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# $P2Y_2$ receptors activate neuroprotective mechanisms in astrocytic cells

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**Abstract**

Mechanical or ischemic trauma to the CNS causes the release of nucleotides and other neurotransmitters into the extracellular space. Nucleotides can activate nucleotide receptors that modulate the expression of genes implicated in cellular adaptive responses. In this investigation, we used human 1321N1 astrocytoma cells expressing a recombinant $P2Y_2$ receptor to assess the role of this receptor in the regulation of anti-apoptotic (*bcl-2 and bcl-xl*) and pro-apoptotic (*bax*) gene expression. Acute treatment with the $P2Y_2$ receptor agonist UTP up-regulated *bcl-2* and *bcl-xl*, and down-regulated *bax*, gene expression. Activation of $P2Y_2$ receptors was also coupled to the phosphorylation of cyclic AMP responsive element binding protein that positively regulates *bcl-2* and *bcl-xl* gene expression. Cyclic AMP responsive element decoy oligonucleotides markedly attenuated the UTP-induced increase in *bcl-2* and *bcl-xl* mRNA levels. Activation of $P2Y_2$ receptors induced the phosphorylation of the pro-apoptotic factor Bad and caused a reduction in *bax/bcl-2* mRNA expression ratio. All these signaling pathways are known to be involved in cell survival mechanisms. Using cDNA microarray analysis and RT–PCR, $P2Y_2$ receptors were found to up-regulate the expression of genes for neurotrophins, neuropeptides and growth factors including nerve growth factor 2; neurotrophin 3; glia-derived neurite-promoting factor, as well as extracellular matrix proteins CD44 and fibronectin precursor – genes known to regulate neuroprotection. Consistent with this observation, conditioned media from UTP-treated 1321N1 cells expressing $P2Y_2$ receptors stimulated the outgrowth of neurites in PC-12 cells. Taken together, our results suggest an important novel role for the $P2Y_2$ receptor in survival and neuroprotective mechanisms under pathological conditions.

**Keywords:** astrocytes, gene expression, $P2Y_2$ nucleotide receptor, purinergic signaling.

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Astrocytes are involved in normal brain functions including neuronal development, synaptic activity, synaptic plasticity and homeostatic control of the extracellular environment (Fields and Stevens-Graham 2002). Mechanical or ischemic trauma in the brain causes astrocytes to undergo reactive gliosis, a process characterized by increased expression of glial fibrillary acidic protein (GFAP) and cell proliferation. Reactive astrocytes secrete neurotrophic factors that can contribute to neuronal regeneration after injury, and proteases and protease

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*Abbreviations used*: 1321N1-$P2Y_2$ cells, human 1321N1 astrocytoma cells transfected with $P2Y_2$ nucleotide receptor cDNA; BSA, bovine seum albumin; CRE, cyclic AMP responsive element; CREB, cyclic AMP responsive element binding protein; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FCIII, fetal clone III serum; *fn*, fibronectin precursor; *gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *gdnpf*, glia-derived neurite-promoting factor; GFAP, glial fibrillary acidic protein; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; NGF, nerve growth factor; *ngf-2*, nerve growth factor-2; *nt-3*, neurotrophin-3; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; SDS, sodium dodecyl sulfate.

 

inhibitors that modulate neurite growth and offer neuronal protection (Goss *et al*. 1998). However, responses of astrocytes to mechanical and physical trauma are variable and depend upon the interplay of local factors and signals emanating from surrounding damaged tissue. Nonetheless, reactive astrogliosis has been implicated in the progression of neurodegenerative diseases and neuronal cell death (Zhang *et al*. 1998).

Nucleotides released from damaged cells into the extracellular space activate G protein-coupled P2Y and ionotropic P2X nucleotide receptors leading to an increase in intracellular $Ca^{2+}$ levels and the activation of mitogen-activated protein kinase (MAPK) signaling pathways (Gendron *et al*. 2003). It has been suggested that nucleotides acting through P2Y receptors can play trophic roles in the development and regeneration of tissue in the nervous system (Neary and Abbracchio 2001) whereas the P2X receptors may mediate neurodegenerative events including astrogliosis (Franke *et al*. 2001; James and Butt 2002; Le-Feuvre *et al*. 2002). P2Y receptors have diverse functions, including the regulation of platelet aggregation, muscle contraction, inflammation, neurotransmission, insulin secretion and epithelial ion transport, although little is known about the neuroprotective pathways for P2Y receptors.

The Bcl-2 family of proteins are key regulators of apoptosis that play a central role in dictating cell fate in response to diverse stimuli (Adams and Cory 1998; Chao and Korsmeyer 1998). Although all members share certain structural homology, they are functionally diverse. For example, bcl-2 and bcl-xl inhibit apoptosis whereas bax and Bad promote apoptosis. Consequently, the level of expression and, particularly, the ratio of anti-apoptotic proteins to pro-apoptotic proteins are believed to play a critical role in determining whether cells survive or die (Oltvai *et al*. 1993; Adams and Cory 1998). Anti-apoptotic bcl-2 and bcl-xl proteins show widespread expression in the nervous system where they play an important role in neuronal survival (Merry and Korsmeyer 1997). There is compelling evidence that increased expression of genes of the Bcl-2 family can protect against neuronal damage and delay neuronal death, and that endogenous tolerance to transient forebrain ischemia depends on the expression of *bcl-2* and *bcl-xl* genes (Davies 1995; Zhu *et al*. 1999). In the present study, we demonstrate that activation of $P2Y_2$ receptors induces *bcl-2* and *bcl-xl* expression in a cyclic AMP responsive element binding protein (CREB)-dependent manner, thus triggering survival-signaling cascades in human astrocytic cells. In addition, activation of $P2Y_2$ receptors stimulated the expression of genes implicated in nervous system development, neuronal migration, differentiation and survival, and the formation and function of synapses. These novel observations suggest that $P2Y_2$ receptors may regulate important neuroprotective mechanisms under pathological conditions in the human brain.

## Materials and methods

### Materials

UTP, apyrase and rat nerve growth factor (NGF) were obtained from Sigma-Aldrich (St Louis, MO, USA). PD98059, SB203580, LY294002, wortmannin and etoposide were purchased from Calbiochem (San Diego, CA, USA). The anti-polyclonal phosphoprotein antibodies to Akt $Thr^{308}$, CREB $Ser^{112}$, Bad $Ser^{112}$, Bad $Ser^{136}$, Bad $Ser^{155}$, p38 $Thr^{180}/Tyr^{182}$, p42/p44 MAPK–extracellular signal-regulated kinase (ERK)1/2 $Thr^{202}/Tyr^{204}$, and anti-polyclonal antibodies to Akt, CREB, Bad, p38, p42/p44 MAPK–ERK1/2 and rabbit IgG conjugated to horseradish peroxidase were purchased from Cell Signaling Technology (Beverly, MA, USA).

### Cell culture and transfection

Human 1321N1 astrocytoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Carlsbad, CA, USA) containing 5% (v/v) fetal clone III serum (FCIII) (Invitrogen), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. The retroviral vector pLXSN was used for stable expression of the $P2Y_2$ receptor constructs in 1321N1 cells, as described previously (Erb *et al*. 1995). Briefly, the recombinant $P2Y_2$-pLXSN constructs were used to transfect PA317 amphotrophic packaging cells for production of the viral vectors. Then, 1321N1 cells were infected with the viral vectors and selected for neomycin resistance with 1 mg/mL G418 (Invitrogen). Rat pheochromocytoma PC-12 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in F-12K medium (American Type Culture Collection) containing 15% (v/v) horse serum (American Type Culture Collection), 2.5% (v/v) FCIII, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin. Rat immortalized astrocytes (DITNC cells) were cultured in DMEM containing 5% (v/v) FCIII, 5% (w/v) glucose, 100 U/mL penicillin and 100 μg/mL streptomycin.

### RNA isolation

Total RNA was isolated using the TRIZOL® reagent (Invitrogen) following the manufacturer's instructions. Briefly, the cells were treated with agonists, pelleted by centrifugation and lysed with the TRIZOL® reagent by repeated pipeting. Cell homogenates were incubated for 5 min at 21°C to allow complete dissociation of the nucleoprotein complexes. After a chloroform extraction, the RNA-containing aqueous phase was recovered and mixed with isopropyl alcohol. Precipitated RNA was washed once with 75% (v/v) ethanol and centrifuged at 8200 *g* for 5 min at 4°C. RNA pellets were resuspended in Rnase-free water and RNA was quantified by UV spectrophotometry. Removal of DNA was accomplished by treatment with Dnase I (Rnase free; Roche Diagnostics Corporation, Indianapolis, IN, USA). The RNA was precipitated its integrity determined by 1% (w/v) agarose gel electrophoresis.

