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Cell cycle regulation of astrocytes by extracellular nucleotides and fibroblast growth factor-2

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Abstract

Extracellular ATP enhances the mitogenic activity of fibroblast growth factor-2 (FGF2) in astrocytes, but the molecular mechanism underlying this synergistic interaction is not known. To determine whether the potentiating effect of extracellular ATP involves cell cycle control mechanisms, we have measured the expression of cyclins that are induced in different phases of the cell cycle in primary cultures of rat cortical astrocytes. We found that ATP potentiated the ability of FGF2 to stimulate expression of cyclin D1, a regulator of cell cycle entry, as well as cyclin A, a regulator of DNA replication. Because FGF2 and P2 purinergic receptors are coupled to extracellular signal regulated protein kinase (ERK), a key member of a signaling cascade that regulates proliferation, we also investigated the role of ERK in regulating cyclin expression induced by FGF2 and ATP. We found that the potentiating effect of ATP on cyclin expression was significantly reduced by U0126, an inhibitor of MEK, the upstream activator of ERK. P2 receptor agonist studies revealed that UTP enhanced FGF2-induced cyclin expression and mitogenesis whereas 2-methylthioADP was ineffective. By contrast, 2′,3′-*O*-(4-benzoyl)-benzoyl-ATP markedly inhibited FGF2-induced mitogenesis. Consistent with opposing effects of P2Y and P2X receptors on mitogenesis, UTP stimulated a transient activation of ERK whereas BzATP stimulated a more sustained ERK signal. These findings suggest that signaling by P2Y receptors, most likely of the purine/pyrimidine subtype, enhance the ability of FGF2 to stimulate entry into a new cell cycle, as well as DNA replication, by an ERK-dependent mechanism, whereas signaling by P2X receptors, possibly the P2X7 subtype, inhibits FGF2-induced mitogenesis in astrocytes. Interactions between P2Y, P2X and polypeptide growth factor signaling pathways may have important implications for CNS development as well as injury and repair.

Introduction

Although normally quiescent, astrocytes in the adult nervous system have the ability to proliferate. In particular, astrocyte proliferation in adult brains has been observed after breakdown of the blood brain barrier such as occurs after stab wounds and head trauma [1–6]. In addition, astrocytes can act as neural stem cells in the normal and regenerating brain [7]. Studies of cell cycle regulatory proteins in astrocytes have demonstrated that addition of epidermal growth factor (EGF) to confluent astrocytes resulted in the formation of multiple cell layers and elevated levels of cyclin D1 [8] which is induced in response to mitogenic signals. Cell cycle entry and progression is regulated by the expression of proteins termed cyclins [9]. Cyclin levels are low in quiescent cells but increase in response to mitogenic growth factors during distinct phases of the cell cycle. For example, cyclin D1 is increased during entry and progression through G1 phase while cyclin A is increased in S phase during which time DNA is replicated. The expressed cyclins bind to and activate enzymes termed cyclin-dependent protein kinases, thereby leading to changes in phosphorylation of key proteins that control various phases of the cell cycle. It was suggested that changes in the expression of cell cycle regulatory proteins may be involved in the increased proliferation observed in the gliosis that occurs after injury to the adult CNS [8].

Fibroblast growth factor-2 (FGF2) is increased after CNS injury (e.g., [10]) and can stimulate the gliotic response in cultured astrocytes (e.g., [11]) and *in vivo* [12]. When applied to mechanically wounded astrocytes, FGF2 enhanced proliferation, stellation and cell migration [13]. ATP is also released upon tissue injury and may contribute to gliosis [14]. When ATP or other nucleotide receptor agonists were added to astrocytes in culture, key hallmarks of gliosis were observed, i.e., increases in proliferation,

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stellation and glial fibrillary acidic protein (GFAP) [15–17]. *In vivo*, GFAP and astrocyte proliferation were increased after infusion of an ATP analog into rat brain [18]. These effects of ATP were blocked by nucleotide receptor antagonists, thereby indicating that P2 purinergic receptors mediate the gliotic actions of ATP *in vivo*.

Extracellular ATP can enhance the mitogenic activity of polypeptide growth factors in several cell types (reviewed in [19]). In astrocytes, we found that extracellular ATP markedly potentiated FGF2-induced mitogenesis [20]. However, little is known about the interactive effects of extracellular nucleotides and growth factors on cell cycle regulatory mechanisms, particularly in cells of the central nervous system. Here we report for the first time that extracellular ATP and UTP enhance FGF2-induced expression of cyclins involved in cell cycle entry and progression through the G1 and S phases of the cell cycle by a mechanism involving extracellular signal regulated protein kinase (ERK). We also demonstrate that BzATP inhibits the ability of FGF2 to stimulate mitogenesis, thereby suggesting that P2Y and P2X receptors exert opposing effects on FGF2-induced proliferation in astrocytes.

