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European Journal of Pharmacology 361 (1998) 119–127

ejp

Modulation of the effects of extracellular ATP on $[Ca^{2+}]_i$ in rat brain microvacular endothelial cells

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Received 30 March 1998; revised 10 August 1998; accepted 11 September 1998

Abstract

This study examined the intracellular regulation of signal transduction initiated by activation of the $P2Y_2$ purinoceptor in a cultured rat brain microvascular endothelial cell line (RBE4). Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was monitored in single cells, using FURA-2 fluorimetry. As previously described [Nobles, M., Revest, P.A., Couraud, P.-O., Abbott, N.J., 1995. Characteristics of nucleotide receptors that cause elevation of cytoplasmic calcium in immortalized rat brain endothelial cells, RBE4, and in primary cultures. Br. J. Pharmacol., 115, 1245–1252], extracellular ATP (100 μM, 20 s) evoked a transient increase in intracellular free calcium concentration ($[Ca^{2+}]_i$). The amplitude of the Ca^{2+} transient evoked by ATP decreased with successive applications (desensitisation), as expected for a P2 purinoceptor. The modulation of the Ca^{2+} signal downstream to the activation of the ATP receptor was investigated, using agents selected for their ability to interfere with the intracellular pathways activated by ATP. The amplitude of the Ca^{2+} transient observed on the second application of ATP was compared in the presence and absence of these agents. The Ca^{2+} transient triggered by ATP was decreased by the inhibitor of nitric oxide synthesis, *N*-omega-nitro-L-arginine methyl ester (L-NOARG). The inhibition induced by 100 μM L-NOARG was reversed by coapplication of the permeant cGMP analogue 8-brcGMP (100 μM). 8-BrcGMP caused a transient increase in $[Ca^{2+}]_i$ when applied alone, and a dose-dependent inhibition of the increase in $[Ca^{2+}]_i$ elicited by ATP. Indomethacin, an inhibitor of prostaglandin synthesis, inhibited the response to ATP. The inhibition caused by 10 μM indomethacin was reversed by coapplication of the permeant analogue of cAMP, 8-brcAMP (100 μM). 8-BrcAMP caused a transient rise in $[Ca^{2+}]_i$ when applied alone, and a dose-dependent inhibition of the Ca^{2+} response evoked by ATP. The non-permeant cyclic nucleotides cAMP and cGMP did not affect the desensitising response to ATP, nor did they reverse the inhibitory actions of L-NOARG or indomethacin. It is concluded that cyclic nucleotides, nitric oxide, and prostaglandin synthesis pathways are able to interact with the Ca^{2+} second messenger pathway in rat brain endothelial cells activated by extracellular ATP.

Keywords: Endothelial cell, brain; Cyclic nucleotide; Ca^{2+}, intracellular; P2 purinoceptor

1. Introduction

A $P2Y_2$ purinoceptor (according to the gene-based terminology, Communi and Boeynaems, 1997) has been identified in rat brain microvascular endothelial cells, both in primary cultures and in the immortalised cell line RBE4 (Nobles et al., 1995). The activation of this purinoceptor by ATP is coupled to a transient increase in intracellular calcium ($[Ca^{2+}]_i$) that persists when extracellular Ca^{2+} is chelated with EGTA, consistent with Ca^{2+} mobilisation from intracellular stores.

Stimulation of purinergic receptors results in the activation of phosphoinositidase C that generates several intracellular second messengers, primarily inositol phosphates and 1,2-diacylglycerol (Boarder et al., 1995). While inositol trisphosphate (IP_3) releases Ca^{2+} from intracellular stores, diacylglycerol in concert with membrane phospholipids and Ca^{2+} activates protein kinase C (Berridge, 1987). However, other second messengers are also produced in response to P2 purinoceptor activation (reviewed in Boarder et al., 1995). Elevation of $[Ca^{2+}]_i$ can cause stimulation of phospholipase A_2 and nitric oxide synthase, resulting respectively in increased synthesis of prostaglandin I_2 (prostacyclin) and nitric oxide (NO). P2 purinoceptors have also been linked to changes in cAMP concentration, both an increase and a decrease in cAMP concentration having been reported (reviewed in Boarder et al., 1995).

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0014-2999/98/$ - see front matter
PII: S0014-2999(98)00671-2

In a number of cell types, modulation of signal transduction in a membrane receptor pathway by downstream mechanisms has been demonstrated. In some systems, the cyclic nucleotides cAMP and cGMP have been shown to modify the receptor-mediated increase in $[Ca^{2+}]_i$ (Yamanishi et al., 1983; Zavoico and Feinstein, 1984; Nakashima et al., 1986). Thus, in human platelets, cAMP suppressed the increase in $[Ca^{2+}]_i$ elicited by thrombin (Zavoico and Feinstein, 1984), while cAMP and cGMP dramatically reduced the ability of thapsigargin to release Ca^{2+} from intracellular compartments (Brüne and Ulrich, 1992). In chick pineal cells, agents that increase intracellular cAMP have been shown to cause mobilisation of Ca^{2+} from intracellular stores (D'Souza and Dryer, 1994).

