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Characterization of the Ca^{2+} responses evoked by ATP and other nucleotides in mammalian brain astrocytes

Carlo Centemeri, Chiara Bolego, [1]Maria P. Abbracchio, Flaminio Cattabeni, Lina Puglisi, *Geoffrey Burnstock & Simonetta Nicosia

Institute of Pharmacological Sciences, University of Milan, Milan, Italy and *Department of Anatomy and Developmental Biology, University College London, London

1 This study was aimed at characterizing ATP-induced rises in cytosolic free calcium ion, $[Ca^{2+}]_i$, in a population of rat striatal astrocytes loaded with the fluorescent Ca^{2+} probe Fura2, by means of fluorescence spectrometry.

2 ATP triggered a fast and transient elevation of $[Ca^{2+}]_i$ in a concentration-dependent manner. The responses of the purine analogues 2-methylthio-ATP (2-meSATP), adenosine-5′-O-(2-thiodiphosphate) (ADPβS), as well as uridine-5′-triphosphate (UTP) resembled that of ATP, while α,β-methylene-ATP (α,β-meATP) and β,γ-methylene-ATP (β,γ-meATP) were totally ineffective.

3 Suramin (50 μM) had only a minor effect on the ATP response, whereas pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) (5 μM) significantly depressed the maximum response.

4 Extracellular Ca^{2+} did not contribute to the observed $[Ca^{2+}]_i$ rise: removing calcium from the extracellular medium (with 1 mM EGTA) or blocking its influx by means of either Ni^{2+} (1 mM) or Mn^{2+} (1 mM) did not modify the nucleotide responses.

5 Furthermore, after preincubation with 10 μM thapsigargin, the nucleotide-evoked $[Ca^{2+}]_i$ increments were completely abolished. In contrast, 10 mM caffeine did not affect the responses, suggesting that thapsigargin-, but not caffeine/ryanodine-sensitive stores are involved.

6 Both application of the G-protein blocker guanosine-5′-O-(2-thiodiphosphate) (GDPβS) (1 mM) and preincubation with pertussis toxin (PTx) (350 ng ml^{-1}) partially inhibited the nucleotide-mediated responses. Moreover, the phospholipase C (PLC) inhibitor U-73122, but not its inactive stereoisomer U-73343 (5 μM), significantly reduced the ATP-evoked $[Ca^{2+}]_i$ rise.

7 In conclusion, our results suggest that, in rat striatal astrocytes, ATP-elicited elevation of $[Ca^{2+}]_i$ is due solely to release from intracellular stores and is mediated by a G-protein-linked P2Y receptor, partially sensitive to PTx and coupled to PLC.

Keywords: Rat brain astrocytes; ATP; P2 receptor; cytosolic Ca^{2+}; Fura2

Introduction

Adenosine 5′-triphosphate (ATP) released into the extracellular space in the mammalian central nervous system (CNS) acts as an intercellular signalling molecule (for reviews, see Abbracchio *et al*., 1996; Burnstock, 1996). These highly specific actions are mediated by the activation of either ligand-gated P2X-receptors (which mediate the fast responses) or G-protein-coupled P2Y-receptors (which mediate the slower metabotropic responses, Abbracchio & Burnstock, 1994; Abbracchio, 1997). In brain, receptors have been localized to both neurones and non-excitable cells (such as astroglia, oligodendroglia and microglia, Abbracchio *et al*., 1995a; Abbracchio, 1997), suggesting that synaptically released ATP may also serve as a physiological mediator of neurone-to-glia communication (Barres, 1991). ATP and other nucleotides are also massively released in brain following a variety of insults, including trauma and ischaemia, which has suggested their involvement in the onset and maintenance of reactive astrogliosis (Neary *et al*., 1996).

In both excitable and non-excitable cells, ATP produces increases in cytosolic free calcium ions, $[Ca^{2+}]_i$ (El-Moatassim *et al*., 1992; Ho *et al*., 1995). In dorsal spinal cord astrocytes, these increases are fully dependent on release from intracellular stores, probably via activation of phospholipase C (PLC) and production of inositol-tris-phosphate (IP_3) (Ho *et al*., 1995). At least 2 different PLC-linked P2-receptors are expressed by these cells (Ho *et al*., 1995): a P2Y-receptor exclusively sensitive to ATP and a P2U receptor which responds to both ATP and uridine-5′-triphosphate (UTP) ($P2Y_1$ and $P2Y_2$ receptors, respectively, according to the nomenclature proposed by Abbracchio & Burnstock, 1994. A detailed pharmacological characterization of these receptors in astrocytes from mammalian brain is still lacking.

By utilizing primary cultures from rat corpus striatum, we have demonstrated that ATP analogues induce long-term trophic effects on astrocytic cells, such as a marked elongation of astrocytic processes, which suggests induction of cell differentiation. Such an action may be relevant to the astrocytic activation occurring in reactive astrogliosis, and apparently involves a G-protein-coupled P2 receptor (Bolego *et al*., 1997). In several instances, early changes in $[Ca^{2+}]_i$ have been related to induction of cell differentiation (Finkbeiner, 1993).

The present work was undertaken to characterize pharmacologically the Ca^{2+} responses to ATP and other nucleotides in astrocytes from mammalian brain, and to investigate the relationship between early $[Ca^{2+}]_i$ increases and the long-term morphological changes induced by ATP on these cells (described in the accompanying paper by Bolego *et al*.).

