British Journal of Pharmacology (1997) 121, 1692–1699

Characterization of the signalling pathways involved in ATP and basic fibroblast growth factor-induced astrogliosis

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1 A brief challenge of rat astrocytes with either α,β-methyleneATP (α,β-meATP) or basic fibroblast growth factor (bFGF) resulted, three days later, in morphological differentiation of cells, as shown by marked elongation of astrocytic processes. The P2 receptor antagonist suramin prevented α,β-meATP- but not bFGF-induced astrocytic elongation. Similar effects on astrocytic elongation were also observed with ATP and other P2 receptor agonists (β,γmeATP, ADPβS, 2meSATP and, to a lesser extent, UTP).

2 Pertussis toxin completely abolished α,β-meATP- but not bFGF-induced effects. No effects were exerted by α,β-meATP on cyclic AMP production; similarly, neomycin had no effects on elongation of processes induced by the purine analogue, suggesting that adenylyl cyclase and phospholipase C are probably not involved in α,β-meATP-induced effects (see also the accompanying paper by Centemeri *et al*., 1997). The tyrosine-kinase inhibitor genistein greatly reduced bFGF- but not α,β-meATP-induced astrocytic elongation.

3 Challenge of cultures with α,β-meATP rapidly and concentration-dependently increased [^{3}H]-arachidonic acid (AA) release from cells, suggesting that activation of phospholipase A_2 (PLA_2) may be involved in the long-term functional effects evoked by purine analogues. Consistently, exogenously added AA markedly elongated astrocytic processes. Moreover, various PLA_2 inhibitors (e.g. mepacrine and dexamethasone) prevented both the early α,β-meATP-induced [^{3}H]-AA release and/or the associated long-term morphological changes, without affecting the astrocytic elongation induced by bFGF. Finally, the protein kinase C (PKC) inhibitor H7 fully abolished α,β-meATP- but not bFGF-induced effects.

4 Both α,β-meATP and bFGF rapidly and transiently induced the nuclear accumulation of Fos and Jun. Both c-fos and c-jun induction by the purine analogue could be fully prevented by pretreatment with suramin. In contrast, the effects of bFGF were unaffected by this P2 receptor antagonist.

5 It was concluded that α,β-meATP- and bFGF-morphological differentiation of astrocytes occurs via independent transductional pathways. For the purine analogue, signalling involves a G_i/G_o protein-coupled P2Y-receptor which may be linked to activation of PLA_2 (involvement of an arachidonate-sensitive PKC is speculated); for bFGF, a tyrosine kinase receptor is involved. Both pathways merge on some common intracellular target, as suggested by induction of primary response genes, which in turn may regulate late response genes mediating long-term phenotypic changes of astroglial cells.

6 These findings implicate P2 receptors as novel targets for the pharmacological regulation of reactive astrogliosis, which has intriguing implications in nervous system diseases characterized by degenerative events.

Keywords: Rat brain astrocytes: glial fibrillary acidic protein (GFAP); P2 receptors; basic fibroblast growth factor (bFGF) receptors; primary response genes; astrocytic differentiation

Introduction

Astroglial cells are recognized to play crucial roles in both central nervous system (CNS) development (Rakic, 1991), neurotransmission (Barres, 1991), and in the aetiopathology of neurodegenerative diseases (Eddleston & Mucke, 1993). Astrocytes react to various types of injury with astrogliosis, a reaction characterized by increased expression of the astroglial specific marker glial fibrillary acidic protein (GFAP) and elongation of GFAP-positive astrocytic processes. These morphological changes are taken as an index of the astroglial cell differentiation occurring in every type of astrogliosis, even in the absence of astrocytic proliferation (e.g. foetal anoxia, Hatten *et al*., 1991). Reactive astrogliosis may play a role in post-traumatic brain recovery (Hatten *et al*., 1991; Weinstein *et al*., 1991), as well as in inflammatory demyelinating diseases, AIDS dementia, and such neurodegenerative diseases as Alzheimer's (Eddleston & Mucke, 1993). Characterization of the endogenous factors that regulate astroglial cell function and differentiation has therefore important implications in nervous system pathophysiology.

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In the nervous system, purines have well characterized functions as neurotransmitters and neuromodulators (Williams & Jacobson, 1995; Burnstock, 1996; Abbracchio, 1997), that are mediated by specific receptors for nucleosides (P1 receptors) or nucleotides (P2 receptors, further subdivided in ligand-gated P2X and G-protein-coupled P2Y receptor families) (Abbracchio & Burnstock, 1994; Surprenant *et al*., 1995). In brain, adenosine 5′-triphosphate (ATP) has been implicated as a physiological mediator of neurone-to-glia communication (Barres, 1991; Abbracchio *et al*., 1995a), and, based on massive release of ATP and other nucleotides in ischaemic and injured brain, a trophic role for this nucleotide in the initiation and maintenance of reactive astrogliosis has been suggested (for review, see Neary *et al*., 1996). In particular, we have previously demonstrated that a long-term (3 day) exposure of rat brain primary cultures to the relatively hydrolysis-resistant ATP analogue α,β-methyleneATP (α,β-meATP) resulted in morphological differentiation of astroglial cells, as shown by a concentration-dependent elongation of GFAP-positive processes (Abbracchio *et al*., 1995b). Qualitatively comparable effects were induced on the same cells by a classic trigger of reactive astrogliosis, such as basic fibroblast growth factor

(bFGF). Suramin selectively counteracted α,β-meATP- but not bFGF-induced astrocytic differentiation, suggesting that both the purine analogue and the polypeptidic growth factor can promote the formation of reactive astrocytes through the activation of distinct transduction pathways (Abbracchio *et al*., 1995b).

