ACTIVATION OF $P2X_7$ RECEPTORS STIMULATES THE EXPRESSION OF $P2Y_2$ RECEPTOR mRNA IN ASTROCYTES CULTURED FROM RAT BRAIN

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Under pathological conditions brain cells release ATP at concentrations reported to activate $P2X_7$ ionotropic receptor subtypes expressed in both neuronal and glial cells. In the present study we report that the most potent $P2X_7$ receptor agonist BzATP stimulates the expression of the metabotropic ATP receptor $P2Y_2$ in cultured rat brain astrocytes. In other cell types several kinds of stimulation, including stress or injury, induce $P2Y_2$ expression that, in turn, is involved in different cell reactions. Similarly, it has recently been found that in astrocytes and astrocytoma cells $P2Y_2$ sites can trigger neuroprotective pathways through the activation of several mechanisms, including the induction of genes for antiapoptotic factors, neurotrophins, growth factors and neuropeptides. Here we present evidence that $P2Y_2$ mRNA expression in cultured astrocytes peaks 6 h after BzATP exposure and returns to basal levels after 24 h. This effect was mimicked by high ATP concentrations (1 mM) and was abolished by $P2X_7$-antagonists oATP and BBG. The BzATP-evoked $P2Y_2$ receptor up-regulation in cultured astrocytes was coupled to an increased UTP-mediated intracellular calcium response. This effect was inhibited by oATP and BBG and by $P2Y_2$siRNA, thus supporting evidence of increased $P2Y_2$ activity. To further investigate the mechanisms by which $P2X_7$ receptors mediated the $P2Y_2$ mRNA up-regulation, the cells were pre-treated with the chelating agent EGTA, or with inhibitors of mitogen-activated kinase (MAPK) (PD98059) or protein kinase C, (GF109203X). Each inhibitor significantly reduced the extent to which BzATP induced $P2Y_2$ mRNA. Both BzATP and ATP (1 mM) increased ERK1/2 activation. $P2X_7$-induced ERK1/2 phosphorylation was unaffected by pre-treatment of astrocytes with EGTA whereas it was inhibited by GF109203X. Phorbol-12-myristate-13-acetate (PMA), an activator of PKCs, rapidly increased ERK1/2 activation. We conclude that activation of $P2X_7$ receptors in astrocytes enhances $P2Y_2$ mRNA expression by a mechanism involving both calcium influx and PKC/MAPK signalling pathways.

In addition to its well-known intracellular role, ATP is released under a variety of physiological and pathological conditions (1-3) where it acts as a fast intercellular signalling molecule through cell surface P2 receptors.

The P2 purinergic receptor family has been divided into metabotropic (P2Y) and ionotropic (P2X) classes (4). The P2Y (1, 2, 4, 11, 12, 13,

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14) receptors are G-protein coupled and the P2X receptors ($P2X_{1-7}$) are ligand-gated ion channels (5-6). The former are responsible for Ca^{2+} release, largely from intracellular stores, whereas the latter are responsible for Ca^{2+} entry from the extracellular environment.

The $P2X_7$ receptor subtype is an unusual member of the ionotropic P2 family in that it is a non-desensitising, non-selective cation channel with low affinity for ATP (7-9). The $P2X_7$ receptor was first identified and characterised in cells of hematopoietic lineage. Its activation has been implicated in proliferation, giant cell formation, degranulation and cytolytic cell death (8, 10-11). Recently $P2X_7$ receptors have been found on other cell types, including glia (11-17).

Previously, we reported the first evidence of the presence of $P2X_7$-like activity in astrocytes cultured from rat brain (12). In these cells the activation of $P2X_7$ sites by BzATP, the most potent agonist for this ionotropic ATP receptor: i) increased release of adenine-based purines; ii) triggered a sustained and dose-dependent increase of intracellular calcium concentration ($[Ca^{2+}]i$); iii) was unable to cause a rise in $[Ca^{2+}]i$ in the presence of the extracellular chelating agent EGTA; and, iv) made a significant proportion of astrocytes permeable to the fluorescent dye Lucifer Yellow (MW 463) (12). These effects were abolished when cells were pre-treated with the $P2X_7$ receptor antagonist oxidised ATP (oATP). Support for the presence of $P2X_7$ receptors on astrocytes was strengthened further by evidence that $P2X_7$ receptor mRNA is expressed in these cells (18).

$P2X_7$ receptors have been found in Schwann cells (19), human Muller cells (20) and neurons (21). In these cells, and in astrocytes, activation of $P2X_7$ receptor has been reported to regulate the expression and production of cytokines, chemokines and cysteinyl leukotrienes (13-14) as well as neuronal transmission; pore formation and cytotoxicity have been less consistently observed. Moreover, it has been recently shown in astrocytes that ATP increases the production of 2-arachidonylglycerol (22), an agonist for endocannabinoid receptors known to exert a neuroprotective activity, and attenuates the LPS-induced release of tumor necrosis factor alpha thus restraining the glia-mediated inflammatory response (23), and that these effects occur via $P2X_7$ activation.

There is growing interest in the possibility that of the P2Y metabotropic ATP receptors the $P2Y_2$ subtypes play a neuroprotective role. $P2Y_2$ receptors have been reported to be important in the development of the nervous system, neuronal migration, differentiation and survival. In epithelial cells, vascular smooth muscle cells and cardiomyocyte P2Y2 receptors are usually expressed at lower levels than $P2Y_1$ receptors; but they are markedly up-regulated in response to different kinds of stress or injury, and are involved in different kinds of cellular reactions such as intimal hyperplasia (24), T-cell differentiation (25) and increased cardiac inotropism (26). In astrocytes and astrocytoma cells $P2Y_2$ receptors have recently been found to activate neuroprotective mechanisms by stimulating the expression of genes for antiapoptotic factors (bcl-2 and bcl-xl), neurotrophins, growth factors and neuropeptides (27).

We previously reported that astrocytes release substantial amounts of adenine-based purines under different kinds of stimulation (1, 28). Moreover, in response to ischemia, Rudolphi and colleagues have shown that the extracellular concentration of ATP dramatically rises by up to 200-fold, reaching concentrations able to stimulate $P2X_7$ receptors.

In the present study we report that activation of $P2X_7$ receptors on cultured astrocytes from rat brain increases levels of $P2Y_2$ receptor mRNA, and that either calcium or PKC and ERK1/2 signalling are involved in this response.

MATERIALS AND METHODS

Materials

2'-3'-O-(4 Benzoylbenzoyl)adenosine 5'triphosphate (BzATP), oxidized ATP (oATP), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N',-tetracetic acid (EGTA), sulfinpyrazone, dimethyl sulfoxide (DMSO), Brillant Blue G, 3-(4.5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) and poly-D-lysine, were from Sigma (Sigma-Aldrich, Milan, Italy), {1-[2-(5-Carboxyoxazol-2yl)-6aminobenzofuran-5-oxyl]-2(2'-amino-5'-methyl phenoxy)-ethane-,N,N'N'-tetracetic Acid Pentaacetoxymethyl Ester} (fura-2/AM), Bisindolylmaleimide I (GF109203X), 2-(2-amino-3-methoxy phenyl)-4H-1-benzopyran-4-one (PD98059) were purchased from Calbiochem (Calbiochem-Novabiochem Corp., CA, USA). RNase-free DNase-1 was

purchased from Roche (Monza, Italy), RETROscript™ was from Ambion Inc. (Texas, USA), PCR reaction buffer, deoxy-dNTPs and AmpliTaqGold DNA polymerase were supplied by Perkin-Elmer (Branchburg, NJ, USA), oligonucleotide primers were from MGW-Biotech (Ebersberg, Germany), Hybond N nylon membranes, megaprime DNA labelling system, [5-^{3}H]uridine, [alpha^{32}P]-dCTP, donkey anti-rabbit HPR-conjugated secondary antibody and chemiluminescence detection system (ECL) were from Amersham Biosciences (Milan, Italy). PVDF membranes were from Bio-Rad Laboratories (Milan, Italy). Rabbit phospho-ERK1/2 antibody, New England Biolabs, was from Celbio (Milan, Italy). Dulbecco's modified Eagle medium (DMEM), horse serum (HS) and TRIzol Reagent were from GibcoBRL (Life Technologies, Milan, Italy). Disposable materials for tissue cultures were purchased from NUNC (Mascia Brunelli, Milan, Italy)