### RT–PCR

RT–PCR was performed with total RNA isolated as described above. After Dnase I treatment, 2 μg RNA was used for cDNA synthesis with a Reverse Transcription System kit from Promega (Promega, Madison, WI, USA) following the manufacturer's instructions. The reactions were performed with a Perkin–Elmer Gene-Amp® PCR System 2400 (Perkin-Elmer, Boston, MA, USA) at 42°C for 60 min

and reactions were stopped by cooling at 4°C. PCR was performed using 2 μL cDNA template from the RT reactions in a total volume of 25 μL PCR Master Mix (Promega). The following cDNAs were amplified with 1 μM of the indicated sets of primers: human *bcl-2* (accession number M13994; primers corresponding to 1799–1823 and 2139–2165 nt), human *bcl-xl* (accession number Z23115; primers corresponding to 381–402 and 903–922 nt) and human *bax* (accession number L22473; primers corresponding to 172–195 and 516–537 nt, as designed by Santos-Beneit and Mollinedo 2000). The following cDNAs were amplified with 500 nM of the indicated sets of primers: human nerve growth factor 2 (*ngf-2*) (accession number X53655; primers corresponding to 85–104 and 294–313 nt), human neurotrophin 3 (*nt-3*) (accession number M37763; primers corresponding to 117–136 and 499–518 nt), human CD44 (*cd44*) (accession number AY101192; primers corresponding to 362–381 and 869–888 nt), human glia-derived neurite-promoting factor (*gdnpf*) (accession number A03911; primers corresponding to 102–122 and 620–640 nt) and human fibronectin precursor (*fn*) (accession number X02761; primers corresponding to 4409–4429 and 4934–4953 nt). Rat *bcl-2* cDNA was amplified with 500 nM of the indicated set of primers (accession number NM_016993; primers corresponding to 640–660 and 949–969 nt). Primers for human glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) cDNA (accession number BC014085; primers corresponding to 881–906 and 1460–1481 nt) and for rat *gapdh* (accession number BC059110; primers corresponding to 497–516 and 785–804 nt) were used as positive controls for the presence and integrity of the cDNA. The conditions for PCR amplification of cDNAs were as follows: an initial denaturation step at 95°C for 5 min and a final 7-min extension step at 72°C for all cDNAs unless otherwise indicated; 30 cycles of denaturation at 95°C for 30 s, annealing at 69°C for 30 s and extension at 72°C for 90 s, and a final 15-min extension step at 72°C for *bcl-2*, *bcl-xl* and *bax*; 30 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min for *ngf-2*, *nt-3*, *cd44*, *gdnpf* and *fn*; annealing at 60°C for 1 min for human *gapdh*; annealing at 62°C for 1 min for rat *bcl-2*; annealing at 52°C for 1 min for rat *gapdh* and extension at 72°C for 1 min, and a final 7-min extension step at 72°C. Each PCR product (23 μL) was electrophoresed in 1 × TAE buffer (40 mM Tris acetate and 1 mM EDTA) on 1% (w/v) agarose gels containing 0.6 μg/mL ethidium bromide. A 100-bp DNA ladder (Promega) was used as a marker for cDNA fragment size. The fluorescence of bands was quantified using Bio-Rad Versa Doc™ and QuantityOne© software (Bio-Rad, Philadelphia, PA, USA) in a Macintosh workstation (Cupertino, CA, USA).

### Treatment of cells in culture with cyclic AMP responsive element (CRE) oligonucleotides

CRE **decoy** oligonucleotides containing the palindromic CRE *cis*-element TGACGTCA, which allows self-hybridization and formation of a duplex hairpin that competes with CRE enhancers for binding of transcription factors, were used to inhibit CRE-directed transcription *in vivo*, as previously described by Park *et al*. (1999). The sequences of the CRE decoy and control phosphorothioate oligonucleotides (Invitrogen) were as follows: CRE decoy, 5′-TGACGTCATGACGTCATGACGTCA-3′; control nonsense sequence, 5′-CTAGCTAGCTAGCTAGCTAGCTAG-3′.

Cells ($1 \times 10^5$ cells/well) were plated in six-well plates containing growth medium without antibiotics at 37°C. After 24 h, culture medium was replaced with serum-free medium containing 200 nM CRE decoy or control oligonucleotides in the presence of Lipofectamine 2000 (Invitrogen) to increase the delivery of oligonucleotides to the cells. After an additional 18 h, cells were washed, and treated as indicated in the figure legends. Cells were then harvested at the indicated time and *bcl-2* and *bcl-xl* mRNA expression was measured by RT–PCR, as described above.

### Western blot analysis

Human 1321N1 cells expressing the $P2Y_2$ receptor (1321N1-$P2Y_2$ cells) were grown to 80% confluence in six-well plates and incubated at 37°C in DMEM with 5% (v/v) FCIII serum. Before the experiment, cells were incubated at 37°C for 18 h in DMEM supplemented with 0.5% (w/v) bovine serum albumin (BSA) (Fisher Scientific, Hampton, NH, USA). Receptor ligands or inhibitors were added in serum-free media with 0.5% (w/v) BSA. After incubation for the time indicated in the figure legends, the cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with 50 μL lysis buffer [25 mM Tris-HCl, pH 7.4, 25 mM NaCl, 1 mM $Na_3VO_4$, 10 mM NaF, 10 mM $Na_4P_2O_7$, 25 mM β-glycerophosphate, 25 mM *p*-nitrophenylphosphate, 0.5 mM EGTA, 0.5% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin and 10 nM okadaic acid]. The cell extracts were centrifuged (8200 *g* for 10 min at 4°C) to remove insoluble material. Some 15 μL 3 × Laemmli sample buffer [187.5 mM Tris-HCl, pH 6.8, 6% (w/v) sodium dodecyl sulfate (SDS), 1.8% (v/v) β-mercaptoethanol and 0.003% (w/v) bromophenol blue] was added to 30 μL of supernatant. The mixture was heated for 5 min at 96°C, and 100 μg of cell lysate protein, determined by Bradford's assay (Bradford 1976), was subjected to SDS–polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes for western analysis.

Immunodetection of phosphorylated p38 on nitrocellulose membranes was performed using a 1 : 1000 dilution of rabbit anti-human phospho-p38 IgG (Cell Signaling Technology) as the primary antibody and a 1 : 2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody (Cell Signaling Technology) in Tris-buffered saline (TBS) [50 mM Tris-HCl, pH 7.6, 0.15 M NaCl and 0.1% (v/v) Tween 20] supplemented with 5% (w/v) non-fat dry milk. Phosphorylated proteins were visualized by chemiluminescence and quantitated using the Super-Signal West Dura Luminol/Enhancer Solution (Pierce, Woburn, MA, USA) and GS-525 Molecular Imager and MultiAnalyst™ software (Bio-Rad Laboratories, Hercules, CA, USA). For normalization of the signals, the membranes were stripped of antibodies by incubating for 15 min at room temperature in Re-Blot stripping solution (Chemicon, Temecula, CA, USA), washed with TBS, and reprobed with a 1 : 1000 dilution of rabbit anti-human p38 as the primary antibody.

Bad, CREB and protein kinase B (PKB)/Akt were detected by western analysis using rabbit anti-mouse Bad (1 : 1000 dilution), rabbit anti-human CREB (1 : 1000 dilution) and rabbit anti-mouse Akt (1 : 1000 dilution) antibodies. Detection of phosphorylated proteins was performed using rabbit anti-mouse phospho-Akt (1 : 1000 dilution), rabbit anti-human phospho-CREB (1 : 1000 dilution), rabbit anti-mouse phospho-Bad $Ser^{112}$ (1 : 1000 dilution), rabbit anti-mouse phospho-Bad $Ser^{155}$ (1 : 1000 dilution) and rabbit anti-mouse phospho-Bad $Ser^{136}$ (1 : 500 dilution).