The aim of the present study has been the characterization of the signalling mechanisms utilized by purine derivatives and bFGF in inducing astroglial cell differentiation. To do so, we have tested the effects of a number of pharmacological agents (which are known to interact with various transductional pathways at different levels) on the elongation of GFAP-positive processes induced by brief challenges with either purine analogues or bFGF. Moreover, since challenge of astrocytes with various stimuli is accompanied by a rapid and specific induction of primary response genes (Arenander *et al*., 1989; Morgan & Curran, 1991; Eddleston & Mucke, 1993) that play a key role in mediating the long-term phenotypic changes of these cells (Neary *et al*., 1996), we have studied the induction of the c-fos and c-jun proto-oncogene products in cultures exposed to either α,β-meATP or bFGF.

Methods

Cell culture and experimental protocol

Rat brain primary cultures were prepared by a slight modification of the previously described method (Abbracchio *et al*., 1994; 1995b). Cells were plated in serum-supplemented medium; after 24 h (day 1 in culture), the serum-supplemented medium was replaced with a chemically defined serum-free medium, containing 0.02 μM progesterone, 0.12 iu ml^{-1} insulin, 0.03 μM sodium selenite, 5 mg ml^{-1} transferrin, 100 mM putrescine, 0.08% bovine serum albumin (BSA) and 5 mg ml^{-1} glucose. At day 2 in culture, cells were challenged with the various pharmacological agents (purine or pyrimidine analogues or bFGF, as described in the legends of the tables or figures). Contact time for P2 receptor agonists and bFGF was 60–120 min, as indicated in the legends of the figures and tables. With the exception of pertussis toxin (PTx), which required a 18 h preincubation period, all other agents (suramin, mepacrine, dexamethasone, H7, neomycin or genistein) were added to the cells 30 or 60 min before the challenge with the differentiating agents, as indicated. After the challenge, cultures were washed, placed in chemically defined serum-free medium and grown for 3 additional days before fixing and immunostaining with an anti-GFAP antibody (see below). In selected experiments where the induction of primary response genes was evaluated, immediately after the challenge with the differentiating agents, cells were placed in chemically defined serum-free medium and fixed after 0–300 min (see below). A phenotypic characterization of cultures grown under the above described experimental conditions showed that astroglial cells account for more than 95% of total cell number; no significant labelling with specific neuronal markers (e.g. microtubule-associated protein 2; MAP-2) was obtained (data not shown).

Immunofluorescence staining and analysis of GFAP positive processes

Astrocytes were identified as previously described (Abbracchio *et al*., 1994) by indirect immunofluorescence by use of rabbit anti-GFAP immunoglobulins (1 : 500; overnight at room temperature), sequentially followed by incubation with biotinylated donkey anti-rabbit secondary antibody (1 : 250; 1 h at room temperature), and streptavidin-fluorescein (1 : 100; 1 h at room temperature). All antibodies were diluted in antibody-diluting solution containing 0.1% Triton X-100, 0.1% sodium azide and 0.01% BSA in phosphate-buffered saline (PBS). Labelled cells were examined by a Zeiss fluorescence microscope equipped with a fluorescein filter. The length of GFAP-positive astrocytic processes was measurred with an image video system consisting of a Macintosh computer (equipped with NIH Image 1.47 software) and video camera connected to the microscope.

Assay of adenylyl cyclase activity

Adenylyl cyclase activity was assayed by the conversion of [^{32}P]-ATP to [^{32}P]-adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP), as previously described (Abbracchio *et al*., 1989). Cultures were grown in 100 × 20 mm Petri dishes (about 2×10^6 cells/dish). At day 2 in culture, cells were scraped from culture dishes, homogenized in 80 mM Tris-maleate, pH 7.4, containing 2 mM dithiothreitol (10 strokes with a Dounce homogenizer), centrifuged (15 min at 11,500 × *g*) and the pellets were resuspended in the same buffer. Aliquots (approximately 15–20 μg of protein/sample) were incubated for 15 min at 33°C in the absence (basal activity) or presence of increasing concentrations of α,β-meATP, in medium containing 0.5 mM ATP, 0.2 mM EGTA, 2 mM $MgCl_2$, 1 mM cyclic AMP, 80 mM Tris-maleate (pH 7.4), 10 μM guanosine 5′-triphosphate (GTP), 0.2 mM papaverine to inhibit cyclic AMP-phosphodiesterases, a regenerating system consisting of 6.3 mM creatine phosphate and 60 μg/sample creatine phosphokinase, approx. 10^4 d.p.m./sample [^{3}H]-cyclic AMP to measure chromatographic recovery, 1 μCi/sample [^{32}P]-ATP and 2 iu ml^{-1} adenosine deaminase (ADA) to degradate endogenous adenosine. In some experiments, to evaluate possible inhibitory effects of ATP analogues on cAMP formation, better basal adenylyl cyclase activity was stimulated by addition of the direct activator forskolin (10 μM final concentration). After the incubation, samples were stopped by the addition of 2% sodium dodecyl-sulphate, 1.3 mM cyclic AMP and 40 mM ATP and boiled for 4 min. [^{32}P]-cyclic AMP was purified by double-column chromatography on AG-50X4 and alumina columns, as previously described (Salomon *et al*., 1974). Results were calculated as pmol cyclic AMP $min^{-1}mg^{-1}$ protein.