Astrocyte cultures

Primary cultures of astrocytes were prepared from 18 to 19-day old rat fetuses as previously reported (29) (work approved by the Animal Research Ethics Board of the University of Chieti and carried out in accordance with the European Animal Care Guidelines). After decapitation the cortices were quickly removed and pooled into 2 ml Dulbecco Modified Eagle's Medium (DMEM) with added glucose (6g/l), $NaHCO_3$ (3.7 g/l) and horse serum (20%). The tissue was mechanically dissociated and the cell suspension obtained was centrifuged at 200 g for 3 min. The resultant pellet was re-suspended in the growth medium (indicated above) containing also 5 mM L-leucine methyl ester in order to restrain contamination by microglia (30). Cells were seeded onto poly-D-lysine (10 μg/ml) coated flasks and maintained at 37°C in a humidified incubator under 95% air and 5% CO_2. Growth medium was renewed after 24 hrs and subsequently every 3 days, with the serum concentration being diminished to 5%. At the 7th and 13th days *in vitro* (DIV), the cells were shaken for 3 h at 80 rpm on a plate shaker to minimise microglia contamination. For Northern Blot, Western Blot analysis and calcium measurements confluent primary cultures of astrocytes at the 14th DIV were trypsinised (0.025% trypsin/0.04% EDTA dissolved in PBS, 10-20 min, 37°C) and re-plated at a concentration of 1.5 x 10^6 cells/ dish (100 mm Petri dishes).

The assays on astrocytes were performed 4 days after re-plating. More than 98% of the cultured cells were identified as astrocytes as previously reported (12).

3-(4.5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay

Cell viability was determined by the MTT assay. Astrocytes were seeded into 96-well plates at a density of 10.000 cells/well, and treated as described in the figure legend. MTT was dissolved in PBS at a concentration of 5 mg/ml. Twenty microliters of this MTT stock solution was added to each well and the plates were incubated for 4 h at 37°C. After the removal of the supernatant, 200 μl of dimethyl sulfoxide (DMSO) was added and the plate was agitated for a minimum of 15 min to solubilize the formazan crystals. The plates were read at 540 nm on a Titertek Multiscan microElisa reader.

RNA isolation

For RNA determination, astrocytes in 100 mm dishes were incubated for 24 h in a medium containing 1% FBS to equilibrate the cells and then treated with the various agents for the times and concentrations indicated in the text and figure legends.

Total RNA was isolated from cells using TRIzol reagent following the supplier's instructions. The resulting RNA pellet was finally washed with 70% ice-cold ethanol, air-dried and re-dissolved in 30 μl diethyl-pyrocarbonate (DEPC)-treated water. The RNA concentration was determined spectrophotometrically.

RT-PCR analysis

10 μg RNA was treated with 10 U RNase-free DNase I to remove any genomic DNA contaminants. cDNA was synthesized using random hexamers as primers by using RETROscript ™ according to the manufacturer's instructions. The resultant cDNA (2 μl) was amplified in a 50 μl reaction volume containing 2 U Taq polymerase, dNTP (200 μM each), 1.5 mM $MgCl_2$, 5 μl 10X polymerase chain reaction buffer and primers used at final concentration of 1 mM. The following primers were used: $P2Y_2$ forward 5'-TTTGCTCCCTTTT-3' and reverse 5'-GTTGCCTTAGATACGATTCC-3'; $P2Y_4$ forward 5'-ATGGACTCATGGCCCGGCGA-3' and reverse 5'-CTTGGTGGGTGTCCGCCCAC-3'; $P2Y_6$ forward 5'-TATAACTACGCCAGAGGGGACCAC-3' and reverse 5'-GGCGGGCCATGCGACAGTAG-3'. The mixture was overlaid with 25 μl of mineral oil to prevent evaporation. Temperature cycling was as follows: 1 cycle at 94°C for 5 min and 30 cycles at 94°C for 1min, 55°C for 1 min, and 72°C for 1 min, followed by 72°C for 10 min. PCR products were detected by electrophoresis on a 1% agarose gel containing ethidium bromide.

$P2Y_2$ small interfering RNA

SiRNA transfection of the rat $P2Y_2$ receptor (NM_017255) and non-specific control RNA (NSC) duplexes was performed in primary cultures of astrocytes from rat brain cortex at 60-70% confluence. siRNA duplex specific for the $P2Y_2$ receptor was chemically synthesized (http:

//www.Dharmacon.com), as well as their non-specific control (NSC). ON-TARGETplus SMARTpool siRNA (L-091224-00 ORF) were used.

Transfection of siRNAs for targeting endogenous genes was carried out using Oligofectamine (Invitrogen, Life Technologies) according to the manufacturer's instructions (100 nM siRNA in a 100 cm dish). Briefly, astrocytes at 60-70% confluence were cultured for 1 day in culture medium without antibiotics. 1 nmol siRNA was diluted in 175 μl optimem for 5 min at room temperature. In the meantime 3 μl oligofectamine was mixed in 15 μl optimem for 5 min. RNA interference was performed by mixing diluted oligofectamine and siRNA for 30 min at room temperature and then adding this solution to cells. The same procedure was performed on other dishes using a siRNA-scrambled (NSP= non-specific control). Cells were incubated at 37°C for 24 hours. Twenty-four hours after transfection, the cells were lysed and assayed for gene silencing.

Northern blot analysis

RNA (15 μg /lane) was fractionated on formaldehyde denaturing 1% agarose gel and blotted overnight onto Hybond N nylon membrane. The filter was then UV cross-linked in UV Stratalinker 1800 (Stratagene). The blots were pre-hybridized at least 8 hours at 42° in a solution containing 50% formamide, 5x Denhardt's Solution, 0.1% SDS and 100 μg / ml salmon sperm DNA. Hybridization of cDNA probes was performed in a buffer of the same composition containing 10^6 cpm/ml of radiolabelled probe. cDNAs, obtained by extracting PCR products from agarose gel slices (Millipore, Bedfore, MA USA) were radiolabelled by the method of random priming using [alpha^{32}P]-dCTP. After hybridization, the membranes were washed in 0.1X SSC containing 0.1% SDS at 50°C and exposed for 2 days to Kodak Biomax MS autoradiography film at -80°C with intensifying screen. Subsequent to hybridization with $P2Y_2$ cDNA probe, Northern blots were hybridized with a probe for GAPDH to allow correction for the recovery of RNA in each sample. Experiments were performed at least in triplicate. For the purpose of quantification, autoradiograms were scanned using laser densitometry. $P2Y_2$ mRNA signals were normalized against GAPDH content by determining the ratio of the respective optical densities.

Calcium measurements

Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured with the fluorescent indicator Fura-2 as described previously (12). In brief, cultures were washed twice with phosphate-buffered saline and cells were detached from the flasks with a 0.025% trypsin-EDTA solution (37°C, 5 min). Cells were then washed twice by centrifugation in a standard buffer containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1mM KH_2PO_4, 1 mM $CaCl_2$, 5.5 mM glucose and 20 mM HEPES, pH 7.4. 3 μM of the cell-permeable acetoxy methyl ester of Fura-2 and 250 μM sulfinpyrazone were then added to the cell suspension for 30 min at 37°C. After centrifugation at room temperature, cells were re-suspended in the standard buffer containing 250 μM sulfinpyrazone. $[Ca^{2+}]_i$ measurements were carried out in a thermostatically regulated and magnetically stirred fluorometer cuvette at a density of $1x10^6$ cells/ml. Fura-2 fluorescence was monitored with excitation wavelengths at 340 and 380 nm and the emission wavelength at 510 nm by a FluoroMax ™ spectrofluorometer. The 340/380 nm fluorescence signalling ratios (R) were converted off-line to an estimate of $[Ca^{2+}]_i$ using the following formula: $[Ca^{2+}]_i = Kd\ \beta$ (R-Rmin)/(Rmax-R). Calibrations were obtained by adding 0.1% Triton X-100 to solutions containing 1.5 mM $CaCl_2$ (Rmax), or no added Ca^{2+} (Rmin). Kd (a dissociation constant of Fura-2 for Ca^{2+}) was assumed to equal 224 nm according to Grynkiewicz et al. (1985) and β (equivalent to 380 nm at Rmin/380 nm at Rmax) was 4.2.

