinoceptor antagonist), and the desensitization with repeated application of ATP, are all evidence for a P_2 type purinoceptor. The fact that the degree and pattern of desensitization were unchanged in calcium-free medium shows that the effects are exerted at the receptor and transduction level, and are not influenced by refilling of the stores.

Several observations suggest that the predominant nucleotide receptor on RBE4 cells is a P_{2U} receptor, activated by both ATP and UTP: ATP and UTP were equally effective in raising $[Ca^{2+}]_i$, the actions of ATP and UTP were not additive; cross desensitization was observed between ATP and UTP; and the desensitization obtained with ATP and with UTP showed the same amplitude.

The P_{2U} receptor that we have identified on RBE4 cells is similar to that described in neutrophils and monocytes (Cowen *et al.*, 1989) and on anterior pituitary cells (Davidson *et al.*, 1990). The same pharmacological characteristics were obtained: ATPγS was effective in elevating $[Ca^{2+}]_i$, but ADP was markedly less effective than ATP (or UTP). Moreover, the observation that the ATP analogue, α,β meATP was less effective than ATP in stimulating intracellular calcium release is consistent with a nucleotide receptor classification as described by O'Connor (1991).

In our hands, 2meS-ATP proved to be an antagonist of the rise in $[Ca^{2+}]_i$ induced by ATP: when co-applied with 2meS-ATP, ATP was markedly less effective in elevating $[Ca^{2+}]_i$ in all cells tested ($n=8$). In astrocytes, Pearce & Langley (1994) have reported a decrease in ATP-stimulated [^{3}H]-phosphoinositide accumulation in the presence of 2meS-ATP.

Reactive Blue 2 at low concentrations (<50 μM) has been reported to display some degree of selectivity towards the P_{2Y} purinoceptor (Kennedy, 1990). In RBE4 cells, this compound appears to be a good antagonist of the ATP response mediated via the ATP-UTP receptor. A similar observation has been

reported in a clonal cell line of brain endothelial cells (Frelin *et al.*, 1993). Complete inhibition required preincubation of the cells with Reactive Blue 2 for 500s. This effect appears specific for purinoceptors since under similar conditions, Reactive Blue 2 was unable to affect the cellular response to histamine.

A separate ADP receptor has been described together with a P_{2U} receptor in osteoblastic cells (Reimer & Dixon, 1992) and a clonal brain endothelial cell line (Frelin *et al.*, 1993). We were not able to obtain an additive response to ATP and ADP, nor a response to ADP on cells desensitized to the action of ATP or of UTP. However, the rate of desensitization obtained with ADP was less than that with ATP (or UTP), and cells desensitized to the action of ADP were still able to respond, to a smaller extent, to ATP, showing that the P_{2U} receptor is not fully desensitized by ADP. The lower desensitization observed with ADP could be linked to its lower efficacy.

Comparison of RBE4 cells and primary cultures

The more limited study of the pharmacology of the primary cultured rat brain endothelial cells reported here shows a close similarity between the behaviour of the primary and RBE4 cells. This adds to the evidence from cell biological and physiological characterization, that RBE4 cells retain most features of the primary cultured rat brain microvascular endothelium (Abbott *et al.*, 1995). Since many of the features are also present *in vivo*, these results confirm that for physiological and pharmacological studies, the RBE4 cells and primary cultured endothelial cells are good models of the brain endothelium *in vivo*.

Comparison with studies on a clonal cell line by Frelin et al. (1993)

There are some interesting differences in the response to nucleotides. A plateau phase in the calcium response was seen in the clonal cells, sensitive to extracellular calcium, but not in the primary cultures or RBE4 cells. The plateau has been attributed to calcium entry from the external medium. As a plateau phase is characteristic of large vessel endothelial cells (Carter *et al.*, 1988), it is possible that the clonal cell line is derived from large cerebral vessels (arteries or veins) rather than capillaries, or represents a de-differentiated state with similar properties.

In RBE4 cells, the similarity of the decline in the calcium transients to repeated nucleotide application in normal and calcium-free medium, showing that the response to ATP is independent of calcium entry during the period of the experiment, confirms the absence of significant calcium entry during each nucleotide-mediated calcium transient.

The present conclusions concerning the presence of an ATP-UTP receptor in brain endothelial cells are broadly similar with those of Frelin *et al.* (1993). However they reported an additional ADP receptor (see also Vigne *et al.*, 1994), not seen in the present study.

