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# The pharmacology of nucleotide receptors on primary rat brain endothelial cells grown on a biological extracellular matrix: effects on intracellular calcium concentration

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**1** Brain capillary endothelial cells express a variety of nucleotide receptors, but differences have been reported between culture models. This study reports examination of nucleotide receptors on primary cultured rat brain capillary endothelial cells (RBCEC) grown on a biological extracellular matrix (ECM) to produce a more differentiated phenotype.

**2** Fura-2 fluorescence ratio imaging was used to monitor intracellular free calcium concentration $[Ca^{2+}]_i$. ATP, UTP, and 2-methylthioATP (2-MeSATP) increased $[Ca^{2+}]_i$ to similar levels, while 2-MeSADP, ADP and adenosine gave smaller responses.

**3** Removal of extracellular calcium caused no significant change in the $[Ca^{2+}]_i$ response to 2-MeSATP, evidence that the response was mediated by a metabotropic (P2Y) receptor.

**4** All cells tested responded to ATP, UTP, 2-MeSATP and ADP, while 63% responded to adenosine and 50% to 2-MeSADP. No cells responded to $\alpha,\beta$-methyleneATP. Cells grown on rat tail collagen instead of ECM gave smaller and less uniform $[Ca^{2+}]_i$ responses, suggesting that the differentiating effect of the ECM contributed to a more uniform receptor profile.

**5** The $[Ca^{2+}]_i$ response to the $P2Y_1$-selective agonist 2-MeSADP was abolished in the presence of the subtype-selective antagonist adenosine 3′-phosphate 5′-phosphosulphate (PAPS).

**6** The $P2Y_2$ antagonist suramin completely blocked the response to ATP and inhibited the response to UTP by 66%.

**7** The $A_1$ subtype-selective adenosine receptor agonist $N^6$-Cyclopentyladenosine (CPA) gave a small but characteristic $[Ca^{2+}]_i$ response, while $A_{2A}$ and $A_{2B}$ subtype-selective agonists failed to generate $[Ca^{2+}]_i$ changes.

**8** The results are consistent with the presence on RBCEC of a $P2Y_2$-like receptor coupled to phospholipase C, and a $P2Y_1$-like receptor mobilizing intracellular $Ca^{2+}$. The role of multiple nucleotide receptors in the function of the brain endothelium is discussed.

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**Keywords:** Blood-brain barrier; endothelium; ATP; purinergic; nucleotide; extracellular matrix

**Abbreviations:** $\alpha,\beta$-MeATP, $\alpha,\beta$-methyleneATP; CGS-21680, 2-p-(2-carboxyethyl) phenethylamino-5′-N-ethylcarboxamido adenosine; CPA, $N^6$-cyclopentyladenosine; 2-MeSADP, 2-methylthio ADP; 2-MeSATP, 2-methylthio ATP; NECA, N ethylcarboxamidoadenosine; PAPS, adenosine 3′-phosphate 5′-phosphosulphate

## Introduction

The blood-brain barrier is formed by the endothelial cells of cerebral microvessels. The barrier function derives from the extremely tight *zonulae occludentes* (tight junctions) joining adjacent cells, effectively sealing the paracellular pathway, combined with a number of specific transporter and enzymatic mechanisms that regulate the passage of molecules across the cells (Abbott & Romero, 1996; Tamai & Tsuji, 1996; Grant *et al*., 1998). Receptors on the brain endothelial cells are involved in the regulation of several aspects of the physiological function of the blood-brain barrier, including release of agents across the luminal and abluminal membranes to control blood clotting and smooth muscle tone respectively (Boarder & Hourani, 1998), regulating the permeability of the tight junctions (Abbott & Revest, 1991; Abbott, 2000), and controlling specific transcellular solute transport (O'Donnell *et al*., 1995). Differences have been reported between the pharmacology of brain and non-brain endothelium (reviewed in Boarder & Hourani, 1998), consistent with the special functions of the blood-brain barrier.

Studies of the receptors and signal transduction pathways of the brain endothelium *in situ* are complicated by the presence of other cell types (e.g. neurons, smooth muscle, astrocytes, mast cells), making it difficult to establish the site and mechanisms of action of applied chemical agents (Abbott, 2000). Several *in vitro* models of brain endothelium have been used including non-passaged primary culture, passaged cells and immortalized cell lines, but variability in the findings suggests that differences in preparative and culture methods exert a significant influence on the receptor phenotype observed. It is thus difficult on the basis of studies on a single preparation to draw conclusions about the situation *in vivo*.

Purine and pyrimidine receptors are of particular interest in relation to the function of the blood-brain barrier, having been shown not only to regulate the release of prostacyclin ($PGI_2$) and nitric oxide from the brain endothelium (Boarder

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& Hourani, 1998), but also to control blood-brain barrier permeability (Olesen, 1989). Natural ligands for nucleotide receptors, including ATP, ADP and UTP, can be released from a number of cell types in the region of the vessel wall, such as platelets on the blood side, and smooth muscle cells and neurons on the brain side. Moreover, the endothelium itself can release ATP (Gordon, 1986) able to act on nucleotide receptors on astrocytes and neurons (reviewed in Nobles *et al*., 1995), so the endothelium is able to act as both target and source of nucleotide signals.

Early studies on unpassaged (P0) primary cultured rat brain endothelial cells showed an elevation in cytoplasmic calcium concentration ($[Ca^{2+}]_i$) on application of purinergic agents, with potency in the order ATP > ADP > AMP, and desensitization to repeated application of ATP, characteristic of P2 receptors (Revest *et al*., 1991). Subsequent studies on these cells showed that UTP and ATP were equally effective, suggesting presence of a P2U subtype (Nobles *et al*., 1995). More detailed characterization of the P2U receptor on the immortalized rat brain endothelial cell line RBE4 generated by transfection of second passage (P2) primary cultured rat brain endothelium showed no evidence for a separate receptor sensitive to ADP, but responses to the agonists $\alpha,\beta$-methyleneATP ($\alpha,\beta$-MeATP) and 2-methylthioATP (2-MeSATP) in a proportion of cells suggested presence of other nucleotide receptors (Nobles *et al*., 1995).