Western blot analysis

Western blot analysis was used to evaluate MAPK activation. Cells in 100 mm dishes were incubated for 24 h in serum-free medium to equilibrate the cells and then treated as indicated. At the end of the indicated incubation times, cells were harvested at 4°C in buffers specific to the assays (25 mM Tris buffer, pH 7.4, containing 150 mM NaCl, 100 µM sodium orthovanadate, 1.5 mM MgCl2, 1.0 mM EDTA, 1% NP40, 10% glycerol, 1 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin). After sonication, samples were centrifuged at 14,000 rpm for 5 min and an aliquot of each supernatant was processed for the assessment of protein concentration by the Bradford method. Samples were diluted in SDS-bromophenol blue buffer and boiled for 5 min before loading. Electrophoresis was performed in 12% SDS-PAGE using 10 µg total protein per lane. After separation, proteins were transferred onto a PVDF membrane using a mini trans-blot transfer cell (Bio-Rad Laboratories). After blocking, membranes were incubated with polyclonal primary antibody (rabbit phospho-ERK1/2 antibody, final dilution 1:1000) for 1 h at room temperature and then repeatedly washed and exposed to donkey anti-rabbit HPR-conjugated secondary antibody for 1 h at room temperature (final dilution 1:2500). Immunocomplexes were visualised using the enhancing chemiluminescence detection system (ECL). To determine the equal loading of samples per lane, at the end of each experiment the blots were stripped and re-probed with a rabbit anti-β-actin polyclonal antibody (Santa Cruz Biotechnology),

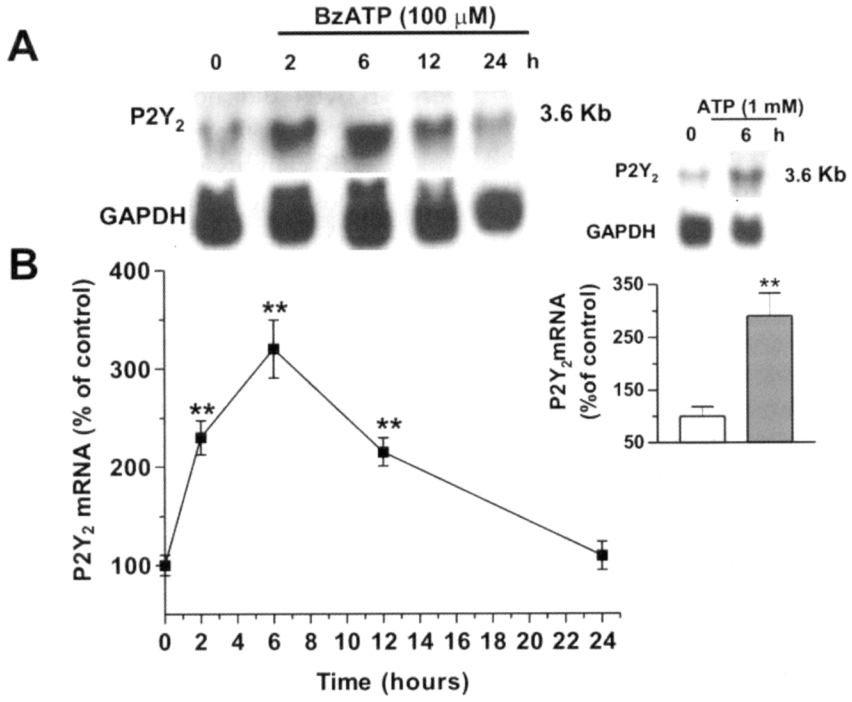

Fig. 1. *A representative Northern blot showing the time course of induction of $P2Y_2$ receptor mRNA in serum-starved cultured rat astrocytes is shown in panel A. Serum-starved astrocytes (as described in Materials and Methods) were exposed to BzATP (100 μM) and RNA extracted at the indicated times. In panel B changes in mRNA levels for the $P2Y_2$ receptor were normalized to those of GAPDH mRNA and expressed as percentage of mRNA levels in untreated cultures. Each value represents mean ± S.E.M. of three independent experiments (**p < 0.005; Student's t test).*
*Inset, a representative Northern blot showing the effect of ATP (1 mM) on $P2Y_2$ receptor mRNA expression in cultured astrocytes. The serum-starved cells were treated for 6 h with ATP and then total RNA was extracted for Northern Blot analysis. Values from densitometric analysis were normalized to those of GAPDH mRNA and expressed as percentage of mRNA levels in untreated cultures (N = 3, ** p< 0.005; Student's t test).*

according to the manufacturer's instructions.

Statistical Analysis

The statistical significance was established by analysing data by Student's two-tailed t-test, using the program Prism version 3.03 (GraphPad Software, San Diego, CA, USA) with $p < 0.05$ considered to be significant

RESULTS

BzATP and ATP enhanced the expression of $P2Y_2$ receptor mRNA in rat brain cultured astrocytes

To investigate whether the activation of $P2X_7$ receptors induced the expression of the $P2Y_2$ gene, cultured astrocytes from rat brains were treated with BzATP, the most potent $P2X_7$ agonist with an affinity about 10-fold higher than ATP itself (31-32) for 0-24 h (Fig. 1 A and B). BzATP was used at a concentration of 100 μM, which we previously reported to maximally increase purine release (12) and cysteinyl leukotriene efflux from the same cell types (13). Since no really specific antibodies are currently available (33), the $P2Y_2$ receptor expression was evaluated at the RNA level.

As previously reported (34), total RNA was extracted from the cells and the level of $P2Y_2$ mRNA was analyzed by Northern blotting. BzATP rapidly enhanced the expression of $P2Y_2$ mRNA whereas that of GAPDH, chosen as housekeeping gene, remained constant as shown by the representative Northern blot in Fig. 1 A. A significant increase was found at 2 h and the BzATP-mediated up-regulation of this metabotropic ATP/UTP receptor reached a peak level at 6 h. As shown in Fig. 1 B, the densitometric analysis revealed that the peak level of BzATP- induced $P2Y_2$ mRNA expression was 320

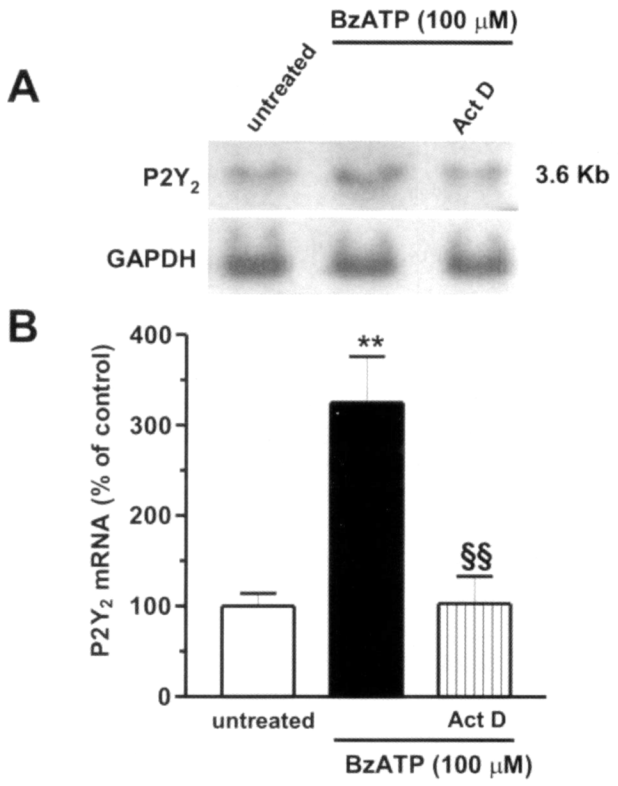

Fig. 2. *Effect of the inhibitor of RNA polymerase, Actinomycin D (Act D), on BzATP-mediated induction of $P2Y_2$ receptor mRNA in cultured rat astrocytes evaluated by Northern blot analysis. Serum-starved cells were pre-treated with Act D (10 μg/ml) for 1 h and then treated with 100 μM BzATP for 6 h. A representative experiment is reported in panel A. In panel B changes in mRNA levels for $P2Y_2$ receptors were normalized to those of GAPDH mRNA and expressed as percentage of mRNA levels in untreated cultures. Each value represents mean ± S.E.M. of three independent experiments (**$p < 0.005$ vs untreated cells; §§$p < 0.005$ vs BzATP-treated cells; Student's t test).*