Physiological significance of nucleotide receptors

The physiological relevance of the nucleotide receptors on brain microvascular endothelial cells is of interest. The following discussion incorporates observations reviewed by Burnstock & Kennedy (1985), Seifert & Schultz (1989), Ralevic & Burnstock (1991), O'Connor (1992), Burnstock (1993), Illes & Nöremberg (1993) and Toda *et al.* (1993).

Brain endothelial cells *in situ* will be exposed to nucleotides from both the blood and the brain side. From the blood side, ATP, ADP and UTP are released from aggregating platelets, acting on the luminal endothelial membrane. From the brain side, ATP can be released from neurones, including non-adrenergic, non-cholinergic endings on cerebral vessels, and will be broken down to ADP and adenosine. ATP can be released from the endothelium itself in response to shear stress, and from vascular smooth muscle especially in inflammation, raising the possibility of positive feedback loops involving ATP release and ATP action on the two cell types. UTP is present in all cells including those of the central nervous system and may be released by cell damage and hypoxia. However, the recent evidence for specific UTP receptors on neurones may indicate a more physiological role in neurotransmission or modulation. Thus, ATP, ADP and UTP may be released into the vicinity of the brain endothelial cells in both normal and pathological situations.

In response to ATP and UTP, endothelial cells release nitric oxide and prostacyclin, both able to cause smooth muscle cell relaxation, with prostacyclin having an additional anti-platelet aggregation activity. The anti-platelet activity is likely to be important thoughout the microvascular bed, whereas the smooth muscle action will be relevant in the arteriolar segment of the cerebral circulation. ATP is one of several inflammatory mediators capable of increasing the permeability of the blood-brain barrier (Abbott & Revest, 1991); experiments on mesenteric microvascular endothelium show that tight junction opening following ATP-mediated elevation of cytosolic calcium and endothelial contraction is a possible mechanism (He *et al.*, 1990; Curry, 1992). The shorter duration of the calcium transient (no plateau) in brain capillary endothelial cells compared with that reported for other endothelial cells (Carter *et al.*, 1988) may be related to the relative resistance to opening of the blood-brain barrier by inflammatory mediators.

The ability of endothelial cells to release ATP, and the presence on astrocytes of P_2 receptors (Magoski & Walz, 1992; Kastritsis *et al.*, 1992), leads to the idea that brain endothelial cells may communicate with perivascular glial cells via ATP. By an analogous argument, both ATP and nitric oxide may be used for communication between endothelial cells and neurones. Thus ATP may play a role in several communicating and feedback loops involving the endothelium, smooth muscle, neurones and astrocytes. It is likely that as more is known about the physiological functions of UTP, equivalent mechanisms involving UTP will emerge. Detailed understanding of the effects of endothelial activation by ATP and UTP will depend on knowledge of the local concentrations of these agents achieved in physiological conditions, and of the effects of the second messenger and calcium mobilization systems involved.

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References

ABBOTT, N.J., COURAUD, P.-O., ROUX, F. & BEGLEY, D.J. (1995). Studies on an immortalized brain endothelial cell line: characterization, permeability and transport. In *New Concepts of the Blood-Brain Barrier*. ed. Greenwood, J., Begley, D.J., Segal, M.B. & Lightman, S. New York: Plenum Press, (in press).

ABBOTT, N.J., HUGHES, C.C.W., REVEST, P.A. & GREENWOOD, J. (1992). Development and characterisation of a rat endothelial culture: towards an *in vitro* blood-brain barrier. *J. Cell. Sci.*, **103**, 23–38.

ABBOTT, N.J. & REVEST, P.A. (1991). Control of brain endothelial permeability. *Cerebrovasc. Brain Metabol. Rev.*, **3**, 1–34.

BARNARD, E.A., BURNSTOCK, G. & WEBB, T.E. (1994). G protein-coupled receptors for ATP and other nucleotides: a new receptor family. *Trends Pharmacol. Sci.*, **15**, 67–70.

BEGLEY, D.J., CHEN, Z.D., ROLLINSON, C. & ABBOTT, N.J. (1994). Activity of p-glycoprotein (multidrug resistance protein) in cultured immortalized rat brain microvascular endothelial cells (RBE4). *J. Physiol.*, **480**, 9P.

BURNSTOCK, G. (1978). A basis for distinguishing two types of purinergic receptors. In *Cell Membrane Receptors for Drugs and Hormones: a Multidisciplinary Approach.* ed. Straub, R.W. & Bolis, L. pp. 107–118. New York: Raven Press.