We have therefore investigated whether the $P2Y_2$ receptor-dependent Ca^{2+} signal described in brain endothelial cells is also subject to intracellular control by a co-produced second messenger. In order to study the downstream regulation of the $P2Y_2$ receptor of brain endothelium, cells were challenged with a first ATP application which led to the activation of the intracellular machinery that we aimed to modify by the use of different substances.

In the present study, we report evidence for a role of cAMP and cGMP in the regulation of the increase in $[Ca^{2+}]_i$ evoked by ATP in a rat brain endothelial cell line, RBE4.

2. Materials and methods

2.1. Cell cultures

RBE4 cells were provided by Dr. Pierre-Olivier Couraud and Dr. Francoise Roux. The RBE4 cell line was originally established by transfecting pure second passage primary cultures of rat brain microvascular endothelial cells with the 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, and blood-brain barrier markers (Roux et al., 1993, 1994; Durieu-Trautmann et al., 1993a; Abbott et al., 1995). Cells were stored at −70°C or in liquid nitrogen in 95% foetal calf serum (Sigma) 5% dimethyl sulfoxide (DMSO, Aldrich), at a concentration of $3–5 \times 10^6$ cells ml^{-1}. When needed, cells were thawed, then centrifuged at 1500 RPM for 10 min. The pellet obtained was resuspended in culture medium containing alpha minimum essential medium Eagle (MEM/F10 (1:1), Gibco), 10% foetal calf serum, 1 ng ml^{-1} basic fibroblast growth factor (Sigma), 300 μg ml^{-1} Geneticin (Sigma), and seeded at a density of 10^4 cells cm^{-2}. After thawing, cells were passaged at least once in plastic flasks (to allow recovery) before seeding onto glass coverslips treated with rat-tail collagen cross-linked with carbodiimide (Aldrich), as previously described (Nobles and Abbott, 1996). Cells were studied between passage 30 and 60, and used during the two days following seeding (preconfluent cultures).

2.2. Intracellular Ca^{2+} measurements

RBE4 cells were loaded with FURA-2 (Molecular Probes) by incubation with the membrane permeant acetomethyl-ester (10 μM in HEPES buffer, 2% bovine serum albumin, 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 (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 an HEPES buffer (composition in mM: NaCl 140, $MgCl_2$ 1, $CaCl_2$ 1.5, KCl 5, D-glucose 10, HEPES 10; pH 7.4; room temperature). Fluorescence was monitored in single cells. Prior to the experimental protocol, cells were superfused for ~15 min, in order to wash out excess FURA-2. To avoid the problem of bleaching of FURA-2, the total duration of an experiment did not exceed 1000 s. In view of problems in calibrating intracellular Ca^{2+} measurements (reviewed in Williams and Fay, 1990), changes in intracellular Ca^{2+} concentration are reported as the change in ratio of fluorescence intensity (ΔFR) measured at the peak of the response to agonists. As the K_d for FURA-2 is 225 nM, the normal resting $[Ca^{2+}]_i$ is well below half-saturation for the dye and allows reasonable accuracy of measurements in the range of 1–10 μM (Cobbold and Rink, 1987). The relationship between fluorescence intensity and $[Ca^{2+}]_i$ is linear between 500 nM and 10 μM (Poenie, 1990).

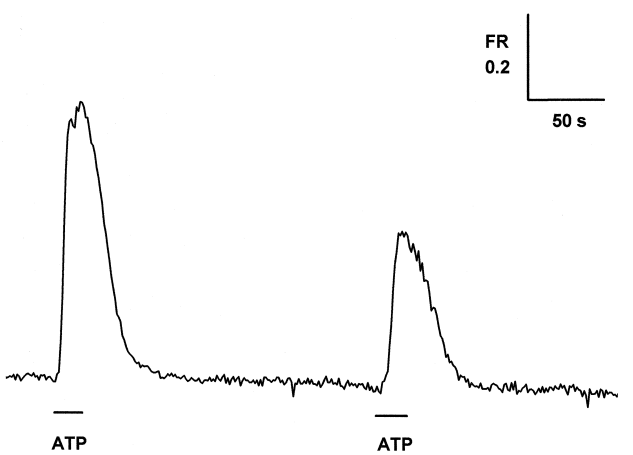

Fig. 1. Desensitisation of the ATP response in RBE4 cells. Trace representing the response of an RBE4 cell to sequential applications of ATP (100 μM, 20 s) separated by 150 s. This trace is representative of 16 experiments.

Table 1
$[Ca^{2+}]_i$ response (ΔFR = fluorescence ratio) to sequential applications of ATP (100 μM, 20 s) separated by 150–200 s

	A	B	C	D
	ΔFR_{ATP1}	ΔFR_{ATP2}	Percentage of initial response	Percentage inhibition
Control	0.57 ± 0.05	0.30 ± 0.04	53 ± 4.7	
L-NOARG	0.78 ± 0.08	0.29 ± 0.08	34.2 ± 8.4 *	35.5
L-NOARG + Br-cGMP	0.87 ± 0.02	0.48 ± 0.02	54.1 ± 9.2	no inhibition
L-NOARG + cGMP	0.67 ± 0.05	0.23 ± 0.01	35.4 ± 4.2 *	33.2

The first application (ATP1) was always made in control conditions, while the second application (ATP2) was made either in control conditions ($n = 16$), or (test) in the presence of L-NOARG 100 μM ($n = 6$), L-NOARG 100 μM + brcGMP 100 μM ($n = 3$), or L-NOARG 100 μM + cGMP 100 μM ($n = 3$). Means ± S.E.M., * $P < 0.05$ compared to ATP in control buffer. Column C was the mean calculated from $\Delta FR_{ATP2}/\Delta FR_{ATP1}$ of individual experiments. Column D was calculated from $(C_{control} - C_{test}/C_{control}) \times 100$.