A series of studies on the B7 clone of passaged rat brain endothelial cells introduced by Vigne *et al*. (1989) demonstrated two types of metabotropic nucleotide receptors, one (P2U) sensitive to ATP and UTP coupled to phospholipase C (PLC), and an ADP- and 2-MeSATP-sensitive $P2Y_1$-like receptor increasing mobilization of a thapsigargin-sensitive calcium pool, but independent of inositol phosphates (Frelin *et al*., 1993; Vigne *et al*., 1994a). These studies were pursued in the B10 clone lacking the $P2U/P2Y_2$ receptor, where ATP was shown to act as a weak antagonist on the $P2Y_1$-like receptor (Feolde *et al*., 1995; Vigne *et al*., 1998b).

Boarder and co-workers reported studies on primary cultured (P0 to P10) human brain endothelial cells (Purkiss *et al*., 1994) in which ATP and UTP gave a small stimulation of PLC but 2-MeSATP, ATP$\gamma$S and $\beta,\gamma$-methylene-ATP had no effect. Their subsequent studies on primary cultured rat brain endothelial cells (Albert *et al*., 1997) showed evidence for a $P2Y_2$-like receptor coupled to PLC, $Ca^{2+}$ and mitogen-activated protein kinase (MAPK) pathways, a $P2Y_1$-like receptor linked to $Ca^{2+}$ mobilization but independent of inositol phosphates, and a further response to ATP by an unidentified receptor producing a rise in cyclic AMP in the presence of forskolin. They observed some differences in the percentage of cells responding to ATP, ADP, UTP and 2-MeSATP but it was not clear whether this reflected the *in vivo* condition.

There have been significant recent advances in understanding nucleotide receptors, with the cloning of several members of the P2X (ionotropic) family of ligand-gated channels, and of the P2Y (metabotropic) family of G-protein coupled receptors; however, it has proved difficult to identify the cloned receptor(s) with particular pharmacological phenotypes naturally expressed in tissues (King *et al*., 1998; Vigne *et al*., 2000). Thus for the brain endothelium, further pharmacological studies of nucleotide receptors are needed to investigate the phenotype regulating blood-brain barrier physiology.

We have recently found that primary cultured rat brain endothelial cells grown on a biological extracellular matrix (ECM) produced by corneal endothelial cells grow more rapidly and uniformly to confluence, and show a more uniform spindle-shaped cell morphology characteristic of differentiated brain endothelial cells (Dömötör *et al*., 1998). The cells are sufficiently homogeneous in physiological properties for resolution of the role of the plasmalemmal $Na^+-Ca^{2+}$ exchange transporter in regulating basal $[Ca^{2+}]_i$, and in returning $[Ca^{2+}]_i$ to the basal level after elevation in response to a brief extracellular pulse of ATP (Dömötör *et al*., 1999). This preparation has proved ideal for a more detailed pharmacological characterization and separation of nucleotide receptors on brain endothelial cells.

The results allow clear pharmacological identification and separation of $P2Y_2$-like and $P2Y_1$-like receptors, and in addition reveal a small calcium response to adenosine. The cells grown on the biological ECM showed a greater uniformity of pharmacological profile and larger calcium responses than cells grown on rat tail collagen. This study suggests a role for extracellular matrix constituents in regulating the differentiated pharmacological phenotype of brain endothelial cells, and indicates the presence of multiple types of nucleotide receptor, with distinct functions, on the *in vivo* blood-brain barrier.

## Methods

### *Cell culture*

Procedures for obtaining cell cultures were in accordance with Guidelines for Use of Laboratory Animals of the Semmelweis University, Budapest. Three- to four-month-old Wistar rats (~300 g) were decapitated. Primary cultures of rat brain microvascular endothelium prepared by the method of Abbott *et al*. (1992) were grown on glass coverslips coated with bovine corneal endothelial extracellular matrix; for assessment of the effect of ECM, cells were grown for comparison on rat tail collagen (see below). Cells were characterized immunocytochemically as endothelial by detection of factor VIII-related antigen. The culture medium was based on a standard Dulbecco's medium composed of Dulbecco's modified Eagle's medium, 20% plasma-derived adult bovine serum (Sigma), 75 $\mu$g ml$^{-1}$ endothelial cell growth supplement, 100 i.u. ml$^{-1}$ penicillin / 100 $\mu$g ml$^{-1}$ streptomycin, 2 mM glutamine, 80 $\mu$g ml$^{-1}$ heparin grade I and supplement containing vitamin C, glutathione, insulin, transferrin and selenium. Chemicals were purchased from Sigma Aldrich, Hungary. Cells were used after culturing for 5–9 days, when they were close to confluence.

### *Preparation of biological extracellular matrix*

This procedure was a modification of the method described by Gospodarowicz (1984), as detailed previously (Dömötör *et al*., 1998). Briefly, fresh bovine eyes kept on ice were obtained from an abattoir; primary cultures of corneal endothelium were prepared, passaged 2–3 times and finally cultured on untreated glass coverslips. After reaching confluence, cells were lysed to expose the extracellular matrix by treating the preparations with 20 mM $NH_4OH$ in distilled water. Matrix-coated coverslips were kept under sterile conditions in isotonic salt solution for up to 3 months at 4°C.

### *Preparation of rat tail collagen coated coverslips*

Following sterilization with 70% ethanol and flaming, the glass coverslips were coated with 0.4 mg/ml rat tail collagen

as previously described (Abbott *et al*., 1992). They were fixed in wet ammonia vapour for 10 min, then washed three times in Hanks' Balanced Salt Solution (HBSS) before the addition of cells.

### *Measurement of $[Ca^{2+}]_i$*

To monitor changes in $[Ca^{2+}]_i$, cells on coverslips were incubated for 1 h at 37°C with 6 $\mu$M fura-2 AM in Dulbecco's modified Eagle's medium. The coverslips were transferred to a perfusion chamber fitted to the stage of an inverted Nikon Diaphot 200 microscope and superfused with normal HEPES buffer at 37°C for 10 min prior to experiments. All drugs and chemicals at 37°C were introduced close to the cells of interest *via* a glass multitube system (tip diameter 0.5 mm; perfusion rate constant at 10 $\mu$l $s^{-1}$). $[Ca^{2+}]_i$ was measured by digital image fluorescence microscopy (objective, Fluor 40/1.3; Nikon). Excitation wavelengths were 340 and 380 nm, generated by a polychromator illumination system with a resolution of 12 nm (Visitron Sv. GmbH, Pucheim, Germany). Fluorescence emission was monitored at 510 nm. Digital imaging and data analysis were carried out using a $512 \times 512$ frame transfer CCD camera (Princeton Instruments) and Metafluor software (Universal Imaging Corp., West Chester, PA, U.S.A). A fluorescence ratio image (340/380 nm) was acquired every 2 s. In each experimental condition fluorescence was monitored in cells from two to three coverslips (20–30 cells in frame). Fluorescence ratios were converted to free $[Ca^{2+}]_i$ using the equation described by Grynkiewicz *et al*. (1985). The maximum ($R_{max}$) and minimum ($R_{min}$) ratio value and the ratio of fluorescence for $Ca^{2+}$-bound/$Ca^{2+}$-free dye measured at 380 nm ($S_{f2}/S_{b2}$) were determined using an *in vitro* calibration method and were corrected for viscosity (Poenie, 1994). Experimental results are expressed as $[Ca^{2+}]_i$ or fluorescence ratio as indicated.