Methods

Cultures

Astrocytic cultures were prepared from 7 day-old rats as previously described (Abbracchio *et al*., 1994). The cell suspension was diluted to a final concentration of about 6×10^5 viable cells ml^{-1}; 2.5 ml were placed into 35 mm dishes (each containing 5 poly-L-lysine-coated glass coverslips). Cultures were maintained in a 5% CO_2 incubator at 37°C to allow cells to adhere

[1]Author for correspondence at: Institute of Pharmacological Sciences, Via Balzaretti 9, 20133 Milan, Italy

to the dishes. After 24 h, the serum-supplemented medium was replaced with a chemically defined serum-free medium (see Bolego *et al*., 1997). Cultures were maintained for further 24 h in this medium before the Ca^{2+} measurements were performed.

Measurement and calculation of $[Ca^{2+}]_i$

Fura2 acetoxymethylester (Fura2/AM) in dimethylsulphoxide (DMSO) was diluted to 10 μM with HEPES-buffered saline [HBS (mM): NaCl 145, $MgCl_2$ 1, KCl 5, HEPES 10, glucose 10 and $CaCl_2$ 1; pH 7.4], supplemented with 0.03% Pluronic F-127. Astrocytes adherent to glass coverslips were incubated with this Fura2/AM solution for 75 min at 25°C in the dark. The cells were then rinsed 3 times with HBS and kept in the same solution.

$[Ca^{2+}]_i$ was monitored by single excitation fluorimetry (Perkin-Elmer LS5). Each coverslip was placed in the fluorimeter cuvette, containing 1.5 ml of HBS (or Ca^{2+}-free HBS) and thermostated at 30°C. Fluorescence was recorded at 340 nm excitation and 505 nm emission, except for the data shown in Figure 4d (see below).

The experiments depicted in Figure 4b and d were performed in the absence of extracellular calcium; in Figure 4b, extracellular Ca^{2+} was replaced by 1 mM EGTA, cells were exposed to ATP and subsequently 2 mM $CaCl_2$ was added. In Figure 4d, Ca^{2+} was replaced by 1 mM Mn^{2+} and the excitation wavelength was manually alternated between 340 and 360 nm (Ca^{2+}-insensitive fluorescence) every 3–4 s; Ca^{2+} release from intracellular stores is presented as the fluorescence increase at 340 nm, while the drop at 360 nm reveals Mn^{2+} influx through the plasma membrane (Merrit *et al*., 1989).

To evaluate $[Ca^{2+}]_i$, the calibration was performed on each sample: Fura2 leakage was estimated by addition of 0.2 mM $MnCl_2$, followed by 0.5 mM CaDTPA. To obtain the maximal fluorescence (F_{max}), 10 mM $CaCl_2$, 4-Br-A23187 (2.3 μM) and digitonin (100 μM) were added sequentially. Finally, $MnCl_2$ (20 mM) was added to record the autofluorescence of the system. Intracellular Ca^{2+} values were obtained from the observed fluorescence (F) according to Tsien *et al*. (1982), Grynkiewicz *et al*. (1985) and Hesketh *et al*. (1983). F,F_{min} and F_{max} values were corrected for autofluorescence (i.e., fluorescence variations upon additions to unloaded astrocytes).

Data analysis

The concentration-response curves were analysed and drawn by means of the computer programme ALLFIT (De Lean *et al*., 1978), which calculates the lower and upper plateaux, the slope and the EC_{50}, as well as their s.e. The programme also allows comparison of two or more curves. Mean data were fitted and the statistical significance of the parameter difference was evaluated by means of the F test for the extra sum of squares, taking $P<0.05$ as statistically significant.

Student's t test for unpaired data was used to compare two means.

Each data point was obtained from a single coverslip. All data are mean ± s.e. mean of results from at least 3 separate experiments performed at least in duplicate (except for data in Tables, where % coefficient of variation is shown).

Materials

Fura2/AM was from Molecular Probes and was stored in anhydrous DMSO at −80°C. 4-Br-A23187 (Sigma) and digitonin (Merck) were dissolved in DMSO. Pluronic F-127 (trade mark of BASF Wyandotte) was from Molecular Probes. Solutions for $[Ca^{2+}]_i$ assay were prepared with water for high performance liquid chromatography. Hank's Balanced Salt Solution (HBSS), HBSS without calcium and magnesium, FCS, Medium 199, PBS and glucose solution were purchased from Gibco BRL. Trypsin, DNAase I, ATP, α,β-methylene-ATP (α,β-meATP), β,γ-methylene-ATP (β,γ-meATP), 2-methylthio-ATP (2-meSATP), adenosine-5′-O-(2-thiodiphosphate) (ADPβS), guanosine-5′-O-(2-thiodiphosphate) (GDPβS), uridine-5′-triphosphate (UTP), pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), 5′-*p*-fluorosulphonyl-benzoyladenosine and caffeine (Sigma) were dissolved in HBS. Thapsigargin (Sigma) and U-73122 (1-[6-[[17β-3-methoxyestra-1-3-5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione) and U-73343 (1-[6-[[17β-3-methoxyestra-1-3-5(10)-trien-17-yl]amino]hexyl]-2,5-pyrolidine-dione) (kindly provided by J. Neary, University of Miami, School of Medicine) were dissolved in DMSO. The final concentration of DMSO in the samples (max 0.1%) had no effect on calcium levels. Pertussis toxin (PTx) was dissolved in 50% glycerol. Suramin (dissolved in HBS) was a kind gift from Bayer.