Materials and methods

Cell culture and treatment

Primary cultures of astrocytes were obtained from neonatal rat (Fischer) cerebral cortices as previously described [20]; the experimental procedure was approved and monitored by the Animal Studies Subcommittee at the Miami VA Medical Center and the Animal Care and Use Committee, University of Miami School of Medicine. Cells were seeded at densities of 1.5 and 4 million cells per 60 mm and 100 mm dishes, respectively; cells were not replated before use. At least 95% of the cell population was astrocytes, as determined by staining with cell-specific markers [20]. Experiments were conducted with 4–5-week-old cultures. Prior to treatment, cells which had been maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum were shifted to the quiescent phase by incubation in serum-free DMEM for 48 h. Stock solutions of ATP and other nucleotides (Sigma Chemical Co., St. Louis, MO) and FGF2 (recombinant human FGF2; R&D Systems, Minneapolis, MN) were divided into single-use aliquots and stored at −80 °C.

Immunoblotting

Cyclin expression was measured by immunoblotting. After treatment with FGF2 and/or nucleotides, cells were rinsed twice in ice-cold PBS and lysed in a buffer containing 20 mM Tris, pH 7.0, 0.27 M sucrose, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 1 μg/ml pepstatin A, 4 μM leupeptin, 0.3 U/ml aprotinin, 100 μg/ml 4-(2-aminoethyl)-benzenesulfonylfluoride (AEBSF) and 1% NP-40. Protein concentrations were determined by the Coomassie micro method (Bio Rad Laboratories, Hercules, CA) with bovine serum albumin as standard. Lysates containing equal amounts of protein (50 μg) were subjected to SDS-polyacrylamide gel electrophoresis [21] using 11% (w/v) acrylamide and transferred to nitrocellulose filters with a Genie electrophoretic blotter (Idea Scientific Inc., Minneapolis, MN) for 1 h at 12 V in a transfer buffer containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol. Membranes were incubated with a blocking solution containing 20 mM Tris, pH 7.7, 137 mM NaCl, 0.1% (v/v) Tween 20 (TTBS) and 5% (w/v) nonfat dry milk for 1 h at room temperature, rinsed in TTBS, and then incubated for 1 h at room temperature with specific antibodies (anti-cyclin D1, 1/500 dilution; anti-cyclin A, 1/200 dilution; or β-actin, 1/50,000 dilution) in blocking solution containing 0.02% sodium azide. Polyclonal antibodies recognizing cyclin D1 (sc-718) and cyclin A (sc-596) were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, and monoclonal antibodies recognizing β-actin were obtained from Sigma Chemical Co., St. Louis, MO. Following three rinses in TTBS, membranes were incubated for 1 h at room temperature with peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ) diluted 1/5000 in blocking solution. Membranes were washed three times in TTBS, and cyclin D1, cyclin A and β-actin were detected by ECL (Super-Signal West Femto Maximum Sensitivity Substrate; Pierce Biotechnology, Rockford, IL) with Kodak Biomax film (Eastman Kodak Company, Rochester, NY). Films were scanned and densitometrically analysed using Molecular Analyst Software (Bio-Rad Laboratories, Hercules, CA). In some cases, membranes were blocked and probed according to the manufacturer's instructions for analysis with an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NB) using Alexa Fluor 680-labeled anti-rabbit IgG (Molecular Probes, Eugene, OR) or IRDye 800 labeled anti-mouse IgG (Rockland Immunochemicals, Gilbertsville, PA) secondary antibodies. For quantification, cyclin/actin ratios were measured to correct for minor loading differences. To compare levels of cyclin expression among different experiments, data were calculated as fold stimulation compared to FGF2.

DNA synthesis

^{3}H-Thymidine incorporation was measured as previously described [20]. Data were expressed as cpm/mg protein. Protein concentrations were determined by the modified Lowry procedure with bovine serum albumin as standard [22].

ERK1/2 phosphorylation

Quiescent cultures were treated at 37 °C with vehicle, UTP or BzATP for the times indicated. Cells were lysed in SDS sample buffer [21] and protein concentrations determined by the modified Lowry procedure [22]. Cell lysates, containing 35 μg of protein, were subjected to SDS-PAGE

(11% acrylamide gel) and Western Blot analysis was performed using specific phospho-ERK monoclonal antibody (Thr202/Tyr204; 1/5000 dilution) from Cell Signaling Technology, Beverly, MA, and total-ERK polyclonal antibody (1/5000 dilution) from Santa Cruz Biotechnology, Santa Cruz, CA. For visualization, membranes were blocked and probed according to the manufacturer's instructions for analysis with an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NB) using IR-Dye 800 conjugated anti-mouse IgG (1/5000 dilution) and IR-Dye 700DX conjugated anti-rabbit IgG (1/10,000 dilution) obtained from Rockland Immunochemicals, Gilbertsville, PA. Intensities of phospho-ERK1/2 and total ERK1/2 bands were quantified and ratios of phospho-ERK1/2/total ERK1/2 were calculated and compared to control (vehicle-treated) cultures.

Statistical analyses

Data were analyzed by Student's *t*-test for two groups or repeated measures ANOVA for multiple groups followed by *post-hoc* comparisons (Bonferroni test) using an Instat software package (GraphPad Software, San Diego, CA, USA). Replicate experiments were conducted with cultures from different seedings.