### *Solutions and drugs*

Normal HEPES buffer contained (mM): NaCl, 135; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1.2; glucose, 10; and HEPES, 20; pH adjusted to 7.4. For calcium-free experiments $CaCl_2$ was omitted and replaced by 1.5 mM EGTA. All chemicals were obtained from Sigma Aldrich, Hungary.

### *Data analysis and statistics*

Data analysis was performed with Sigmaplot 4.0 Software. Results are given as means $\pm$ s.e.mean. Statistical significance was evaluated by Student's *t*-test and differences with $P<0.05$ were taken as significant.

## Results

### *Extracellular ATP-induced $[Ca^{2+}]_i$ elevation*

The present study used brief (8 s) pulses of ATP which we have previously shown caused a $[Ca^{2+}]_i$ transient resulting from mobilization of $Ca^{2+}$ from intracellular stores, with no contribution from influx of extracellular $Ca^{2+}$ (Dömötör *et al*., 1999). The first pulse of 100 $\mu$M ATP increased $[Ca^{2+}]_i$ from the basal level of $166.5 \pm 3.6$ nM ($n=30$) to a peak of $791.2 \pm 27.7$ nM ($n=30$). Two further applications of ATP at 70 s intervals gave progressively smaller $Ca^{2+}$ transients, respectively 68% and 53% of the initial response ($n=30$), showing the desensitization characteristic of nucleotide receptors (Barnard *et al*., 1996) as previously observed in primary cultured and immortalized rat brain endothelial cells (Revest *et al*., 1991; Nobles *et al*., 1995).

### *The effect of nucleotide agonists on $[Ca^{2+}]_i$*

Agents used for this investigation included nucleotide analogues chosen to distinguish between P2Y receptors (agonist potency in natural tissues 2-MeSATP > ATP$\gamma$S > ATP $\geqslant$ ADP > > $\alpha,\beta$-MeATP, UTP) and P2U receptors (UTP = ATP $\geqslant$ ATP$\gamma$S > > ADP, 2-MeSATP, $\alpha,\beta$-MeATP), and other agents that have proved valuable in identifying cloned P2Y-like proteins: $P2Y_1$ (2-MeSATP > ADP) and the two main candidates for the P2U phenotypic receptor, $P2Y_2$ (ATP = UTP, suramin-sensitive) and $P2Y_4$ (UTP $\geqslant$ ATP, suramin insensitive) (King *et al*., 1998). Some agents need to be used with caution: thus $\alpha,\beta$-MeATP has been used as a stereotypic P2X receptor agonist, but it acts on only some P2X subfamilies (Fredholm *et al*., 1997), and is also a potent agonist of a metabotropic receptor in rat astrocytes (Abbracchio *et al*., 1999). 2-MeSATP is an agonist at rat $P2Y_1$ receptors (Filippov *et al*., 2000), but it also acts at some P2X receptor subtypes (Fredholm *et al*., 1997).

To characterize the nucleotide receptor subtypes present on the surface of primary RBCE cells we challenged the cells with 8 s pulses of ATP and a number of other agonists and measured $[Ca^{2+}]_i$ transients. All drugs were applied at 100 $\mu$M concentration and the results are shown as changes in the fluorescence ratio ($\Delta$FR) in Figure 1a. The $[Ca^{2+}]_i$ transient in response to UTP was slightly larger than that produced by ATP ($\Delta$FR $0.82 \pm 0.017$ and $0.74 \pm 0.018$ respectively, $n=140$, $P<0.05$). The response to 2-MeSATP was not significantly different from that to ATP ($\Delta$FR $0.69 \pm 0.04$, $n=90$) but the response to ADP ($\Delta$FR $0.39 \pm 0.04$, $n=60$) was significantly smaller ($P<0.05$). The potent $P2Y_1$ receptor agonist 2-MeSADP gave a smaller response than ATP, but not significantly different from the response to ADP ($\Delta FR = 0.36 \pm 0.02$, $n=50$). Since ADP does not act on $P2Y_2$ receptors (Brown *et al*., 1995), the ADP-induced $[Ca^{2+}]_i$ elevation in our system is further evidence for activation of a $P2Y_1$-like receptor. $\alpha,\beta$-MeATP did not cause $[Ca^{2+}]_i$ elevation in the RBCE cells (data not shown), while 100 $\mu$M adenosine gave a small but characteristic $[Ca^{2+}]_i$ response ($\Delta$FR $0.19 \pm 0.05$, $n=50$) which was significantly smaller than the ATP-induced $[Ca^{2+}]_i$ elevation ($P<0.05$).

All cells tested responded to ATP, UTP, 2-MeSATP and ADP, while adenosine and 2-MeSADP caused an increase in $[Ca^{2+}]_i$ in 63% and 50% of the cells, respectively. To confirm that the 2-MeSATP-induced $[Ca^{2+}]_i$ response was due to metabotropic and not ionotropic receptor activation, we repeated the experiment in calcium-free medium (Figure 1b). The absence of external calcium did not affect the 2-MeSATP-evoked $[Ca^{2+}]_i$ transient; in calcium-containing medium the increase in $[Ca^{2+}]_i$ was $454 \pm 27.2$ nM ($n=37$), while in calcium-free medium it was $442 \pm 27.56$ nM ($n=36$). These results indicate a relatively uniform receptor profile within the population of RBCE cells grown on the biological matrix, and suggest that under these conditions these primary endothelial cells express at least three different nucleotide receptor subtypes: P2U-like, $P2Y_1$-like, and an adenosine receptor.