Results

Pharmacological characterization of ATP-induced $[Ca^{2+}]_i$ elevation

In a pure population (at least 95% astroglial cells) of rat striatal astrocytes, ATP triggered a fast and transient elevation of cytosolic calcium levels, $[Ca^{2+}]_i$, from a basal level of 223.7 ± 7.4 ($n=20$) to 420.7 ± 24.5 nM at the highest concentration of ATP tested (1 mM) ($n=5$). The effect was concentration-dependent (Figure 1), with $EC_{50}=43\pm37$ μM and was not apparently affected by apyrase; indeed, 30 min preincubation with 100 μM 5′-*p*-fluorosulphonyl-benzoyladenosine, a well known inhibitor of apyrase (Marti *et al*., 1996), did not significantly modify the response to 100 μM ATP ($[Ca^{2+}]_i$ increase over basal: 1.8 ± 0.2 and 1.6 ± 0.1 in the absence and presence of the inhibitor, respectively; $n=3$).

To characterize the pharmacological profile of the receptor involved, we evaluated the effect of different receptor agonists and antagonists. The effect of ATP was mimicked by the purine analogues 2-methylthio-ATP (2-meSATP) and adenosine-5′-O-(2-thiodiphosphate) (ADPβS), as well as by uridine-5′-triphosphate (UTP). The parameters of the curves are shown in Table 1. In contrast, adenosine was devoid of any effect on $[Ca^{2+}]_i$ (data not shown).

Figure 2 shows that 100 μM α,β-methylene-ATP (α,β-meATP) and 100 μM β,γ-methylene-ATP (β,γ-meATP) were unable to elicit $[Ca^{2+}]_i$ elevation in a sample which responded to ATP ($n=4-5$).

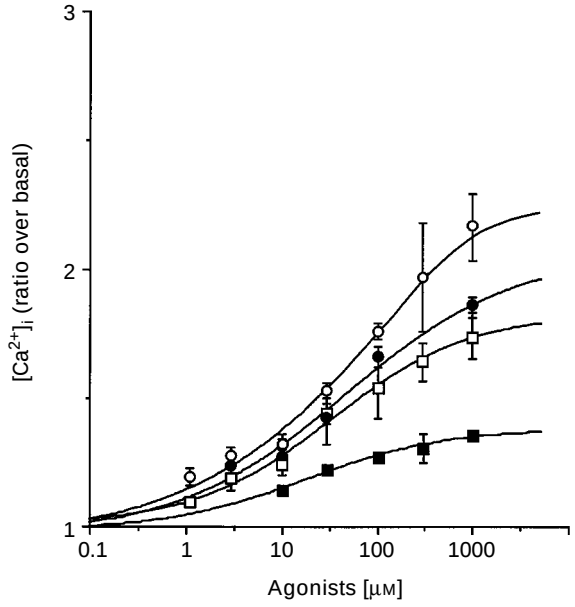

Figure 1 Rat striatal astrocytes. Concentration-response curves for 2-meSATP (○, $n=3-5$), ATP (●, $n=5-8$), ADPβS (□, $n=3-5$) and UTP (■, $n=3-6$) on $[Ca^{2+}]_i$. Each point is the mean of the peak response to the agonists at each concentration; vertical lines show s.e.mean.

Table 1 Parameters of the concentration-response curves for $[Ca^{2+}]_i$ elevation by the agonists

	EC_{50} (μM) (95%CL)	Slope (95%CL)	E_{max} (s/b) (95%CL)
2-me-SATP	69 (48–100)	0.54* (0.45–0.64)	2.41 (1.6–3.6)
ATP	43 (1.6–1390)	0.55* (0.14–2.2)	2.01 (1.3–3.0)
ADPβS	33 (14–76)	0.60* (0.44–0.83)	1.82 (1.7–2.0)
UTP	20 (0.61–658)	0.63 (0.06–7.1)	1.37 (1.0–1.8)

Data are from Figure 1; *$P<0.05$ vs unit. E_{max}: maximum response attained; s/b: ratio of stimulated over basal values. CL: confidence limits.