### RNA Prepararion and microarray analysis

Total RNA (50 μg) isolated from 1321N1-$P2Y_2$ cells that had been treated with UTP for 2 h was used in a 20-μL reverse transcription reaction, as described in the BD Atlas™ SpotLight™ Labeling Kit (BD Biosciences Clontech, Palo Alto, CA, USA) to synthesize biotin-labeled probes with a pooled set of primers complementary to genes represented on the Atlas™ Human cDNA Neurobiology Array (BD Biosciences Clontech) of 588 human neurobiology-related cDNA segments spotted on a nylon membrane. The biotin-labeled probes were purified by passage over a NucleoSpin Extraction Spin Column (BD Biosciences Clontech) and used to detect UTP-induced changes in gene expression with the Atlas™ Neurobiology Array membrane as described in the BD SpotLight™ Chemiluminescent Hybridization and Detection Kit (BD Biosciences Clontech). Each array membrane was prehybridized with continuous agitation for 1 h at 42°C in 5 mL hybridization solution (SpotHyb™ Buffer; BD Biosciences Clontech) containing 0.1 mg/mL sheared salmon testes DNA (Sigma). Hybridization with biotin-labeled cDNA probes was performed overnight in 5 mL SpotHyb™ Buffer at 42°C. The next day, membranes were washed with continuous agitation at 60°C in 2 × saline sodium citrate buffer (SSC), 1% (w/v) SDS (four times, each for 30 min) and then at 48°C in 0.1 × SSC (twice for 30 min). Probe detection and signal visualization were performed as described in the BD SpotLight™ Chemiluminescent Hybridization and Detection Kit. The membranes were mounted, wrapped in plastic and exposed to a PhosphorImager chemi-screen for 2 days at room temperature. The exposed screen was scanned on a GS-525 Molecular Imager using the MultiAnalyst™ software. The digital images were then analyzed using Atlas Vision 3.0 software (BD Biosciences Clontech).

### Confocal immunofluorescence microscopy

PC-12 cells were plated on to Laboratory-Tek chamber slides (Nalge Nunc Int., Rochester, NY, USA) at a density of $1 \times 10^5$ cells/mL and incubated for 24 h in F-12K medium supplemented with 15% (v/v) horse serum and 2.5% (v/v) FCIII. The cells were washed with PBS and incubated for an additional 24 h in cell-conditioned serum-free F-12K medium supplemented with 0.5% (w/v) BSA, or with 100 μM rat NGF, a positive control for neurite outgrowth. The cell-conditioned medium was obtained from $1 \times 10^6$ cells/mL 1321 N1-$P2Y_2$, or untransfected ($P2Y_2$ receptor null) 1321N1 cells treated for 24 h in the presence or absence (control) of 100 μM UTP. Serum-free F-12K medium containing 100 μM rat NGF supplemented with 0.5% BSA was used as positive control for neurite outgrowth stimulation. Cells were fixed in PBS containing 3.7% formaldehyde for 5 min, washed with PBS, permeabilized with 0.1% (v/v) Triton X-100 in PBS for 3 min, and washed three times with PBS. Fixed cells were incubated for an additional 20 min with Alexa-fluor 488-conjugated phalloidin to visualize F-actin (Molecular Probes, Eugene, OR, USA) and washed. Images were acquired using a Zeiss (Thornwood, NY, USA) LSM-5 Pascal scanning confocal microscope equipped with an Alpha-Fluar 100 × 1.45 DIC oil immersion objective. A 488-nm beam from an argon laser was used for the excitation of Alexa-fluor 488. Emission from Alexa-fluor 488 was detected through a BP505-530 filter. Final image composites were created using Zeiss LSM5 PASCAL Image software, version 3.2.

### Cell proliferation assay

Human 1321N1-$P2Y_2$ cells were seeded on 96-well plates (BD Biosciences Clontech) at a density of $1 \times 10^3$ cells/well in a final volume of 100 μL. Proliferation was measured using a 5-bromo-2′-deoxyuridine incorporation immunoassay kit following the manufacturer's instructions (Roche Diagnostics Corporation).

### Statistical analysis

One-way multiple Tukey comparison post-test ANOVA and unpaired Student's *t*-test were used for comparison of multiple groups and two groups respectively. $p < 0.05$ between control and experimental groups was considered to be statistically significant. All analyses were performed with InStat software, version 3.06 (GraphPad Software Inc., San Diego, CA, USA).

## Results

### $P2Y_2$ nucleotide receptor expressed in 1321N1 astrocytoma cells mediates transient up-regualtion of *bcl-2* and *bcl-xl* and down-regulation of *bax* gene expression

$P2Y_2$ nucleotide receptor activation by UTP in 1321N1-$P2Y_2$ cells caused the transient dose-dependent up-regulation of mRNA for the anti-apoptotic factors *bcl-2* and *bcl-xl*, and a down-regulation of mRNA for pro-apoptotic *bax* (Figs 1a and b). Addition of carrier buffer by itself produced a minimal response that could be eliminated by pretreatment of the cells with the ATP-degrading enzyme apyrase (Fig. 1a). It is plausible that shear stress causes the release of small amounts of nucleotides that modestly activate $P2Y_2$ signaling in an autocrine fashion as previously described by Lazarowski *et al*. (1997). Immortalized rat astrocytes (DITNC cells) that express endogenous $P2Y_2$ receptors (Gendron *et al*. 2003) also exhibited UTP-induced up-regulation of *bcl-2* mRNA (Fig. 1c). The *bax/bcl-2* mRNA expression ratio decreased significantly after exposure of 1321N1-$P2Y_2$ cells to 100 μM UTP for more than 2 h, in contrast to an increase in the *bax/bcl-2* mRNA expression ratio with the antiproliferative drug etoposide (Fig. 1d). The increase in anti-apoptotic versus pro-apoptotic gene expression in response to UTP suggests that the $P2Y_2$ nucleotide receptor may be a mediator of cell survival responses.

### CREB phosphorylation mediates *bcl-2* and *bcl-xl* gene transcription in response to $P2Y_2$ receptor activation

Treatment of 1321N1-$P2Y_2$ cells with 100 μM UTP for 30 min increased levels of phospho-CREB and phospho-ATF-1 (activation transcription factor-1) (Fig. 2a). Consistent with the identification of a CRE in the *bcl-2* gene promoter region (Riccio *et al*. 1999), we found that a single-stranded phosphorothioate oligodeoxynucleotide comprising a CRE palindrome (i.e. triplet copies of TGACGTCA, a CRE consensus sequence) inhibited 100 μM UTP-stimulated *bcl-2* and *bcl-xl* gene expression in 1321N1-$P2Y_2$ cells (Fig. 2b). Transfection with a control nonsense oligonucleotide did not affect $P2Y_2$