Measurement of [^{3}H]-arachidonic acid (AA) release

[^{3}H]-AA release was measured by utilizing a standardized methodology (Bruner & Murphy, 1990; Stella *et al*., 1994; Petroni *et al*., 1994) with slight modifications. Briefly, astrocytes cultured in 6-well dishes were incubated overnight with [^{3}H]-AA (0.3 μCi ml^{-1}) added to the serum-free culture medium. After two washes with PBS w/o calcium and magnesium (0.1% albumin), cultures were incubated at 37°C with medium containing either α,β-meATP (at various concentrations, in the absence or presence of mepacrine, as indicated) or 2 mM glutamate (as a positive control for AA release from cells of the astrocytic lineage, Stella *et al*., 1994). Incubation time was 15 min; as expected (Bruner & Murphy, 1990), [^{3}H]-AA release increased linearly with time up to 30 min. After incubation, the culture medium was collected, centrifuged at 3,000 r.p.m. for 5 min and radioactivity estimated in an aliquot of supernatants. Adhering cells were washed twice with cold PBS, lysed by addition of 0.1% Triton-X100 for 10 min, scraped and counted to allow quantification of incorporated radioactivity. For each experimental sample, results are calculated as amount of radioactivity released in the medium/total amount of radioactivity ([^{3}H]-AA released + [^{3}H]-AA incorporated) and expressed as % of basal control release.

Fos and Jun immunostaining

In experiments aimed at evaluating induction of primary response genes, at different times after the challenge, cells were fixed with 4% paraformaldehyde, and washed three times (10 min each) with PBS, permeabilized with 0.1% Triton X-100 in PBS, and incubated overnight at room temperature with either anti-Fos (1 : 80; Sambucetti & Curran, 1986) or anti-Jun (1 : 80, Angel *et al*., 1988) immunoglobulins, followed by biotinylated donkey anti-rabbit secondary antibody (1 : 250; 1 h at room temperature) and streptavidin-fluorescein (1 : 100; 1 h at

room temperature). Labelled cells were examined with a Zeiss fluorescence microscope equipped with a fluorescein filter. Cultures were examined with a 20× objective lens and the number of cells showing Fos or Jun nuclear immunoreactivity quantified and expressed as % of total cell scored.

Materials and drug preparation

With a few exceptions (see below), all pharmacological agents were obtained from Sigma Chemicals Co. Trypsin, α,β-meATP, β,γ-meATP, bFGF, pertussis toxin (PTx), neomycin were dissolved in Hanks' balanced salt solution (HBSS) without calcium and magnesium. Concentrated solution of DNAase I, transferrine, putrescine, sodium selenite and BSA were prepared in sterile bidistilled water. Genistein, phorbol-12-13-dibutyrate (PDBu) and 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7) were dissolved in dimethylsulphoxide (DMSO) to obtain 10 mM solutions; dilutions were then performed in HBSS without calcium and magnesium. Progesterone was dissolved in ethanol to obtain a 10 mM solution and then diluted in water. Suramin, a kind gift from Bayer, was dissolved in HBSS without calcium and magnesium. HBSS, HBSS without calcium and magnesium, FCS, Medium 199, PBS tablets and 20% glucose solution were purchased from Gibco BRL, Life Technologies (European Division). All the radiolabelled compounds ([^{3}H]-AA; [^{3}H]-cyclic AMP and [^{32}P]-ATP) were obtained from NEN-Dupont, Italy. The ionic exchange resin AG50X4 was purchased from BioRad Laboratories, Italy. Adenosine deaminase and creatine phosphate were obtained from Boehringer Mannheim, Germany.

Statistical analysis

For astrocytic elongation, values for the total length of processes/cell and the mean length/processes were obtained from 50 cells/condition. Statistical analysis was performed on the data from at least 3 different experiments (minimum 150 cells/condition) by use of either one-way ot two-way ANOVA followed by either Sheffee's or Fisher's tests for multiple comparison. In experiments where single agents were compared to controls, statistical analysis was performed with Student's *t* test. For Fos and Jun experiments, at least 300 cells/condition/experiment were analysed. Statistical analysis of time-courses of c-fos and c-jun induction was performed with the Bonferroni correction. For all other experiments, statistical analysis was performed on the data from 3–6 different experiments (run in triplicate or quadriplicate) by either one-way ot two-way ANOVA followed by either Sheffe's or Fisher's tests for multiple comparison.

Results

Characterization of the long-term trophic actions consequent to brief challenges with either P2-receptor agonists or bFGF

A 120 min challenge of cultures with either 10 μM α,β-meATP or 1 ng ml^{-1} bFGF resulted, three days later, in a highly significant increase of the mean length of astrocytic processes/cell with respect to control cultures (Table 1). Similar effects were obtained after a 1 h challenge (data not shown). α,β-meATP-induced elongation of processes was completely prevented by suramin, which did not effect either the length of processes *per se* (data not shown) or block the astrocytic elongation induced by bFGF (Table 1). Statistically significant elongation of astrocytic processes with respect to control cultures was also observed after a 2 h challenge of cultures with ATP and with a variety of other P2 receptor agonists, including uridine 5′-triphosphate (UTP) (Table 2). For all compounds, maximal effects were obtained at 10 μM (except for UTP – see legend of Table 2). No further increases of elongation were observed at higher P2 receptor agonist concentrations (data not shown).

