

P2Y₂ receptor-mediated proliferation of C₆ glioma cells *via* activation of Ras/Raf/MEK/MAPK pathway

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1 Extracellular purine and pyrimidine nucleotides have been implicated in the regulation of several cellular functions including mitogenesis. In this study, experiments were conducted to characterize the P2Y receptor on C₆ glioma cells responsible for stimulating cell proliferation associated with mitogen-activated protein kinase (MAPK) activation.

2 UTP and ATP produced a similar effect on [³H]-thymidine incorporation in a time- and concentration-dependent manner, suggesting the involvement of P2Y₂ receptor in mediating proliferation of C₆ glioma cells.

3 In response to UTP, both p42 and p44 MAPK were activated in a time- and concentration-dependent manner using Western blot analysis with an anti-phospho-p42/p44 MAPK antibody. The phosphorylation reached maximal levels after 5 min and declining by 30 min.

4 Pretreatment with pertussis toxin (PTX) did not change these responses to UTP. Both DNA synthesis and phosphorylation of MAPK in response to UTP were attenuated by tyrosine kinase inhibitors, genistein and herbimycin A, protein kinase C (PKC) inhibitors, staurosporine and GF109203X, and removal of Ca²⁺ by addition of BAPTA/AM plus EGTA.

5 UTP-induced [³H]-thymidine incorporation and p42/p44 MAPK phosphorylation was completely inhibited by PD98059 (an inhibitor of MEK1/2). Furthermore, we showed that overexpression of dominant negative mutants of Ras (RasN17) and Raf (Raf-301) completely suppressed MEK1/2 and p42/p44 MAPK activation induced by ATP and UTP.

6 These results conclude that the mitogenic effect of UTP is mediated through a P2Y₂ receptor that involves the activation of Ras/Raf/MEK/MAPK pathway. UTP-mediated MAPK activation was modulated by Ca²⁺, PKC, and tyrosine kinase associated with cell proliferation in cultured C₆ glioma cells.

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Abbreviations: BCA, bicinchoninic acid; CPA, N⁶-cyclopentyl adenosine; DMEM, Dulbecco's modified Eagle's medium; EC₅₀, concentration required for half-maximal stimulation; ECL, enhanced chemiluminescence; F-12, Ham's nutrient mixture F-12; FBS, foetal bovine serum; IP₃, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; $\alpha\beta$ -MeATP, α,β -methylene ATP; β,γ -MeATP, β,γ -methylene ATP; MEK1/2, MAPK kinase; 2MeSATP, 2-methylthio ATP; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin

Introduction

ATP is released from cells and acts in a well-established physiological role as an extracellular signalling molecule (Burnstock, 1997). Receptors for extracellular ATP were subdivided into P2X and P2Y receptors on the basis of pharmacological studies using isolated preparations from several species (Burnstock & Kennedy, 1985). These receptor subtypes differed in their transduction mechanisms; P2X receptors are transmitter-gated ion channels whereas P2Y receptors are members of the G protein-coupled receptor superfamily (Fredholm *et al.*, 1997). So far, P2X receptor subtypes have been subclassified mainly as P2X₁ to P2X₇ (Surprenant *et al.*, 1996). The stable analogue of ATP, α,β -MeATP, is a potent agonist for the P2X₁ receptor (Burnstock

& Kennedy, 1985). P2Y receptors have been subclassified as P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors (Hourani & Hall, 1994; Communi *et al.*, 1997). The P2Y₁ receptor is activated by adenine nucleotides and not by uridine nucleotides. ADP is the most potent natural agonist for this receptor, and whether or not ATP is an agonist for this receptor remains uncertain (Leon *et al.*, 1997). The P2Y₂ receptor is activated equipotently by ATP and UTP but is activated, at most only poorly, by diphosphate nucleotides (Nicholas *et al.*, 1996). The P2Y₄ receptor is activated by UTP and weakly, if at all, by ATP, UDP, or ADP (Nicholas *et al.*, 1996). The P2Y₆ receptor is UDP-sensitive and is activated weakly, if at all, by UTP, ATP, or ADP (Nicholas *et al.*, 1996). The P2Y₁₁ receptor has been shown to be activated by ATP and ADP, but not by UTP or UDP (Communi *et al.*, 1997). In several cell types, P2Y receptors are G protein coupled receptors which are associated with phospholipase C (PLC) activation with subsequent increases in inositol 1,4,5-trisphosphate (IP₃) formation, intracellular Ca²⁺ release, diacylgly-

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cerol production, and activation of PKC (Boarder *et al.*, 1995; Harden *et al.*, 1995; Yang *et al.*, 1997).

ATP has been shown to increase [³H]-thymidine incorporation in Swiss 3T3 (Huang *et al.*, 1989), A431 (Huang *et al.*, 1989), and renal mesangial cells (Huwilder & Pfeilschifter, 1994). However, ATP-induced cell proliferation in C₆ glioma cells needs to be investigated. It is conceivable that phosphorylation of cellular proteins by protein kinases plays an important role in regulation of cellular functions including cell growth, migration, differentiation, and response to extracellular stimuli. Cell proliferation induced by growth factors can be divided into at least two distinct classes that act through different signal transduction pathways. One is activated by receptors that contain an intrinsic protein tyrosine kinase such as those activated by PDGF and EGF (Pouyssegur & Seuwen, 1992). Tyrosine phosphorylation of cytosolic protein has been shown to be important in relaying extracellular messages to the nucleus, thus promoting cell division (Malarkey *et al.*, 1995). The activation of MAPKs, in particular, the p42 and p44 MAPK isoforms, seems to be a key component in growth signal transduction through tyrosine kinase receptors such as the PDGF- β receptor (Blenis, 1993). The other pathway is activated by G protein-coupled receptors (Davis, 1993; Lange-Carter *et al.*, 1993). Several studies reveal that the cellular growth mediated by the intracellular signal pathway for the G protein-linked receptor also involves MAPKs (Pouyssegur & Seuwen, 1992; Davis, 1993). These MAPKs are activated during proliferation and cell cycle transition triggered by various stimuli (Blenis, 1993). Thus, MAPKs are indeed important integrators of G protein-coupled receptor- and tyrosine kinase receptor-mediated signals for cell growth. Moreover, many growth factor receptors have been shown to activate a signal transduction pathway that includes MAPK kinase (MEK) and MAPK (Davis, 1993; Lange-Carter *et al.*, 1993; Marshall, 1995). The requirement for the activation of the Ras, Raf, MEK, and MAPK, associated with cell proliferation, had been demonstrated for receptor tyrosine kinases such as PDGF in several cell types (Blenis, 1993; Post & Brown, 1996). Utilizing selective expression of dominant negative mutants, Ras, Raf, or MEK has further been shown to play a key step toward MAPK activation (Stacey *et al.*, 1991; Schaap *et al.*, 1993; Abdellatif *et al.*, 1998).