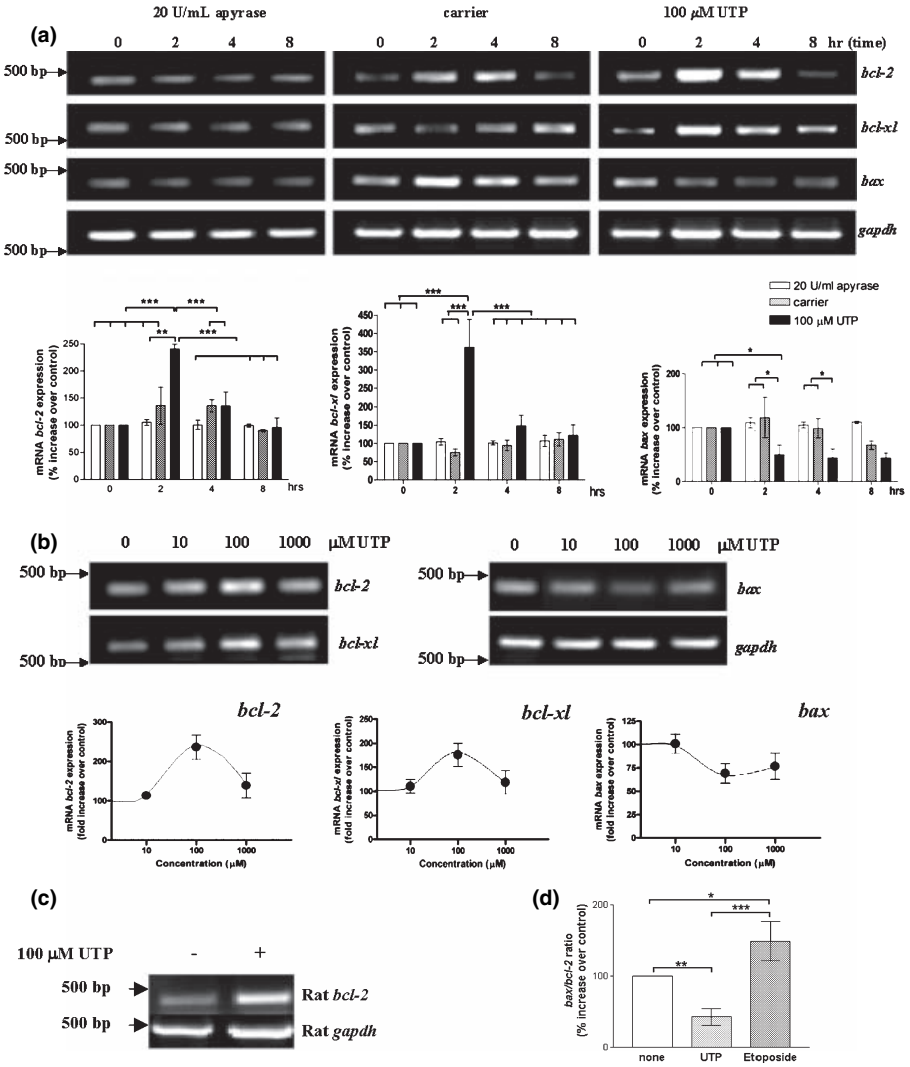


**Fig. 1** $P2Y_2$ receptor activation alters *bcl-2*, *bcl-xl* and *bax* gene expression. (a) Serum-starved 1321N1-$P2Y_2$ cells not treated and pretreated with 20 U/mL apyrase for 30 min were exposed to UTP (100 μM) or carrier buffer for 2–8 h, and then subjected to RT–PCR to determine *bcl-2, bcl-xl*, *bax* and *gapdh* mRNA levels. A representative experiment is shown in the top panel. Cells treated with carrier buffer only displayed a minimal response that was eliminated by apyrase pretreatment for 30 min. In the bottom panel, changes in mRNA levels for *bcl-2*, *bcl-xl* and *bax* were normalized to *gapdh* mRNA levels and mean ± SEM values ($n = 3$) were expressed as a percentage of mRNA levels at the zero time point. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ (one-way ANOVA). (b) 1321N1-$P2Y_2$ cells were treated with 10 μM, 100 μM or 1000 μM UTP for 2 h and then subjected to RT–PCR to determine levels of *bcl-2, bcl-xl*, *bax* mRNA. Changes in mRNA levels for *bcl-2*, *bcl-xl* and *bax* were normalized to *gapdh* mRNA levels and are shown as mean ± SEM values ($n = 3$). (c) DITNC cells were treated for 2 h with 100 μM UTP, and *bcl-2* and *gapdh* mRNA levels were determined. (d) *bax/bcl-2* mRNA expression ratios normalized to *gapdh* mRNA levels were determined for 1321N1-$P2Y_2$ cells exposed to 100 μM UTP or 100 μM etoposide for 2 h. Values are mean ± SEM ($n = 3$) expressed as a percentage of responses in untreated cells. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ (one-way ANOVA).

receptor-induced up-regulation of *bcl-2* and *bcl-xl* gene expression (Fig. 2b). These results suggest that $P2Y_2$ receptor-mediated up-regulation of *bcl-2* and *bcl-xl* gene expression is dependent upon transcriptional activation by a CREB protein.

### Activation of $P2Y_2$ nucleotide receptor induces the phosphorylation of the pro-apoptotic protein Bad

Activation of $P2Y_2$ receptor by 100 μM UTP in 1321N1-$P2Y_2$ cells caused a time-dependent increase in the phosphorylation of Bad on $Ser^{112}$ (Fig. 3) with a maximal response occurring within 20 min. Phosphorylation of Bad on $Ser^{112}$ is known to mediate the pro-apoptotic effects of Bad by interfering with the binding of *bax* to *bcl-2* or *bcl-xl* (Chao and Korsmeyer 1998). We could not detect a change in phosphorylation of Bad on $Ser^{136}$ or $Ser^{155}$, other residues known to regulate Bad activity (Scheid *et al*. 1999; Lizcano *et al*. 2000) after 1321N1-$P2Y_2$ cells had been activated with UTP (data not shown).

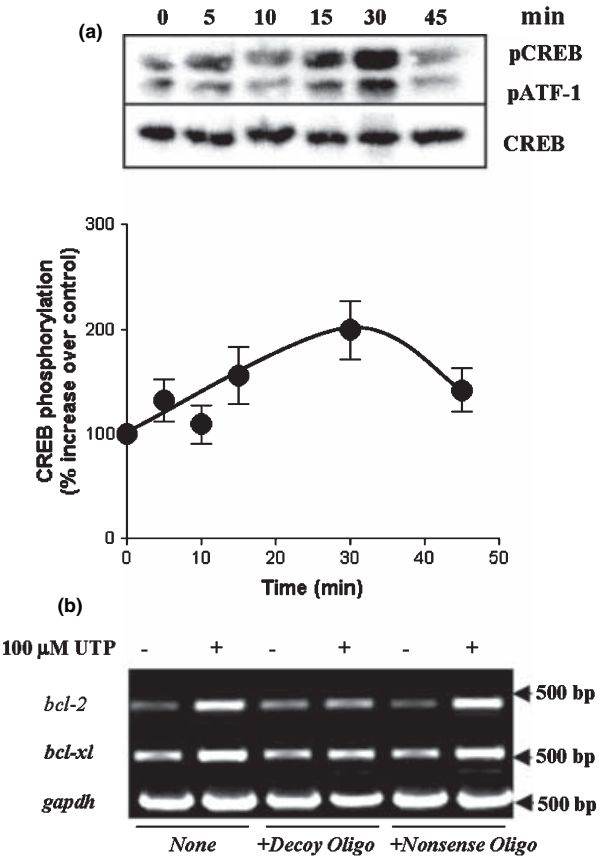


**Fig. 2** Involvement of CREB proteins in the $P2Y_2$ receptor-mediated up-regulation of *bcl-2* and *bcl-xl* gene expression. (a) Western blot analysis for phospho-CREB (pCREB), phospho-ATF-1 (pATF-1) and total CREB was performed with cell extracts from 1321N1-$P2Y_2$ cells incubated with 100 μm UTP for 0–45 min. A representative blot is shown in the upper panel. In the lower panel, phospho-CREB was normalized to total CREB and mean ± SEM values ($n = 3$) were expressed as a percentage of the response in the absence of UTP. (b) Cells ($1 \times 10^5$) were pretreated overnight with 200 nM CRE decoy (+ Decoy Oligo) or control nonsense oligonucleotide (+ Nonsense Oligo) in the presence of Lipofectamine 2000 and then exposed to 100 μm UTP for 2 h; *bcl-2*, *bcl-xl* and *gapdh* mRNA expression was determined by RT–PCR and quantitative analysis of digitized data.

### $P2Y_2$ receptors mediate the activation of signaling pathways known to regulate CREB protein activity

Activation of 1321N1-$P2Y_2$ cells with 100 μM UTP caused phosphorylation of p38 (Fig. 4), a stress-activated protein kinase known to phosphorylate CREB (Deak *et al*. 1998), and this response reached a maximum 10 min after addition of UTP (Fig. 4). Inhibition of p38 with 40 μM SB203580 or the MEK/ERK MAPK signaling cascade with 20 μM PD98059 significantly attenuated $P2Y_2$ receptor-induced CREB phosphorylation in 1321N1-$P2Y_2$ cells (Fig. 5a). $P2Y_2$ receptor-induced up-regulation of *bcl-2* and *bcl-xl* gene expression was also sensitive to inhibition by SB203580 and PD98059 (Fig. 5b), confirming a direct linkage between $P2Y_2$ receptors, stress-activated protein kinase and MAPK signaling cascades and the regulation of CREB/*bcl-2*- and CREB/*bcl-xl*-mediated survival responses.

### $P2Y_2$ receptors mediate activation of phosphatidylinositol 3-kinase (PI3K) and Akt

Treatment of 1321N1-$P2Y_2$ cells with 100 μM UTP induced a time-dependent increase in the phosphorylation of Akt (Fig. 6a), an activator of CREB (Du and Montminy 1998). Consistent with the recognition of Akt as a downstream substrate of PI3K (Coffer *et al*. 1998), we observed that CREB phosphorylation in response to $P2Y_2$ activation in 1321N1-$P2Y_2$ cells was inhibited by the PI3K inhibitors LY294002 and wortmanin (Fig. 5a).