Results

Expression of cyclin D1 and cyclin A

To determine whether interactive effects of extracellular ATP and FGF2 involve cell cycle regulation, we measured the expression of cyclins that are induced in different phases of the cell cycle. Quiescent, primary cultures of rat cortical astrocytes were treated with ATP (100 μM), FGF2 (25 ng/ml) or a combination of ATP and FGF2. Expression of cyclin D1, a cell cycle regulatory protein induced in G1 phase in response to stimulation by growth factors, was measured by immunoblotting and identified by co-migration with a positive control (Figure 1A). Blots were also probed with anti-actin antibodies as a loading control. Cyclin D1 expression was stimulated by FGF2 (Figure 1A). At the level of detection, ATP alone was without effect. However, when cells were treated with both ATP and FGF2, the expression of cyclin D1 was potentiated. An analysis of variance revealed an overall significant difference among the groups and planned comparisons revealed that the ATP + FGF2 group was significantly different ($P < 0.05$) from the FGF2 group (Figure 1B). These results suggest that extracellular ATP can enhance the ability of FGF2 to stimulate entry of astrocytes into the cell cycle.

To determine whether extracellular ATP also enhances the effect of FGF2 on entry and progression of astrocytes through S phase, cyclin A expression was measured. Quiescent, primary cultures of rat cortical astrocytes were treated with ATP (100 μM), FGF2 (25 ng/ml) or a combination of ATP and FGF2. Cyclin A expression was measured by immunoblotting and identified by co-migration with a positive control (Figure 2A). Blots were probed with anti-actin antibodies as a loading control. We found that ATP also potentiated the ability of FGF2 to stimulate expression of cyclin A (Figure 2A). An analysis of variance from group data revealed an overall significant difference and planned comparisons revealed that the ATP + FGF2 group was significantly different ($P < 0.01$) from the FGF2 group (Figure 2B). These results indicate that extracellular ATP also enhances the ability of FGF2 to induce the expression of cyclin A, a protein involved in entry and progression through the S phase, the DNA replication stage of the cell cycle.

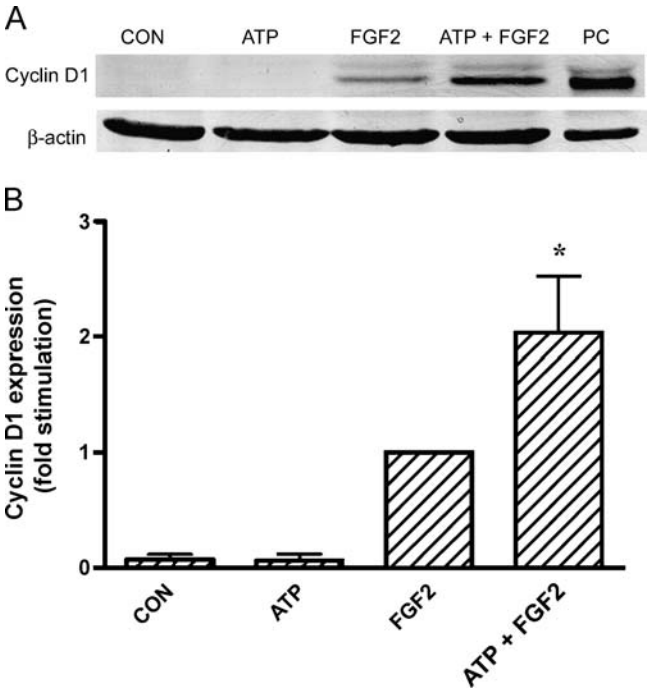

Figure 1. Extracellular ATP enhances cyclin D1 expression induced by FGF2. A) Quiescent, primary cultures of rat cortical astrocytes were treated with ATP (100 μM), FGF2 (25 ng/ml) or a combination of ATP and FGF2 for 20 h. Lysates were subjected to SDS-PAGE, and cyclin D1 expression was measured by immunoblotting. Cyclin D1 was identified by co-migration with a positive control (PC). Blots were also probed with anti-actin antibodies as a loading control. ATP potentiated the ability of FGF2 to stimulate expression of cyclin D1. B) Results (mean ± SEM) from three independent experiments in which astrocyte cultures were treated as described in (A). FGF2-induced cyclin D1 expression was significantly enhanced by extracellular ATP (*$P < 0.05$).

Enhancement of FGF2-induced cyclin expression by extracellular ATP involves ERK signaling

To investigate the mechanism by which extracellular ATP enhances cyclin expression induced by FGF2, we focused on ERK, a key member of a signaling pathway that regulates mitogenesis [23]. Both FGF2 and extracellular ATP stimulated ERK in rat cortical astrocytes [24–26], and blockade of the ERK cascade inhibited ATP-induced mitogenesis in astrocytes [27]. To determine whether the potentiation of FGF2-induced cyclin D1 expression by extracellular ATP in primary cultures of