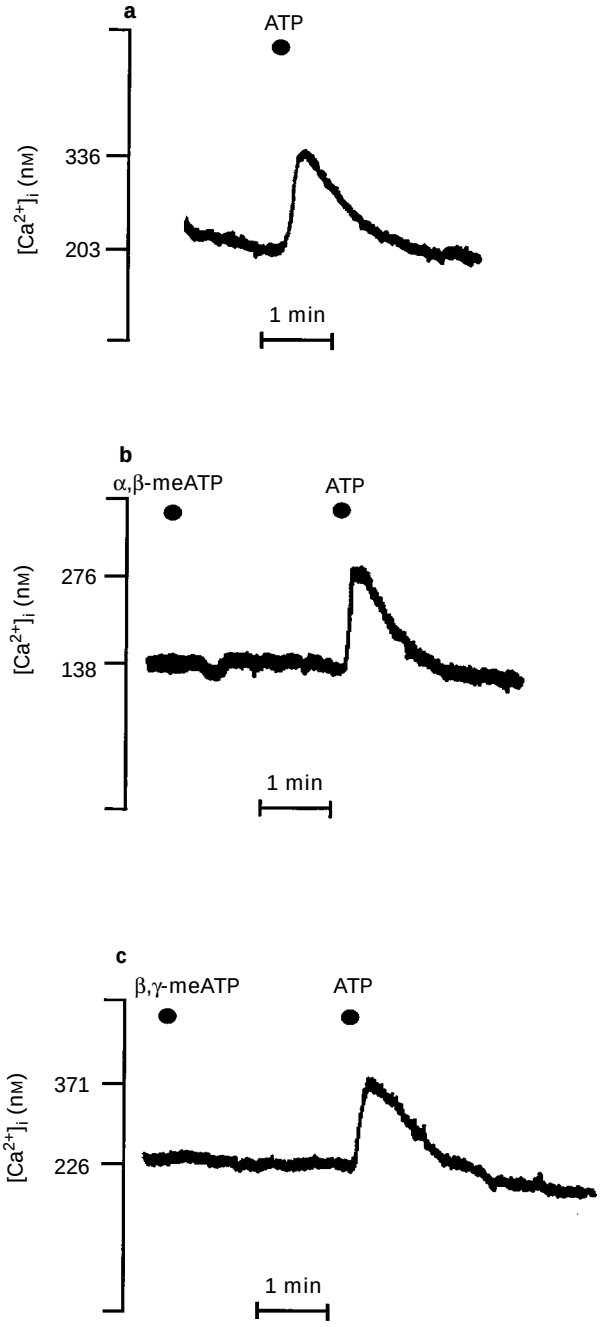

Figure 2 Rat striatal astrocytes. Effects of P2-purinoceptor agonists on $[Ca^{2+}]_i$. (a) Representative tracing showing $[Ca^{2+}]_i$ rise evoked by 100 μM ATP ($n=20$). (b) and (c) The lack of effect on $[Ca^{2+}]_i$ of 100 μM α,β-meATP ($n=4$) and 100 μM β,γ-meATP ($n=5$), respectively, in samples which responded to a subsequent stimulation with ATP.

The effect of the P2 antagonists suramin and pyridoxal phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), preincubated for 5 min, was evaluated: while suramin (50 μM) had only a minor effect on the concentration-response curve for ATP (Figure 3), the same concentration of PPADS completely abolished the ATP-induced $[Ca^{2+}]_i$ rise at any concentration tested (data not shown). At 5 μM, PPADS markedly decreased the upper plateau, the difference being statistically significant ($P<0.05$). The parameters of the ATP concentration-response curves in the presence and absence of antagonists are shown in Table 2.

Finally, since ATP has been demonstrated to induce differentiation of rat striatal astrocytes (Abbracchio *et al*., 1995b; Bolego *et al*., 1997), the effect on $[Ca^{2+}]_i$ of bFGF (basic fibroblast growth factor), a typical trigger for differentiation in these cells, was investigated. bFGF at 1 ng ml^{-1} was unable to affect $[Ca^{2+}]_i$ (data not shown).

Source of Ca^{2+}

Different experimental approaches were used to evaluate whether ATP-induced $[Ca^{2+}]_i$ elevation was due to either influx from the extracellular space or release from the intracellular

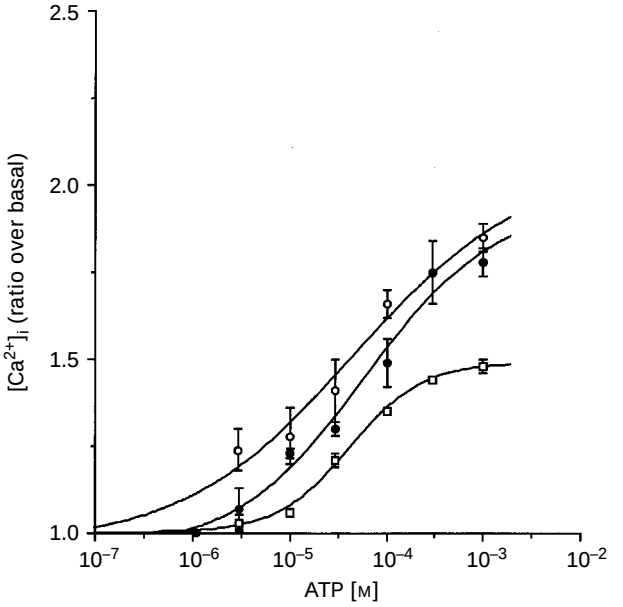

Figure 3 Rat striatal astrocytes. Effect of 50 μM suramin and 5 μM PPADS on ATP-induced $[Ca^{2+}]_i$ rise. The antagonists were preincubated for 5 min before the addition of ATP. Concentration-response curves of ATP in the presence (●, suramin, $n=3-5$; □, PPADS, $n=4-6$) or absence (○) of antagonists. Each point is the mean of the peak response to the agonists at each concentration; vertical lines show s.e.mean.