2.3. Drugs

ATP, *N*-omega-nitro-L-arginine methyl ester (L-NOARG), 8-bromo cGMP (8-brcGMP), 8-bromo cAMP (8-brcAMP), cAMP, chlorophenylthio-cAMP (CPTcAMP) and cGMP were from Sigma. These drugs were diluted in buffer. Indomethacin (Sigma) was solubilized in ethanol, and diluted in buffer, the final concentration of ethanol not exceeding 0.01%. All agonists were routinely presented as a 20-s superfusion pulse, unless specified.

2.4. Data analysis and statistics

The results are given as means ± S.E.M. Statistical significance was evaluated by Student's *t*-test, with $P < 0.05$ being taken as significant.

3. Results

3.1. Desensitisation of the Ca^{2+} response

The Ca^{2+} response to activation of the $P2Y_2$ purinoceptor present on RBE4 cells is transient and decreases with repeated applications (Nobles et al., 1995; and Fig. 1). A 20-s pulse of extracellular ATP (100 μM) caused a transient increase in fluorescence ratio (ΔFR) of 0.57 ± 0.05 ($n = 16$). The response to a second application of ATP, 150–200 s later, was of similar shape, but with a peak of 53 ± 4.7% of the initial response (Table 1).

Agents were evaluated for their ability to interfere with the ATP-mediated Ca^{2+} response. Since activation of the release of second messengers (IP_3, nitric oxide, prostaglandins, cyclic nucleotides) downstream of the purinoceptor requires the activation of the receptor by ATP, the following protocol was used. Cells were first challenged with ATP (100 μM, 20 s), then agents able to interfere with nitric oxide synthesis (L-NOARG) or prosytaglandin synthesis (indomethacin) were applied for 150–200 s immediately after the first 20-s ATP signal, before the second 20-s ATP pulse. The effects of the agents tested were assessed on the shape and amplitude of the second response to ATP. The initial response to ATP also acted as a useful internal control for normalisation of responses between cells.

Since desensitisation of the response to the second ATP challenge occurred (see above), the effect of an agent will be superimposed on this desensitisation. For this reason, the effect of agent was calculated from the change in ΔFR to the second ATP challenge, compared with control, expressed as a percentage. For individual experiments, the Ca^{2+} response to the second ATP pulse (ATP2) was calculated as a percentage of the response to the first ATP pulse (ATP1), i.e., $(\Delta FR_{ATP2}/\Delta FR_{ATP1}) \times 100\%$. Thus the reference (control) response was the mean percentage $(\Delta FR_{ATP2\ control}/\Delta FR_{ATP1\ control} \times 100)$ % in control solution. The percentage inhibition in the presence of an agent was then calculated from the mean percentage change in response during the second ATP application (see Tables 1 and 2).

Table 2
$[Ca^{2+}]_i$ response (ΔFR = fluorescence ratio) to sequential applications of ATP (100 mM, 20 s) separated by 150–200 s

	A	B	C	D
	ΔFR_{ATP1}	ΔFR_{ATP2}	Percentage of initial response	Percentage inhibition
Control	0.57 ± 0.05	0.30 ± 0.04	53 ± 4.7	
Indo	0.61 ± 0.08	0.22 ± 0.03	39.7 ± 3.7 *	25.1
Indo + Br-cAMP	0.68 ± 0.1	0.37 ± 0.05	57 ± 4.6	no inhibition
Indo + cAMP	1 ± 0.2	0.41 ± 0.02	34.1 ± 5.4 *	35.7

The first application (ATP1) was always made in control conditions, while the second application (ATP2) was made either in control conditions ($n = 16$), or (test) in the presence of indomethacin 10 μM ($n = 7$), indomethacin 10 μM + brcAMP 100 μM ($n = 8$), or indomethacin 10 μM + cAMP 100 μM ($n = 3$). Means ± S.E.M., * $P < 0.05$ compared to ATP in control buffer.

3.2. Effect of an NO synthase inhibitor on the ATP-induced increase in $[Ca^{2+}]_i$

L-NOARG is an inhibitor of nitric oxide synthesis. A concentration of 100 μM L-NOARG caused an inhibition of 35.5% in the response to the second ATP challenge ($n = 6$, $P < 0.05$ compared to control conditions, Fig. 2A and Table 1). The shape of the Ca^{2+} transient was otherwise unaffected. There was no measurable effect on $[Ca^{2+}]_i$ at the time of application of L-NOARG (Fig. 2A).