The important role of glial cells in various pathological conditions in brain was first recognized by Del-Rio Hortega (1932). One of the characteristics of glial cells is their activation at a very early stage in response to injury (Gehrmann *et al.*, 1995). Glia activation often precedes reactions of any other cell type in the brain. Furthermore, glial cells have receptors for signalling molecules such as ATP (Langosch *et al.*, 1994), and can react ATP with changes in their extracellular ionic milieu (Langosch *et al.*, 1994) and by activation of transcriptional mechanism (Priller *et al.*, 1995). An understanding of intercellular signalling pathways for glia proliferation and activation could form a rational basis for targeted intervention on glial reactions to injuries in the brain. C₆ glioma cells cloned from a rat glial tumour were widely used as a cell culture model to study physiological functions. However, whether the activation of MAPK by ATP associated with cell proliferation of C₆ glioma cells has not been fully elucidated.

In this study, the experiments were undertaken to investigate the effect of ATP on activation of Ras/Raf/MEK/MAPK pathway, associated with cell proliferation of

C₆ glioma cells. In order to characterize the purinoceptor receptor subtype mediating mitogenic effects in C₆ cells, we examined the responses to ATP and its analogues selective for different receptor subtypes.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (F-12) medium, OPTI-MEM I medium, Lipofectamine Plus reagent, and foetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD, U.S.A.). [³H]-Methyl thymidine, Hybond C membrane, and enhanced chemiluminescence (ECL) Western blotting detection system were from Amersham (Buckinghamshire, England). Fura-2/AM was from Molecular Probes (Eugene, OR, U.S.A.). PhosphoPlus p42/p44 MAPK and phosphoPlus MEK1/2 antibody kits were from New England Biolabs (Beverly, MA, U.S.A.). p42 MAPK antibody was from Santa Cruz (Santa Cruz, CA, U.S.A.). Genistein, herbimycin A, staurosporine, GF109203X, BAPTA/AM, PD98059, and SB203580 were from Calbiochem (San Diego, CA, U.S.A.). Bicinchoninic acid (BCA) protein assay reagent was from Pierce (Rockford, IL, U.S.A.). Enzymes and other chemicals were from Sigma (St Louis, MO, U.S.A.).

Cell culture

The C₆ glioma cells were obtained from ATCC (CCL 107) and established in monolayer culture in DMEM/F-12 medium supplemented with 10% FBS and antibiotics (100 U ml⁻¹ penicillin G, 100 μ g ml⁻¹ streptomycin, and 250 ng ml⁻¹ fungizone). Cells were maintained in continuous culture at 37°C in a humidified atmosphere containing 5% CO₂. At subconfluence, cultures were replated by enzymatic dissociation with trypsin/EDTA.

[³H]-Thymidine incorporation and cell proliferation

Cells were plated onto 24-well culture plates and grown to confluence. Cells were growth-arrested by incubation in serum-free DMEM/F-12 for 48 h. Confluent, growth-arrested cells were used because cells can be synchronized in G₀/G₁ phase of the cell cycle and at this baseline, minimally incorporate [³H]-thymidine. After 48 h in serum-free media, the cells were stimulated with ATP and its analogues. After 6 h of stimulation, cells were labelled with 1 μ Ci ml⁻¹ of [³H]-thymidine for another 18 h in the presence of agonists. The experiments were terminated by washing the cells with cold PBS, precipitation of acid-insoluble materials with 10% TCA, and extraction of the DNA with 0.1 N NaOH. The precipitants were filtered through Whatman GF/B filters and washed three times with cold PBS using a cell harvester. The radioactivity was counted using a scintillation counter (Beckman LS5000TA, Fullerton, CA, U.S.A.).

Plasmids and transfection

The plasmids encoding RasN17 and Raf-301 (dominant negative mutants of Ras and Raf-1), cloned into pJ3H and pKRSPA, respectively, were kindly provided by Dr H.-F. Yang Yen (Institute of Molecular Biology, Academia Sinica). The dominant negative mutant of MEK1, MEK K97R, was a gift from Dr K.-L. Guan at the University of Michigan and

subcloned into pAS-CYH. All plasmids were prepared using QIAGEN plasmid DNA preparation kits.

C₆ glioma cells were plated at 3×10^5 cells ml⁻¹ in 12-well culture plates for 24 h, reaching about 80% confluence. Cells were washed once with PBS and once with serum-free DMEM, and 0.5 ml of serum-free OPTI-MEM I medium was added to each well. The DNA PLUS-Lipofectamine reagent complex was prepared according to the instructions of the manufacturer. The amount of transfected plasmid was kept constant (2 µg of RasN17 or Raf-301 for each well). The DNA PLUS-Lipofectamine reagent complex was added to each well, then incubated at 37°C for 5 h. At that time, 0.5 ml of OPTI-MEM I medium containing 20% FBS was added and incubated for 19 h. After 24 h of transfection, the cells were washed twice with PBS and maintained in DMEM/F-12 containing 10% FBS for 48 h. Cells were then washed once with PBS and incubated with serum-free DMEM/F-12 for 24 h before treatment with either ATP or UTP.

Preparation of cell extracts and Western blot analysis of MAPK isoforms

For experiments, cells were plated in 100-mm dishes and made quiescent at confluence by incubation in fresh DMEM/F-12 for 48 h. Growth-arrested C₆ glioma cells were incubated with or without UTP at 37°C for various times. When inhibitors were used, they were applied 1 h prior to the addition of UTP. After incubation, the cells were rapidly washed with ice-cold PBS, scraped and collected by centrifugation at $1000 \times g$ for 10 min. The collected cells were lysed with ice-cold lysis buffer containing (mM): Tris-HCl 25, pH 7.4, NaCl 25, NaF 25, sodium pyrophosphate 25, sodium vanadate 1, EDTA 2.5, EGTA 2.5, 0.05% Triton X-100, 0.5% SDS, 0.5% deoxycholate, 0.5% NP-40, 5 µg ml⁻¹ leupeptin, 5 µg ml⁻¹ aprotinin, and PMSF 1. The lysates were centrifuged at $45,000 \times g$ for 1 h at 4°C to yield the whole cell extract. The protein concentration was determined by the BCA reagents according to the manufacturer's instructions. Samples (100 µg protein) were denatured and subjected to SDS-PAGE using a 10% running gel. Proteins were transferred to nitrocellulose membrane and the membrane was successively incubated at room temperature with 5% BSA in TTBS (Tris-HCl 50 mM, NaCl 150 mM, 0.05% Tween 20, pH 7.4) for 1 h. The phosphorylation of MEK1/2 and p42/p44 MAPK isoforms were identified and quantified by Western blot analysis using anti-phospho-MEK1/2 or anti-phospho-p42/p44 MAPK polyclonal antibody kit according to the manufacturer's instructions. Briefly, membranes were incubated overnight at 4°C with the anti-phospho-MEK1/2 or anti-phospho-p42/p44 MAPK polyclonal antibody used at a dilution of 1:1000 in TTBS. Membranes were washed with TTBS four times for 5 min each, incubated with a 1:1500 dilution of anti-rabbit horseradish peroxidase antibody for 1 h. Following each incubation, the membrane was washed extensively with TTBS. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL (Amersham International).