Table 2 Parameters of ATP concentration-response curves for $[Ca^{2+}]_i$ elevation in the absence and presence of PPADS or suramin

	EC_{50} (μM) (95%CL)	Slope (95%CL)	E_{max} (s/b) (95%CL)
ATP	51.4 (2.2–1196)	0.52* (0.19–1.4)	2.04 (1.5–2.8)
ATP + 5μM PPADS	42.4 (11–162)	1.16 (0.34–3.9)	1.49† (1.2–1.8)
ATP + 50μM suramin	62.6 (13–295)	0.79 (0.41–1.5)	1.89 (1.6–2.2)

Data are from Figure 2; *$P<0.05$ vs unit; †$P<0.05$ vs ATP alone. E_{max}: maximum response attained; s/b: ratio of stimulated over basal values. CL: confidence limits

organelles. Figures 4 shows that the removal of Ca^{2+} from the extracellular medium (b) did not significantly affect the response to 100 μM ATP ($n=4$, compare with (a)), indicating that the response is caused predominantly by discharge from the intracellular stores. Accordingly, the subsequent addition of 2 mM $CaCl_2$ caused only a minor increment in $[Ca^{2+}]_i$ (which occurred also in the absence of agonist, data not shown). The same pattern of response was obtained with 100 μM ADPβS, 30 μM 2-meSATP and 1 mM UTP (data not shown). The second approach (c) utilized 1 mM Ni^{2+}, which blocks Ca^{2+} entry (Fox *et al*., 1987; Tsien *et al*., 1988): in this case as well, ATP-induced $[Ca^{2+}]_i$ elevation was not affected, confirming the lack of Ca^{2+} influx from the extracellular space ($n=4$); the same applied to ADPβS and UTP (data not shown). Finally, the hypothesis that ATP triggers almost exclusively Ca^{2+} release was confirmed by using Mn^{2+}, which enters the cells through Ca^{2+} channels. Figure 4d indicates that there was no divalent cation influx upon ATP addition, while Ca^{2+} discharge did take place ($n=4$).

Characterization of the intracellular Ca^{2+} pool

Thapsigargin (10 μM), which inhibits the endoplasmic reticulum (ER) Ca^{2+}-ATPase (Thastrup *et al*., 1989), causes emptying of an ER store and consequent increase in $[Ca^{2+}]_i$ (Figure 5b); application of 100 μM ATP after thapsigargin was almost ineffective (compare Figure 5b with Figure 5a), indicating that ATP released Ca^{2+} from the thapsigargin-sensitive pool ($n=3$). Thapsigargin abolished the response to 100 μM ADPβS, 30 μM 2-meSATP and 1 mM UTP, as well (data not shown). In contrast, 10 mM caffeine (Ehrlich *et al*., 1994) was without effect on $[Ca^{2+}]_i$ (Figure 5c), suggesting that these cells might not possess a caffeine/ryanodine-sensitive store; accordingly, the subsequent addition of ATP (Figure 5c) yielded a response indistinguishable from the control one (Figure 5a, $n=3$).

Nature of the coupling between the ATP receptor and $[Ca^{2+}]_i$ elevation

The effect of guanosine-5′-O-(2-thiodiphosphate) GDPβS, a GDP analogue known to inhibit the function of heterotrimeric G-proteins (Cassel *et al*., 1979) even in intact cells (Sim *et al*., 1995), is shown in Figure 6: GDPβS (1 mM) markedly inhibited the response triggered by 100 μM ATP, thus suggesting that this agonist elicits $[Ca^{2+}]_i$ elevation through a G-protein mediated process. In addition, pertussis toxin (PTx, 350 ng ml^{-1}, Figure 7) significantly reduced ATP-evoked $[Ca^{2+}]_i$ increase (57% inhibition, $P<0.001$, $n=3$).

U-73122, an inhibitor of phospholipase C (PLC; Smith *et al*., 1990) at 5 μM was able to abolish the effect of 100 μM ATP on $[Ca^{2+}]_i$ (Figure 8a), which therefore is likely to be mediated by products of phosphatydyl inositol turnover. U-73343 (5 μM), the inactive stereoisomer of U-73122, did not affect the response to ATP (Figure 8b, $n=3$).

Discussion

In the present study we showed that rat striatal astrocytes possess ATP receptors coupled to $[Ca^{2+}]_i$ elevation. ATP-induced increase in $[Ca^{2+}]_i$ has been demonstrated in many different cell types (Burnstock, 1993), and is believed to mediate many of the physio-pathological actions of this purine. In particular, in astroglial cells, ATP has been shown to affect $[Ca^{2+}]_i$, with different molecular mechanisms; in some cell types (Jahromi *et al*., 1992; Salter & Hicks, 1994), ATP-elicited $[Ca^{2+}]_i$ increase was rapid, transient and monophasic, usually due to discharge of the ion from intracellular stores and possibly mediated through the formation of inositol thisphosphate (IP_3). In other systems, the pattern of response was biphasic (Neary *et al*., 1991; Kastritis *et al*., 1992; Lin *et al*., 1992; Walz *et al*., 1994), consisting of both Ca^{2+} release from the stores