Furthermore, UTP-induced Bad phosphorylation at $Ser^{112}$ was completely inhibited by pretreatment of 1321N1-$P2Y_2$ cells with wortmanin (Fig. 6b). Surprisingly, the PI3K inhibitor LY294002 had only a minor attenuating effect on UTP-induced Bad phosphorylation at $Ser^{112}$ in 1321N1-$P2Y_2$ cells (Fig. 6b). In contrast, the MEK1/2 inhibitor PD98059 significantly reduced Bad phosphorylation at $Ser^{112}$ in 1321N1-$P2Y_2$ cells treated with UTP compared with levels in cells treated with UTP in the absence of PD98059 (Fig. 6b). Taken together, these results indicate that Akt and MEK, but not PI3K, are necessary components of the $P2Y_2$ receptor-mediated signaling pathway to Bad phosphorylation at $Ser^{112}$.

### $P2Y_2$ receptor activation induces the proliferation of human 1321N1-$P2Y_2$ cells

It has been suggested that the P2Y/ERK pathway is important for ATP-induced cell proliferation because PD98059 blocks mitogenesis induced by extracellular ATP in rat (Neary *et al*. 1999) and human (Neary *et al*. 1998) astrocytes. As we have shown that activation of $P2Y_2$ receptors triggers survival-signaling cascades in human astrocytic cells, and to further investigate the potential role of these receptors in trophic responses, we treated 1321N1-$P2Y_2$ cells with 100 μM UTP for 24 h and measured their proliferation by probing 5-bromo-2′-deoxyuridine incorporation. Activation of $P2Y_2$ receptors by UTP resulted in a significant ($p = 0.0167$) increase in DNA synthesis (absorbance at 450 nm 0.14 ± 0.01; $n = 14$) compared with that in untreated cells (0.090 ± 0.015; $n = 14$).

### $P2Y_2$ receptor activation induces the expression of neurologically related genes

A cDNA array containing genes of neurological interest was used to determine potential gene targets for $P2Y_2$ receptor-mediated signaling. Results are shown in Fig. 7 and Table 1. Stimulation of 1321N1-$P2Y_2$ cells with 100 μM UTP for 2 h up-regulated the expression of transcription factors such as brain-specific homeobox/POU domain proteins 2 and 5,

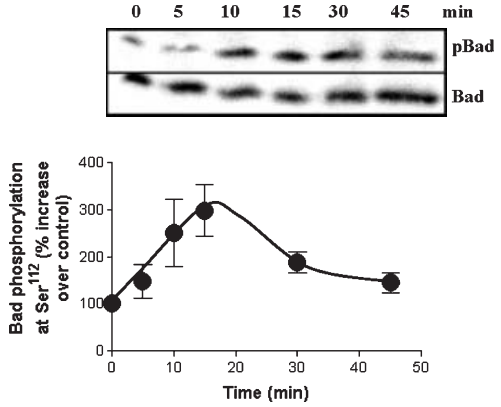


**Fig. 3** $P2Y_2$ receptor-dependent Bad phosphorylation at $Ser^{112}$. Human 1321N1 cells expressing $P2Y_2$ receptors were plated at a density $0.5 \times 10^6$ cells/well in six-well plates and incubated with 100 μM UTP for the indicated times at 37°C. Phosphorylated Bad (pBad) was detected by western blot analysis in whole-cell lysates with an anti-phospho-$Ser^{112}$ Bad antibody. A representative blot is shown in the upper panel. Phospho-$Ser^{112}$ Bad was normalized to total Bad protein and mean ± SEM values ($n = 3$) were expressed as a percentage of Bad phosphorylation at 0 min (lower panel).

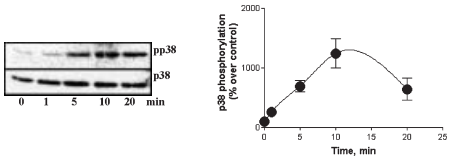


**Fig. 4** Time course of p38 phosphorylation mediated by the $P2Y_2$ receptor. Human 1321N1-$P2Y_2$ cells were plated at a density $0.5 \times 10^6$ cells/well in six-well plates and incubated with 100 μM UTP for the indicated time at 37°C. Phosphorylated p38 (pp38) was detected by western blot analysis in whole-cell lysates with anti-phospho-p38 antibodies. A representative blot is shown in the left panel. Phospho-p38 was normalized to total p38 protein and the mean ± SEM values ($n = 3$) were expressed as a percentage of p38 phosphorylation at 0 min (right panel).

nervous-system specific octamer-binding transcription factors N-oct3, N-oct5A and N-oct5B, and c-jun/AP-1. Activation of $P2Y_2$ receptors also enhanced the expression of genes for neurotrophins, neuropeptides and growth factors including neurotrophin precursors and neurotrophic factors (*ngf-2*, *gdnpf*, *nt-3*, *nt-4*, *nt-5*, *nt-6*), astrocyte GFAP, epidermal growth factor receptor, and phospholipase $A_2$ precursor. Some of these genes, including astrocyte GFAP and recognition molecules such as tenascin and laminin, are increased in reactive astrocytes after CNS injury (Ridet *et al*. 1997), suggesting that $P2Y_2$ receptor activation may play a physiological/neuroprotective role in astrocyte signaling and nerve tissue regeneration. Other genes differentially up-regulated by $P2Y_2$ receptor activation included those encoding extracellular communication proteins and matrix proteins:

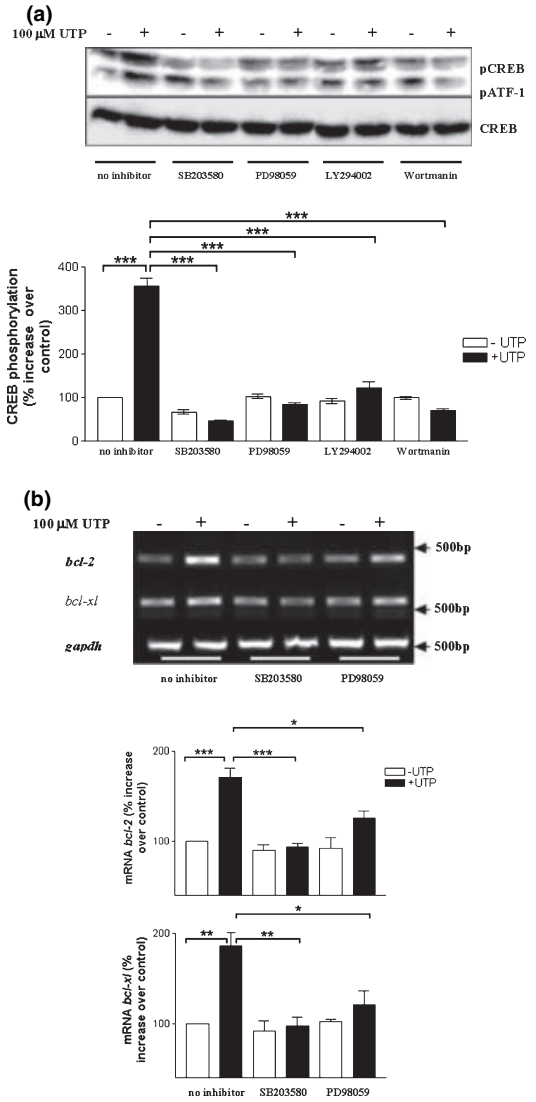


**Fig. 5** Role of PI3K, MEK1/2 and p38 activation in $P2Y_2$ receptor-mediated phosphorylation of CREB and up-regulation of *bcl-2* and *bcl-xl*. Human 1321N1-$P2Y_2$ cells were plated at a density of $0.5 \times 10^6$ cells/well and incubated for 1 h with 40 μM SB203580, 20 μM PD98059, 100 μM LY294002 or 2 μM wortmanin followed by 100 μM UTP for 30 min at 37°C, and western analysis was performed on whole-cell lysates. (a) Representative western blot for phospho-CREB (pCREB), phospho-ATF-1 (pATF-1) and total CREB is shown in the upper panel. In the lower panel, phospho-CREB was normalized to total CREB and mean ± SEM values ($n = 3$) were expressed as a percentage of the response in the absence of UTP. (b) Serum-starved 1321N1-P2Y cells were incubated for 1 h with 40 μM SB203580 or and 20 μM PD 98059, followed by 100 mM UTP for 2 h, and *bcl-2, bcl-xl* and *gapdh* mRNA expression was determined by RT–PCR. A representative experiment is shown in the top panel. In the bottom panel, changes in mRNA levels for *bcl-2* and *bcl-xl* were normalized to *gapdh* mRNA levels and the mean ± SEM values ($n = 3$) were expressed as a percentage of mRNA levels in untreated controls. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ (one-way ANOVA).