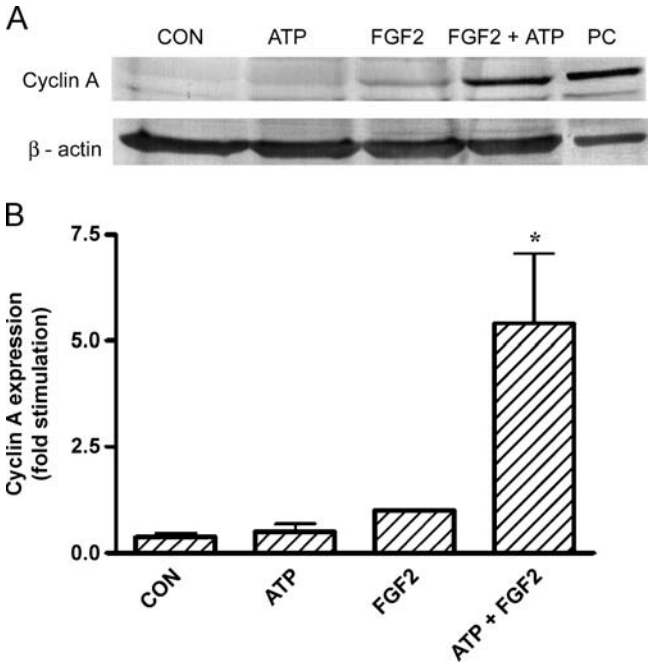

Figure 2. Extracellular ATP enhances cyclin A expression induced by FGF2. (A) Quiescent, primary cultures of rat cortical astrocytes were treated with ATP (100 μM), FGF2 (25 ng/ml) or a combination of ATP and FGF2 for 20 h. Lysates were subjected to SDS-PAGE, and cyclin A expression was measured by immunoblotting. Cyclin A was identified by co-migration with a positive control (PC). Blots were also probed with anti-actin antibodies as a loading control. ATP potentiated the ability of FGF2 to stimulate expression of cyclin A. (B) Results (mean ± SEM) from four independent experiments in which astrocyte cultures were treated as described in (A). FGF2-induced cyclin A expression was significantly enhanced by extracellular ATP (*$P < 0.01$).

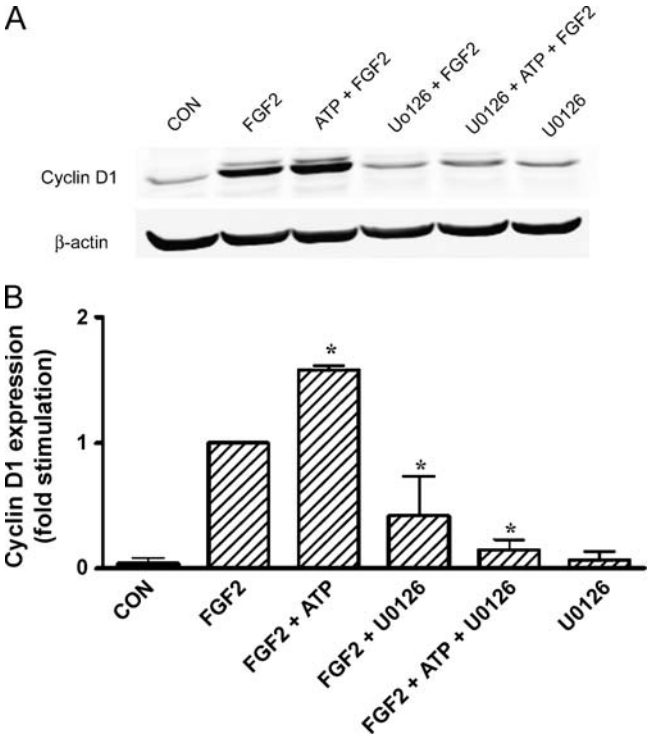

Figure 3. ERK mediates the enhancement of cyclin D1 expression induced by FGF2 and extracellular ATP. To determine whether the potentiation of FGF2-induced cyclin D1 expression by extracellular ATP was mediated by ERK, U0126 (10 μM, 30 min), an inhibitor of MEK, the upstream activator of ERK, was added to quiescent, primary cultures of rat cortical astrocytes prior to treatment with ATP (100 μM), FGF2 (25 ng/ml) or a combination of ATP and FGF2 for 20 h. (A) Lysates were subjected to SDS-PAGE, and cyclin D1 expression was measured by immunoblotting. (B) Results (mean ± SEM) from three independent experiments in which astrocyte cultures were treated as described in (A). Blockade of the ERK cascade significantly inhibited the expression of cyclin D1 induced by FGF2 as well as the potentiation of FGF2-induced cyclin D1 by extracellular ATP (*: FGF2 + ATP compared to FGF2, $P < 0.05$; FGF2 + U0126 compared to FGF2, $P < 0.05$; FGF2 + ATP + U0126 compared to FGF2 + ATP, $P < 0.001$).

astrocytes was mediated by ERK, we utilized U0126, an inhibitor of MEK, the upstream activator of ERK. U0126 (10 μM) was added to quiescent, primary cultures of rat cortical astrocytes 30 min prior to treatment with ATP (100 μM), FGF2 (25 ng/ml) or a combination of ATP and FGF2. When the ERK cascade was blocked, the up-regulation of cyclin D1 by FGF2 was significantly inhibited ($P < 0.05$) (Figures 3A and B). Importantly, blockade of the ERK cascade also significantly inhibited the ability of extracellular ATP to enhance cyclin D1 expression induced by FGF2 ($P < 0.001$) (Figures 3A and B).