Analysis of data

Concentration-effect curves were fitted and EC₅₀ values were estimated using the Prism Program (GraphPad, San Diego, CA, U.S.A.). Data were expressed as the means \pm s.e.mean and analysed with Student's *t*-test at a 0.01 level of significance. Error bars were omitted when they fell within the symbol representing values.

Results

Extracellular ATP- and UTP-stimulated DNA synthesis

To determine the mitogenic effect of ATP-induced DNA synthesis, we tested the ability of ATP and its analogues to stimulate [³H]-thymidine uptake in C₆ glioma cells. ATP and UTP stimulated [³H]-thymidine incorporation in a time-dependent manner (Figure 1A). Onset of the effects of ATP and UTP on [³H]-thymidine incorporation was slow, a significant increase in this response being observed by 4 h after addition of [³H]-thymidine in the presence of these agonists at a concentration of 1 mM, and a maximal effect occurring by 24 h. Furthermore, ATP, UTP, ADP, and UDP produced a concentration-dependent increase in [³H]-thymidine incorporation into serum-deprived, quiescent C₆ glioma cells (Figure 1B). The concentrations of ATP, UTP, ADP, and UDP that produced a half maximal increase (EC₅₀) in [³H]-thymidine incorporation were 77 ± 15 , 21 ± 12 , 110 ± 25 , and

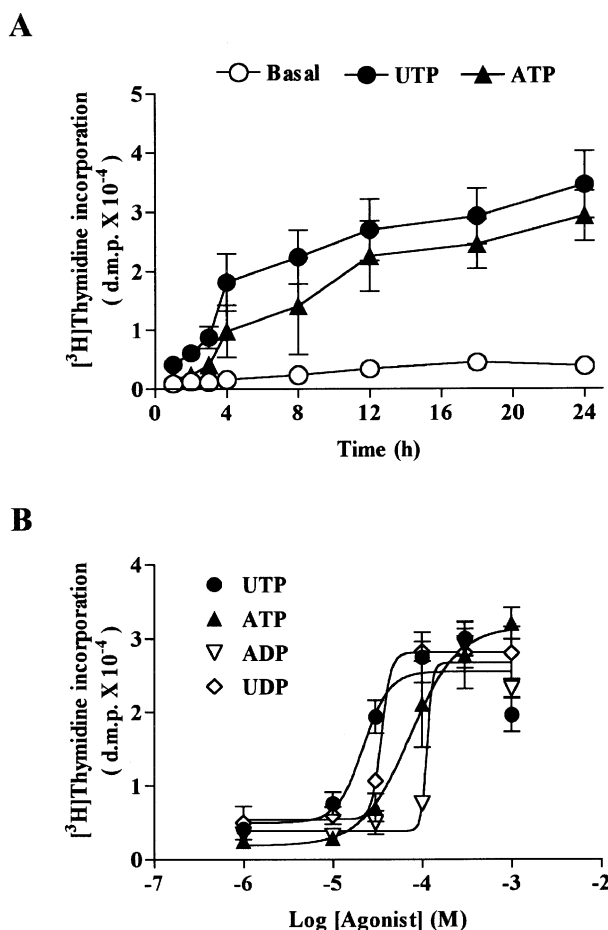


Figure 1 [³H]-Thymidine incorporation induced by ATP and UTP in C₆ glioma cells. (A) For time course, after 48 h in serum-free medium, the cells were stimulated with vehicle (basal), ATP or UTP at a concentration of 100 µM. The cells were labelled with 1 µCi ml⁻¹ [³H]-thymidine for the times indicated in the continuous presence of agonists. (B) For concentration dependence, the cells were stimulated with various concentrations of ATP, UTP, ADP or UDP. After stimulation for 6 h, cells were labelled with 1 µCi ml⁻¹ [³H]-thymidine for another 18 h in the presence of agonists. The incorporation of [³H]-thymidine was determined as described in Methods. Data are expressed as the means \pm s.e.mean of three separate experiments determined in triplicate.

$34 \pm 11 \mu\text{M}$, $n=3$, respectively. ATP (1 mM) and UTP (1 mM) stimulated [³H]-thymidine incorporation to approximately 9.7 ± 1.2 and 9.6 ± 0.9 folds, over the basal level (2850 ± 220 d.p.m. per well), respectively. Whereas P1 receptor agonists AMP and N⁶-cyclopentyl adenosine (CPA), P2X₁ receptor agonists α,β -methylene ATP (α,β -MeATP) and β,γ -methylene ATP (β,γ -MeATP) as well as the P2Y₁ receptor agonist 2-methylthio ATP (2MeSATP) did not cause any significant DNA synthesis (data not shown). These results suggest that the mitogenic effects of ATP and UTP were triggered by activation of P2Y₂ receptors.

Extracellular UTP stimulated MAPK isoforms activation

MAPKs, another group of components in the signal transduction pathway, have shown to be activated during stimulation of cell proliferation. Therefore, we determined whether UTP activated MAPKs, in C₆ glioma cells. Tyrosine phosphorylation of p42 and p44 MAPK isoforms was monitored by Western blot with polyclonal antisera reactive for the tyrosine phosphorylated state of these two forms of MAPK. As shown in Figure 2, UTP (100 μM) stimulated a marked increase in the levels of p42 and p44 kDa phosphorylation within 2 min. Densitometric analysis of the blot revealed that at 5 min, UTP induced a 3.0 ± 0.4 and 3.6 ± 1.5 fold increase in p42 and p44 MAPK isoforms, respectively, above the basal level. After 30 min, phosphorylation declined below the basal level. Parallel blots run as controls that used antibody against the total p42 MAPK did not show any change (Figure 2). Moreover, as demonstrated in Figure 3, the UTP-induced phosphorylation of p42 and p44 MAPK isoforms was concentration-dependent. Densitometric analysis of the blot indicated that the maximal effect was achieved with 100 μM UTP. Similar results were obtained with ATP (data

not shown). The following experiments were performed using UTP.