Table 1 Effect of suramin on α,β-meATP- and bFGF-induced elongation of GFAP positive processes

Condition	*Mean length of processes/cell* (μm)
Control	134.9±6.6
α,β-meATP	206.8±7.8*
α,β-meATP + suramin	135.5±6.6°
bFGF	234.7±9.7*
bFGF + suramin	216.5±9.0†

Cells were challenged for 2 h with either α,β-meATP (10 μM) of bFGF (1 ng ml^{-1}), washed, grown for 3 additional days in chemically defined serum-free medium and immunostained for GFAP as described in detail in Methods. Suramin (10 μM) was added to cultures 60 min before the differentiating agents. Results are the mean±s.e.mean of 150 cells/experimental condition from three independent experiments. *$P<0.05$ vs control; °$P<0.05$ vs α,β-meATP, not statistically different with respect to control; †$P<0.05$ vs control, not statistically different with respect to bFGF alone (ANOVA followed by Fisher's test for multiple comparison). Suramin alone was not different from control.

Table 2 Effect of various P2 receptor agonists on the length of GFAP positive processes

Condition	*Mean length of processes/cell* (μm)
Control	115.1±4.8
ATP	188.1±6.3*
β,γmeATP	198.8±8.9*
ADPβS	162.8±7.4*
2-meSATP	147.8±7.6*
UTP	142.0±6.3*

Cells were challenged for 2 h with the indicated nucleotides (all at a 10 μM concentration, except for UTP which was tested at 100 μM), washed, grown for 3 additional days in chemically defined serum-free medium and immunostained for GFAP as described in detail in Methods. Results are the mean±s.e.mean of at least 150 cells/experimental condition from three independent experiments. *$P<0.05$ vs control, one was ANOVA followed by Fisher's test for multiple comparison.

Characterization of the transductional pathways involved in purine- and bFGF-induced astrocytic differentiation

The G_i/G_o protein inactivator PTx completely abolished α,β-meATP- but not bFGF-induced elongation of astrocytic processes (Table 3). Conversely, the tyrosine-kinase inhibitor genistein significantly reduced the effects produced by bFGF, whereas it did not significantly affect the astrocytic elongation induced by α,β-meATP (Table 4). Neomycin had no effect on the astrocytic elongation induced by either agent (data not shown), apparently ruling out the involvement of phospholipase C; this is supported by the demonstration that, in parallel cultures maintained under identical experimental conditions, neither α,β-meATP, nor β,γ-meATP (nor the polypeptidic growth factor bFGF) had any effects on Ca^{2+} release from intracellular stores (see Figure 2 of the accompanying paper Centemeri *et al*., 1997).

Inhibition of adenylyl cyclase activity (Boyer *et al*., 1993) did not play any role in α,β-meATP-induced effects; in fact, whereas forskolin significantly stimulated cyclic AMP production (43.1±5.9 pmol cyclic AMP min^{-1} mg^{-1} prot. with respect to a basal activity of 9.9±2.7, mean±s.e.mean from 3 independent experiments run in quadriplicate, $P<0.001$, ANOVA followed by Scheffe's *F* test for multiple comparison), no significant changes of forskolin-stimulated cyclic AMP production were induced by α,β-meATP over a wide range of concentrations (36.7±3.8, 42.78±3.37, 39.3±3.2 and

39.4±3.8 pmol cyclic AMP min^{-1} mg^{-1} prot. in the presence of 10 nM, 100 nM, 1 μM and 10 μM α,β-meATP, respectively; mean±s.e.mean of 3 experiments run in triplicate).

α,β-meATP concentration-dependently increased the release of [^{3}H]-AA from [^{3}H]-AA-labelled cultures (Figure 1). Release of [^{3}H]-AA by α,β-meATP was already statistical significant (117.5±5.1% of unstimulated release, $P<0.002$ with respect to basal, mean±s.e.mean of 19 determinations from 5 independent experiments) at the concentration (10 μM) which induced the morphological differentiation of cells (Tables 1–3). In parallel samples, glutamate, a previously characterized trigger for [^{3}H]-AA release in rat astroglial cells (Stella *et al.*, 1994), elicited a 46.5±7.6% increase of arachidonate release ($P<0.0001$ with respect to basal release, mean±s.e.mean of 15 determinations from 5 independent experiments). Therefore, 100 μM α,β-meATP appeared to be equipotent with 2 mM glutamate in releasing AA (Figure 1). Consistently, exogenously administered arachidonic acid (1 μM) induced the morphological differentiation of astroglial cells (mean length of processes/cell: 293.5±12.6 μm with respect to 106.6±5.8 μm in control cultures, mean±s.e.mean from 3 experiments run in triplicate, $P<0.05$ ANOVA followed by Scheffe's test).

To confirm a role for phospholipase A_2 (PLA_2) in α,β-meATP-induced effects, both the early [^{3}H]-AA release (Figure 1) and the long-term morphological changes induced by the purine analogue on these cells (Table 5) were shown to be completely prevented by pre-exposure of cultures to a PLA_2 inhibitor such as mepacrine (10 μM), which, in contrast, did not affect bFGF-induced morphological changes (Table 5). Inhibition of α,β-meATP-induced astrocytic elongation by mepacrine was concentration-dependent and maximal at 10 μM (data not shown). Mepacrine also blocked the astrocytic differentiation induced by β,γmeATP (data not shown). Similar effects were also obtained with other inhibitors of PLA_2, such as monoalide (data not shown) and dexamethasone (10 μM), which completely counteracted the elongation of astrocytic processes induced by the purine analogue (mean length of processes/cell in the presence of both α,β-meATP and dexamethasone: 149.5±6.8, $P<0.05$ vs 200.3±8.5 in α,β-meATP-treated cultures and not statistically different with respect to 144.4±6.1 in control cultures; mean±s.e.mean of 3 experiments run in triplicate, ANOVA followed by Scheffe's test for multiple comparison).