Because blockade of the ERK cascade inhibited cyclin D1 expression induced by FGF2 and extracellular ATP, and because cyclin A is expressed subsequent to cyclin D1, we reasoned that inhibition of the ERK cascade should also reduce cyclin A expression. To test this, U0126 (10 μM) was added to quiescent, primary cultures of rat cortical astrocytes 30 min prior to treatment with ATP (100 μM), FGF2 (25 ng/ml) or a combination of ATP and FGF2 (Figures 4A and B). We found that blockade of the ERK cascade significantly inhibited the expression of cyclin A induced by FGF2 ($P < 0.05$) as well as the potentiation of FGF2-induced cyclin A by extracellular ATP ($P < 0.001$). These findings indicate that the ERK cascade plays an important role in mediating the co-mitogenic activity of extracellular ATP.

P2Y and P2X receptors exert opposing effects on FGF2-induced mitogenesis

To investigate the type(s) of P2 purinergic receptors involved in the synergistic effects of extracellular ATP on cyclin expression induced by FGF2, we conducted studies with UTP and 2methylthioADP (2MeSADP), agonists of $P2Y_{2/4}$ and $P2Y_1$ receptors, respectively. Quiescent astrocyte cultures were treated with FGF2 (25 ng/ml), ATP (100 μM), UTP (100 μM), 2MeSADP (10 μM) or a combination of FGF2 and each nucleotide. We found that cyclin A expression was enhanced by combined treatment of astrocytes with FGF2 and UTP (Figure 5A). By contrast, 2MeSADP was ineffective. Similar results were obtained for cyclin D1. To extend these studies, we investigated the effects of UTP and 2MeSADP on FGF2-induced astrocyte mitogenesis. We found that FGF2-induced DNA synthesis was also potentiated by UTP, but not by 2MeSADP (Figure 5B). Thus, these studies were consistent with those of cyclin A and D1 and suggested a role for $P2Y_{2/4}$ receptors. To investigate further the type of P2 receptor

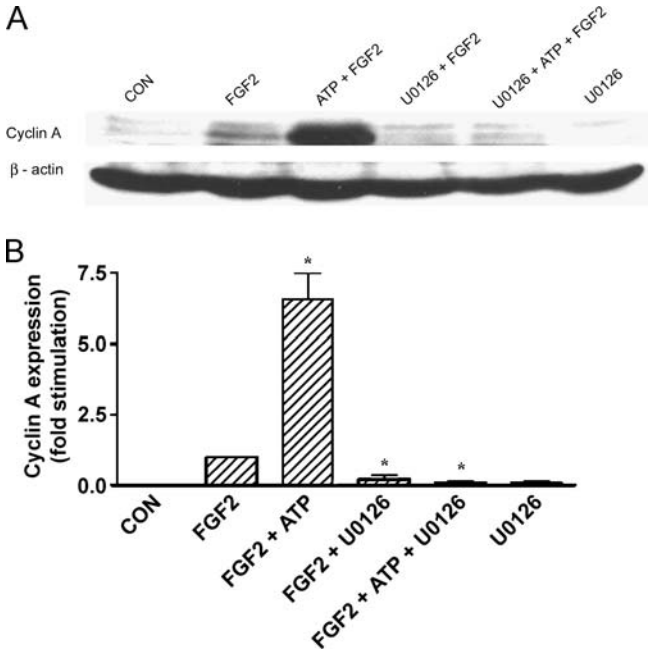

Figure 4. Blockade of the ERK cascade inhibits cyclin A expression induced by FGF2 and extracellular ATP. (A) U0126 (10 μM, 30 min) was added to quiescent, primary cultures of rat cortical astrocytes prior to treatment with ATP (100 μM), FGF2 (25 ng/ml) or a combination of ATP and FGF2 for 20 h. Lysates were subjected to SDS-PAGE, and cyclin A expression was measured by immunoblotting. (B) Results (mean ± SEM) from three independent experiments in which astrocyte cultures were treated as described in (A). Blockade of the ERK cascade significantly inhibited the expression of cyclin A induced by FGF2 as well as the potentiation of FGF2-induced cyclin A by extracellular ATP (*: FGF2 + ATP compared to FGF2, $P < 0.001$; FGF2 + U0126 compared to FGF2, $P < 0.05$; FGF2 + ATP + U0126 compared to FGF2 + ATP, $P < 0.001$).