BURNSTOCK, G. (1990). Overview. Purinergic mechanisms. *Ann. N.Y. Acad. Sci.*, **603**, 1–17.

BURNSTOCK, G. (1993). Hypoxia, endothelium and purines. *Drug Devel. Res.*, **28**, 301–305.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2 purinoceptors? *Gen. Pharmacol.*, **16**, 433–440.

BURNSTOCK, G. & WARLAND, J.J. (1987). P_2-purinoceptors of two subtypes in the rabbit mesenteric artery: Reactive Blue 2 selectively inhibits responses mediated via the P_{2Y} but not the P_{2X} purinoceptor. *Br. J. Pharmacol.*, **90**, 383–391.

CARTER, T.D., HALLAM, T.J., CUSACK, N.J. & PEARSON, J.D. (1988). Regulation of P_{2Y}- purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. *Br. J. Pharmacol.*, **95**, 1181–1190.

COWEN, D.S., LAZARUS, H.M., SHURIN, S.B., STOLL, S.E. & DUBYAK, G.R. (1989). Extracellular adenosine triphosphate activates calcium mobilization in human phagocytic leucocytes and neutrophil/monocyte progenitor cells. *J. Clin. Invest.*, **83**, 1651–1660.

CURRY, F.E. (1992). Modulation of venular microvessel permeability by calcium influx into endothelial cells. *F.A.S.E.B. J.*, **6**, 2456–2466.

DAVIDSON, J.S., WAKEFIELD, I.K., SHNIUS, U., VAN DER MERVE, P.A. & MILLAR, R.P. (1990). A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. *Endocrinology*, **126**, 80–87.

DURIEU-TRAUTMANN, O., BOURDOULOUS, S., ROUX, F., BOURRE, J.M., STROSBERG, A.D & COURAUD, P.O. (1993a). Immortalized rat brain microvessel endothelial cells: II. Pharmacological characterization. *Adv. Exp. Med. Biol.*, **331**, 205–210.

DURIEU-TRAUTMANN, O., FEDERICI, C., CREMINION, C., FOIGNANT-CHAVEROT, N., ROUX, F., STROSBERG, A.D. & COURAUD, P.-O. (1993b). Nitric oxide and endothelin secretion by brain microvessel endothelial cells: regulation by cyclic nucleotides. *J. Cell. Physiol.*, **155**, 104–111.

EDWARDS, F.A. & GIBB, A.J. (1993). ATP - a fast neurotransmitter. *F.E.B.S. Lett.*, **325**, 86–89.

FRELIN, C., BREITTMAYER, J.P. & VIGNE, P. (1993). ADP induces inositol phosphate-dependent intracellular Ca^{2+} mobilization in brain capillary endothelial cells. *J. Biol. Chem.*, **268**, 8787–8792.

GREENBERG, S., DI VIRGILIO, F., STEINBERG, T.H. & SILVERSTEIN, S.C. (1988). Extracellular nucleotides mediate Ca^{2+} fluxes in J774 macrophages by two distinct mechanisms. *J. Biol. Chem.*, **263**, 10337–10343.

HALL, D.A. & HOURANI, S.M.O. (1993). Effects of analogues of adenine nucleotides on increases in intracellular calcium mediated by P_{2T}-purinoceptors on human blood platelets. *Br. J. Pharmacol.*, **108**, 728–733.

HE, P., PAGAKIS, S.N. & CURRY, F.E. (1990). Measurement of cytoplasmatic calcium in single microvessels with increased permeability. *Am. J. Physiol.*, **258**, H1366–1374.

HOYLE, C.H.V. & BURNSTOCK, G. (1991). ATP receptors and their physiological roles. In *Adenosine in the Nervous System.* ed. Stone, T.W. pp. 43–76. London: Academic Press.

ILLES, P. & NÖRENBERG, W. (1993). Neuronal ATP receptors and their mechanism of action. *Trends Pharmacol. Sci.*, **14**, 50–54.

KASTRITSIS, C.H.C., SALM, A.K. & MCCARTHY, K. (1992). Stimulation of the P_{2Y} purinergic receptor on type 1 astroglia in inositol phosphate formation and calcium mobilization. *J. Neurochem.*, **58**, 1277–1284.

KENNEDY, C. (1990). P_1- and P_2- purinoceptor subtypes - an update. *Arch. Int. Pharmacodyn. Ther.*, **303**, 30–50.