± 51% of the untreated cells (Fig. 1 B). A similar effect was found by adding high ATP concentrations (1 mM) to the astrocyte medium as shown in the inset of Fig. 1. When cells were pre-treated for 1 h with actinomycin D (Act D) (10 μg/ml), an inhibitor of RNA polymerase, BzATP was ineffective (Fig. 2 A and B) indicating that *de novo* transcription is required for the $P2Y_2$ induction.

The MTT assay was used to estimate cell viability after BzATP exposure. As shown in Fig. 3, astrocytes were challenged with the agonist (50-300 μM) for up to 72 h. Control values were determined at each time interval and were unchanged throughout the treatment period ($p > 0.05$). Exposure to 100 μM BzATP did not significantly reduce viability of astrocytes for up to 24 h of treatment. However, the MTT viability measure was already reduced by 12 h exposure to the agonist added to the culture medium at a concentration of 300 μM (Fig. 3)

To confirm that the BzATP-induced $P2Y_2$ up-regulation was mediated by the $P2X_7$ receptor, the cells were treated with oxidized ATP (oATP) or with Brilliant Blue G (BBG), which are known to be selective and potent inhibitors of $P2X_7$ sites.

The cells were pre-treated with oATP (300 μM) for 3 h or with BBG (1 μM) for 30 min and then exposed to BzATP for 6 h. As shown in Fig. 4, both the antagonists almost abolished the BzATP stimulated $P2Y_2$ mRNA expression.

We previously reported that astrocytes challenged with BzATP (100 μM) stimulated the release of adenine-based purines from these cells (12). Moreover, we recently published that ATP along with UTP, the two naturally occurring agonists for $P2Y_2$ receptors, increased the levels of $P2Y_2$ mRNA in cultured rat brain astrocytes (34). To assess whether the effect of BzATP was mediated by the induced release of ATP, the ATP scavenger apyrase (30 U / ml) was added to the medium 15 min before the application of the $P2X_7$ agonist. As shown in Fig. 5, the response of the cells to 100 μM BzATP was unaffected by the presence of the enzyme.

Previous pharmacological studies demonstrated that in astrocytes $P2Y_2$ receptor activation is linked to an increase in $[Ca^{2+}]_i$. In cultured astrocytes, incubated for 24 h in a serum free medium, we recently reported (34) that 100 μM UTP increased $[Ca^{2+}]_i$ (Fig. 6 A) (28 ± 7 nM over the basal value). As shown in Fig. 6A, this increase was significantly higher in astrocytes pre-treated for 6 h with 100 μM BzATP and then harvested for $[Ca^{2+}]_i$ measurement (135 ± 24 nM over the basal value). When the cells were pre-treated with the $P2X_7$ antagonists oATP (300 μM, for 2 h) or BBG (1 μM for 30 min) the effect of BzATP was abolished (Fig. 6 A). Finally $P2Y_2$ receptor siRNA ($P2Y_2R$ siRNA) was used to inhibit the expression of the receptor. RT-PCR showed that siRNA significantly reduced $P2Y_2$ without affecting the expression of $P2Y_4$, the

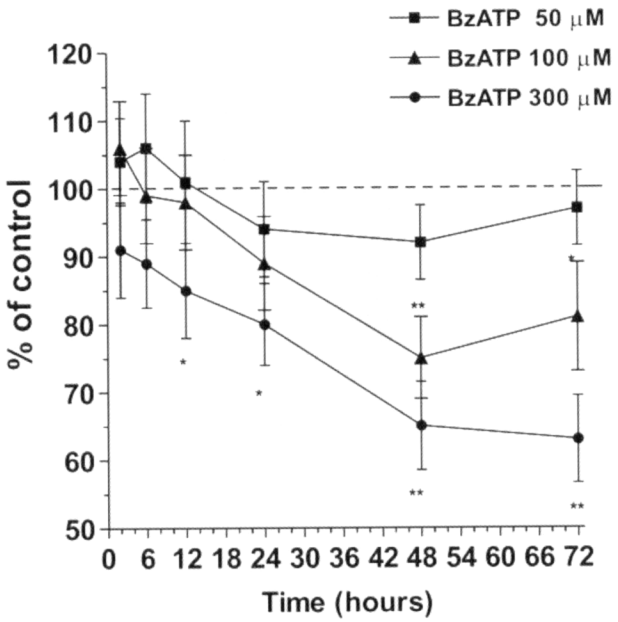

Fig. 3. *MTT dye-reduction analysis of the effect of BzATP (50-300 μM) on astrocyte cell viability. Cultured rat brain astrocytes were grown to confluence and then treated with the $P2X_7$ agonist for the indicated times as reported in Materials and Methods. Each value represents the mean ± S.E.M (N = 15, *p< 0.05 and **p< 0.005 vs control; Student's t test).*

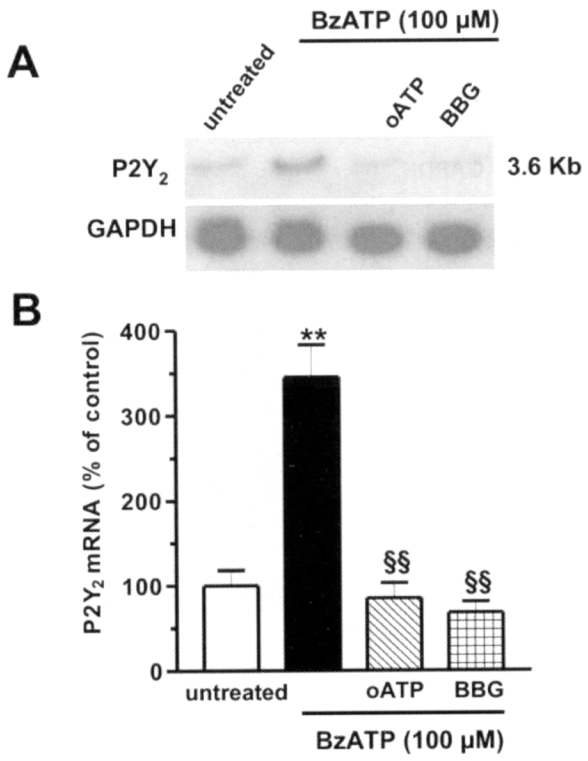

Fig. 4. *Effect of $P2X_7$ antagonists oATP and BBG on BzATP-mediated induction of $P2Y_2$ receptor mRNA in cultured rat astrocytes evaluated by Northern blot analysis. Serum-starved cells were pre-treated with oATP (300 μM) for 2 h or with BBG (1 μM) for 30 min and then treated with 100 μM BzATP for 6 h. A representative experiment is reported in panel A. In panel B changes in mRNA levels for $P2Y_2$ receptors were normalized to those of GAPDH mRNA and expressed as percentage of mRNA levels in untreated cultures. Each value represents mean ± S.E.M. of four independent experiments (**p< 0.005 vs untreated cells, §§p< 0.005 vs BzATP-treated cells; Student's t test).*

other UTP-preferring P2Y receptor (Fig. 6 B). Pre-treatment of the cultured cells with $P2Y_2$RsiRNA counteracted the UTP-induced $[Ca^{2+}]_i$ increase in astrocytes pre-treated for 6 h with 100 μM BzATP (Fig. 6 A).