Figure 2 shows the peak $[Ca^{2+}]_i$ signals as a function of the concentration of agonists; ATP, UTP and 2-MeSATP were added in the concentration range 1-1000 $\mu$M. The maximal $[Ca^{2+}]_i$ responses were reached at 100 $\mu$M agonist concentra-

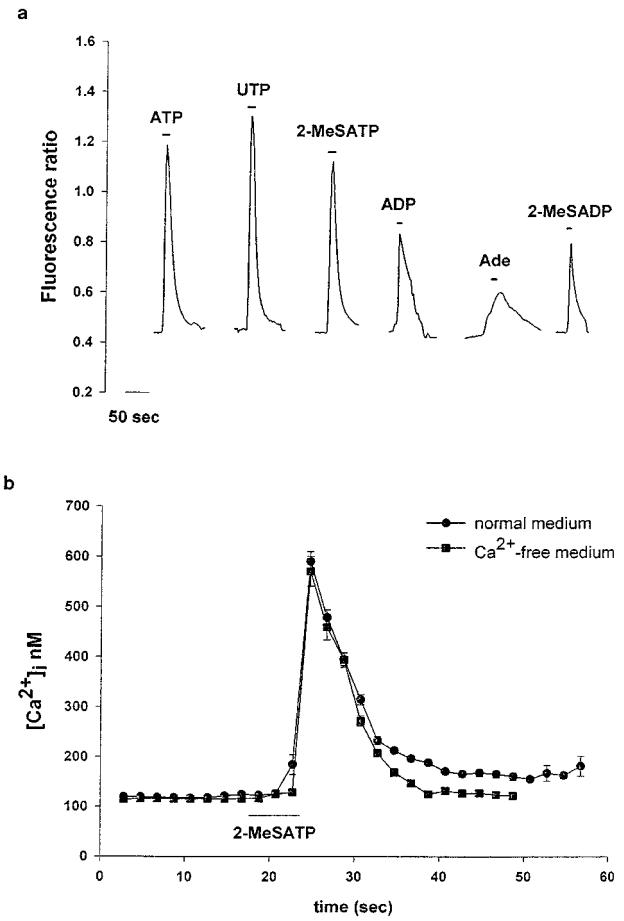


**Figure 1** $[Ca^{2+}]_i$ response of RBCEC cells to nucleotide receptor agonists. (a) Representative traces of $[Ca^{2+}]_i$ changes detected in RBCE cells on stimulation with different agonists, expressed as fluorescence ratio. Pulses of ATP ($n=140$), UTP ($n=147$), 2-MeSATP ($n=90$), 2-MeSADP ($n=50$), ADP ($n=60$) or adenosine (Ade) ($n=50$) were applied at 100 $\mu$M and for 8 s as indicated. For each agonist, a fresh coverslip was used. (b) The change in $[Ca^{2+}]_i$ caused by 2-MeSATP (100 $\mu$M, 8 s) in $Ca^{2+}$-free medium (442$\pm$27.5 nM, $n=36$) was not significantly different from the response in normal medium (454$\pm$27.2 nM, $n=37$).

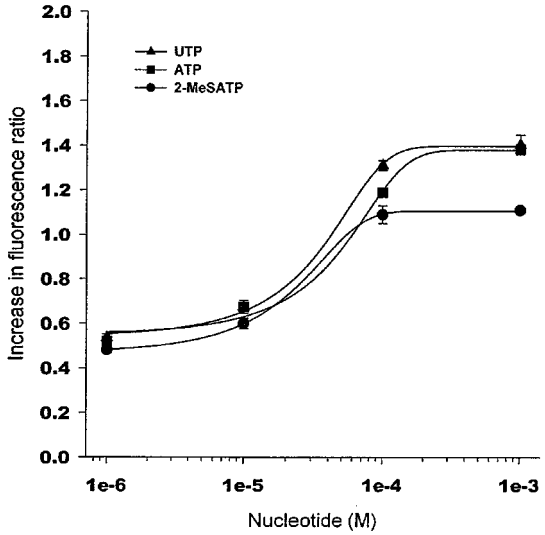


**Figure 2** $[Ca^{2+}]_i$ response (fluorescence ratio) as a function of agonist concentration. Endothelial cells were simulated with ATP, UTP or 2-MeSATP for 8 s, and the fluorescence ratio measured at the peak of the $[Ca^{2+}]_i$ signal. Curves were fitted with the Hill equation, with no parameters fixed. Results are mean$\pm$s.e.mean obtained in three different experiments, $n>50$ number of cells. s.e.mean is not shown where it is smaller than the symbol.

tion. The results with ATP and UTP are similar to those reported for RBE4 cells (Nobles *et al.*, 1995); concentration-response curves for 2-MeSATP have not been previously reported for primary brain endothelial cell preparations.

### *Co-existence of $P2Y_2$ and $P2Y_1$-like purinoreceptors on primary brain endothelial cells*

In order to investigate whether ATP and UTP are acting on the same receptors, the Fura 2-loaded cells were challenged with ATP (100 $\mu$M) and when the ATP-induced $[Ca^{2+}]_i$ signal had returned approximately to the baseline, cells were stimulated with UTP (100 $\mu$M) in the continuing presence of ATP. UTP caused a negligible $[Ca^{2+}]_i$ elevation under these conditions (Figure 3a). By contrast, the response to 2-MeSATP (100 $\mu$M) was not affected by the continuing presence of ATP (Figure 3b). When ATP and UTP were applied together (100 $\mu$M each, 10 s, $n=40$) the $[Ca^{2+}]_i$ increase was not significantly different from that caused by ATP (100 $\mu$M, 10 s, $n=140$) or UTP (100 $\mu$M, 10 s, $n=50$) alone, however when ATP was applied together with 2-MeSATP (100 $\mu$M each, 10 s, $n=50$) $[Ca^{2+}]_i$ increased to a significantly higher level than that measured after a challenge with ATP alone ($P<0.05$; Figure 4). These results are further evidence that both P2U-like and $P2Y_1$-like receptors are present on these primary cultured brain capillary endothelial cells.