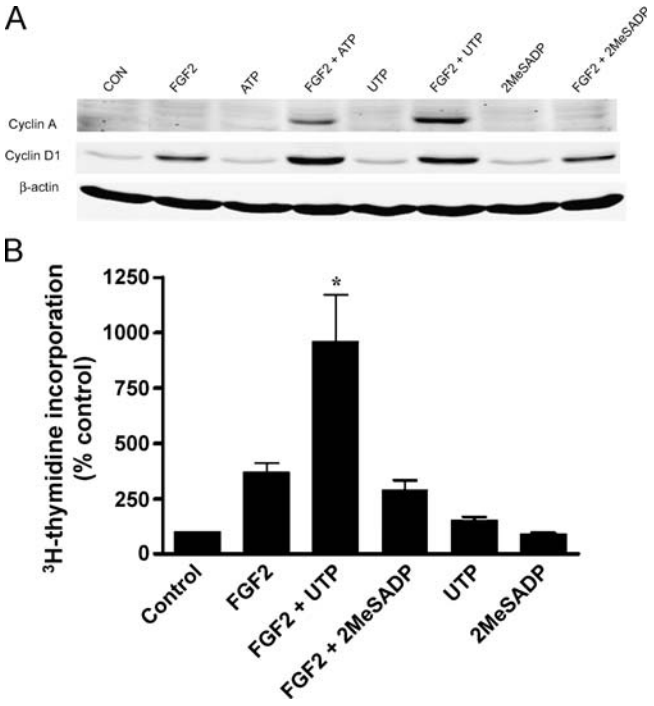

Figure 5. P2Y purine/pyrimidines receptors mediate the synergistic effects of extracellular ATP on FGF2-induced cyclin expression and mitogenesis. (A) Quiescent, primary cultures of rat cortical astrocytes were treated with ATP (100 μM) UTP (100 μM), 2MeSADP (10 μM), FGF2 (25 ng/ml) or a combination of nucleotides and FGF2 for 20 h. Lysates were subjected to SDS-PAGE, and cyclin A expression was measured by immunoblotting. Blots were also probed with anti-actin antibodies as a loading control. UTP and ATP, but not 2MeSADP, potentiated the ability of FGF2 to stimulate expression of cyclin A. Similar results were obtained in three independent experiments. (B) Quiescent, primary cultures of rat cortical astrocytes were treated with ATP (100 μM) UTP (100 μM), 2MeSADP (10 μM), FGF2 (25 ng/ml) or a combination of nucleotides and FGF2, and DNA synthesis was measured as described in Materials and methods. ^{3}H-Thymidine incorporation in control cultures was 15,714 ± 3568 cpm/mg protein (mean ± SEM; $n = 6$). FGF2-induced DNA synthesis was significantly enhanced by UTP (*$P < 0.05$).

involved, we conducted studies with pyridoxalphosphate-6-azophenyl-2′,4′disulphonic acid (PPADS), an antagonist of UTP-stimulated, $P2Y_2$ receptors in astrocytes [28, 29]. We found that when astrocytes were treated with 50 μM PPADS 15 min prior to addition of UTP, DNA synthesis stimulated by UTP was reduced by 85% ($n = 3$). We also investigated the effect of PPADS on the ability of UTP to enhance FGF2-induced mitogenesis but found that PPADS partially inhibited the mitogenic effect of FGF2 in the absence of UTP (data not shown), perhaps suggesting that basal extracellular nucleotides contribute to FGF2 mitogenic activity, as reported for other cellular responses [30]. Collectively, the effects of UTP on FGF2-induced cyclin expression and mitogenesis, together with the inhibitory effect of PPADS on UTP-stimulated DNA synthesis, suggest a role for P2Y receptors, possibly $P2Y_2$, in mediating the potentiating effects of extracellular nucleotides on FGF2 responses in astrocytes.

$P2X_7$ receptors have also been linked to cellular proliferation in a subpopulation of lymphoid cells [31] and in Jurkat cells [30], although activation of these ionotropic P2X receptors can also lead to apoptosis in several cell types, including lymphocytes [33] and microglia [34]. Because functional $P2X_7$ receptors are expressed in astrocytes [35–38], we examined the effect of stimulating these receptors on FGF2-induced mitogenesis. We found that activation of $P2X_7$ receptors by 2′,3′-*O*-(4-benzoyl)-benzoyl-ATP (BzATP; 100 μM) reduced FGF2-induced mitogenesis by over 90% in primary cultures of rat cortical astrocytes (Figure 6). The decrease in DNA synthesis was not due to cytotoxicity as measured by trypan blue exclusion. For example, the percentage of trypan blue positive cells in cultures untreated or treated with 100 μM BzATP, 25 ng/ml FGF2 or the combination of BzATP and FGF2 for 22 h did not exceed 6%, whereas in cultures treated with staurosporine, approximately 75% of the cells were trypan blue positive after 22 h ($n = 3$). Similar results were obtained with a tetrazolium-based test of cell viability (data not shown). Thus, the results presented here, together with those presented previously [20] suggest that P2Y receptors and P2X receptors exert opposing effects on FGF2-induced cellular proliferation in astrocyte cultures.