Calcium, MAPK and PKC involvement in the BzATP-induced stimulation of $P2Y_2$ receptor mRNA

We previously reported that in cultured rat brain astrocytes BzATP induced a dose-dependent increase in $[Ca^{2+}]_i$ which was markedly reduced when cells were treated with the extracellular calcium chelating agent EGTA (12). To evaluate whether $P2Y_2$ up-regulation induced by the $P2X_7$ agonist was controlled by calcium influx, the glial cells were pre-treated with 2 mM EGTA for 15 min. As shown in Fig. 7 A and B, chelation of extracellular calcium reduced by 68 ± 12% the $P2Y_2$ mRNA induction mediated by BzATP.

Since it has been reported that $P2X_7$ activation is able to stimulate ERK1/2 phosphorylation and PKC activity in different cell types including astrocytes, human astrocytoma cells and type-2 astrocytes cell line RBA-2 (14, 35-36), the experiments were also carried out in the presence of PKC and mitogen-activated protein kinase (MAPK) inhibitors PD98059 (30 μM) and GF109203X (10 μM) (Fig. 7 C and D). Both drugs significantly reduced the BzATP-stimulated expression of ERK1/2 by 63 ± 9% and 59 ± 11% respectively.

Role of calcium and PKC on ERK1/2 activation by BzATP

To investigate further whether in our experimental

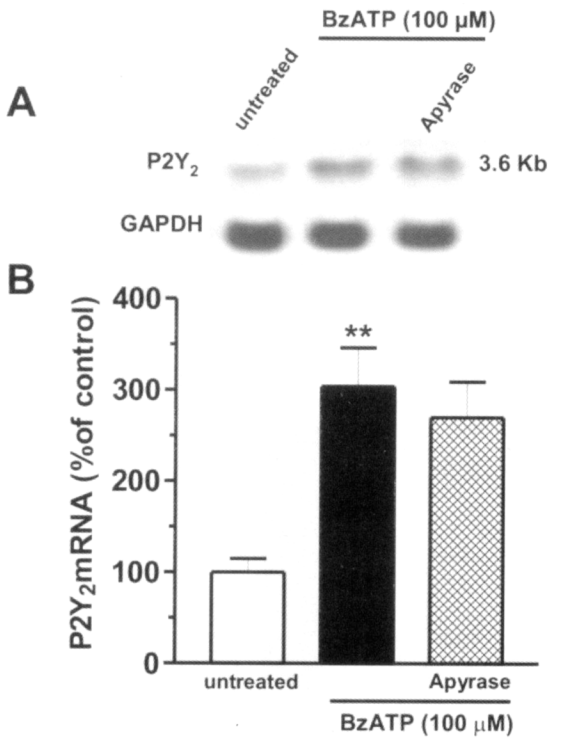

Fig. 5. *Effect of the ATP scavenger enzyme Apyrase (Apy) on BzATP-mediated induction of $P2Y_2$ receptor mRNA in rat cultured astrocytes evaluated by Northern blot analysis. Serum-starved cells were pre-treated with Apy (30 U/ml) for 15 min and then treated with 100 μM BzATP for 6 h. A representative experiment is reported in panel A. In panel B changes in mRNA levels for $P2Y_2$ receptors were normalized to those of GAPDH mRNA and expressed as percentage of mRNA levels in untreated cultures. Each value represents mean ± S.E.M. of three independent experiments (**p < 0.005 vs untreated cells; Student's t test).*

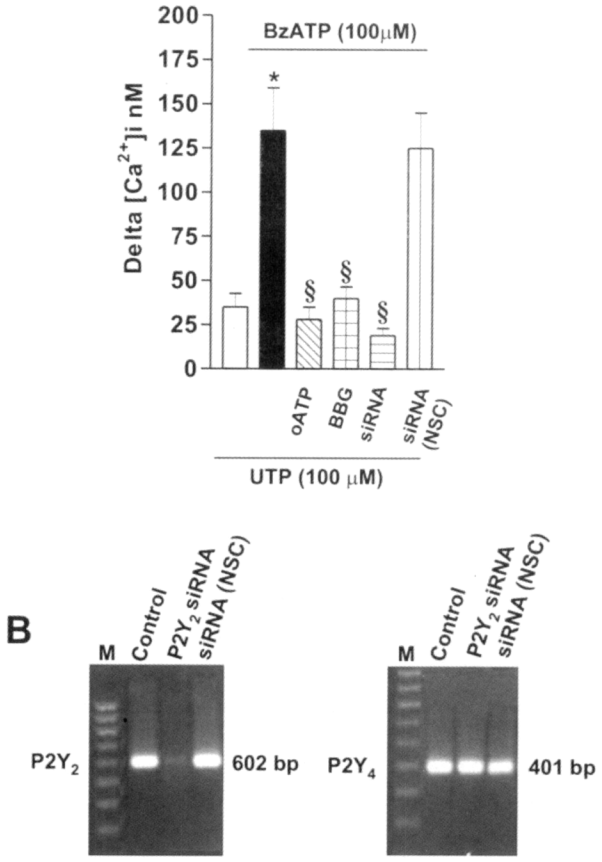

Fig. 6. *A) Intracellular calcium increase evoked by 100 μM UTP in serum-starved cells (control) or in serum-starved cells pre-treated with BzATP (100 μM) for 6 h in the absence or in the presence of oATP (300 μM), BBG (1 μM) or in astrocytes transfected with 100 nM $P2Y_2$ siRNA or with 100 nM siRNA (NSC). $[Ca^{2+}]_i$ was evaluated in Fura-2 pre-loaded serum-starved astrocytes as described in Materials and Methods. Delta calcium represents the net increases in $[Ca^{2+}]_i$ calculated by subtracting the basal level of $[Ca^{2+}]_i$ from peak levels. Data are the means ± S.E.M. from three determinations. (* p< 0.05 vs untreated cells, § p< 0.05 vs BzATP-treated cells; Student's t test). B) $P2Y_2$ siRNA-reduced $P2Y_2$ receptor mRNA expression in rat brain astrocytes. The cells at 60-70% of confluence were incubated for 1 day in OPTIMEM without antibiotics and treated with 100 nM $P2Y_2$ receptor siRNA or Non Specific Control siRNA (NSC) plus 3 μl Oligofectamine. 24 hours after transfection, total RNA was isolated and expression of $P2Y_2$ and $P2Y_4$ receptors was determined by RT-PCR as described in Materials and Methods. Lane 1 M DNA ladder; Lane 2 untreated control; Lane 3 $P2Y_2$ receptor siRNA; Lane 4 siRNA (NSC).*

conditions BzATP stimulated MAPK signalling, the time course of expression of phosphorylated ERK1/2 (p-ERK1/2) was analysed by Western blotting. As shown in Fig. 8 A and B the application of BzATP (100 μM) caused a rapid and reversible increase in p-ERK1/2. The signal increased as early as 5 min, peaked at 30 min. Also, when the cells were treated with 1 mM ATP for 30 min a similar increase in pERK1/2 was found (inset of Fig. 9).

Pre-treatment of astrocytes with oATP (300 μM for 2 h) or with BBG (1 μM for 30 min) blocked the activation of ERK1/2 induced by the $P2X_7$ agonist (Fig. 9 A and B).