3.3. Reversal of the effect of L-NOARG by 8-brcGMP

ATP has been shown to cause an increase in NO release in non-brain endothelial cells (Bogle et al., 1991), while NO donor molecules have been shown to cause an increase

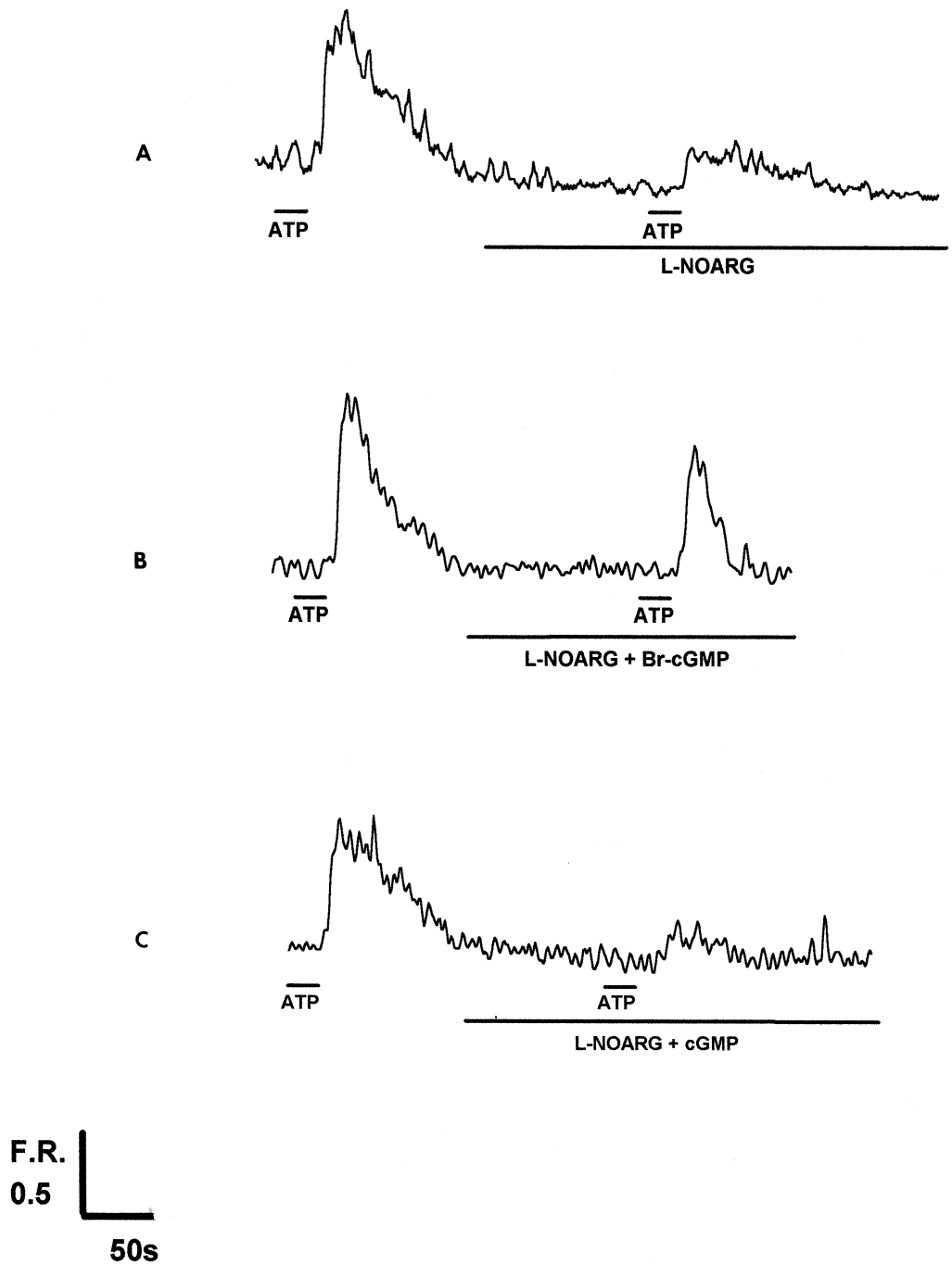

Fig. 2. Effect of NO synthase inhibitor and of cGMP on the increase in $[Ca^{2+}]_i$ induced by ATP. (A) Time-course of the effect of ATP 100 μM, followed by a 175-s incubation with L-NOARG 100 μM, followed by a second ATP challenged in the continuing presence of L-NOARG. This trace is representative of six experiments. (B, C) Similar experiments but with either brcGMP (B) or cGMP (C) co-applied with L-NOARG. Br-cGMP but not cGMP was able to reverse the inhibitory effect of L-NOARG on the response to ATP.

in $[cGMP]_i$ in brain endothelial cells (Marsault and Frelin, 1992). We therefore investigated the effect of cGMP on the L-NOARG-mediated inhibition of the response to ATP, using the membrane permeant and nonhydrolysable analogue 8-brcGMP.

The inhibitory effects of L-NOARG were counteracted by coapplication of the membrane permeant cGMP analogue, 8-brcGMP. In the simultaneous presence of 100 μM L-NOARG and 100 μM 8-brcGMP, the response to the second ATP challenge was not significantly different from that in control conditions ($n = 3$, Fig. 2B and Table 1).

The membrane impermeant cGMP had no effect on the response to ATP in the presence of L-NOARG (Fig. 2C, Table 1, $n = 3$, inhibition 33.2%, not significantly different from the inhibition seen in the presence of L-NOARG alone). As the membrane permeant analogue 8-brcGMP was effective in blocking the action of L-NOARG, but the membrane impermeant cGMP was not, this indicates that the target of the nucleotide is likely to be intracellular.