We used a further pharmacological manœuvre to check the expression of a $P2Y_1$-like receptor subtype on the rat RBCE cells. Using the subtype-selective antagonist adenosine 3′-phosphate 5′-phosphosulphate (PAPS, Bültmann *et al.*, 1998), we challenged the cells with 2-MeSADP (Figure 5a,b). In the presence of PAPS (5 $\mu$M), 2-MeSADP (100 $\mu$M, 8 sec, $n=37$) failed to increase $[Ca^{2+}]_i$ (Figure 5a,b), confirming the presence of a $P2Y_1$-like receptor.

### *Pharmacological differentiation between rat $P2Y_2$ and $P2Y_4$ receptors*

The cloned rat $P2Y_2$ and $P2Y_4$ receptors are both equally sensitive to ATP and UTP (King *et al.*, 1998). Suramin, a selective antagonist of $P2Y_2$-purinoceptors (King *et al.*, 1998; Bogdanov *et al.*, 1998) was tested on the $[Ca^{2+}]_i$ elevation evoked by ATP (Figure 6a). After a first challenge with 100 $\mu$M ATP for 10 s, the cells were perfused with medium containing 100 $\mu$M suramin for 100 s, then ATP (100 $\mu$M, 10 s) was applied again in the presence of the antagonist. As shown in Figure 6a, ATP failed to increase $[Ca^{2+}]_i$ under these conditions. When perfusion was switched back to a normal medium lacking suramin, the $[Ca^{2+}]_i$ response to ATP (100 $\mu$M, 10 s) partially recovered. For control cells we used fragments of the same coverslips broken prior to the experiments, and cells were challenged with ATP under identical conditions but in the absence of suramin. The inset in Figure 6a shows that the third ATP-induced $Ca^{2+}$-peak was not significantly different from the third control response, but suramin completely blocked the ATP-induced $[Ca^{2+}]_i$ elevation. 100 $\mu$M suramin inhibited the $[Ca^{2+}]_i$ elevation evoked by UTP (100 $\mu$M) by 66% ($n=38$, $P<0.05$, Figure 6b and inset). The inhibitory effect of suramin on the ATP-induced $[Ca^{2+}]_i$ elevation was more pronounced in the present study than in previous studies on the RBE4 brain endothelial cell line (Nobles *et al.*, 1995).

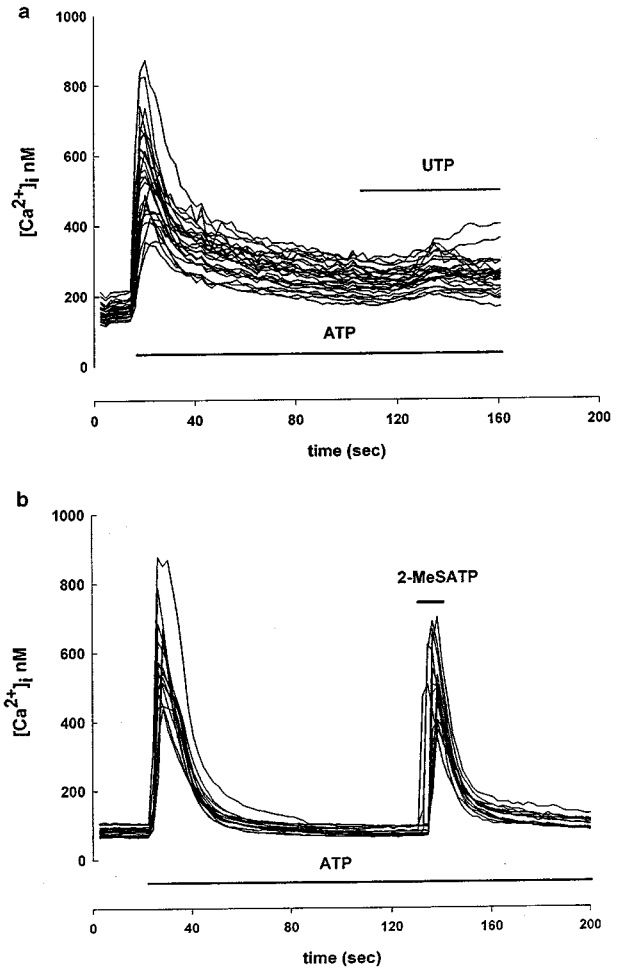


**Figure 3** Cross-desensitization between nucleotide agonists. (a) ATP (100 $\mu$M) was tested first and when the $[Ca^{2+}]_i$ levels returned approximately to the baseline, UTP (a) or 2-MeSATP (b) was added in the continuing presence of 100 $\mu$M ATP ($n=25$ for a; $n=20$ for b).

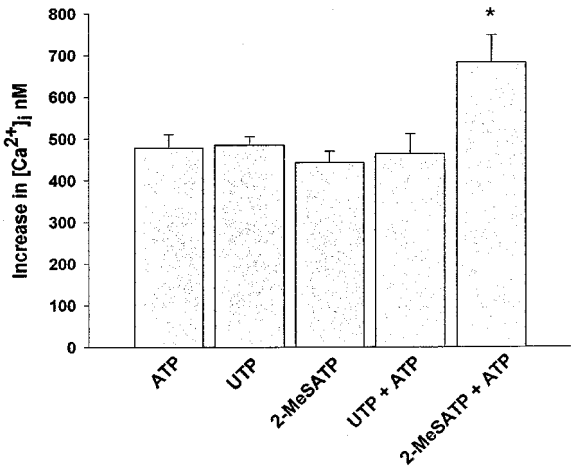


**Figure 4** Co-existence of P2U-like and $P2Y_1$-like receptors in primary brain capillary endothelial cells. Differences between the agonist-evoked peak and resting $[Ca^{2+}]_i$ levels are shown. ATP (100 $\mu$M, 10 s, $n=140$), UTP (100 $\mu$M, 10 s, $n=50$) and 2-MeSATP (100 $\mu$M, 10 s, $n=90$) alone, or a mixture of ATP/UTP (100 $\mu$M for each nucleotide, 10 s, $n=40$) or a mixture of ATP/2-MeSATP (100 $\mu$M for each nucleotide, 10 s, $n=50$) were applied as indicated. Data are means$\pm$s.e.mean $^*P<0.05$, significantly different as compared with response to ATP or 2-MeSATP alone.