Effects of pertussis toxin and tyrosine kinase inhibitors on UTP-induced DNA synthesis and MAPK phosphorylation

To examine whether the effect of UTP on DNA synthesis and p42/p44 MAPK activation was mediated through the activation of a receptor coupled to a pertussis toxin (PTX)-sensitive G protein, C₆ glioma cells were pretreated with 100 ng/ml PTX for 24 h and then stimulated with UTP. As shown in Figure 4, pretreatment of these cells with PTX did not significantly attenuate the UTP-induced [³H]-thymidine incorporation ($P > 0.01$, $n=3$) and p42/p44 MAPK activation ($P > 0.01$, $n=3$), indicating that the effect of UTP on these responses was mediated through a PTX-insensitive G protein.

To determine whether the effect of UTP on DNA synthesis and p42/p44 MAPK activation was mediated through activation of tyrosine kinase, C₆ glioma cells were treated with 10 μM genistein or herbimycin A for 1 h and then stimulated with 100 μM UTP. As shown in Figure 4, pretreatment of these cells with genistein and herbimycin A also inhibited the UTP-induced [³H]-thymidine incorporation ($P < 0.01$, $n=3$) and p42/p44 MAPK activation ($P < 0.01$, $n=3$), suggesting the implication of tyrosine kinase in these responses.

Effect of PKC on UTP-induced DNA synthesis and MAPK phosphorylation

In order to determine whether PKC activation is involved in DNA synthesis and p42/p44 MAPK activation in response to UTP, C₆ glioma cells were pretreated with PKC inhibitors staurosporine (1 μM) or GF109203X (1 μM) for 1 h and then

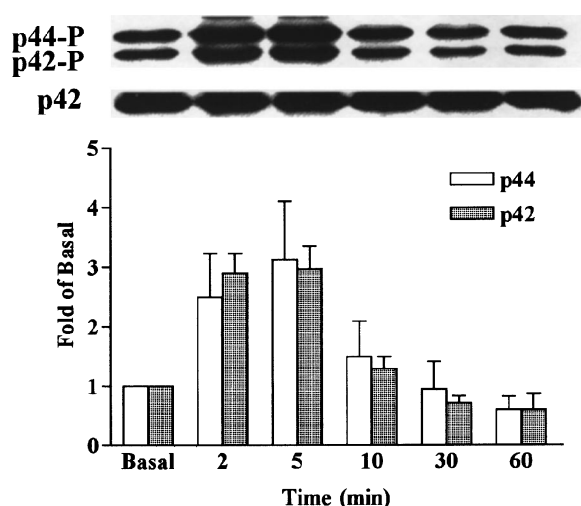


Figure 2 Time course of UTP-stimulated activation of p42 and p44 MAPK isoforms in C₆ glioma cells. The cells were grown to confluence, made quiescent by serum-deprivation for 48 h and incubated with 100 μM UTP for 2–60 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with anti-phospho-p42/p44 MAPK or total p42 MAPK (as a control) polyclonal antibody. Bands were visualized by an ECL method and quantified by a densitometer. Similar results were obtained in three independent experiments.

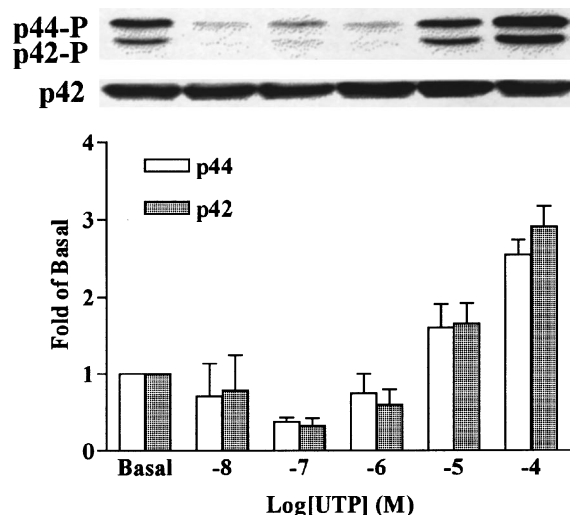


Figure 3 Concentration-dependence of UTP-stimulated activation of p42 and p44 MAPK isoforms in C₆ glioma cells. The cells were grown to confluence, made quiescent by serum-deprivation for 48 h and incubated with various concentrations of UTP for 5 min. The cell lysates were subjected to 10% SDS–PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with anti-phospho-p42/p44 MAPK or total p42 MAPK (as a control) polyclonal antibody. Bands were visualized by an ECL method. Similar results were obtained in three independent experiments.