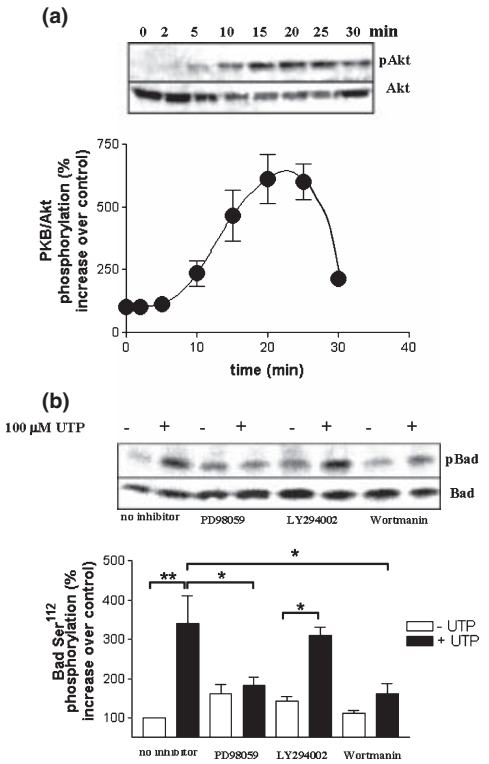


**Fig. 6** Role of Akt, MEK and PI3K in $P2Y_2$ receptor-mediated signal transduction to Bad. (a) Human 1321N1-$P2Y_2$ cells were plated at a density $1 \times 10^6$ cells/well and incubated with 100 μM UTP for indicated time at 37°C. Whole-cell lysates were prepared and phosphorylated Akt (pAkt) was detected by western blot analysis. A representative blot is shown in the upper panel. In the lower panel phospho-Akt was normalized to total Akt and mean ± SEM values ($n = 3$) were expressed as a percentage of the response at 0 min. (b) Human 1321N1-$P2Y_2$ cells were treated with the MEK1/2 inhibitor PD98059 (20 μM) or the PI3K inhibitors LY294002 (100 μM) or wortmanin (2 μM) for 1 h followed by treatment with 100 μM UTP for 5 min. Cell extracts were prepared and Bad phosphorylated at $Ser^{112}$ (pBAD) was detected by western blot analysis. A representative blot is shown in the upper panel. In the lower panel Bad phosphorylation at $Ser^{112}$ was normalized to total Bad and mean ± SEM values ($n = 3$) were expressed as a percentage of the response in the absence of UTP. $^{**}p < 0.01$, $^{*}p < 0.05$ (one-way ANOVA).

ephrin A3 precursor, CD44, guanine nucleotide-binding protein G(I)/G(S)/G(T) β subunit 1, fibronectin precursor, laminin γ1 subunit precursor and laminin B2 subunit. Furthermore, the cytoskeleton/motility protein cytoplasmic β-actin was up-regulated by $P2Y_2$ receptor activation. To confirm the results obtained with the cDNA microarrays, we showed by RT–PCR that selected genes (i.e. *ngf-2*, *nt-3*, *gdnpf*, *cd44* and *fn*) were up-regulated upon treatment of 1321N1-$P2Y_2$ cells with UTP (Fig. 7c).

Because the cDNA microarray data suggested that UTP-treated 1321N1-$P2Y_2$ cells might induce gene expression of trophic factors that are essential for neuroprotection and neurogenesis, we determined whether UTP-treated 1321N1-$P2Y_2$ cells were releasing factors that could stimulate neuronal differentiation and neurite formation in PC-12 cells, a neuronal cell precursor (Greene and Tischler 1976). Using immunofluorescence confocal microscopy (Fig. 8), we showed that conditioned medium obtained from 1321N1-$P2Y_2$ cells treated with 100 μm UTP for 24 h could enhance neurite outgrowth, comparable to the effects on PC-12 cells of treatment with rat NGF (100 μm) for 24 h. Neurite outgrowth in PC-12 cells did not occur with conditioned media from untreated 1321N1-$P2Y_2$ cells nor from untransfected ($P2Y_2$ receptor null) 1321N1 cells treated with UTP.

## Discussion

Astroglial cells are known to play a critical role in neuromodulation, neuroprotection, pH maintenance and axon guidance control during development, homeostasis preservation and blood brain barrier maintenance in the CNS (Kimelberg and Norenberg 1989). The molecular mechanisms underlying these processes have not been well defined, but the data presented here are consistent with the involvement of $P2Y_2$ nucleotide receptors in the regulation of neuroprotective responses. Nucleotides can be released into the extracellular space from injured brain cells (Neary *et al*. 1996) or stimulated nerves (North 2002) where they can act on specific P2 nucleotide receptors to produce trophic effects in astrocytes. There is widespread expression of $P2Y_2$ receptors throughout different brain regions in neurons and glial cells (Jimenez *et al*. 2000; Lenz *et al*. 2000; John *et al*. 2001; Moorea *et al*. 2001; Zhu and Kimelberg 2001; Fam *et al*. 2003; Gallagher and Salter 2003; Neary *et al*. 2003). In astrocytes, $P2Y_2$ receptors couple to signal transduction pathways that regulate the mobilization of intracellular calcium and the activation of protein kinase cascades including the MAPKs, ERK1/2 (Neary *et al*. 1999; Erb *et al*. 2001). ERK1/2 signaling has been implicated in the activation of transcription factors CREB and c-Fos by traumatic brain injury (Yang *et al*. 1994). Furthermore, CREB activation by phosphorylation at $Ser^{133}$ can be induced by growth factors (Neary *et al*. 1996; Mao and Wang 2002) and extracellular nucleotides have been shown to activate CREB. Phosphorylation of CREB at $Ser^{133}$ can be induced by extracellular signals such as glutamate (Mao and Wang 2002), growth factors (Deak *et al*. 1998) and extracellular nucleotides (Wagstaff *et al*. 2000; Molliver *et al*. 2002). CREB activation has been linked to signaling cascades that include ERK 1/2 protein kinase A, protein kinase C, $Ca^{2+}$calmodulin-dependent protein kinase, p38, the non-receptor tyrosine kinase src, PI3K/Akt and the pp90 ribosomal S6 kinase family (Imprey *et al*. 1998; Pierrat *et al*. 1998; Cammarota *et al*. 2001). CREB activation has been shown to regulate many neuronal functions, including neuronal excitation (Moore *et al*. 1996), development (Imaki