### *Adenosine receptor characterization*

To investigate which adenosine receptor subtype is present on RBCEC we challenged the cells with subtype-specific adenosine receptor agonists. 2-p-(2-Carboxyethyl) phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS-21680), the $A_{2A}$ receptor specific agonist (Poulsen & Quinn, 1998) failed to induce $[Ca^{2+}]_i$ changes at 100 nM concentration (data not shown). N-ethylcarboxamidoadenosine (NECA), the $A_{2B}$ receptor agonist (Dubey *et al*., 2000) at 50 nM concentration also caused no change in $[Ca^{2+}]_i$ (data not shown). The $A_1$ receptor agonist $N^6$ - Cyclopentyladenosine (CPA) at 100 nM concentration caused a $\sim$100 nM change in $[Ca^{2+}]_i$ in 40% of the cells tested ($n=37$, Figure 7). This $[Ca^{2+}]_i$ elevation is about 25% of the ATP-induced $[Ca^{2+}]_i$ change, which is similar to the response to adenosine (Figure 1a).

### *Comparison with RBCE cells grown on rat tail collagen*

Growth of cells on ECM appeared to give more uniform responses within the endothelial cell population (percentage cells responding to each agonist) than previously observed with the RBE4 cell line (Nobles *et al*., 1995) or with primary cultured rat brain endothelial cells grown on collagen (Albert *et al*., 1997). In order to test whether the difference could be due to the differentiating effect of ECM, selected agonists were tested on RBCE cells grown on rat tail collagen instead of ECM (Table 1). Cells grown on rat tail collagen gave smaller responses than those grown on ECM, moreover, only 66% responded to 2-MeSATP, and none responded to adenosine (compared with 100 and 63% respectively from cells grown on ECM).

## Discussion

A number of different experimental models have been used to examine the nucleotide sensitivity of endothelial cells, both *in vivo* and *in vitro*. They include the isolated mesenteric arterial bed of the rat (Ralevic & Burnstock, 1996), the *in situ* mesenteric microvessels of the frog (He *et al*., 1996), human placental cotyledons (Ralevic *et al*., 1997), and well-studied endothelial culture preparations from pulmonary artery, HUVEC and adrenal microvessel. Preparations of brain endothelium investigated with nucleotides include *in situ* pial microvessels of the frog and rat (Olesen, 1989), isolated and cannulated penetrating arterioles of the brain surface (Janigro *et al*., 1996), and cell cultures using primary cells, clonal populations of passaged cells, and immortalized cell lines (Albert *et al*., 1997; Vigne *et al*., 1994a; Nobles *et al*., 1995). The studies in primary cultures have used cells between P0 to P10.

The present demonstration of $P2U/P2Y_2$ receptors on primary cultured rat brain endothelial cells agrees with the findings of Nobles *et al*. (1995) and Albert *et al*. (1997), suggesting that these cells when isolated and grown in similar ways show reproducible expression of this receptor. However, although Boarder and co-workers observed $Ca^{2+}$ responses to 2-MeSATP suggesting presence of a $P2Y_1$-like receptor in primary cultured rat brain endothelial cells (Albert *et al*., 1997), they found no detectable $P2Y_1$ mRNA using RT–PCR (Anwar *et al*., 1999). Vigne *et al*. (2000) provided functional evidence for $P2Y_1$ receptors on B7 and B10 clones of passaged rat brain endothelial cells. The present observation of additive responses to 2-MeSATP and ATP, the response to 2-MeSADP and inhibition of the response to 2-

**Table 1** Comparison of $[Ca^{2+}]_i$ responses of rat brain capillary endothelial cells

| Agonist | *Nobles* et al., *1995* RBE4 % cells responding | *Nobles* et al., *1995* RBE4 $\Delta FR/\Delta FR_{ATP}$ % | *Albert* et al., *1997* primary RBCEC % cells responding | *Albert* et al., *1997* primary RBCEC $\Delta FR/\Delta FR_{ATP}$ % | *Sipos* et al., *this study* primary RBCEC grown on ECM % cells responding | *Sipos* et al., *this study* primary RBCEC grown on ECM $\Delta FR/\Delta FR_{ATP}$ % | *Sipos* et al., *this study* primary RBCEC grown on rat tail collagen % cells responding | *Sipos* et al., *this study* primary RBCEC grown on rat tail collagen $\Delta FR/\Delta FR_{ATP}$ on ECM % |
|---|---|---|---|---|---|---|---|---|
| ATP | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 43 |
| UTP | 100 | 92 | 71 | 100 | 100 | 105 | 98 | 42 |
| ADP | 100 | 58 | 84 | 100 | 100 | 72 | 100 | 41 |
| 2-MeSATP | 17 | 75 | 70 | 100 | 100 | 102 | 66 | 51 |
| Adenosine | no data available | – | no data available | – | 63 | 30 | 0 | 0 |

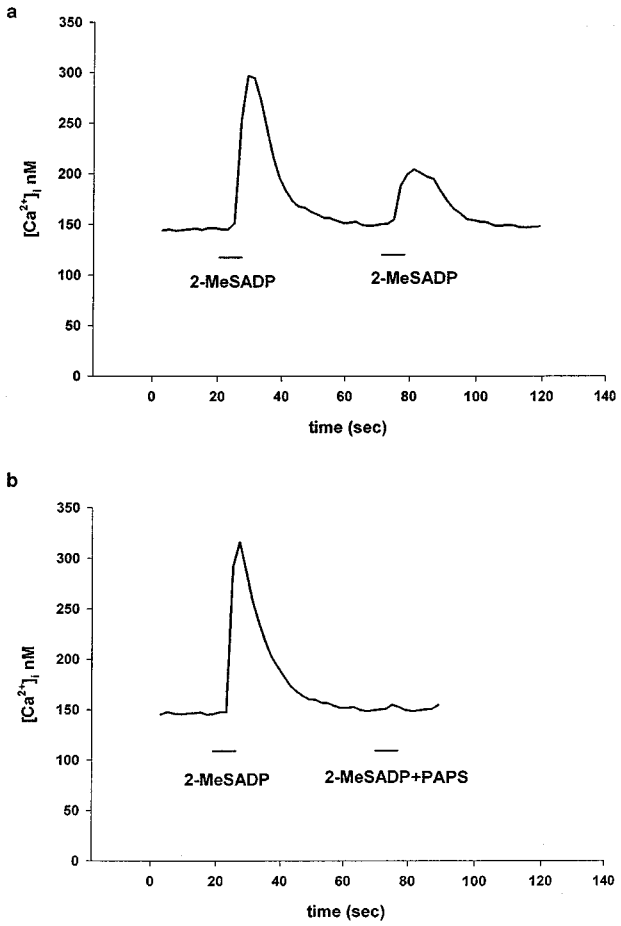


**Figure 5** Effect of $P2Y_1$ receptor agonist and antagonist on $[Ca^{2+}]_i$ in RBCE cells. (a) Response of RBCE cells ($n=37$) to 100 $\mu$M 2-MeSADP repeated at intervals of 50 s. (b) 2-MeSADP (100 $\mu$M) was first tested alone, then 50 s later cells were challenged with 100 $\mu$M 2-MeSADP together with 5 $\mu$M PAPS ($n=35$). 2-MeSADP was applied for 10 s as indicated; each trace is representative of two separate experiments.