3.4. Effect of 8-brcGMP on the increase in $[Ca^{2+}]_i$ induced by ATP

To characterise further the involvement of cGMP in signal transduction elicited by ATP, we tested the action of 8-brcGMP on the response to ATP. A concentration of 0.1 μM 8-brcGMP (150–200 s) had no effect on the $[Ca^{2+}]_i$ of RBE4 cells ($n = 7$), nor on the degree of desensitisation of the ATP response (data not shown). In a concentration range of 1 μM to 100 μM, 8-brcGMP was itself able to cause an increase in $[Ca^{2+}]_i$, with a 150–200 s pulse of 8-brcGMP eliciting a single transient increase in $[Ca^{2+}]_i$ (Fig. 3A). The rise in $[Ca^{2+}]_i$ elicited by 8-brcGMP was not dose-dependent in the range of concentrations tested. After incubation of cells for 150–200 s with 8-brcGMP, the response to the second ATP pulse was decreased. In a range of concentrations from 1 μM to 100 μM 8-brcGMP, this inhibition was a function of the concentration of 8-brcGMP (Fig. 3B), with complete inhibition of the response to ATP found for concentrations of 8-brcGMP greater than 10 μM (Fig. 3B).

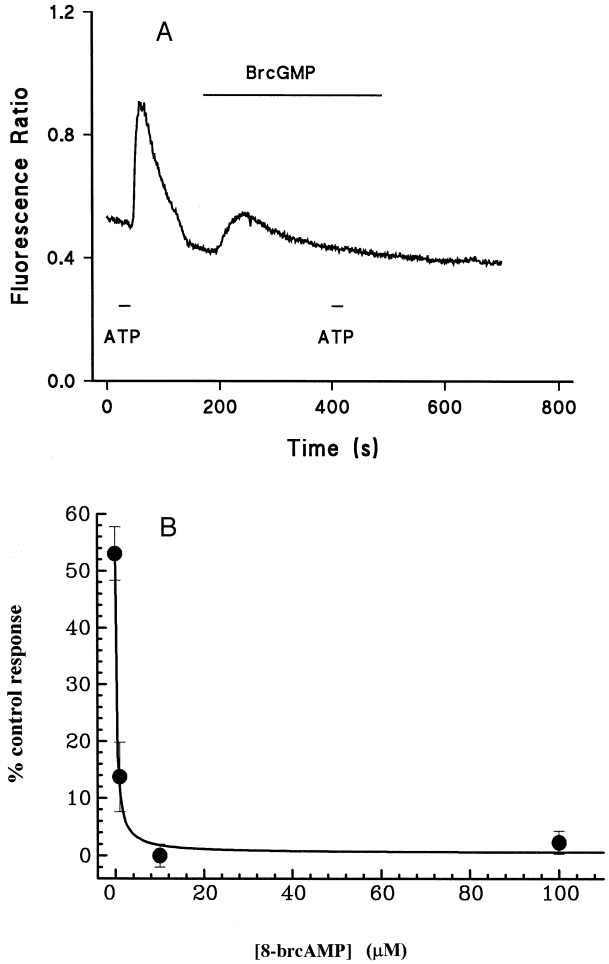

Fig. 3. Effect of 8-brcGMP on the increase in $[Ca^{2+}]_i$ induced by ATP. (A) An experiment in which ATP (100 μM, 20 s) was tested alone, then cells were superfused for 200 s with 100 μM 8-brcGMP, and ATP was tested again in the continuing presence of 8-brcGMP. The trace is representative of three experiments. (B) Concentration-dependent inhibition of the ATP-induced increase in fluorescence ratio caused by increasing concentrations of 8-brcGMP. Means ± S.E.M., $n = 3$.

3.5. Effects of agents which interfere with the metabolism of arachidonic acid on the ATP-induced increase in $[Ca^{2+}]_i$

Phospholipids can be hydrolysed to arachidonic acid by phospholipase A_2. Once released, arachidonic acid can follow two pathways, the cyclooxygenase pathway leading to the formation of prostaglandins and thromboxanes, and the lipooxygenase pathway producing leukotrienes and other unsaturated hydroxy acids. The cyclooxygenase pathway can be blocked by the anti-inflammatory drug indomethacin, while the lipooxygenase pathways can be blocked by nordihydroguaiaretic acid (NDGA). ATP has been shown to activate the release of arachidonic acid in astrocytes via the activation of a P2Y receptor (Bruner and Murphy, 1992). We have tested the effects of inhibition of the cyclooxygenase pathway on the ATP-induced release of $[Ca^{2+}]_i$ in RBE4 cells.

The results are presented in Fig. 4A and Table 2. Indomethacin caused an inhibition of the response to ATP. A concentration of 10 μM indomethacin caused a 25% inhibition of the second response to ATP ($n = 4$, $P < 0.05$, Fig. 4A and Table 2).