KIRKPATRICK, K.A. & BURNSTOCK, G. (1992). Evidence that the inhibition of ATP release from sympathetic nerves by adenosine is a physiological mechanism. *Gen. Pharmacol.*, **23**, 1045–1050.

LUSTIG, K.D., SHIAU, A.K., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 5113–5117.

MAGOSKI, N.S. & WALZ, W. (1992). Ionic dependence of a P_2-purinoceptor mediated depolarization of cultured astrocytes. *J. Neurosci. Res.*, **32**, 530–538.

NOBLES, M. & ABBOTT, N.J. (1994). Development of methods for growing brain endothelial cells on glass. *J. Physiol.*, **480**, 4P.

NOBLES, M., REVEST, P.A., COURAUD, P.O. & ABBOTT, N.J. (1994). Evidence for nucleotide receptors in immortalized rat brain capillary endothelial cells. *J. Physiol.*, **475**, 64P.

O'CONNOR, S.E. (1992). Recent developments in the classification and functional significance of receptors for ATP and UTP, evidence for nucleotide receptors. *Life Sci.*, **50**, 1657–1664.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

PEARCE, B. & LANGLEY, D. (1994). Purine- and pyrimidine-stimulated phosphoinositide breakdown and intracellular calcium mobilisation in astrocytes. *Brain Res.*, **660**, 329–332.

RALEVIC, V. & BURNSTOCK, G. (1991). Effects of purines and pyrimidines on the rat mesenteric arterial bed. *Circ. Res.*, **69**, 1583–1590.

REIMER, W.J. & DIXON, S.J. (1992). Extracellular nucleotides elevate $[Ca^{2+}]_i$ in rat osteoblastic cells by interaction with two receptor subtypes. *Am. J. Physiol.*, **263**, C1040–C1048.

REVEST, P.A., ABBOTT, N.J. & GILLESPIE, J.I. (1991). Receptor-mediated changes in intracellular $[Ca^{2+}]$ in cultured rat brain endothelial cells. *Brain Res.*, **549**, 159–161.

ROUX, F., DURIEU-TRAUTMANN, O., BOURRE, J.M., STROSBERG, A.D. & COURAUD, P.O. (1993). Immortalized rat brain microvessel endothelial cells: I. Expression of blood-brain barrier markers during angiogenesis. In *Frontiers in Cerebral Vascular Biology: Transport and its Regulation.* ed. Drewes, L.R. & Betz, A.L. pp. 201–204. New York: Plenum Press.

ROUX, F., DURIEU-TRAUTMANN, O., CHAVEROT, N., CLAIRE, M., MAILLY, P., BOURRE, J.M., STROSBERG, A.D. & COURAUD, P.O. (1994). Regulation of gamma-glutamyl transpeptidase and alkaline phosphatase activities in immortalized rat brain microvessel endothelial cells. *J. Cell. Physiol.*, **159**, 101–113.

SCHILCKER, E., URBANEK, E. & GOTHBERT, M. (1989). ATP, alpha, beta methylene ATP and suramin as tools for characterization of vascular P_{2X} receptors in the pithed rat. *J. Auton. Pharmacol.*, **9**, 357–366.

SEIFERT, R. & SCHULTZ, G. (1989). Involvement of pyrimidinoceptors in the regulation of cell functions by uridine and by uracil nucleotides. *Trends Pharmacol. Sci.*, **10**, 365–369.

TODA, N., AYAJIKA, K. & OKAMURA, T. (1993). Cerebroarterial relaxations mediated by nitric oxide derived from endothelium and vasodilator nerve. *J. Vasc. Res.*, **30**, 61–67.

VIGNE, P., FEOLDE, E., BREITTMAYER, J.P. & FRELIN, C. (1994). Characterization 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.

WIKLUND, N.P. & GUSTAFFSON, L.E. (1988). Indications for P_2-purinoceptor subtypes in guinea pig smooth muscle. *Eur. J. Pharmacol.*, **148**, 361–370.

WILLIAMS, D.A. & FAY, F.S. (1990). Intracellular calibration of the fluorescent calcium indicator FURA-2. *Cell Calcium*, **11**, 75–83.

YU, N. & TURNER, J.T. (1991). Functional studies in human submandibular duct cell line, HGS-PA, suggest a second salivary gland receptor subtype for nucleotides. *J. Pharmacol. Exp. Ther.*, **259**, 1344–1350.

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