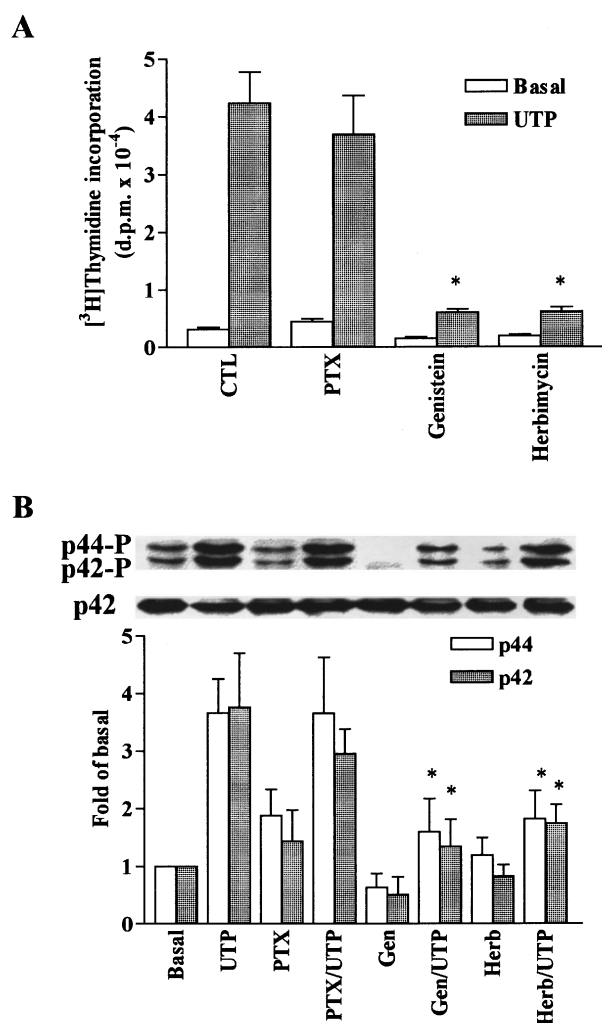


Figure 4 Involvement of G protein and tyrosine kinase in DNA synthesis and MAPK phosphorylation induced by UTP in C₆ glioma cells. The cells were preincubated with pertussis toxin (PTX, 100 ng ml⁻¹, 24 h), genistein (Gen, 10 μ M, 1 h), or herbimycin A (Herb, 10 μ M), 1 h, and then stimulated with vehicle or 100 μ M UTP. (A) For DNA synthesis, after 6 h incubation, cells were labelled with 1 μ Ci ml⁻¹ [³H]-thymidine for another 18 h in the continuous presence of agonists. The incorporation of [³H]-thymidine was determined as described in Methods. Data are expressed as the means \pm s.e. mean of three separate experiments determined in triplicate. (B) For MAPK experiment, after treatment with these agents, the cells were stimulated with vehicle or 100 μ M UTP for 5 min. The phosphorylation of p42/p44 MAPK was determined as described in Figure 2. Similar results were obtained in three independent experiments. **P* < 0.01, as compared with the control cells exposed to UTP.

stimulated with UTP. As shown in Figure 5, pretreatment of the cells with these PKC inhibitors attenuated [³H]-thymidine incorporation (*P* < 0.01, *n* = 3) and p42/p44 MAPK activation (*P* < 0.01, *n* = 3) in response to UTP. These results suggest that UTP-stimulated [³H]-thymidine incorporation and p42/p44 MAPK activation was mediated through the activation of PKC in C₆ glioma cells.

Effect of Ca²⁺ on UTP-induced DNA synthesis and MAPK phosphorylation

To elucidate whether the UTP-induced increase in [Ca²⁺]_i is implicated in the [³H]-thymidine incorporation and p42/p44 MAPK activation, C₆ glioma cells were preincubated with 30 μ M BAPTA/AM (a potent intracellular Ca²⁺ chelator) and

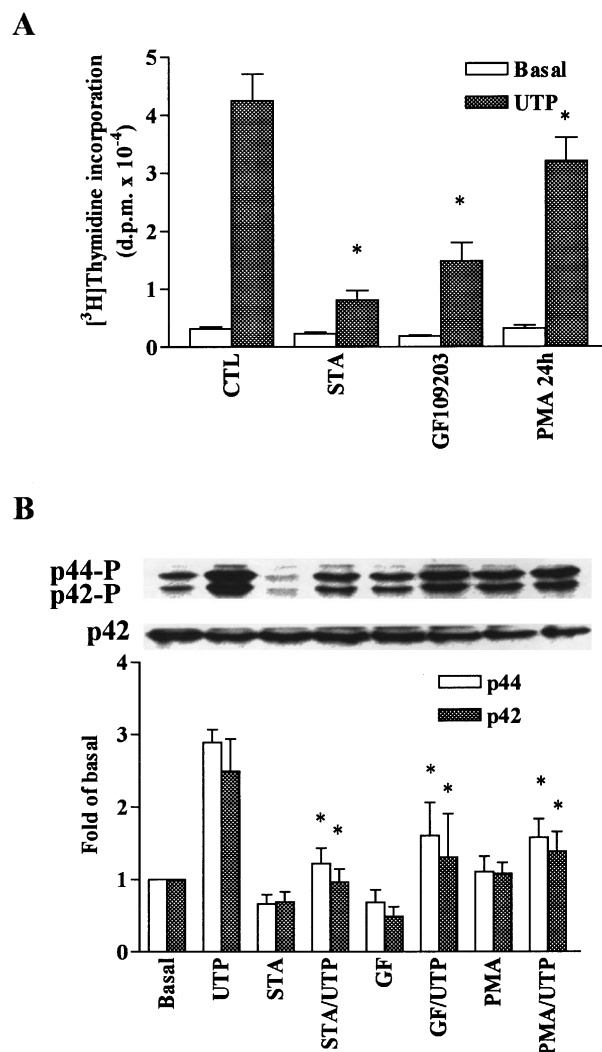


Figure 5 Effects of PKC on DNA synthesis and MAPK phosphorylation induced by UTP in C₆ glioma cells. The cells were preincubated with staurosporine (STA, 1 μ M, 1 h) or GF109203X (GF, 1 μ M, 1 h) and then stimulated with vehicle or 100 μ M UTP. (A) For DNA synthesis, after 6 h incubation, cells were labelled with 1 μ Ci ml⁻¹ [³H]-thymidine for another 18 h in the presence of agonists. The incorporation of [³H]-thymidine was determined as described in Methods. Data are expressed as the means \pm s.e. mean of three separate experiments determined in triplicate. (B) For MAPK experiment, after treatment with these agents, the cells were stimulated with vehicle or 100 μ M UTP for 5 min. The phosphorylation of p42/p44 MAPK was determined as described in Figure 2. Similar results were obtained in three independent experiments. **P* < 0.01, as compared with the control cells exposed to UTP.

5 mM EGTA and then stimulated with UTP. Results in Figure 6 demonstrate that pretreatment of these cells with BAPTA/AM reduced both [³H]-thymidine incorporation (*P* < 0.01, *n* = 3) and p42/p44 MAPK activation (*P* < 0.01, *n* = 3), indicating that Ca²⁺ is required for the UTP-mediated responses.

Effects of MAPK kinase inhibitors on UTP-induced DNA synthesis and MAPK phosphorylation

To ensure that the mitogenic effect of UTP is mediated through the activation of MAPK pathway, the effect of UTP on the p42/p44 MAPK activation and DNA synthesis was examined after treatment of C₆ glioma cells with 10 μ M PD98059 (a synthetic MEK1/2 inhibitor) or 10 μ M SB203580