MeSADP by PAPS, provide clear pharmacological evidence for a $P2Y_1$-like receptor, which is separate from the $P2Y_2$-like receptor. It remains to be established whether this corresponds to the cloned $P2Y_1$ receptor, or represents a novel variant.

The unpassaged primary brain endothelial cultures are derived from freshly isolated microvessels, and are hence likely to reflect rather closely the *in situ* condition. The present study shows that these cells grown on ECM gave greater and more uniform agonist-induced $Ca^{2+}$-response than cells grown on a collagen-coated surface (Table 1); the responses were also more uniform than those reported by Albert *et al.* (1997) from primary cells grown on collagen (Table 1). The introduction of a biological ECM as growth substrate in the present study followed evidence that ECM components can improve the expression of a number of differentiated properties in cell culture (Lin & Bissell, 1993). Specific blood-brain barrier characteristics that are upregulated by ECM include tight junctions (Arthur *et al.*, 1987; Tilling *et al.*, 1998; Hoheisel *et al.*, 1998), P-glycoprotein (Tatsuta *et al.*, 1994) and enzymes including alkaline phosphatase and $\gamma$-glutamyl transpeptidase (Mischeck *et al.*, 1989; Mizuguchi *et al.*, 1994; 1997). As far as we have been able to establish, the present study is the first to show that the differentiation-promoting function of ECM extends to pharmacological phenotype.

In comparison with cells grown on the biological ECM, cells grown on a collagen-coated surface appeared not to express adenosine-sensitive receptors, and a smaller percentage of cells responded to 2-MeSATP. Furthermore, the agonist-induced $[Ca^{2+}]_i$ changes were significantly smaller in cells grown on collagen (Table 1), possibly due to a lower receptor density or altered signal transduction mechanisms. The differences suggest that the complex ECM generated by growing cells acts as a more complete source of local differentiating signals for the brain endothelial cells than simpler ECM preparations such as rat tail collagen or purified Type I collagen (see also Tagami *et al.*, 1992).

Co-existence of P2Y and P2U purinoceptors has been reported on primary bovine aortic endothelial cells (Brown *et al.*, 1995), bovine pulmonary endothelium (Chen *et al.*, 1996) and in primary endothelial cells from adrenal medulla (Mateo *et al.*, 1996). The last study showed that the P2Y purinoceptor activity in primary adrenomedullary endothelial cells was lost after a single passage. Vigne and co-workers showed that both P2U- and $P2Y_1$-like receptors were present in the B7 clone of RBEC (Frelin *et al.*, 1993; Vigne *et al.* 1994a). However, those studies used assays monitoring the behaviour of populations of cells, so gave no information on the percentage of cells expressing a particular receptor type. As the B10 clone of RBEC lacks P2U receptors, it was used for further study of the unusual $P2Y_1$-like receptors which appeared to couple to mobilization of intracellular $Ca^{2+}$ and to inhibition of adenylate cyclase (Feolde *et al.*, 1995; Webb *et al.*, 1996; Vigne *et al.*, 1998a). However, the latter effect was not observed in primary cultured RBEC (Albert *et al.*, 1997).

As shown in Table 1, the response to ATP and UTP of the RBCEC is similar to that previously reported for the RBE4 cell line (Nobles *et al.*, 1995), suggesting similar expression of P2U receptors, but a smaller percentage of RBE4 cells responded to 2-MeSATP, likely to indicate lower expression of a $P2Y_1$-like receptor. Forty per cent of the RBE4 cells also