The protein kinase C (PKC) inhibitor H7 fully abolished α,β-meATP but not bFGF-induced elongation of astrocytic

Table 3 Effect of PTx on α,β-meATP- and bFGF-induced elongation of GFAP-positive processes

Condition	*Mean length of processes/cell (μm)*
Control	145.8±6.7
α,β-meATP	208.2±8.7*
α,β-meATP+PTx	135.1±6.0†
bFGF	260.9±10.2*
bFGF+PTx	230.3±9.5°

Cultures were placed for 18 h in chemically defined serum-free medium containing pertussistoxin (PTx; 350 ng/ml^{-1}) before addition of the differentiating agents (either 10 μM α,β-meATP or 1 ng/ml^{-1} bFGF). After 2 h, cells were washed, grown for 3 additional days in chemically defined serum-free medium and immunostained for GFAP as described in detail in Methods. Results are the mean± s.e.mean of 150 cells/experimental condition from three independent experiments.
*$P<0.05$ vs control; $^{\dagger}P<0.05$ vs α,β-meATP, not statistically different with respect to control; $^{\circ}P<0.05$ vs control, not statistically different with respect to bFGF alone (ANOVA followed by Scheffe's test for multiple comparison). PTx alone was not statistically different with respect to control.

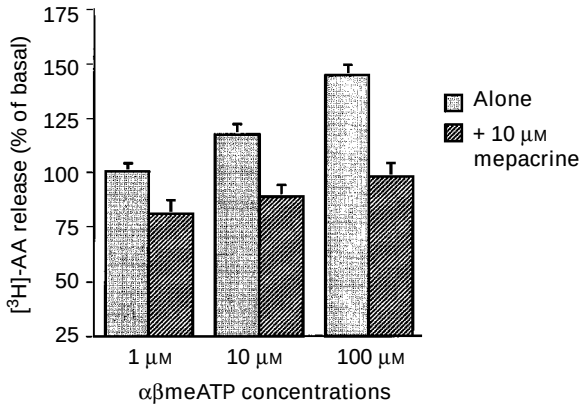

Figure 1 Effect of mepacrine on [^{3}H]-arachidonic acid ([^{3}H]-AA) release evoked by α,βmeATP in rat brain astrocytes. At day 2 in culture, cells were challenged with α,β,meATP (1, 10 or 100 μM) in the absence and presence of 10 μM mepacrine. After 15 min, radioactivity released in the culture medium was collected and analysed as described in Methods. Results are expressed as % of unstimulated [^{3}H]-AA release. Each value represents the mean± s.e.mean of 9–19 determinations from 3–5 independent experiments. Mepacrine alone did not significantly affect basal [^{3}H]-AA release (98.9±4.1% of unstimulated release, $P=0.88$ with respect to control).

Table 4 Effect of genistein on α,β-meATP- and bFGF-induced astrocytic elongation

Condition	*Mean length of processes/cell (μm)*
Control	163.7±7.3
α,β-meATP	209.7±8.2*
α,β-meATP+genistein	185.5±6.6†
bFGF	267.1±9.9*
bFGF+genistein	194.3±8.6°

Cells were pre-exposed to 50 μM genistein for 30 min and then challenged with either α,β-meATP (10 μM) or bFGF (1 ng ml^{-1}). Afterwards, cells were washed, grown for 3 additional days in chemically defined serum-free medium and immunostained for GFAP as described in detail in Methods. Results are the mean±s.e.mean of 150 cells/experimental condition from three independent experiments. *$P<0.05$ vs control; $^{\dagger}P<0.05$ vs control, not statistically different with respect to α,β-meATP alone; $^{\circ}P<0.05$ vs bFGF (ANOVA followed by Scheffe's test for multiple comparison). Genistein alone was not statistically different with respect to control.

Table 5 Effect of mepacrine on α,β-meATP- and bFGF-induced elongation of GFAP-positive processes

Condition	*Mean length of processes/cell (μm)*
Control	151.6±7.2
α,β-meATP	217.7±8.5*
α,β-meATP+mepacrine	163.2±7.7°
bFGF	266.1±9.9*
bFGF+mepacrine	227.7±9.2†

Cells were exposed to 10 μM mepacrine for 60 min before addition of either α,β-meATP (10 μM) or bFGF (1 ng ml^{-1}). After 2 h, cells were washed, grown for 3 additional days in chemically defined serum-free medium and immunostained for GFAP as described in detail in Methods. Results are the mean±s.e.mean of 150 cells/experimental condition from three independent experiments. *$P<0.05$ vs control; $^{\circ}P<0.05$ vs α,β-meATP alone, not statistically different with respect to control; $^{\dagger}P<0.05$ vs control, not statistically different with respect to bFGF alone (ANOVA followed by Scheffe's test for multiple comparison). Mepacrine alone was not different from control.

processes (Table 6). A brief challenge of cultures with 0.1 μM PDBu, a PKC activator, induced a marked elongation of GFAP-positive processes (175.1 ± 9.4 μm in PDBu-exposed cultures with respect to 102 ± 7.6 μm in control cultures, mean $\pm$ s.e.mean of 100 cells, $P < 0.05$).