To determine whether the activation of ERK1/2

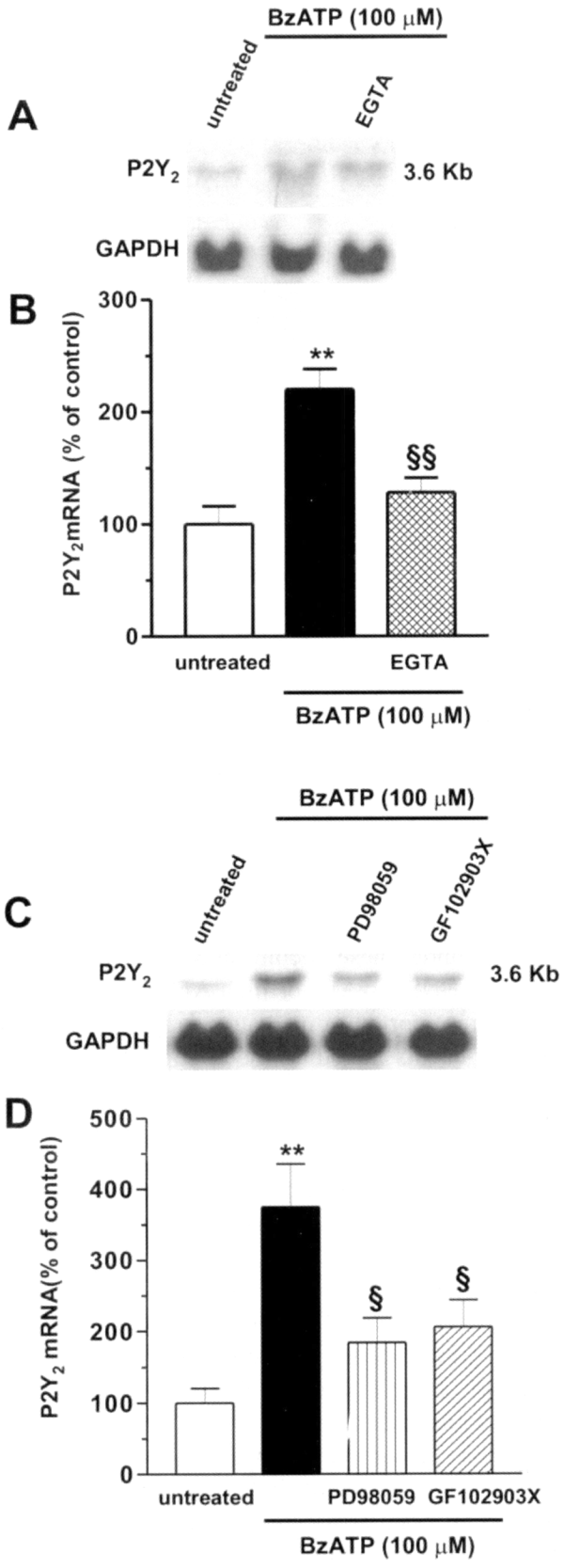

Fig. 7. *Role of calcium, ERK1/2 and PKC activation in BzATP induced up-regulation of $P2Y_2$ receptor mRNA evaluated by Northern blot analysis. Serum-starved astrocytes were pre-treated with: panel A) and B) the extracellular Ca^{2+} chelator EGTA (2 mM) for 15 min, panel C) and D) the MEK inhibitor PD98059 (30 μM) and the PKC inhibitor GF102903X (10 μM) for 30 min and then treated with 100 μM BzATP for 6 h as described in Materials and Methods. Representative experiments are reported in panel A and C respectively. In panels B and D changes in mRNA levels for $P2Y_2$ receptor were normalized to those of GAPDH mRNA and expressed as percentage of mRNA levels in untreated cultures. Data represent mean ± S.E.M. of four independent experiments (**$p< 0.005$ vs untreated cell; §$p< 0.05$ and §$p< 0.05$ vs BzATP-treated cells; Student's t test.).*

induced by BzATP was linked to an increased influx of extracellular Ca^{2+} into the astrocytes, cells were pre-treated with 2 mM EGTA. As shown in Fig. 10 A and B, inhibition was not observed. A significant reduction was only obtained when EGTA was added to the culture medium at higher concentrations (8 mM) (data not shown).

The involvement of PKC was then examined by pre-treating the cultured astrocytes with GF109203X. As shown in Fig. 10 C and D, the PKC inhibitor (10 μM) counteracted the BzATP-stimulated ERK1/2 activation in these cells. As expected, PD98059 (30 μM) used as an internal control, abolished the BzATP-stimulated ERK1/2 activation.

We have recently shown that treating astrocytes with phorbol ester PMA (0.1 μM), a well-known PKC activator, rapidly increased $P2Y_2$ receptor mRNA levels (34). As shown in Fig. 11 A and B, PMA (0.1 μM) significantly stimulated ERK1/2 activation in a time dependent manner thus raising the possibility that PKC and MAPK might act on the same pathway.

DISCUSSION

Contrary to other P2X ionotropic ATP receptors, $P2X_7$ receptors exhibit low affinity for ATP (millimolar range) in the presence of divalent cations (8-9). Steinberg et al (1987) (37) postulated that the physiological agonist for the $P2X_7$ receptor was the tetra-acidic form ATP4- (which in physiological solutions comprises roughly 10% of the total ATP

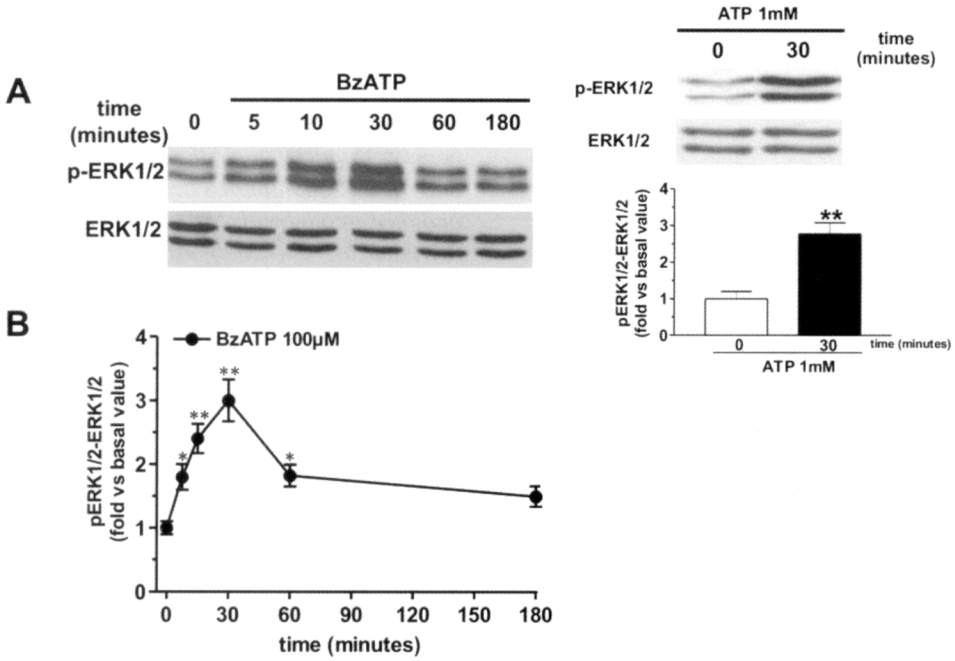

Fig. 8. *Effect of BzATP and of high ATP concentration on ERK1/2 activation in cultured rat brain astrocytes. The cells were treated with 100 μM BzATP (panel A) or with 1 mM ATP (inset) for the time periods indicated. Aliquots (10 μg) of proteins from cell lysates were subjected to specific Western blotting using antibodies against phospho-ERK1/2 as reported in Materials and Methods. Immunoblots, re-probed with antibodies against ERK1/2 to assure equal sample loading, were quantified by densitometric analysis and the values were normalised to ERK1/2 levels (panel B). Data are the mean ± S.E.M. of three independent experiments. (*p< 0.05 and **p< 0.005; Student's t test.).*

present, depending on pH and ion concentration) which was able to stimulate the channel activity (38). Thus the pathophysiological role that these receptors could play *in vivo* has long been questioned.

Even though extracellular ATP levels are thought to be in the picomolar-low nanomolar range in basal conditions, several reports indicate that in different cell types including glia, different kinds of stimuli, such as stretch, osmotic challenge, electrical stimulation and agonist application, can trigger significant ATP release (39).