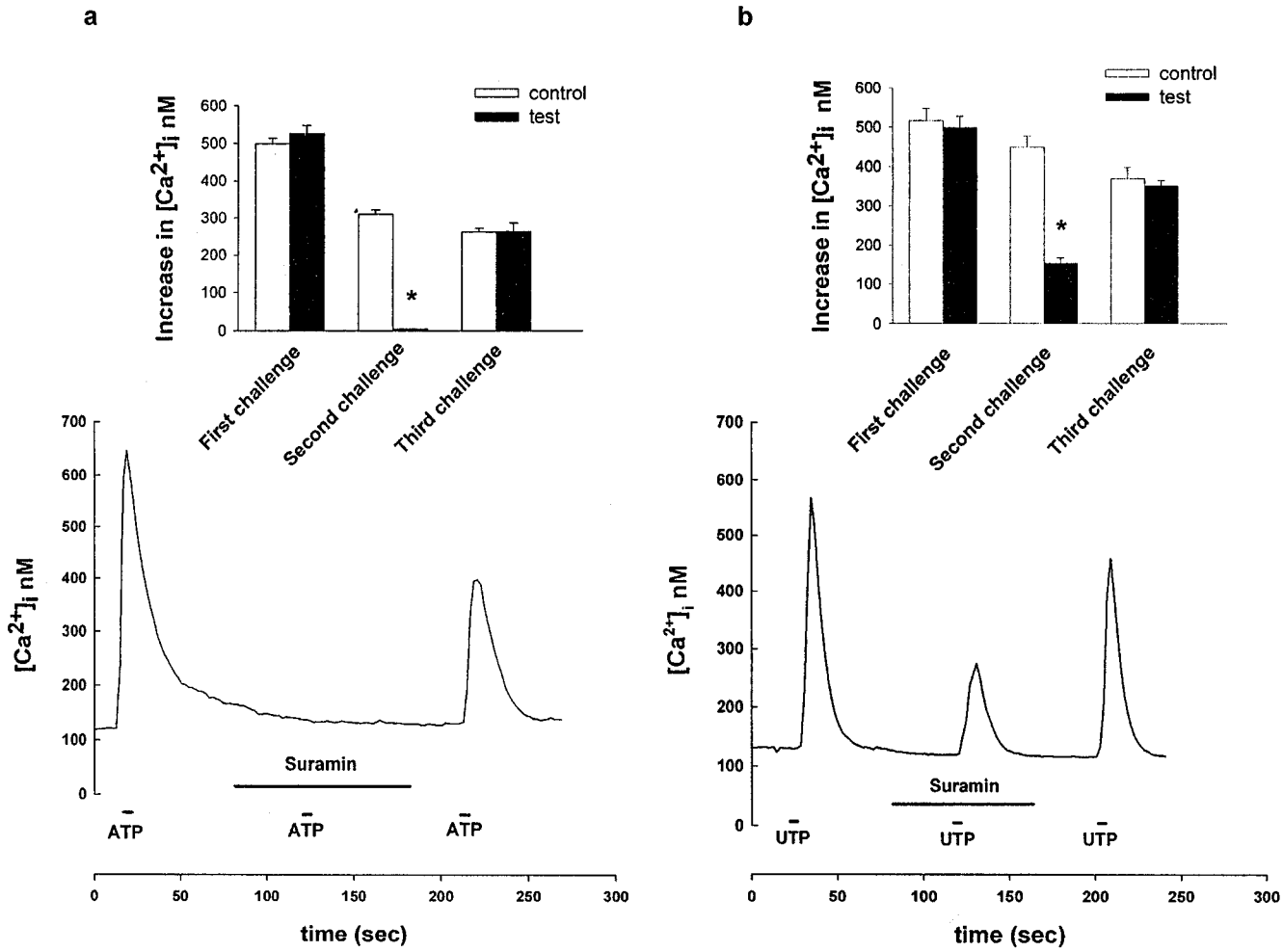


**Figure 6** Effect of Suramin on the ATP- and UTP-evoked $[Ca^{2+}]_i$ increase. (a) ATP or (b) UTP (100 $\mu$M, 10 s, respectively) was first tested alone, then the agonist was added again in the presence of Suramin (100 $\mu$M, 100 s). After removing the antagonist, (a) ATP (100 $\mu$M, 10 s) or (b) UTP (100 $\mu$M, 10 s) was applied again. Each trace is representative of 40–50 cells from three separate experiments. The insets show average changes in $[Ca^{2+}]_i$ (difference between peak and resting $[Ca^{2+}]_i$ level) for controls and for experiments with ATP(a) or UTP(b). Means$\pm$s.e.mean are shown, * significantly different from the respective controls in the absence of the antagonist ($P<0.05$).

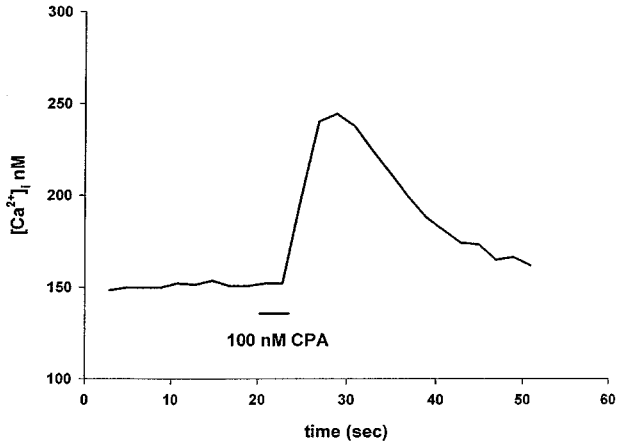


**Figure 7** Activation of $A_1$ type adenosine receptor. Fifty per cent of the RBCE cells tested ($n=38$) responded to the selective $A_1$ agonist CPA (100 nM, 10 s), while other subtype selective agonists (NECA, CGS 21680) failed to increase $[Ca^{2+}]_i$.

responded to $\alpha,\beta$-MeATP, while none of the primary cells responded; Albert *et al.* (1997) showed that all first passage (P1) RBCEC responded to $\alpha,\beta$-MeATP, while unpassaged (P0) cells did not. This comparison suggests that some of the RBE4 cells were expressing the phenotype of early passaged cells.

Adenosine caused a $Ca^{2+}$-response in RBCEC. ATP is readily converted to ADP, AMP and adenosine *in vivo* and *in vitro* through a sequential reaction catalyzed by ecto-ATPases and 5′ nucleotidase (Marcus *et al.*, 1997). However, it is likely that the short application of the drugs (8–10 s) and the large volume of the diluting solution mean that breakdown of ATP or ADP to adenosine made no contribution to the agonist-induced $[Ca^{2+}]_i$ signal under our experimental conditions (see also Vigne *et al.* (1998b)). The small $[Ca^{2+}]_i$ rise in response to adenosine could have resulted from activation of the $P2Y_1$-like receptor, but the fact that the $A_1$-selective agonist gave a similar sized calcium response suggests that both adenosine and CPA exerted their actions *via* an $A_1$ adenosine receptor.

The presence of several types of nucleotide receptors on brain endothelial cells supports the hypothesis that the receptors are designed to respond differently to the natural ligands *in vivo*. $P2Y_1$ and $P2Y_2$ receptors appear to exert their effector actions (such as production and release of $PGI_2$ and NO) by overlapping pathways, including the calcium-mediated activation of endothelial nitric oxide synthase (ecNOS, NO) and activation of MAPK and phospholipase $A_2$ ($PGI_2$). However, the different G

proteins involved allow for subtle differences in feedback control and modulation (Boarder & Hourani, 1998). Reports of an atypical nucleotide receptor coupled to inhibition of adenylate cyclase (Webb *et al*., 1996; see also Vigne *et al*., 1998a,b), and an unidentified ATP receptor activating adenylate cyclase (Albert *et al*., 1997), raise the possibility of additional and even 'brain-specific' signal transduction pathways. They would also be expected to contribute to the growing evidence for 'cross-talk' between second messenger signalling systems in the brain endothelium (Vigne *et al*., 1994b, Nobles & Abbott, 1998). The identification of brain endothelial specific receptors/transduction pathways capable of regulating tight junctional permeability, acting through calcium, cyclic AMP and tyrosine kinase/MAPK cascades would prepare the way for targeted therapies, such as deliberate blood-brain barrier opening for drug delivery, and blood-brain barrier tightening to reverse the permeability increase associated with some neuropathologies including inflammatory conditions, hypoxia and ischaemia (Abbruscato & Davis, 1999; Abbott, 2000).

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