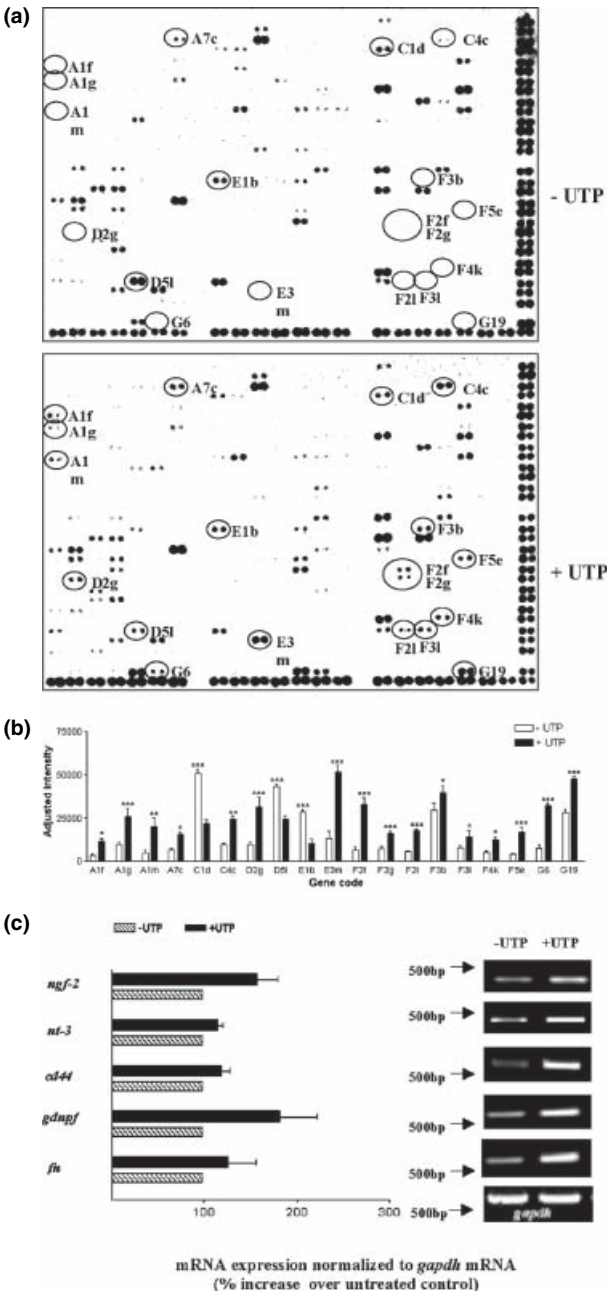


**Fig. 7** Gene expression in 132N1-$P2Y_2$ cells after UTP treatment. (a) Total RNA (50 μg) from 1321N1-$P2Y_2$ cells treated in the presence or absence of 100 μM UTP for 2 h was used to prepare biotin-labeled cDNA for hybridization with Atlas™ Human cDNA Neurobiology Array membranes (BD Biosciences Clontech). Circles indicate genes that are differentially expressed in response to UTP. Abbreviations for indicated gene codes are defined in Table 1. (b) Adjusted intensities (mean ± SEM; $n = 8$) for cells treated in the presence or absence of UTP were calculated as described in Table 1 legend with the use of Atlas Vision 3.0 software (BD Biosciences Clontech). UTP effects were significant at $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ ($n = 8$) (one-way ANOVA). (c) Human 1321N1-$P2Y_2$ cells were treated in the presence or absence of 100 μM UTP for 2 h, and mRNA levels for *ngf-2, nt-3, cd44, gdnpf* and *fn* were quantified by semiquantitative RT–PCR. Values are mean ± SEM of results from three independent experiments. Representative photomicrographs for each gene are shown.

*et al*. 1994) and long-term synaptic plasticity (Silva *et al*. 1998). Recent evidence suggests that CREB activation also might occur in brain damage-resistant hippocampal dentate granule cells (Walton and Dragunow 2000), indicating a role for CREB in nerve survival.

$P2Y_2$ receptors have been shown to activate CREB phosphorylation in rat sensory neurons (Molliver *et al*. 2002) and MCF-7 breast cancer cells (Wagstaff *et al*. 2000). The signal transduction pathways that couple to $P2Y_2$ receptors have been well studied and include: (1) $G_q$-mediated activation of phospholipase C that generates second messengers for intracellular calcium mobilization and protein kinase C activation (Neary and Zhu 1994; King *et al*. 1996; Ralevic and Burnstock 1998; Neary *et al*. 1999; Erb *et al*. 2001); (2) activation of $\alpha_v\beta_3/\beta_5$ integrin signaling cascades via an RGD domain in the $P2Y_2$ receptor that couples the receptor to focal kinases and $G_i/G_o$ proteins (Erb *et al*. 2001); and (3) src-dependent transactivation of growth factor receptors that is mediated by SH3-binding sites in the intracellular C-terminus of the $P2Y_2$ receptor (Liu *et al*. 2004). The possibility that transfected $P2Y_2$ receptors couple through aberrant signal transduction routes is unlikely because the literature provides numerous examples of cell types in which endogenously expressed $P2Y_2$ receptors trigger cellular responses through these signaling pathways (Cowen *et al*. 1990; Soltoff 1998; Soltoff *et al*. 1998; Santiago-Perez *et al*. 2001; Huwiler *et al*. 2002). Thus, vigorous activation of tripartite signaling pathways activated by $P2Y_2$ receptor enables the complex integration of extracellular nucleotide signals that regulates the activities of mitogen- and stress-activated protein kinases including p38, C-Jun N-terminal kinase (JNK) and ERK1/2 (Neary *et al*. 1998, 1999; Paul *et al*. 2000; Erb *et al*. 2001; Santiago-Perez *et al*. 2001; Gendron *et al*. 2003; Liu *et al*. 2004), PI3K (Erb *et al*. 2001) and, undoubtedly, transcription factors that mediate the expression of response-specific genes.

The present study indicates that $P2Y_2$ receptor signaling pathways in human astrocytoma cells stimulate the activation of CREB protein by phosphorylation of $Ser^{133}$, which is known to regulate the nuclear translocation of CREB and modulate CREB-mediated *cis*-activation of gene expression (Enslen *et al*. 1994). Results in the present study indicated that CREB phosphorylation by $P2Y_2$ receptors (Fig. 2) is associated with the up-regulation of *bcl-2* and *bcl-xl*, and the down-regulation of *bax* mRNA expression (Fig. 1), responses that were dependent on p38 or ERK1/2 activities (Figs 5 and 6). Because CRE decoy antisense oligonucleotides that contained CRE *cis*-element and competed with CRE enhancers for binding transcription factors brought about a marked reduction in the UTP-dependent increases in *bcl-2* and *bcl-xl* mRNA levels (Fig. 2b), it is clear that

**Table 1** Genes modulated by $P2Y_2$ receptor activation in 1321N1-$P2Y_2$ cells

| Gene ID code | Protein/gene (Gene Bank) | Gene family | Adjusted intensity[a] | | $P$[b] | $P$[c] |
|---|---|---|---|---|---|---|
| | | | Non-treated | UTP treated | | |
| Up-regulated genes | | | | | | |
| A1f | Brain-specific homeobox/POU domain protein 2, N-oct3;N-oct5A and N-oct5B (Z11933) | Basic transcription factors | 3536.88 | 11 548.38 | 0.0002 | < 0.05 |
| A1g | Brain-specific homeobox/POU domain protein 5 (Z21966) | Basic transcription factors | 9943.5 | 26 088.75 | 0.0025 | < 0.001 |
| A1m | *c-jun* proto-oncogene; transcription factor AP-1 (J04111) | Basic transcription factors | 5044 | 20 185.5 | 0.0235 | < 0.01 |
| A7c | Prostaglandin-$H_2$ D-isomerase precursor (M61900) | Complex lipid metabolism | 6307 | 15 713.5 | < 0.0001 | < 0.05 |
| C4c | Ephrin A3 precursor, EPH-related receptor tyrosine kinase ligand 3 (U14187) | Intracellular transducers/effectors/ modulators | 9664 | 24 372.25 | < 0.0001 | < 0.01 |
| D2g | Guanine nucleotide-binding protein G(I)/G(S)/G(T) β subunit 1 (M36430) | Cytoplasmic organization and cell motility | 9747.5 | 31 734.88 | 0.0016 | < 0.001 |
| E3m | Epidermal growth factor receptor (M34309) | Growth factor and chemokine receptors | 13 253 | 51 691.25 | < 0.0001 | < 0.001 |
| F2f | Neurotrophin 3 precursor; neurotrophic factor; nerve growth factor 2 (X53655; M37763) | Neuropeptides | 6615.5 | 33 097.25 | < 0.0001 | < 0.001 |
| F2g | Neurotrophin 4–6 (M86528 + S41522 + S41540 +S41541) | Neuropeptides | 7121 | 16 132.63 | < 0.0001 | < 0.001 |
| F2l | CD44 antigen hematopoietic form precursor, extracellular matrix receptor-III (M59040) | Cell–cell adhesion receptors | 5715.5 | 17 503.25 | < 0.0001 | < 0.001 |
| F3b | Fibronectin precursor (X02761; K00799; K02273; X00307; X00739) | Extracellular matrix proteins | 29 804.5 | 39 815.5 | 0.0274 | < 0.05 |
| F3l | Laminin γ1 subunit precursor; laminin B2 subunit (J03202) | Extracellular matrix proteins | 7913 | 14 419 | 0.0007 | < 0.05 |
| F4k | Glia-derived neurite-promoting factor (A03911) | Serine protease inhibitor with neurite-promoting activity | 5145 | 12 330.25 | < 0.0001 | < 0.05 |
| F5e | Astrocyte GFAP (J04569) | Intermediate filament proteins | 4118.5 | 16 837.75 | 0.0011 | < 0.001 |
| G6 | Phospholipase $A_2$ (M86400) | Phopholipases and phopshatidylinositol kinases | 7507.5 | 32 360.88 | < 0.0001 | < 0.001 |
| G19 | Cytoplasmic β-actin | Cytoplasmic organization and cell motility | 28 277 | 47 683 | < 0.0001 | < 0.001 |
| Down-regulated genes | | | | | | |
| C1d | Dual-specificity protein phosphatase 7 (Q16829) | Intracellular protein phosphatases | 50 875.5 | 21 625.63 | < 0.0001 | < 0.001 |
| D5l | μ-type opioid receptor (L25119) | G-protein-coupled hormone receptors | 43 286.5 | 24 399.5 | < 0.0001 | < 0.001 |
| E1b | Somatostatin receptor type 4 (D16826) | G-protein-coupled hormone receptors | 28 542.5 | 10 152.5 | < 0.0001 | < 0.001 |

[a]Intensity of gene expression in the presence or absence of 100 μM UTP for 2 h minus the background and multiplied by the normalization coefficient, as determined using cDNA microarray analysis. The background was calculated as the median intensity of the space immediately surrounding a visualized gene. To calculate the normalization coefficient, the ratio of intensity minus background for gene expression in the absence (numerator) or presence (denominator) of UTP was summed for all of the genes in the microarray:

$$\text{Normalization coefficient} = \frac{\sum_{j=1}^{n} (Intensity - Background)_i}{\sum_{j=1}^{n} (Intensity - Background)_j}$$

where $i$ = genes on array 1 (– UTP), $j$ = genes on array 2 (+ UTP) and $n$ = number of genes on the array. [b]Probability of a false positive on individual genes (Student's *t*-test). [c]One-way ANOVA.