Beigi et al. (40) observed that ATP concentrations in the bulk medium of platelets stimulated by thrombin underestimated by at least 1 order of magnitude the ATP concentration near the cell surface. More recently, by using plasma-membrane-targeted luciferase constructs it has been shown that agonist-stimulated ATP release reaches concentrations that are sufficiently high to activate $P2X_7$ receptors (41-42). Thus, considering that more intense and less specific stimulation in the brain caused by injury, ischemia, inflammation or trauma increase ATP release much more dramatically, it seems likely that CNS-$P2X_7$ receptors could be activated under these circumstances.

In the CNS, BzATP or high ATP concentrations have been reported to mediate, via $P2X_7$ receptors, different events including modulation of glutamate and GABA release from spinal cord or hippocampal slices and enhancement of excitatory postsynaptic currents (EPSCs) in the hypoglossal nucleus (43).

In situ astrocytes express $P2X_7$ receptors, thus ATP released after neuronal cell injury could activate these sites. In cultured astrocytes $P2X_7$ stimulation has been shown to increase adenine-based purines (12), to enhance the efflux of cysteinlyl leukotrienes (13), to stimulate the production of TGF-β (44), to induce the expression of MCP-1 (14) and to mediate glutamate release (45).

In the present study we show that BzATP increases the levels of $P2Y_2$ receptor mRNA in cultured rat brain astrocytes as early as 2 h peaking at 6 h, times at which the $P2X_7$ agonists did not modify astrocyte cell viability as shown by MTT

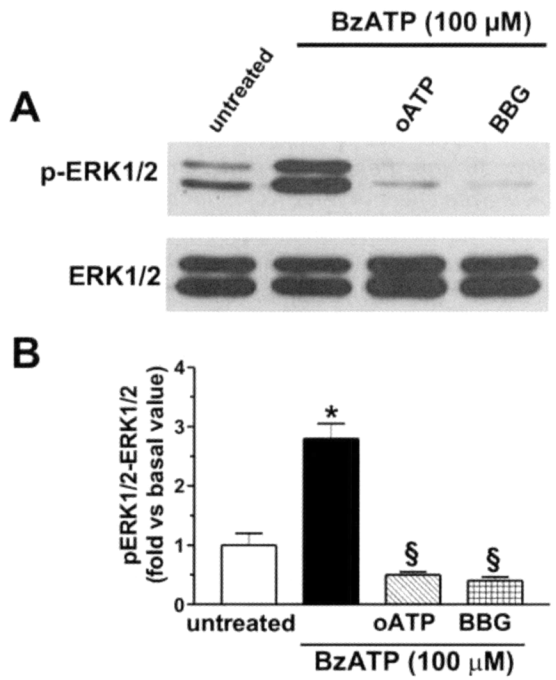

Fig. 9. *Effect of $P2X_7$ antagonists oATP and BBG on BzATP mediated activation of ERK1/2 in cultured rat astrocytes. Panel A): a representative Western blot showing the effect of $P2X_7$ antagonists oATP (300 μM) or with BBG (1 μM) added to the culture medium for 2 h and 30 min respectively before cell exposure to BzATP (100 μM). Aliquots (10 μg) of proteins from cell lysates were subjected to specific Western blotting using antibodies against phospho-ERK1/2 as reported in Materials and Methods. Immunoblots, re-probed with antibodies against ERK1/2 to assure equal sample loading, were quantified by densitometric analysis, the values of which, normalised to ERK1/2 levels, are reported in panel B. Data are the mean ± S.E.M. of three independent experiments. (*p< 0.005 vs untreated cells; §p< 0.005 vs BzATP-treated cells; Student's t test).*

assay. This effect was mimicked by high extracellular ATP concentrations and was inhibited by oATP, a commonly used antagonist that irreversibly blocks the $P2X_7$ receptors, thus providing further evidence of a $P2X_7$ mediated-activity.

However, it has been reported that oATP in the concentration range of 100-300 μM can also partially inhibit $P2X_1$ and $P2X_2$ receptors (46) and can reduce proinflammatory signalling independently of the presence or the activation of P2 receptors. Thus, to confirm the involvement of $P2X_7$ receptor in BzATP-mediated $P2Y_2$ mRNA up-regulation, astrocytes were pre-treated with BBG, a highly selective antagonist for rodent $P2X_7$ receptor with nanomolar affinity (47). As expected, BBG also inhibited the BzATP-induced effect.

As recently reported for UTP and guanosine-mediated $P2Y_2$ receptor mRNA up-regulation, (34) in the presence of the mRNA polymerase inhibitor ActD, BzATP was unable to induce any increase in $P2Y_2$ mRNA levels. This indicates that the $P2X_7$-agonist effect in astrocytes was mediated through *de novo* synthesis rather than through inhibition of the degradation rate of $P2Y_2$ mRNA. The transcriptional induction of the expression of $P2Y_2$ receptors on the astrocyte plasma membranes was linked to an enhanced UTP-mediated Ca^{2+} response, raising the possibility that there is a tight relationship between the level of $P2Y_2$ receptor expression and the rate of activity. These data are in agreement with results obtained by Hou and co-workers (48) in vascular smooth muscle cells. Of P2Y ATP metabotropic receptor subtypes, UTP is the preferential agonist not only for $P2Y_2$ receptors but also for $P2Y_4$ receptors, whereas $P2Y_6$ sites are more sensitive to UDP than UTP (49). Our results show that cell transfection with siRNA for $P2Y_2$ receptors is able to abrogate the UTP-mediated $[Ca^{2+}]i$ increase. Thus the involvement of $P2Y_4$ receptors in the UTP-triggered modification of Ca^{2+} levels seems unlikely.

In our previous study we reported that in cultured astrocytes from rat brain BzATP triggered a $P2X_7$-like response linked to a sustained Ca^{2+} influx (12). An increase in nuclear Ca^{2+} coming from increased cytoplasm Ca^{2+} levels has recently been found to activate nuclear Ca^{2+}-sensitive proteins and subsequently to regulate gene transcription in both neurons and glia (50-51). Our results show that by chelating extracellular Ca^{2+} with 2 mM EGTA, which we reported to abolish the $P2X_7$-like calcium response in cultured astrocytes (12), reduced the BzATP-induced $P2Y_2$ receptor mRNA by 68 ± 12% indicating that other signalling pathways could be involved. In other cell types, stimulation of $P2Y_2$ receptor mRNA expression was mediated not only by an increase in $[Ca^{2+}]i$, but also by MAPK and/or PKC activation (26, 52). In the present study the MAPK and PKC inhibitors, PD98059 and GF109203X, significantly inhibited the BzATP-stimulated $P2Y_2$ mRNA expression in the cultured astrocytes.