Characterization of the early events involved in purine- and bFGF-induced astrocytic differentiation

In control cultures, immunoreactivity for the c-fos and c-jun gene products was faint and non-specifically localized to the cytoplasm (a typical Jun labelling of control cultures is shown in Figure 2a). A brief challenge of cultures with either α,β-meATP or bFGF, utilized at the same concentrations that induced astrocytic elongation, produced a rapid and marked increase of Fos and Jun immunoreactivity, that in these treated cultures was exclusively associated with cell nuclei (Figures 2 and 3). Results of a typical experiment for Jun induction are shown in Figure 2b and 2c for α,β-meATP and bFGF, respectively. Comparable data have been obtained on Fos expression (data not shown). Induction of the nuclear accumulation of the Fos and Jun proteins by either α,β-meATP or bFGF was rapid and transient, as shown by the time course experiments depicted in Figure 3. Significant increases of the number of cells showing Fos- or Jun-positive nuclei were already evident after 1 h of exposure to either α,β-meATP or bFGF (time 0 on the x axis of Figure 3) and peaked after an additional 30–60 min (Figure 3). Induction was significantly higher in treated cultures with respect to controls ($P < 0.05$, Bonferroni correction). Nuclear levels of both Fos and Jun started to decline after 120 min and were back to control values by 180–240 min (Figure 3). Both c-fos and c-jun-induction by α,β-meATP could be completely prevented by pre-exposure of cultures to suramin (Table 7) suggesting that these effects were also mediated by extracellular P2 receptors. In contrast, suramin did not affect induction of these genes by bFGF (Table 7).

Discussion

The present results confirm previous studies from our and other laboratories on the role of ATP in the formation of reactive astrocytes and, for the first time, shed some light on the specific signalling pathways responsible for such long-term trophic effects.

A number of P2 receptor subtypes are expressed by astroglial cells. All these receptors belong to the G-protein-coupled-P2Y receptor subfamily (for review, see Abbracchio, 1997; see also accompanying paper), although they may utilize different effector systems (ibidem). No studies have previously attempted to correlate the short-term responses of ATP to its long-term trophic actions in these cells. The present results show that, in a similar manner to polypeptidic growth factors like bFGF, a brief exposure of astroglial cells to ATP derivatives results, three days later, in marked morphogenic changes, and that this trophic effect is mediated by a P2Y receptor. This receptor is activated by α,β-meATP, a potent agonist at some of the ligand-gated P2X (mainly $P2X_1$ and $P2X_3$) receptors. However, the existence of P2Y receptors responsive to α,β-meATP has been demonstrated previously, (e.g., Windscheif *et al*., 1995), suggesting that the receptor responsible for the

Table 6 Effect of H7 on α,β-meATP- and bFGF-induced elongation of GFAP-positive processes

Condition	*Mean length of processes/cell (μm)*
Control	135.8 ± 6.6
α,β-meATP	213.0 ± 8.9*
α,β-meATP + H7	$147.8 \pm 7.0^{\dagger}$
bFGF	239.2 ± 14.8*
bFGF + H7	$243.7 \pm 10.7^{\circ}$

Cells were exposed to 50 μM H7 for 60 min before addition of either α,β-meATP (10 μM) or bFGF (1 ng ml^{-1}). After 2 h, cells were washed, grown for 3 additional days in chemically defined serum-free medium and immunostained for GFAP as described in detail in Methods. Results are the mean $\pm$ s.e.mean of 150 cells/experimental condition from three independent experiments. *$P < 0.05$ vs control; $^{\dagger}P < 0.05$ vs α,β-meATP, not statistically different with respect to control; $^{\circ}P < 0.05$ vs control, not statistically different with respect to bFGF alone (ANOVA followed by Scheffe's test for multiple comparison). H7 alone was not statistically different from control.

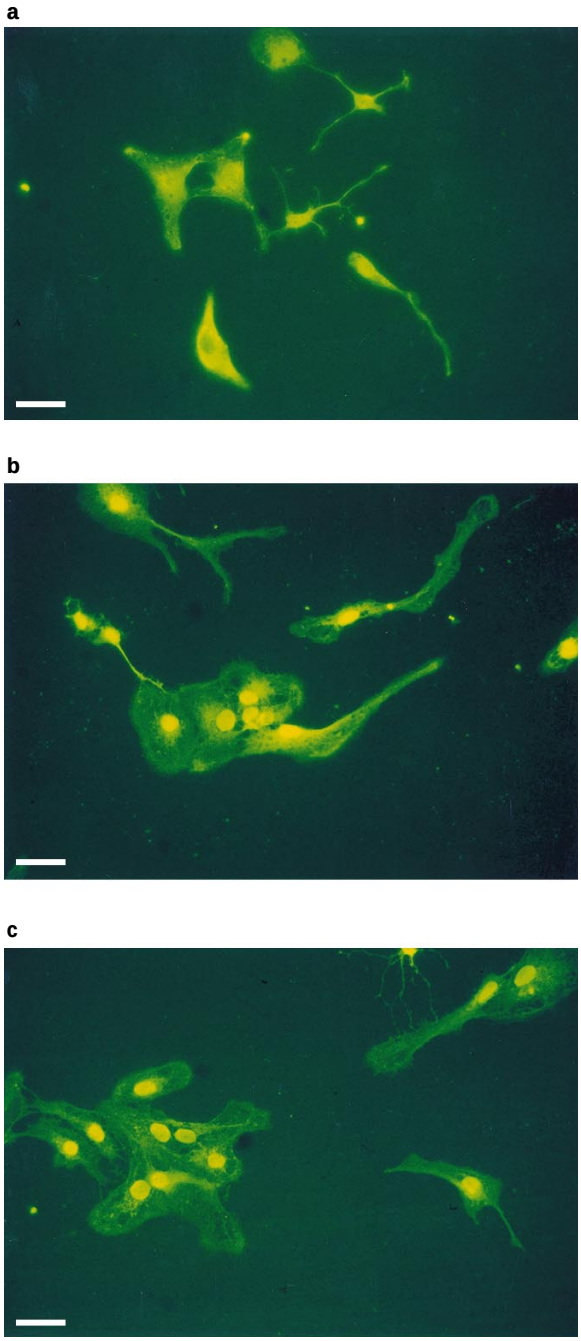

Figure 2 Induction of the nuclear accumulation of the Jun protein by α,β-meATP and bFGF in rat brain astrocytes. At day 2 in culture, cells were exposed to either medium alone (control, a), or 10 μM α,β-meATP (b) or 1 ng ml^{-1} bFGF (c). After 1 h, cells were washed, fixed, immunostained for the Jun protein as described in Methods and analysed under a fluorescence microscope. Micrographs refer to a typical experiment; similar data were obtained in 4 other independent experiments and also for Fos induction. Scale bar: 50 μm.