$P2Y_2$ receptors mediate the activation of the CREB/*bcl-2* pathway.

There is increasing evidence to indicate that pharmacological modulation of the Bcl-2 pathway might interfere with neuronal damage (Kukley *et al*. 2001). In ischemia, the loss of Bcl-2 activity is associated with exacerbated injury (Sato *et al*. 1998), whereas overexpression of this factor protects against injury induced by a variety of lethal stimuli (Davies 1995). To date, Bcl-2 proteins have been reported to be regulated by lithium (Manji *et al*. 1999), estradiol (Dubal *et al*. 1999), ceramide (Chen *et al*. 1986) and brain-derived neurotrophic factor (Schabitz *et al*. 2000). Moreover, a shift in the ratio between pro-apoptotic *bax* and anti-apoptotic *bcl-2* is a critical determinant of susceptibility to apoptosis (Schabitz *et al*. 2000). In our experiments, the *bax/bcl-2* ratio was lower in 1321N1-$P2Y_2$ cells treated with UTP (Fig. 1c) suggesting that $P2Y_2$ receptor activation is anti-apoptotic. Furthermore, UTP also induced the phosphorylation of Bad at $Ser^{112}$, which inactivates this pro-apoptotic member of the Bcl-2 family (Harada *et al*. 1999; Lizcano *et al*. 2000). Phosphorylation of Bad on $Ser^{112}$, $Ser^{136}$ and $Ser^{155}$ (Scheid *et al*. 1999; Lizcano *et al*. 2000) has been shown to regulate Bad binding to 14-3-3 proteins and decrease the affinity of Bad for Bcl-2 and Bcl-xL (Zha *et al*. 1996) thereby propagating an anti-apoptotic response. It is recognized that phosphorylation of Bad on $Ser^{112}$ is dependent upon activation of MAPK/ERK signaling cascades (Fang *et al*. 1999), and our results with $P2Y_2$ receptors support this role for activation of MAPK, as well as PI3K and p38, in the phosphorylation of Bad on $Ser^{112}$.

It is well known that neuron–glia interactions play critical roles in several aspects of the development of the nervous system, including neuronal migration, neuronal and glial differentiation and survival, and synapse formation and function. *In vivo* and *in vitro* studies on neuron–glia interactions in different brain regions suggest that glia express region-specific properties. This specificity is the result of the expression of distinct ion channel activities, neurotransmitter uptake mechanisms, and receptor and cell surface adhesion systems (Hatten *et al*. 1991). Our prediction that $P2Y_2$ receptors play an important role in triggering cell-survival signals led us to examine the expression of differentially regulated genes involved in neuroprotective mechanisms. We identified a number of genes implicated in neuroprotection and neuromodulation whose regulation was under the control of the $P2Y_2$ receptor (Fig. 7; Table 1), including the up-regulation of genes for transcription factors (e.g. homeobox/POU domain protein 5, c-jun/AP-1), and proteins that induce and activate cell cycle progression (Boulon *et al*. 2002) or mediate neuronal survival and regeneration (Herdegen *et al*. 1997). $P2Y_2$ receptors also triggered the up-regulation of genes for cytosolic phospholipase $A_2$ and prostaglandin D synthetase precursor (Fig. 7; Table 1), proteins that mediate the production of prostaglandins in astrocytes that regulate neurotransmission, and immune and inflammatory responses (Goetzl *et al*. 1995).

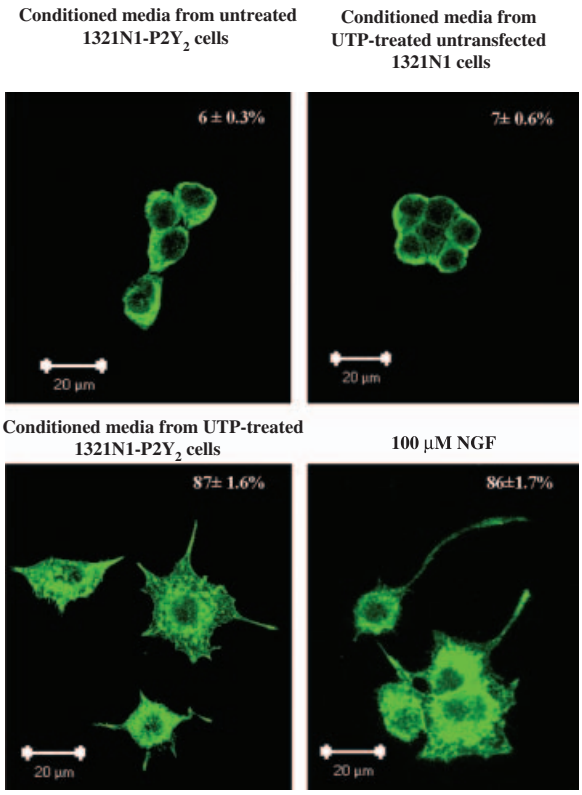


**Fig. 8** Conditioned media from UTP-treated 1321N1-$P2Y_2$ cells stimulates neurite outgrowth in PC-12 cells. PC-12 cells were plated at a density of $1 \times 10^5$ and grown for 24 h in F-12K medium supplemented with 15% (v/v) horse serum and 2.5% (v/v) FCIII. Cells were washed and incubated for 24 h in conditioned medium obtained from 1321N1-$P2Y_2$ or untransfected 1321N1 cells that were incubated in the presence or absence of 100 μM UTP for 24 h. PC-12 cells were also treated with rat NGF (100 μM) for 24 h, as a positive control for stimulation of neurite outgrowth. Neurite outgrowth was visualized with Alexa-fluor 488-conjugated phalloidin. Values are mean ± SEM percentage of cells with neurites from totals of at least 100 cells for each condition from three independent experiments. Effect of conditioned media from 1321N1-$P2Y_2$ cells treated with UTP on neurite outgrowth in PC-12 cells was significant ($p < 0.001$, one-way ANOVA).

We also observed the up-regulation of genes for several neurotrophins and neuropeptides known to potentiate neuronal cell proliferation and differentiation in the CNS (Ghosh and Greenberg 1995). The up-regulation of ephrin A3 precursor mRNA by $P2Y_2$ receptor activation may have significance because ephrins regulate multiple aspects of cell migration and compartment formation during development and glial scar formation (Bundesen *et al*. 2003). Up-regulation of CD44 by $P2Y_2$ receptor activation may relate to the ability of this transmembrane adhesion molecule to couple to the actin cytoskeleton and promote cell migration and growth (Leemans *et al*. 2003). Importantly, our data indicate that

conditioned media from UTP-treated 1321N1 cells promoted neurite outgrowth in PC-12 cells (Fig. 8). Taken together, these data strongly support a model whereby $P2Y_2$ receptors promote the release of neurotrophins and other factors involved in astrocyte growth and differentiation. This, along with stimulation of the CREB/Bcl-2 pathway, may facilitate the survival of neurons.

A better understanding of the signaling mechanisms activated by the $P2Y_2$ nucleotide receptor will be important for developing new strategies for the treatment and management of neurodegenerative diseases. $P2Y_2$ receptor up-regulation occurs in response to stress or injury in blood vessels (Liu *et al*. 2004) and epithelium (Clarke *et al*. 2000), and has been linked to the stimulation of smooth muscle growth (Seye *et al*. 2002; Kumari *et al*. 2003), endothelium-dependent inflammatory responses in macrophages (Liu *et al*. 2004) and immediate early gene responses in T lymphocytes (Koshiba *et al*. 1997). Thus, the novel mechanisms of $P2Y_2$ receptor up-regulation and function in the nervous system warrant further investigation.

## Acknowledgement

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