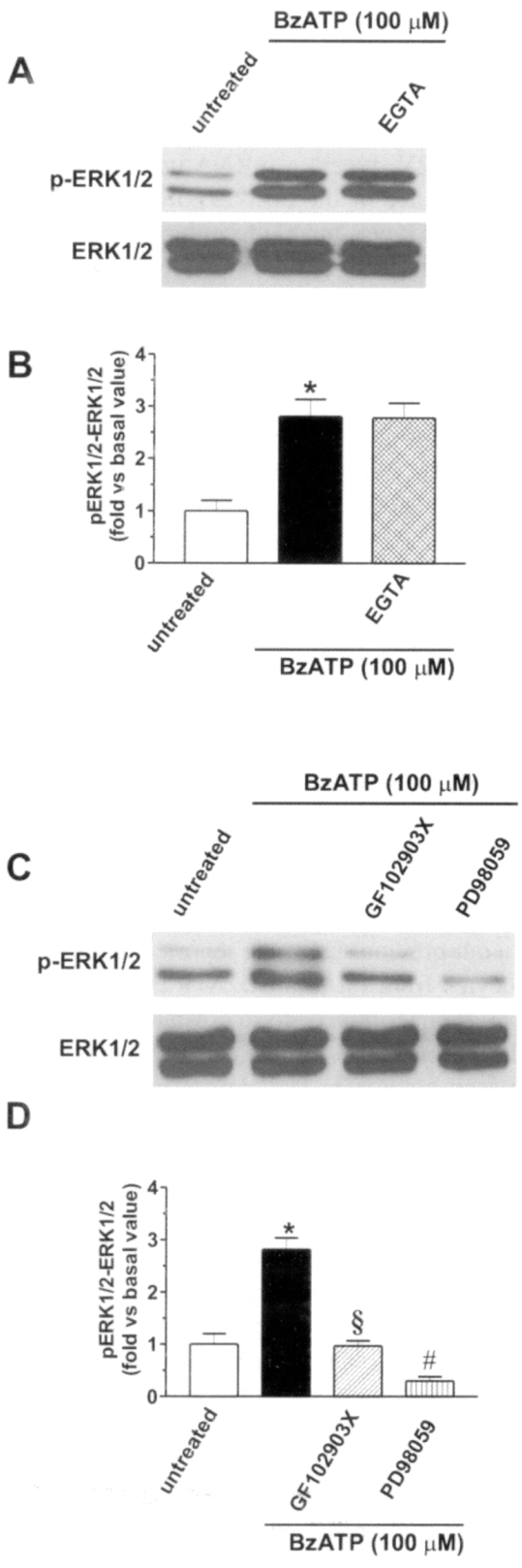

Fig. 10. *Role of calcium and of PKC activation in BzATP mediated activation of ERK1/2. Panel A: a representative Western blot showing the lack of effect of 2 mM EGTA added to the culture medium of serum-starved astrocytes 15 min before cell exposure to BzATP (100 μM) for further 30 min. Panel C: a representative Western blot showing the effect of the PKC inhibitor GF102903X (10 μM) and of the MEK inhibitor PD98059 (30 μM) added to the culture medium for 30 min before cell exposure to BzATP (100 μM). Aliquots (10 μg) of protein from cell lysates were subjected to specific Western blotting using antibodies against phospho-ERK1/2 as reported in Materials and Methods. Immunoblots, re-probed with antibodies against ERK1/2 to assure equal sample loading, were quantified by densitometric analysis, the values of which, normalised to ERK1/2 levels, are reported in panels B and D Data are the mean ± S.E.M. of four independent experiments. (*$p < 0.005$ vs untreated cell, §$p < 0.005$ and #$p < 0.0005$ vs BzATP-treated cells; Student's t test).*

PD98059 and GX109203X caused a 63 ± 9% and 59 ± 11% inhibition respectively. Thus, besides having an effect on calcium, the $P2X_7$-stimulated $P2Y_2$ mRNA expression was also mediated via ERK and PKC signalling pathways.

Our results also show that application of BzATP as well as high ATP concentrations caused a rapid and reversible increase in phosphorylated ERK1/2 within 5 min, in agreement with Panenka et al. and Gendron et al. (14, 35). Treatment of astrocytes with oATP or with BBG inhibited the BzATP-induced ERK1/2 activation, thus confirming the involvement of $P2X_7$ receptors in the response. The effect of GF109203X, which inhibited the BzATP-mediated ERK1/2 phosphorylation indicates that under our experimental conditions the $P2X_7$ agonist may have stimulated PKCs to regulate astrocyte ERK1/2 activation and consequently $P2Y_2$ mRNA up-regulation. This hypothesis is strengthened by the reported effect of PMA on ERK1/2 activation and by our previous finding showing that PMA was able to up-regulate $P2Y_2$ mRNA in the same cells (34).

In contrast, chelation of extracellular Ca^{2+} with 2 mM EGTA, a concentration reported to be able to abolish BzATP-induced increases in $[Ca^{2+}]i$ in these cells (12), was ineffective. This was not surprising given that in other cell types, such as parotid acinar cells ATP/BzATP-induced ERK1/2 activation was not due to the elevation of $[Ca^{2+}]i$ via $P2X_7$ receptor-

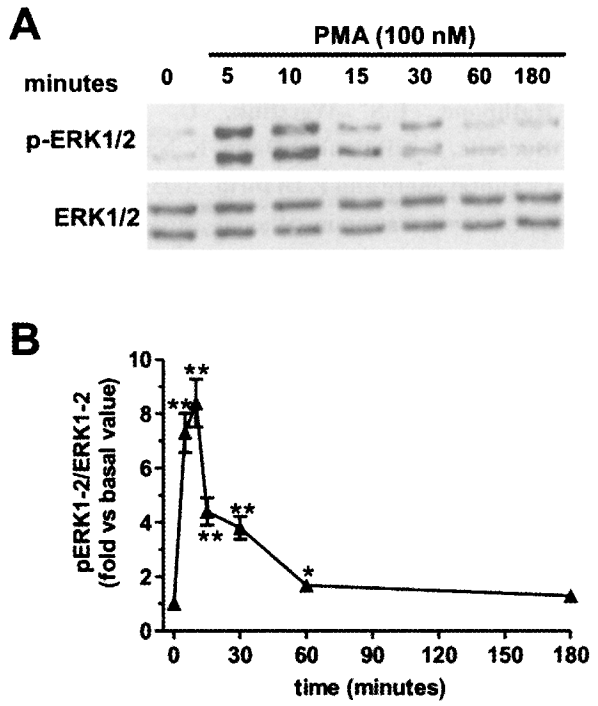

Fig. 11. *Time course of PMA effect on ERK1/2 phosphorylation. Serum-starved astrocytes were treated with 100 nM PMA for the time periods indicated. Aliquots (10 μg) of protein from cell lysates were subjected to specific Western blotting using antibodies against phospho-ERK1/2 as shown in a representative experiment (panel A). Immunoblots were re-probed with antibodies against ERK1/2 to assure equal sample loading and subsequently quantified by densitometric analysis, the values of which, normalised to ERK1/2 levels, are reported in panel B. Data are the mean ± S.E.M. of three independent experiments. (*p< 0.05 and **p< 0.005; Student's t test).*

mediated Ca^{2+} entry. Only when higher EGTA concentrations were used (mM) was an inhibition of BzATP-mediated effect evident. However, in these latter conditions EGTA could also exert a non-specific effect in that it may decrease the pH of the medium, thus inhibiting $P2X_7$ activation (53).

Our results therefore show that BzATP stimulation induces an early up-regulation of astrocyte $P2Y_2$ receptor mRNA via both an increase in $[Ca^{2+}]i$ and activation of the PKC-ERK1/2 pathway. Since the BzATP effect is mimicked by ATP at millimolar concentration, and is counteracted by the $P2X_7$ antagonists oATP and BBG, it is very likely that $P2X_7$ is the receptor involved in the up-regulation of $P2Y_2$ mRNA.

There is increasing evidence that $P2Y_2$ receptors have a neuroprotective role in astrocytes. Activation of this ATP metabotropic receptor subtype has been reported to stimulate the expression of several genes involved in cell differentiation and survival (27) as well as those implicated in nervous system development and neuronal migration (54). Moreover, in human astrocytoma cells stably transfected with human $P2Y_2$ receptor DNA, the receptor activation stimulated the non-amyloidogenic processing of APP, leading to an increased release of sAPPα, a mechanism which may have important neuroprotective implications in neurodegenerative disorders including Alzheimer's disease (55). Despite these findings, little is known of the cellular pathways or factors involved in the regulation of $P2Y_2$ receptor expression. Therefore, the study of the molecular mechanisms underlying changes in $P2Y_2$ mRNA levels in the CNS contributes to a better understanding of their functional role and their regulation.

It could be speculated that when ATP is released at low concentrations, it preferentially activates metabotropic receptors causing a moderate astrocyte reaction. In contrast, when cell injury in the CNS causes release of large amounts of ATP this nucleotide can act as an emergency messenger and stimulate astrocyte $P2X_7$ receptors. In turn, these can trigger several events, including the increase in 2-arachidonylglycerol production (22), the reduction of LPS-induced TNFα release (23) and the up-regulation of $P2Y_2$ receptors, in an attempt to activate also glial-mediated protective mechanisms aimed at limiting the tissue damage.

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