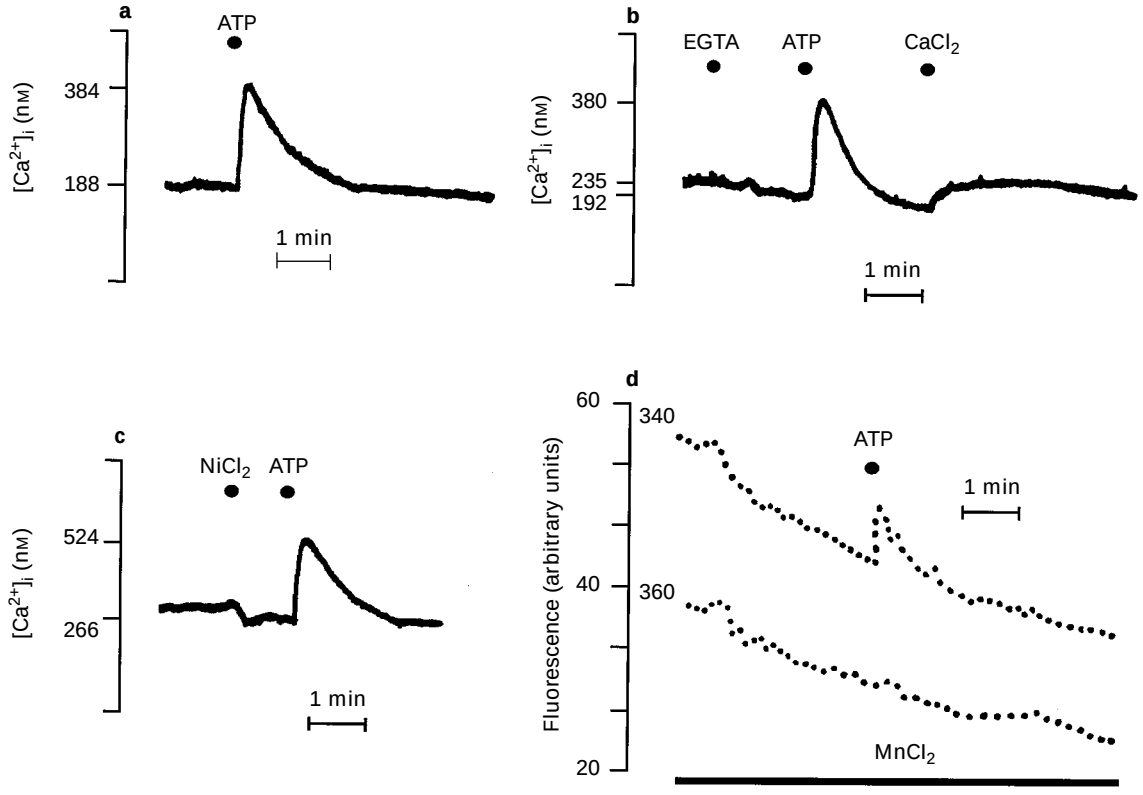

Figure 4 Rat striatal astrocytes. Effect of extracellular Ca^{2+} on the $[Ca^{2+}]_i$ rise induced by 100 μM ATP. (a) Representative trace showing control response to ATP in the presence of 1 mM $[Ca^{2+}]_o$ ($n=35$). (b) Typical trace illustrating the effect of ATP in Ca^{2+}-free medium (1 mM EGTA, $n=4$). After ATP challenge, $[Ca^{2+}]_o$ was restored by addition of 2 mM $CaCl_2$. (c) Representative trace showing ATP response when 1 mM $NiCl_2$ had been added to the extracellular solution ($n=4$). (d) Experimental points showing the responses to ATP in the presence of 1 mM Mn^{2+} ($[Ca^{2+}]_o=0$), recorded at 340 and 360 nm excitation wavelengths ($n=4$).

and influx from the extracellular space. Finally, in a few instances (van den Pol *et al*., 1992; Kastritis *et al*., 1992), ATP has been shown to increase the frequency and amplitude of spontaneous Ca^{2+} oscillations.

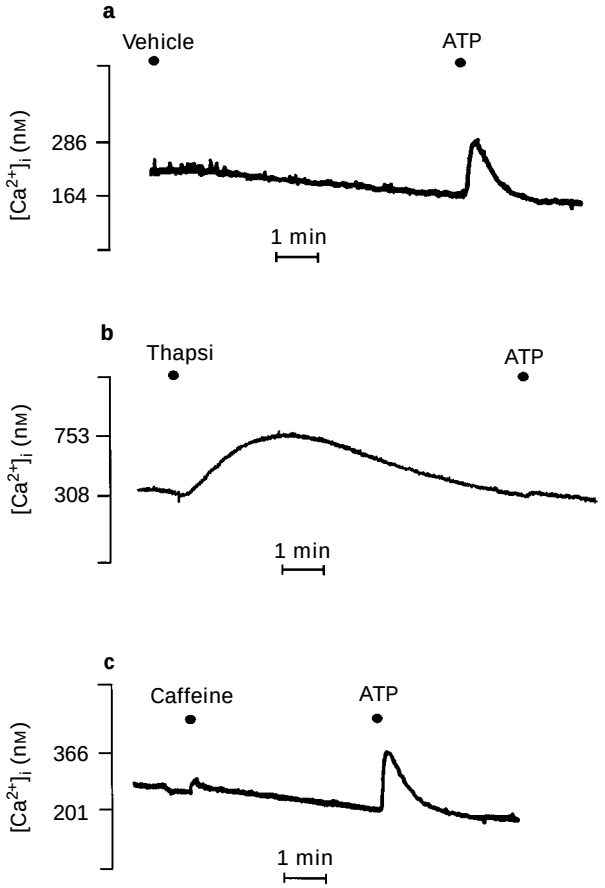

Figure 5 Rat striatal astrocytes. Effect of thapsigargin and caffeine on the $[Ca^{2+}]_i$ elevation caused by 100 μM ATP. (a) Representative trace showing control response to ATP in the presence of vehicle, added before the agonist ($n=3$). (b) Representative trace of the effect of 10 μM thapsigargin (thapsi) on ATP-induced $[Ca^{2+}]_i$ elevation ($n=3$). (c) Typical trace showing an ATP response in the presence of 10 mM caffeine ($n=3$).