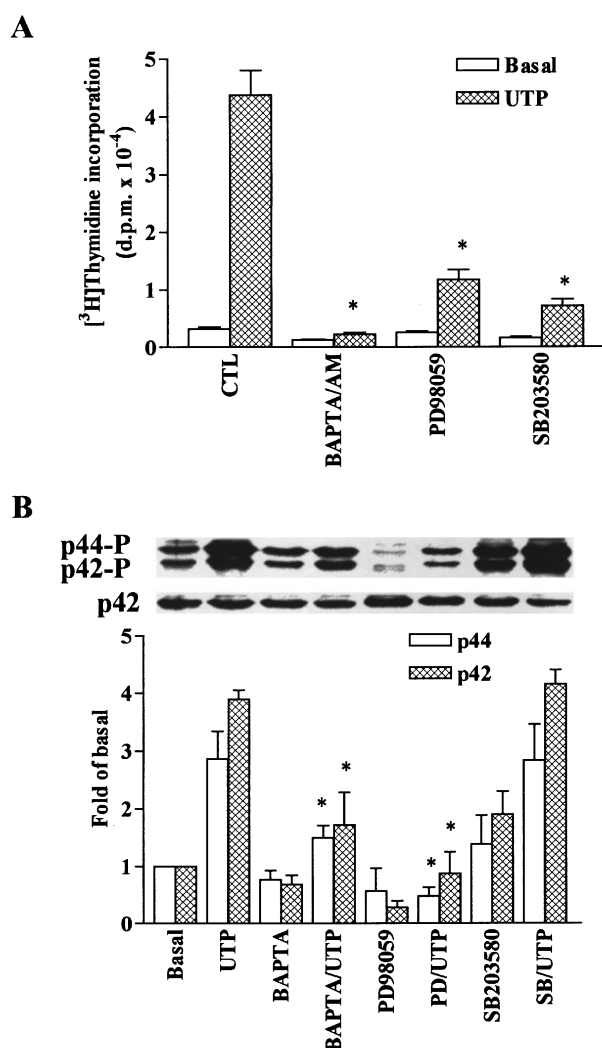


Figure 6 Effects of Ca²⁺ and MAPK kinase inhibitors on DNA synthesis and MAPK phosphorylation induced by UTP in C₆ glioma cells. The cells were preincubated with BAPTA/AM (10 μ M) plus EGTA (5 mM) for 1 h, PD98059 (PD, 10 μ M, 1 h), or SB203580 (SB, 10 μ M, 1 h) and then stimulated with vehicle or 100 μ M UTP. (A) For DNA synthesis, after 6 h incubation, cells were labelled with 1 μ Ci ml⁻¹ [³H]-thymidine for another 18 h in the continuous presence of agonists. The incorporation of [³H]-thymidine was determined as described in Methods. Data are expressed as the means \pm s.e. mean of three separate experiments determined in triplicate. (B) For MAPK experiment, after treatment with these agents, the cells were stimulated with vehicle or 100 μ M UTP for 5 min. The phosphorylation of p42/p44 MAPK was determined as described in Figure 2. Similar results were obtained in three independent experiments. **P* < 0.01, as compared with the control cells exposed to UTP.

(a p38 MEK inhibitor) for 1 h. As shown in Figure 6B, the effect of PD98059 on MAPK phosphorylation was assessed by Western blot analysis. PD98059 completely inhibited the phosphorylation of p42/p44 MAPK isoforms induced by UTP, conforming that MEK1/2 is required for MAPK activation in these cells. This hypothesis was further supported by the results that UTP-induced MEK1/2 activation in a time- and concentration-dependent manner (Figure 7). However, the phosphorylation of these MAPK isoforms was not inhibited by SB203580. In contrast, treatment of C₆ glioma cells with these inhibitors caused a significant inhibition of the UTP-induced [³H]-thymidine incorporation (Figure 6A). The inhibitory effect of SB203580 on UTP-stimulated [³H]-

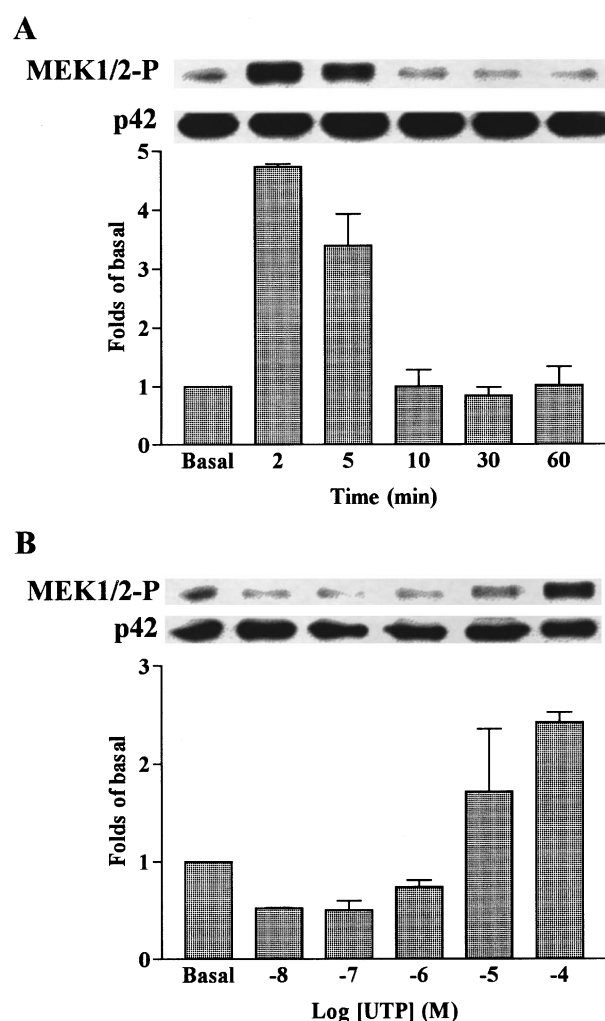


Figure 7 Time course and concentration-dependence of UTP-stimulated activation of MEK1/2 in C₆ glioma cells. The cells were grown to confluence, made quiescent by serum-deprivation for 48 h and incubated with 100 μ M UTP for 2–60 min (A) or various concentrations of UTP for 5 min (B). The cell lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with anti-phospho-MEK1/2 or total p42 MAPK (as a control) polyclonal antibody. Bands were visualized by an ECL method and quantified by a densitometer. Similar results were obtained in three independent experiments.

thymidine incorporation implied that an alternative pathway may be implicated in this mitogenic effect, namely the p38 MAPK pathway.

Activation of MAPK requires Ras and Raf-1

Several lines of evidence have suggested that Ras plays an important role in a variety of cell functions mediated through sequential activation of Raf-1, MEK1/2, and MAPK (Blenis, 1993; Post & Brown, 1996). To elucidate whether the activation of Ras/Raf is required for MAPK phosphorylation induced by ATP and UTP, C₆ glioma cells were transfected with or without a dominant negative Ras (RasN127), Raf (Raf-301), or MEK (MEK K97R), and then treated with ATP or UTP. As shown in Figure 8, both ATP and UTP induced phosphorylation of p42/p44 MAPK in C₆ glioma cells. In addition, transfection with RasN17, Raf-301, or MEK K97R almost completely abolished p42/p44 MAPK phosphorylation