3.6. Effect of membrane-permeant cAMP analogues on the increase in $[Ca^{2+}]_i$ induced by ATP

In some systems, such as the intestinal epithelium and cardiomyocytes, prostaglandins have been shown to activate the synthesis of cAMP (Smith et al., 1987; Church et

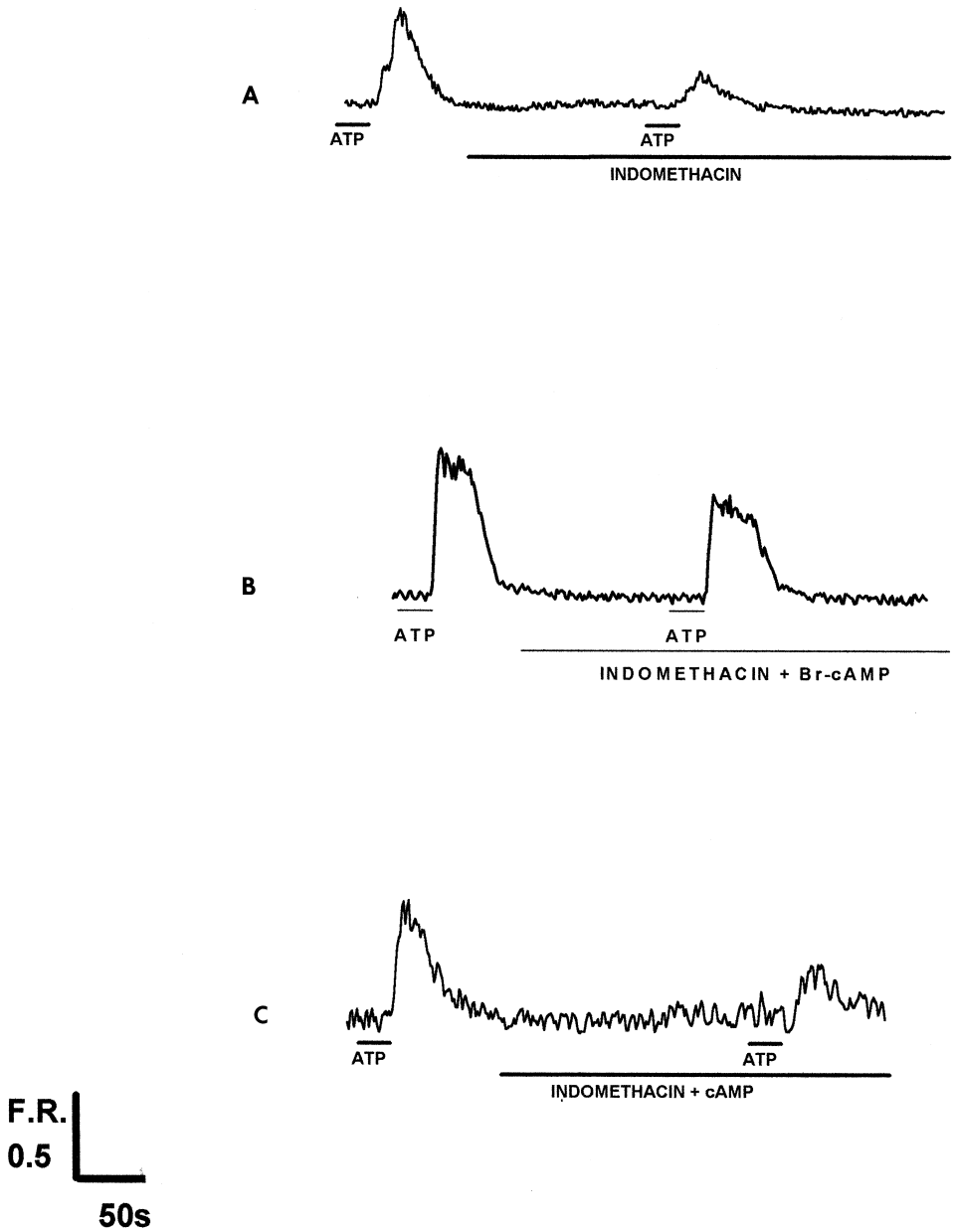

Fig. 4. Effect of inhibition of prostaglandin synthesis on the increase in $[Ca^{2+}]_i$ induced by ATP. (A) Time-course of the effect of ATP 100 μM, followed by a 150-s incubation with indomethacin 10 μM, followed by a second ATP challenge in the continuing presence of indomethacin. This trace is representative of seven experiments. (B, C) Similar experiments but with either brcAMP (B) or cAMP (C) co-applied with indomethacin. Br-cAMP but not cAMP was able to reverse the inhibitory effect of indomethacin on the response to ATP.

al., 1994). Therefore in experiments analogous to those with L-NOARG and exogenous cGMP, we attempted to reverse the effect of indomethacin with a coapplied permeant analogue of cAMP.

A concentration of 100 μM 8-brcAMP coapplied with 10 μM indomethacin for 150–200 s was able to reverse the indomethacin-mediated inhibition of the Ca^{2+} response to ATP ($n = 8$, n.s. compared to ATP alone, $P < 0.05$ compared to ATP in the presence of indomethacin, Fig. 4B and Table 2).

In the combined presence of 10 μM indomethacin and 100 μM cAMP, a 35.7% inhibition of the second response to ATP was observed (Fig. 4C, Table 2, $n = 3$, $P < 0.05$ compared to control, n.s. compared to indomethacin alone). This result indicated that the reversal of the inhibition caused by indomethacin was exclusive to the permeant analogue of the nucleotide, indicating that its target is likely to be intracellular. The transient $[Ca^{2+}]_i$ response to ATP was indistinguishable from the control response.