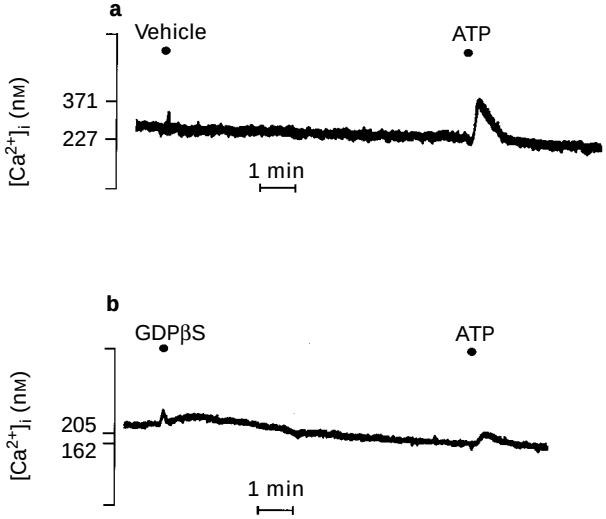

Figure 6 Rat striatal astrocytes. Effect of 1 mM GDPβS on $[Ca^{2+}]_i$ rise induced by 100 μM ATP. (a) Representative trace showing control response to ATP in the presence of vehicle added before the agonist ($n=3$). (b) Representative trace of ATP response 10 min after GDPβS addition ($n=3$).

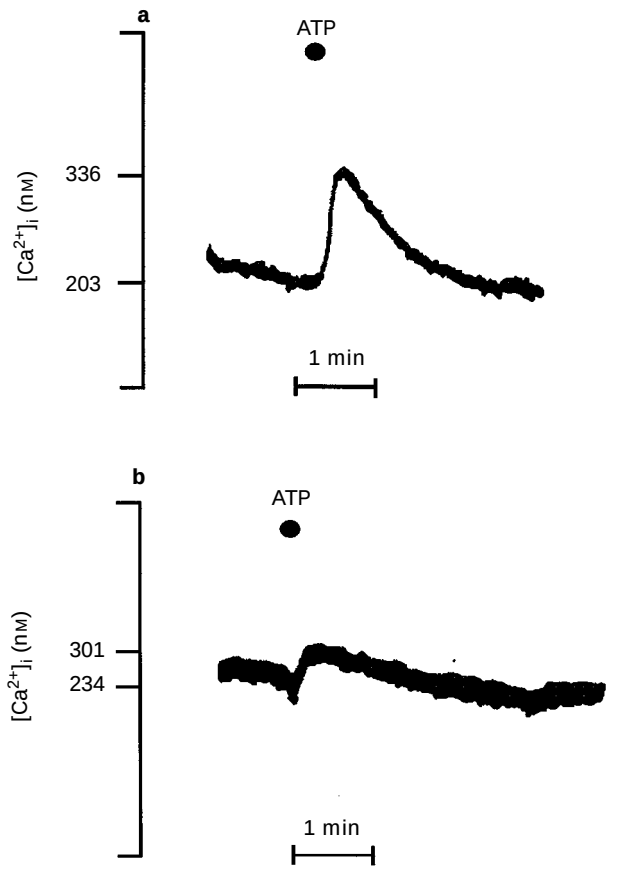

Figure 7 Rat striatal astrocytes. Effect of pertussis toxin (350 ng ml^{-1}), incubated for 18 h, on ATP-induced $[Ca^{2+}]_i$ rise. (a) Representative trace showing $[Ca^{2+}]_i$ rise evoked by 100 μM ATP ($n=3$). (b) Representative trace of ATP response after pretreatment with pertussis toxin ($n=3$).

We demonstrate here that, in rat striatal astrocytes, ATP triggers a rapid, monophasic $[Ca^{2+}]_i$ elevation, which returns to basal levels within 2.5 ± 0.5 min and is due solely to discharge from the intracellular stores; indeed, chelation of extracellular Ca^{2+} with EGTA, or blockade of Ca^{2+} entry with NI^{2+} did not modify the response significantly. Moreover, monitoring of divalent cation entry by means of Mn^{2+} confirmed the lack of Ca^{2+} influx. The intracellular Ca^{2+} pool involved in the nucleotide response overlaps, at least in great part, with the thapsigargin-sensitive stores (Thastrup *et al*., 1989); indeed, depleting such stores with thapsigargin almost completely inhibited the subsequent response to ATP and the other nucleotides.

These responses are mediated by receptors belonging to the P2Y-receptor family, based on the following evidence: (1) ATP and the other nucleotides did not induce influx of Ca^{2+} from the extracellular space, which rules out the intervention of ligand-gated P2X-channels; (2) Ca^{2+} rises were prevented by either GDPβS or PTx, which block G-proteins, consistent with the proposal that all P2Y-receptors are G-protein-coupled receptors (Abbracchio & Burnstock, 1994). In particular, in rat brain astroglial cells, the P2Y-receptors identified in this study are coupled to stimulation of PLC, as suggested by abolition of $[Ca^{2+}]_i$ responses by the PLC inhibitor, U-73122.