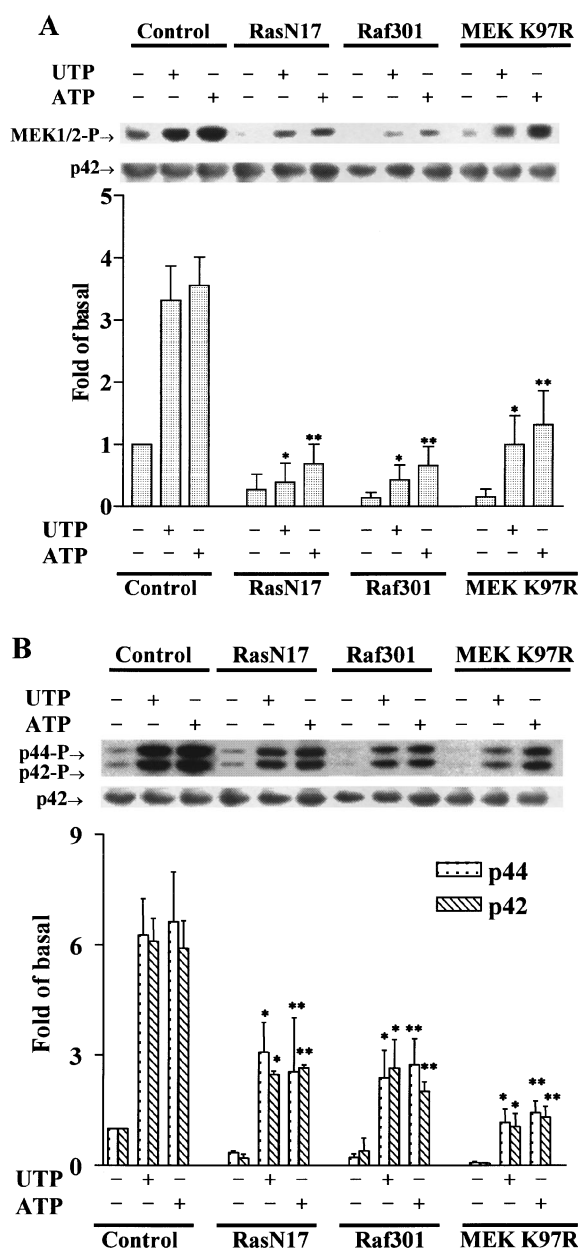


Figure 8 Requirement of Ras, Raf, and MEK1/2 for UTP- and ATP-induced activation of MEK1/2 and p42/p44 MAPK in VSMCs. Cells were transfected with plasmids encoding RasN17, Raf-301, or MEK K97R, and then stimulated with 100 μ M UTP or ATP for 5 min. The cell lysates were subjected to 10% SDS-PAGE and transferred to nitrocellulose membrane. Western blot analysis was performed using an antiserum reactive with the anti-phospho-MEK1/2 or total p42 MAPK (as a control) polyclonal antibody (a) and anti-phospho-p42/p44 MAPK or total p42 MAPK polyclonal antibody (b). Bands were visualized by an ECL method. Data are expressed as the means \pm s.e.mean of three independent experiments of anti-phospho-p42/p44 MAPK in the bar graph. * P < 0.01, as compared with the control cells exposed to UTP; † P < 0.01, as compared with the control cells exposed to ATP.

induced by these two agents, suggesting that ATP and UTP might activate MAPK through a Ras/Raf/MEK-dependent pathway in C₆ glioma cells. Moreover, both ATP and UTP also activated MEK1/2 that is an upstream component of p42/p44 MAPK pathway, these stimulatory effects were almost suppressed by transfection with RasN17, Raf-301, or MEK K97R (Figure 8). These results demonstrated that ATP and UTP, at least in part, activated Ras/Raf/MEK/MAPK pathway in C₆ glioma cells.

Discussion

In the present study, we examined the relative capacity of ATP and its analogues to stimulate proliferation of C₆ glioma cells as measured by the incorporation of [³H]-thymidine. The mitogenic effects of ATP and UTP were concentration-dependent, related to the duration of exposure of cells to stimulus, and presumably mediated by a P2Y₂ receptor. In an attempt to define the subtype of P2Y receptor mediating this effect, we compared the ability of a range of ATP analogues to stimulate mitogenesis. ATP and related analogues act on several cell types *via* receptors subclassified into P1 receptors for adenosine and P2 receptors for ATP and ADP (Burnstock & Kennedy, 1985). The P2 receptors have been further classified into P2X and P2Y (Burnstock & Kennedy, 1985; Fredholm *et al.*, 1997). In this study, α , β -MeATP, β , γ -MeATP, and 2MeSATP had little effect on [³H]-thymidine incorporation, suggesting that the mitogenic effect was not mediated by the P2X₁ or P2Y₁ receptor. Moreover, P1 agonists, AMP and CPA, did not show any mitogenic effect. ATP and UTP possessed similar potency in the mitogenic effect. Therefore, these results suggest that ATP and UTP mediate their mitogenic effects by activating P2Y₂ receptors, which are consistent with the results obtained from several cell types (Neary *et al.*, 1994; Huwilder & Pfeilschifter, 1994; Albert *et al.*, 1997; Soltoff *et al.*, 1998).

To assess the possible mechanisms that might mediate the mitogenic action of ATP, we attempted to pharmacologically analyse some of the potential pathways. Several lines of evidence demonstrate that P2Y₂ receptors couple to a Gq protein in most cell types (Neary *et al.*, 1994; Huwilder & Pfeilschifter, 1994; Albert *et al.*, 1997; Soltoff *et al.*, 1998). This is strengthened by our observation that the UTP-stimulated MAPK activation and [³H]-thymidine incorporation in C₆ glioma cells was not affected by pretreatment with PTX. These findings suggest that the mitogenic effect and activation of p42/p44 MAPK induced by UTP were mediated through a receptor coupled to a PTX-insensitive G protein. The activation of the P2Y₂ receptor is linked to the stimulation of phosphoinositide hydrolysis, which produces two second messengers, diacylglycerol and IP₃ (Boader *et al.*, 1995), which activates PKC and release Ca²⁺ from the intracellular stores, respectively. The activation of PKC and increase of [Ca²⁺]_i seem to account for most of the early proliferative growth-promoting agents coupled to G proteins, such as ATP (Albert *et al.*, 1997) and endothelin (Simonson *et al.*, 1993). Elevation of [Ca²⁺]_i is an important intracellular signal stimulating many cellular responses including mitogenesis. ATP and its analogues have been demonstrated to induce inositol phosphate accumulation and elevation of [Ca²⁺]_i in several cell types (Harden *et al.*, 1995) with striking similarities to our findings in the mitogenesis assay. The mitogenic effects of UTP in our experiments required the presence of extracellular Ca²⁺. This result indicates a role for Ca²⁺ in mediating the mitogenic effect of UTP. This hypothesis was further supported by the results that removal of Ca²⁺ by BAPTA/EGTA significantly attenuated the p42/p44 MAPK activation and [³H]-thymidine incorporation in C₆ glioma cells.