3.7. Effect of 8-brcAMP on the increase in $[Ca^{2+}]_i$ induced by ATP

We observed above that the permeant cGMP analogue not only reversed the inhibitory effect of L-NOARG but

also elicited a Ca^{2+} transient by itself, and inhibited the subsequent responses to ATP. Permeant analogues of cAMP also had these additional effects.

In a concentration range of 1–100 μM, 8-brcAMP (150–200 s) caused a transient increase in $[Ca^{2+}]_i$ ($n = 3$, Fig. 5A). As with 8-brcGMP, the increase in Ca^{2+} was not significantly dose-related, and at all concentrations tested, remained below the response to 100 μM ATP. In order to test whether the increase in $[Ca^{2+}]_i$ seen with brcAMP was reproducible with other cAMP analogues, chlorophenylthio-cAMP (CPT-cAMP) was tested. 100 μM CPT-cAMP caused an increase in ΔFR of 0.23 ± 0.09 ($n = 3$), not significantly different from the response to 100 μM 8-brcAMP, ΔFR of 0.19 ± 0.05 ($n = 4$).

Incubation of cells with 8-brcAMP led to a dose-dependent inhibition of the subsequent response to ATP. The maximal inhibition achieved was notably less than with 8-brcGMP, reaching only 61% for concentrations of 8-brcAMP higher than 10 μM (Fig. 5B). The non-permeant cAMP was unable to induce a Ca^{2+} transient or to affect the size of the transient response to ATP (data not shown).

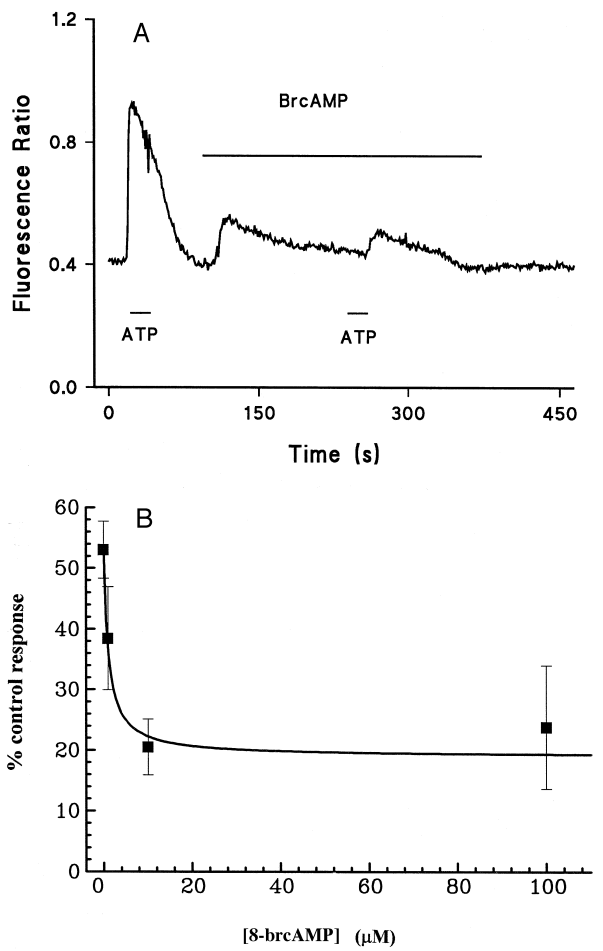

Fig. 5. Effect of 8-brcAMP on the increase in $[Ca^{2+}]_i$ induced by ATP. (A) An experiment in which ATP (100 μM, 20 s) was tested alone, then cells were superfused for 150 s with 100 μM 8-brcAMP, and ATP was tested again in the continuous presence of 8-brcAMP. The trace is representative of eight experiments. (B) Concentration-dependent inhibition of the ATP-induced increase in fluorescence ratio caused by increasing concentration of 8-brcAMP. Means ± S.E.M., $n = 3$.

4. Discussion

4.1. Effect of intracellular cyclic nucleotides on response to extracellular ATP

The sensitivity of a cell to agonists can be regulated by intracellular events downstream of receptor activation. Desensitisation of the $P2Y_2$ receptor occurs to repeated applications of ATP or UTP (Nobles et al., 1995). In the present study, we compared the decline of the response in control conditions to the decline during the application of L-NOARG, indomethacin and cyclic nucleotides.

The Ca^{2+} response of rat brain endothelial cells due to activation of $P2Y_2$ purinoceptors by ATP was reduced by pre-treatment of the cells with the membrane-permeant analogues of cAMP and cGMP (i.e., 8-brcAMP, CPT-cAMP, 8-brcGMP). The cyclic nucleotides must be acting at the intracellular level, since the non-permeant cAMP and cGMP were without effect. The inhibition was dose-dependent, with a maximum at extracellular concentrations of 10 μM cyclic nucleotides. 8-BrcGMP was the most potent inhibitor of the rise in Ca^{2+} evoked by 100 μM ATP, since 100% inhibition was obtained for 10 μM 8-brcGMP, while the maximal inhibition obtained with 8-brcAMP was 61%.