ERK signaling mediates proliferation and differentiation as well as growth arrest, depending on the duration and intensity of ERK activation [39, 40]. Because both P2Y and $P2X_7$ receptors are linked to ERK in astrocytes and

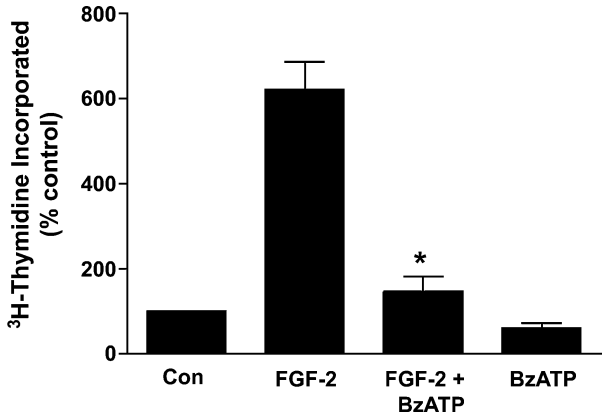

Figure 6. P2X receptors inhibit FGF2-induced mitogenesis in astrocytes. Quiescent, primary cultures of rat cortical astrocytes were treated with BzATP (100 μM), FGF2 (25 ng/ml) or a combination of BzATP and FGF2, and DNA synthesis was measured as described in Materials and methods. FGF2-induced DNA synthesis was significantly inhibited by BzATP (*$P < 0.001$; $n = 6$).

other cell types [24, 32, 35, 41, 42], we investigated the duration and intensity of ERK signaling stimulated by P2Y and $P2X_7$ receptors in astrocytes. Astrocytes were treated with UTP (100 μM) or BzATP (100 μM) for 5, 30, 60, 120, or 180 min, and stimulation of ERK1/2 was determined by dual phosphorylation of threonine 202 and tyrosine 204 residues. We found that UTP stimulated strong ERK1/2 phosphorylation at 5 min which declined thereafter and returned to baseline after 120 min; by contrast, BzATP stimulation of ERK1/2 was less intense at 5 min but remained elevated above baseline at 180 min (Figure 7). Quantification of data from this and an identical, independent experiment revealed that UTP stimulated an approximately 10-fold increase in ERK1/2 phosphorylation at 5 min as compared to about three-fold for BzATP, while at 3 h, no stimulation was observed with UTP but approximately three-fold stimulation was maintained by BzATP. Thus, P2Y receptor activation led to an initially intense but transient activation of ERK whereas activation of ERK by P2X receptors in astrocytes was initially less intense but more sustained. This finding is consistent with studies in other cells in which growth arrest is mediated by a sustained ERK signal whereas proliferation is linked to a transient ERK activation [39, 40].

Discussion

The major findings presented here are (1) extracellular ATP enhances the ability of FGF2 to induce the expression of cyclin D1 and cyclin A, cell cycle regulatory proteins involved in the entry and progression of mammalian cells through the G1 and S phases of the cell cycle, (2) the synergistic effects of extracellular ATP are mediated by signaling from P2Y purine/pyrimidine receptors to ERK, and (3) stimulation of P2X receptors inhibits the mitogenic activity of FGF2. These results may have implications regarding CNS development as well as reactive gliosis that occurs after CNS injuries.

Extracellular ATP has both short-term and long-term biological activities [43]. Of particular interest to nervous system development as well as CNS injury and repair is its ability to function as a trophic factor, either alone or as a co-mitogen in conjunction with polypeptide growth factors [14, 19]. For example, Heppel and colleagues described synergistic interactions between extracellular ATP and growth factors such as EGF and PDGF in fibroblasts and neuroblastoma cell lines [44, 45]. In astrocytes, we found that extracellular ATP markedly potentiated FGF2-induced mitogenesis [20]. However, little is known about the effects of extracellular ATP on cell cycle regulatory mechanisms, particularly in cells of the central nervous system. In a fibroblast cell line, extracellular ATP induced expression of cyclin A [46]. In mouse embryo and human fetus fibroblast cell lines in which placental alkaline phosphatase exerts growth factor-like effects, extracellular ATP potentiated the expression of cyclins A and E induced by placental alkaline phosphatase [47]. FGF2 is a mitogen for many, but not all, cell types. For example, in medial smooth muscle cells, FGF2 stimulated proliferation and expression of cyclin A, but in intimal smooth muscle cells, proliferation was only weakly stimulated by FGF2 and cyclin A expression was not induced [48]. Here we report for the first time that extracellular ATP enhances FGF2-induced expression of cyclins involved in cell cycle entry and progression through the G1 phase (cyclin D1) as well entry and progression through the S phase (cyclin A) of the cell cycle.

Our studies demonstrate that the increased expression of cyclin D1 and cyclin A induced by FGF2 and extracellular

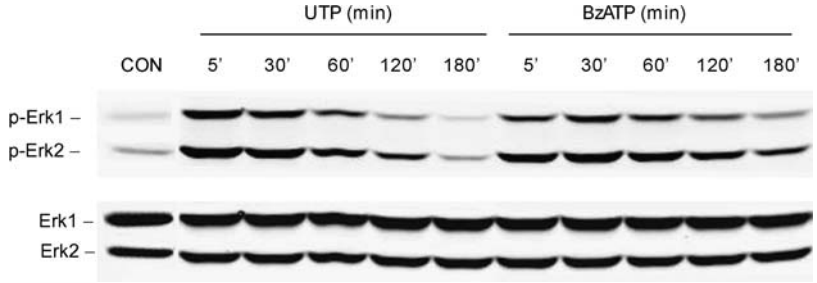

Figure 7. The duration and intensity of ERK phosphorylation differs between P2Y and P2X receptor signaling. Quiescent, primary cultures of rat cortical astrocytes were treated with UTP (100 μM) or BzATP (100 μM) for the times indicated. ERK1/2 phosphorylation was measured as described in Materials and methods. Stimulation of P2Y receptors with UTP elicited an intense but transient ERK phosphorylation whereas stimulation of P2X receptors with BzATP evoked an initially less intense but more sustained ERK phosphorylation. Similar results were obtained in an independent experiment.