Activation of p42/p44 MAPK is known to require both tyrosine and threonine phosphorylations by the dual specificity MEK1/2. Several lines of evidence indicate that complexity in the mechanisms of agonist stimulation of MAPK in cells including the possible involvement of tyrosine kinase upstream of MAPK kinase (Davis, 1993; Blenis, 1993). MAPK isoforms are activated by various growth factors including PDGF (Blenis, 1993; Post & Brown, 1996), EGF (Pouyssegur &

Seuwen, 1992), and ATP (Albert *et al.*, 1997; Soltoff *et al.*, 1998). In this study, we have shown that stimulation with UTP resulted in activation of p42/p44 MAPK in a time- and concentration-dependent manner. These results are consistent with the findings that MAPK isoforms are activated by agonists in several cell types (Pouyssegur & Seuwen, 1992; Albert *et al.*, 1997; Soltoff *et al.*, 1998). Furthermore, we also investigated the implication of a tyrosine kinase in the MAPK cascade of C₆ glioma cells stimulated by UTP, using the tyrosine kinase inhibitors genistein and herbimycin A. The results with tyrosine kinase inhibitors showed that the UTP-induced DNA synthesis and activation of p42/p44 MAPK was significantly attenuated by these inhibitors. These results may imply that the mitogenic response to UTP was dependent on tyrosine kinase.

ATP and UTP have been shown to activate PLC to generate diacylglycerol and elevate PKC activity in several cell types (Harden *et al.*, 1995); this time course of PKC activation appears to be essential for late responses such as proliferation and differentiation (Nishizuka, 1992). PKC is a predominant component in the kinase cascade initiating by ligand attachment to both G protein-coupled receptors and receptors containing intrinsic tyrosine kinase activity. In this study, we further investigated the regulatory mechanisms which are involved in UTP-stimulated [³H]-thymidine incorporation and activation of p42/p44 MAPK by PKC. Pretreatment with the PKC inhibitors, staurosporine and GF109203X, attenuated the UTP-stimulated [³H]-thymidine incorporation and activation of p42/p44 MAPK, indicating that a component of the MAPK signal involves PKC-mediated activation of an intermediate kinase. This is likely to be either Raf-1 which has been shown to be phosphorylated by PKC (Kolch *et al.*, 1993) or possibly MEK1/2 which is also activated in a PKC-dependent manner (Lange-Carter *et al.*, 1993). These results are consistent with studies which examine MAPK activation in response to other G-protein coupled agonists including angiotensin II, vasopressin, and endothelin-1 (Molloy *et al.*, 1993; Malarkey *et al.*, 1995).

Although mitogenic signals from the activation of specific tyrosine kinase-coupled growth factors have been well characterized, the mechanism by which the G protein-coupled receptors activate the components of MAPK pathway is not completely understood. PD98059, a synthetic and highly specific MEK1/2 inhibitor, has been shown to inhibit the activation of p42/p44 MAPK by several stimuli (Alessi *et al.*, 1995; Dudley *et al.*, 1995). Because activation of components in the MAPK cascade originates from stimulation of cells by growth factors, it has been proposed that transmission of the signal along this pathway is required for the induction of mitogenesis. In support of this hypothesis, inhibition of MEK1/2 by PD98059 has been associated with a decrease not only in PDGF-stimulated [³H]-thymidine incorporation in 3T3 cells (Dudley *et al.*, 1995), but also with an attenuation in nerve growth factor-induced differentiation in PC12 cells (Pang *et al.*, 1995). In the current study, pretreatment with PD98059 attenuated the UTP-induced activation of p42/p44 MAPK and DNA synthesis in C₆ glioma cells, revealing that stimulation of

MEK1/2 is required for UTP-induced responses in these cells. In addition, SB203580 showed an inhibitory effect on [³H]-thymidine incorporation but no inhibitory effect on p42/p44 MAPK activation, indicating that an alternate pathway such as p38 MAPK was also involved in the mitogenic effect of UTP in C₆ glioma cells.

It has been well established that growth factors activate phosphorylation of cascade of protein kinases including tyrosine kinases, Ras, Raf-1, MEK and MAPK (Blenis, 1993; Post & Brown, 1996). Several studies have depicted a picture that MAPK could be a convergence point for growth signals originating from tyrosine kinase receptors, G protein-coupled receptors, and cytokines (Blenis, 1993; Post & Brown, 1996). In the proposed signalling pathway, several lines of evidence have suggested that Ras is activated by various stimuli for growth and differentiation and that the activated Ras evoked the phosphorylation cascade of protein kinases including Raf-1, MEK1/2, and MAPK (Kerkhoff & Rapp, 1998; Satoh *et al.*, 1992; Blenis, 1993; Post & Brown, 1996). It has been demonstrated that PDGF-BB-induced cell proliferation may be mediated through the activation of Ras (Arvidsson *et al.*, 1994; Weber *et al.*, 1997). In this study, to elucidate whether Ras is required for UTP-induced activation of MAPKs, C₆ glioma cells were transfected with a dominant negative mutant of Ras (RasN17) that preferentially interacts with guanine nucleotide exchange factors and inhibits Ras functions (Thorburn *et al.*, 1993; Hara *et al.*, 1993). We found that UTP- and ATP-induced p42/p44 MAPK activation were completely suppressed by transfection with RasN17 in C₆ glioma cells, as previously reported in other cell types (Stacey *et al.*, 1991; Abdellatif *et al.*, 1998). Several studies have also shown that activated Ras binds and activates Raf-1, resulting in the activation of MEK1/2 and MAPK (Blenis, 1993; Satoh *et al.*, 1992). Consistent with these reports, we demonstrated that in C₆ glioma cells, UTP- and ATP-induced MAPK activation are completely suppressed by transfection with dominant negative mutants of Raf-1 (Raf-301), suggesting that Raf-1 plays a key role in UTP- and ATP-induced activation of MEK/MAPK cascade in C₆ glioma cells.

In conclusion, we reported here that ATP and UTP appeared to exert their mitogenic effects by binding to a receptor which was coupled to a PTX-insensitive G protein and signalled through Ras/Raf/MEK/MAPK to enhance DNA synthesis in C₆ glioma cells. These results demonstrate that UTP stimulates activation of a MAPK pathway that is regulated by PKC, Ca²⁺, and tyrosine kinase. The mitogenic effects of these nucleotides were triggered by activation of P2Y₂ receptors that subsequently lead to the activation of MAPK isoforms, DNA synthesis and cell proliferation.

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