4.2. Source of Ca^{2+} following activation of $P2Y_2$ receptors

Previous experiments done in Ca^{2+}-free medium (no added $CaCl_2$, 1.5 mM EGTA, see Nobles et al., 1995) have shown that the Ca^{2+} response due to activation of $P2Y_2$ purinoceptor on RBE4 cells is due to the release of Ca^{2+} from intracellular stores. In the present experiments, cyclic nucleotides may be acting primarily by preventing Ca^{2+} mobilisation from intracellular stores. In human platelets, an elevation of either $[cAMP]_i$ or $[cGMP]_i$ was able to decrease the ability of thapsigargin, an inhibitor of store refilling, to release Ca^{2+} from intracellular stores (Geiger et al., 1994). We have been unable to test the direct effects of cyclic nucleotides on Ca^{2+} release in our system, since cyclopiazonic acid (another inhibitor of the Ca^{2+} store ATPase) did not cause a detectable increase in $[Ca^{2+}]_i$ in these brain endothelial cells.

4.3. Effects of cyclic nucleotides on Ca^{2+} mobilisation

In brain capillary endothelial cells in culture, we found that cyclic nucleotides themselves were able to release intracellular Ca^{2+}. Few studies have reported direct effects

of cyclic nucleotides on $[Ca^{2+}]_i$, and some are contradictory. Thus agents that increase $[cAMP]_i$ were shown to cause mobilisation of $[Ca^{2+}]_i$ in chick pineal cells (D'Souza and Dryer, 1994), while Nakashima et al. (1986) found no effect of 8-brcGMP on basal $[Ca^{2+}]_i$ in platelets. To our knowledge, the present study is the first to document direct effects of cyclic nucleotides on intracellular Ca^{2+} at the single cell level. The maximal effects of cyclic nucleotides were, at all concentrations tested, lower than that of 100 μM extracellular ATP (submaximal concentration of ATP). Because of the small effects of cyclic nucleotides on $[Ca^{2+}]_i$, it is unlikely that they deplete the intracellular Ca^{2+} stores. As cyclic nucleotides are able to decrease IP_3 production induced by agonists (Nakashima et al., 1986; Burgess et al., 1989; Campbell et al., 1990; Murthy et al., 1993), an action via inhibition of IP_3 production could be responsible for the present observations. However, further experiments are necessary to resolve this point.

4.4. Inhibition by L-NOARG and indomethacin

The present study shows that regulation of the purinergic response by cyclic nucleotides is dependent on the intracellular concentration of the permeant cyclic nucleotide analogue used. We have shown that the increase in $[Ca^{2+}]_i$ mediated by activation of $P2Y_2$ receptors is decreased by L-NOARG and by indomethacin. L-NOARG is an inhibitor of nitric oxide synthesis, while indomethacin inhibits the cyclooxygenase pathway and prevents further de novo prostaglandin synthesis. In the presence of L-NOARG, NO-dependent stimulation of cGMP production should be blocked, while indomethacin should decrease the prostaglandin-dependent stimulation of cAMP production.

Both L-NOARG (100 μM) and indomethacin (10 μM) caused inhibition of the response to 100 μM ATP. Neither indomethacin nor L-NOARG, at the concentrations chosen, was able to significantly increase $[Ca^{2+}]_i$ of RBE4 cells, so that a direct action on the cellular Ca^{2+} content cannot account for their effects. It has been reported that submicromolar concentrations of indomethacin are able to inhibit cAMP-dependent protein kinases (Kantor and Hampton, 1978). Such a mechanism could explain the recovery of the ATP response in the presence of indomethacin plus 8-brcAMP.

4.5. Interaction between the second messenger pathways

This study suggests a close relationship between the prostanglandin/cAMP pathway, the nitric oxide/cGMP pathway, and the phosphoinositide/Ca^{2+} pathway in brain endothelial cells in culture. Manoeuvres that led to an increase in intracellular cyclic nucleotide levels (by using membrane-permeant derivatives), or that inhibited the prostanglandin or nitric oxide pathways led to perturbations of the Ca^{2+} signal evoked by extracellular ATP. Cross-talk between second messenger pathways in a clonal brain endothelial preparation has been previously reported by Vigne et al. (1994), activated in that study by natriuretic peptide, by isoproterenol, and by endothelin, and also involving cAMP, cGMP and Ca^{2+}-dependent mechanisms. The present study builds on the earlier work, and confirms the generality of cross-talk between second messenger pathways in brain endothelial cells.

Further investigations will be directed at the molecular mechanisms underlying these interactions, and at their physiological consequences at the level of the brain capillaries. It is interesting that the increase in permeability in capillary endothelium in response to inflammatory mediators such as ATP, is correlated with a rise in $[Ca^{2+}]_i$ (He et al., 1990; Abbott and Revest, 1991; He and Curry, 1993), but the increase in permeability is transient (Lum and Malik, 1994). Negative feedback by co-produced second messengers such as cyclic nucleotides may contribute to regulation of the permeability change (Westendorp et al., 1994).

Acknowledgements

We thank Dr. Pierre-Olivier Couraud and Dr. Francoise Roux for the supply of RBE4 cells. This study was supported by the MRC.

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