ATP involves ERK because cyclin expression was attenuated by blocking MEK, the upstream activator of ERK. These findings are consistent with those from studies in fibroblasts that demonstrate that up-regulation of cyclin D1 by PDGF was dependent on ERK activation [49]. Importantly, blockade of the ERK cascade also significantly inhibited the ability of extracellular ATP to enhance FGF2-induced cyclin expression. These findings indicate that the ERK cascade plays an important role in mediating the comitogenic activity of extracellular ATP.

Our studies also indicate that P2Y purine/pyrimidine receptors mediate the potentiating effects of extracellular nucleotides on FGF2-induced cyclin expression and astrocyte mitogenesis, whereas P2X receptors, possibly the $P2X_7$ subtype, inhibit the mitogenic activity of FGF2 in astrocytes. Previous studies demonstrated that rat cortical astrocytes express both P2Y and P2X receptors [24, 36, 38]. For P2Y receptors, RT-PCR studies revealed that primary cultures of cortical astrocytes prepared from one-day-old rat pups contain P2Y1, P2Y2, P2Y4 receptors, but not P2Y6 receptors [24]. A more recent report indicated that P2Y6 receptors are expressed in astrocytes prepared from seven-day-old rats [36]. Differences in subtype expression may be due to differences in developmental patterns of receptor expression or variations in culture conditions. Here we found that UTP, but not 2MeSADP, enhanced cyclin expression stimulated by FGF2. This finding is consistent with the observation that UTP enhanced the ability of FGF2 to stimulate DNA synthesis whereas 2MeSADP was ineffective. In addition, PPADS, an antagonist of UTP-stimulated, $P2Y_2$ receptors in astrocytes [28, 29], attenuated DNA synthesis stimulated by UTP. Interestingly, PPADS also partially reduced FGF2 mitogenic activity in the absence of UTP. This may suggest cross-talk between $P2Y_{2/4}$ receptors and FGF2 receptors, but further studies beyond the scope of this work are needed to investigate this possibility. Collectively, our results suggest that $P2Y_2$ and/or $P2Y_4$ receptors mediate the synergistic effects of extracellular nucleotides on FGF2-induced cyclin expression.

$P2X_7$ receptors are also expressed in cultured astrocytes [35, 36, 38] and are coupled to ERK in these and other cells [32, 35, 37]. Functionally, cellular proliferation was observed upon expression of recombinant $P2X_7$ receptors in lymphoid cells lacking these receptors [31]. In addition, increased proliferation was also noted in Jurkat cells after stimulation of endogenous $P2X_7$ receptors [32]. However, activation of $P2X_7$ receptors leads to apoptosis in several cell types, including lymphocytes [33] and microglia [34]. Here we found that, in cultured astrocytes, the $P2X_7$ receptor agonist BzATP inhibited the mitogenic activity of FGF2. Cytotoxicity experiments indicated that this was not due to a loss of cell viability. The opposing effects of P2X and P2Y receptor stimulation on FGF2-induced mitogenesis might be mediated at least in part by differences elicited in ERK responses. ERK can regulate cellular proliferation, differentiation or growth arrest, depending on the intensity and duration of the response and the cell type [50]. For example, in some cell types a sustained ERK signal is associated with growth arrest whereas a transient signal leads to proliferation [39, 40]. Consistent with this, here we found that stimulation of P2Y receptors elicited an intense but transient ERK signal whereas that evoked by P2X receptors was initially weaker but more sustained. Further studies are needed to explore the effects of P2X receptors on cell cycle regulation and whether their inhibitory effects are due to the expression of cyclin-dependent protein kinase inhibitors.

Because FGF2 and ATP are increased after CNS injury and contribute to the gliotic response, the results presented here may be relevant to the hyperplasia observed in adult brains after trauma and stab wounds. The enhancement of FGF2-induced cyclin expression by extracellular ATP supports the suggestion of Nakatsuji and Miller [8] that changes in the expression of cell cycle regulatory proteins may be involved in the increase in astrocyte proliferation observed after injury to the adult CNS. The reactive astrocytes formed in injured CNS regions impact the re-establishment of neural connections and communication. Since reactive astrocytes produce a variety of molecules that can impede as well as promote axonal regeneration [51, 52], advances in our understanding of the mechanisms that mediate the formation of reactive astrocytes may offer an opportunity to enhance the beneficial, axonal growth-promoting features of reactive astrocytes while attenuating their harmful, growth-inhibiting properties. Thus, an understanding of the mechanisms leading to reactive astrocytes and the glial scar may provide new approaches to restore losses in motor skills and cognitive functions caused by CNS injury.

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