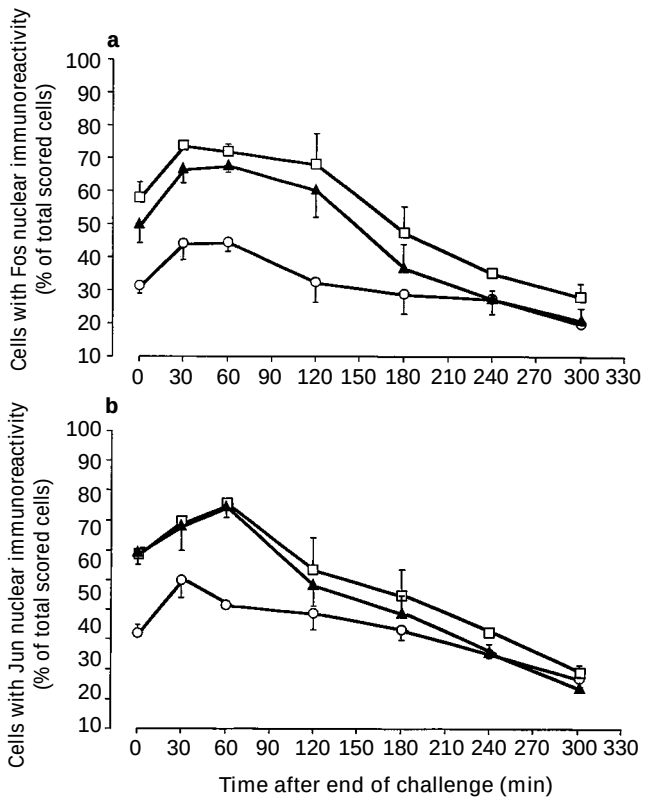

Figure 3 Time-course of α,β-meATP- and bFGF-induction of c-fos and c-jun. At day 2 in culture, cells were challenged with either 10 μM α,β-meATP (▲), or 1 ng ml^{-1} bFGF (□), or chemically defined medium alone (control, ○), for 60 min. Immediately afterwards, the medium was changed, cells were washed, placed in agonist-free medium and fixed after the time intervals shown. Cultures were then immunostained with a Fos (a) or Jun (b) antibody. Data represent the mean of 4 replicates/condition from two independent experiments; vertical lines show s.e.mean. For both bFGF and α,β-meATP, curves were statistically different with respect to corresponding control curve ($P<0.05$, Bonferroni correction).

Table 7 Effect of suramin on α,β-meATP- and bFGF-induced nuclear accumulation of Fos and Jun

Condition	*Cells with nuclear Fos immunoreactivity*	*Cells with nuclear Jun immunoreactivity*
Control	33.3±1.6	33.1±3.8
α,β-meATP	70.3±3.2*	66.1±7.7*
α,β-meATP+suramin	35.6±1.8†	35.0±3.6†
bFGF	69.7±2.2*	68.4±5.3*
bFGF+suramin	63.7±1.9°	59.0±3.6°

At day 2 in culture, cells were pretreated with 10 μM suramin for 60 min before a 1 h challenge with either α,β-meATP (10 μM) or bFGF (1 ng ml^{-1}). Cultures were then washed, placed in chemically defined serum-free medium for 1 h, fixed and immunostained for either Fos or Jun as described in detail on Methods. Results represent the % of cells showing nuclear immunoreactivity over total number of scored cells. Data are the mean±s.e.mean of 4 experiments run in quadruplicate. *$P<0.05$ vs control; $^{\dagger}P<0.05$ vs α,β-meATP, not statistically different with respect to control; $^{\circ}P<0.05$ vs control, not statistically different with respect to bFGF alone (ANOVA followed by Fischer's test for multiple comparison). Suramin alone was not different from control.

trophic actions described here may be either an atypical, or a novel receptor subtype, or a previously uncharacterized isoform of one of the already cloned P2Y receptors (Burnstock & King, 1996). Results of experiments with inhibitors of various transduction pathways suggest that this receptor is probably linked to activation of PLA_2 through a PTx-sensitive G-protein (presumably a G_o/G_i protein). A role for PLA_2 in mediating the differentiating effects of purine analogues on astrocytes is suggested by three lines of evidence: (1) the ability of α,β-meATP to release [^{3}H]-AA from these cells; (2) the ability of exogenously added AA to induce morphological differentiation of cells; (3) the inhibition of both α,β-meATP-induced [^{3}H]-AA and astrocytic elongation by PLA_2 inhibitors such as mepacrine and dexamethasone.