The main purpose of this study was to investigate whether short-term Ca^{2+} responses induced by nucleotides on astroglial cells are involved in their long-term trophic actions (see accompanying paper by Bolego *et al*., 1997). Indeed, early cytoplasmatic Ca^{2+} rises have been implicated in modulation of glial differentiation and proliferation by different agents such as bFGF, PDGF and endothelin (for review see Finkbeiner, 1993). The present results indeed show that neither the non-hydrolysable ATP analogue α,β-meATP nor the polypeptidic

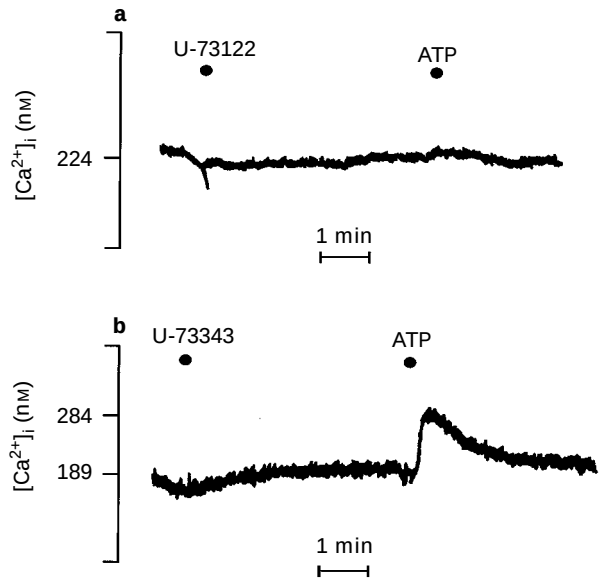

Figure 8 Rat striatal astrocytes. Effect of the PLC inhibitor U-73122 (5 μM, $n=3$) (a) and its inactive stereoisomer U-73343 (5 μM, $n=3$) (b) on ATP-evoked $[Ca^{2+}]_i$ elevation.

growth factor basic fibroblast growth factor (bFGF) could increase $[Ca^{2+}]_i$, despite their ability to trigger morphological differentiation of brain astrocytes (Bolego *et al*., 1997). Therefore, in our system, early changes in $[Ca^{2+}]_i$ are not involved in the induction of morphological differentiation by purine analogues (which is instead mediated by other P2Y-receptors linked to different second messenger systems; see accompanying paper by Bolego *et al*., 1997), although $[Ca^{2+}]_i$ changes might play a role in short-term communication between glial cells or between glia and neurones (Nedergaard, 1994).

There are a number of similarities, but also some interesting differences, between the P2Y-receptors expressed by brain astrocytes and the Ca^{2+}-mobilizing receptors identified by Ho *et al*. (1995) in astrocytes from the spinal cord. Spinal cord astrocytes express at least two different subtypes of P2Y-receptors, one responding to ATP and ATP analogues and linked to the PLCβ/IP_3 pathway (Salter & Hicks, 1995), the other responsive to both ATP and UTP and linked to Ca^{2+} mobilization from thapsigargin-sensitive stores ($P2Y_1$ and $P2Y_2$ receptor, respectively, according to Abbracchio & Burnstock, 1994). In our system as well, both ATP and UTP triggered $[Ca^{2+}]_i$ rises by releasing Ca^{2+} from thapsigargin-sensitive stores. The concentration-response curves for ATP, 2-me-SATP and ADPβS spanned more than two orders of magnitude (slopes significantly different from 1), suggesting that these responses may be mediated by the activation of more than one receptor. Thus, in brain too, at least two different P2-receptors exist, and work is in progress in our laboratory to characterize the receptor subtypes involved and to investigate the cross-reactivity of ATP and UTP. The existence of two receptors might also explain the apparently low potency of some of the agonists, particularly 2-me-SATP. Indeed, the EC_{50} value obtained might be a value intermediate between a higher and a lower EC_{50}, representing an interaction with the two receptors.

In spinal cord astrocytes, both ATP- and UTP-mediated responses were blocked by the P2-receptor antagonists PPADS and suramin. In contrast, in our system, significant antagonism was only observed with PPADS, which significantly depressed the maximum response. The latter may reflect a non-competitive mechanism of the antagonist, or, alternatively, the presence of heterogeneous receptors (see above).

No marked block of the Ca^{2+} responses elicited by ATP was obtained with suramin. However, the slight change in slope (with respect to unit) of ATP curves in the presence and absence of suramin might be related to the above mentioned heterogeneity of ATP receptors: one might hypothesize that suramin blocks only one of the two receptors possibly present. Indeed, suramin-insensitive responses to nucleotides have been demonstrated in a number of tissues, such as vas deferens (von Kügelgen *et al*., 1990) and bladder (Bolego *et al*., 1995). Furthermore, suramin also failed to block the recombinant $P2X_4$-receptor expressed in oocytes (Bo *et al*., 1995; Buell *et al*., 1996).

In conclusion, rat striatal astrocytes possess P2Y receptors coupled to mobilization of Ca^{2+} from intracellular stores; this receptor may be responsible for transducing short-term responses to nucleotides, but is not apparently responsible for the long-term trophic changes induced by these compounds on mammalian brain astrocytes.

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