A role for the other P2 receptors expressed by these cells in the long-term phenotypic changes described here seems unlikely given previous findings. Despite the fact that the prototypical response to P2Y receptor activation in various tissues is stimulation of inositol lipid hydrolysis (Charest *et al*., 1985; Pirotton *et al*., 1987; Fosberg *et al*., 1987; Berrie *et al*., 1989), astroglial P2 receptors coupled to phospholipase C (Salter & Hicks, 1995) do not seem to be involved in the trophic effects described here, since the phospholipase C inhibitor neomycin had no effect on either α,β-meATP- or bFGF-induced elongation of astrocytic processes, and, more importantly, neither purine analogues (α,β-meATP or β,γ-meATP) nor bFGF elicited any increase in $[Ca^{2+}]_i$ in these same cells (Figure 2 of the accompanying paper Centemeri *et al*., 1997), when tested at concentrations that cause marked elongation of astrocytic processes (interestingly, this also suggests that a Ca^{2+}-independent signalling pathway mediates the differentiating effects triggered by polypeptidic growth factors in these cells). P2Y-receptors that inhibit adenylyl cyclase (Boyer *et al*., 1993) were also ruled out based on the lack of effect of α,β-meATP on forskolin-stimulated adenylyl cyclase activity. Of course, we cannot rule out the hypothesis that we may be dealing with the same receptor identified by other authors, i.e. it may simply couple to a different transduction mechanism in our system. Moreover, the possibility that the trophic effects of purine analogues may be mediated by a non specific interaction with growth factor receptors (Voogd *et al*., 1993) was ruled out, based on the demonstration that a tyrosine kinase inhibitor (genistein) had no effect on α,β-meATP-induced elongation of astrocytic processes but markedly reduced astrocytic differentiation by bFGF.

A functional importance for astroglial PLA_2-coupled P2 receptors is suggested by previous results. Astrocytes are believed to represent an important source for eicosanoids in the CNS (Murphy *et al*., 1988; Bruner & Murphy, 1990). Various stimuli, such as ischaemia, anoxia, convulsions and electroconvulsive shock, which all cause activation of astrocytes (Eddleston & Mucke, 1993) also cause increased release of arachidonates (Axelrod *et al*., 1988; Stella *et al*., 1994), which may be involved in the subsequent functional changes of these and neighbouring cells. Activation of an arachidonate-sensitive protein kinase C is speculated (Tanaka & Nishizuka, 1994), consistent with both the morphogenic effects of PDBu in these cells and with the marked reduction of α,β-meATP-induced effects by the PKC inhibitor H7.

The present results are also consistent with data obtained by other authors Neary & coworkers (1994) have shown that both ATP and bFGF are mitogenic for rat cortical astrocytes, and that ATP can activate astrocytic mitogen-activated protein (MAP) kinases via P2 receptors (Neary & Zhu, 1994). The MAP kinase cascade is a key element of signal transduction pathways involved in cellular proliferation and differentiation by both polypeptidic growth factors (for reviews, see: Davis, 1993; Avruch *et al*., 1994) and G-protein-coupled receptors (Clapham & Neer, 1993). MAP kinases affect c-fos induction via phosphorylation of p62, a key transcription factor, that participates in the activation of the serum-response element of the c-fos promoter (Gille *et al*., 1992); MAP kinases also regulate Jun by post-translation phosphorylation (Pulverer *et al*., 1991). ATP and bFGF have also been shown to induce the formation in astrocytes of AP-1 complexes, functional DNA binding protein heterodimers consisting of Fos and Jun families of transcription factors (Neary *et al*., 1994).

Based on all these data, a picture emerges for astrocytic activation where both 'classic' polypeptide growth factors (e.g. bFGF) and novel trophic agents (e.g., ATP), following an interaction with their specific extracellular receptors, can activate distinct and independent transduction pathways that merge at the MAP kinase cascade (Abbracchio *et al*., 1996). Tyrosine-kinase receptors for bFGF activate the *ras-raf* kinase pathway; conversely, P2 receptor signalling to MAP kinase, like other G-protein-coupled receptors (Lange-Carter *et al*., 1993), involves activation of PLA_2 (the present study) and some as yet unknown intracellular target leading to activation of MEK kinase (Neary & Zhu, 1994). Following exposure to both agents, activation of MAP kinase may lead to rapid and transient induction of primary response genes (e.g., c-fos and c-jun, as demonstrated by the present results), which in turn may regulate late response genes mediating long-term phenotypic changes of astroglial cells, such as GFAP induction (Abbracchio *et al*., 1996).

The characterization of the intracellular targets activated by PLA_2-coupled P2 receptors may help in understanding the molecular mechanisms at the basis of astrocytic activation. Also new exciting opportunities could be disclosed to regulate the gliotic response and, thereby, influence the outcome of neurological conditions in stroke patients and in other neurodegenerative diseases characterized by reactive astrogliosis.

The authors wish to thank Dr M. Di Luca (Institute of Pharmacological Sciences, University of Milan, Italy) for useful discussion on the experiments with the PKC inhibitor, Prof. C. Galli, Dr A. Petroni and Dr C. Banfi (Institute of Pharmacological Sciences, University of Milan, Italy) for suggestions on the arachidonic acid experiments, Prof. S. Nicosia (Institute of Pharmacological Sciences, University of Milan, Italy) and Prof. J.T. Neary (University of Miami, School of Medicine, USA) for useful discussion and critical reading of the manuscript. The authors are grateful to Dr A. Faggiotto (Bayer S.p.A. Italia) for kindly providing suramin. M.P.A. is a Partner in the shared-cost project 'Nucleotides, a novel class of extracellular signalling substances in the nervous system' supported by the European Union within the BIOMED 2 programme.

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(Received December 19, 1996
Revised April 16, 1997
Accepted May 2, 1997)