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P_{2Y} purinoceptor subtypes recruit different Mek activators in astrocytes

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1 Extracellular ATP can function as a glial trophic factor as well as a neuronal transmitter. In astrocytes, mitogenic signalling by ATP is mediated by metabotropic P_{2Y} receptors that are linked to the extracellular signal regulated protein kinase (Erk) cascade, but the types of P_{2Y} receptors expressed in astrocytes have not been defined and it is not known whether all P_{2Y} receptor subtypes are coupled to Erk by identical or distinct signalling pathways.

2 We found that the P_{2Y} receptor agonists ATP, ADP, UTP and 2-methylthioATP (2MeSATP) activated Erk and its upstream activator MAP/Erk kinase (Mek). cRaf-1, the first kinase in the Erk cascade, was activated by 2MeSATP, ADP and UTP but, surprisingly, cRaf-1 was not stimulated by ATP. Furthermore, ATP did not activate B-Raf, the major isoform of Raf in the brain, nor other Mek activators such as Mek kinase 1 (MekK1) and MekK2/3.

3 Reverse transcriptase-polymerase chain reaction (RT–PCR) studies using primer pairs for cloned rat P_{2Y} receptors revealed that rat cortical astrocytes express P_{2Y_1}, a receptor subtype stimulated by ATP and ADP and their 2MeS analogues, as well as P_{2Y_2} and P_{2Y_4}, subtypes in rats for which ATP and UTP are equipotent. Transcripts for P_{2Y_6}, a pyrimidine-preferring receptor, were not detected.

4 ATP did not increase cyclic AMP levels, suggesting that $P_{2Y_{11}}$, an ATP-preferring receptor, is not expressed or is not linked to adenylyl cyclase in rat cortical astrocytes.

5 These signal transduction and RT–PCR experiments reveal differences in the activation of cRaf-1 by P_{2Y} receptor agonists that are inconsistent with properties of the P_{2Y_1}, P_{2Y_2} and P_{2Y_4} receptors shown to be expressed in astrocytes, i.e. ATP $\neq$ UTP; ATP $\neq$ 2MeSATP, ADP. This suggests that the properties of the native P_{2Y} receptors coupled to the Erk cascade differ from the recombinant P_{2Y} receptors or that astrocytes express novel purine-preferring and pyrimidine-preferring receptors coupled to the ERK cascade.

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Abbreviations: 2MeSADP, 2-methylthioadenosine diphosphate; 2MeSATP, 2-methylthioadenosine triphosphate; AEBSF, 4-(2-aminoethyl)benzenesulphonylfluoride; DTT, dithiothreitol; Erk, extracellular signal regulated protein kinase; FGF, fibroblast growth factor; GST, glutathione S-transferase; MAPK: mitogen activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MBP, myelin basis protein; Mek, MAPK/Erk kinase; MekK, Mek kinase; PKA, cyclic AMP-dependent protein kinase, PKC, protein kinase C; RT–PCR, reverse transcriptase-polymerase chain reaction; SAPK, stress-activated protein kinase; SDS, sodium dodecyl sulphate; Sek, SAPK/Erk kinase; UTP, uridine 5′-triphosphate

Introduction

Numerous studies have indicated that ATP is a neurotransmitter which can affect both neurones and glial cells through a great variety of purinoceptors (reviewed by Burnstock, 1997; North & Barnard, 1997). ATP mediates several effects in the CNS, such as excitatory neurotransmission (Edwards *et al*., 1992) and activation of voltage-gated Ca^{2+} channels in neurones (reviewed by Bean, 1992) as well as increases in intracellular Ca^{2+} in glial cells (Neary *et al*., 1991; Kastritsis *et al*., 1992; Magoski & Walz, 1992; Bruner & Murphy, 1993; Lyons *et al*., 1994; Salter & Hicks, 1994). ATP is released upon electrical stimulation of rat sensory motor cortex (Wu & Phillis, 1978) and hippocampal slices (Wieraszko *et al*., 1989). ATP can also be co-released from nerve endings with noradrenaline or acetylcholine (reviewed by Burnstock, 1990). In addition, ATP released from damaged or dying cells can stimulate hypertrophic and hyperplastic responses in astrocytes reminiscent of the reactive gliosis that results from brain injury (reviewed by Neary *et al*., 1996). These findings argue in favour of a role for extracellular ATP both in normal neurotransmission and in signalling following lesions of the CNS. Thus, there is considerable interest in defining the receptors and signalling pathways responsible for the physiological and pathological actions of extracellular ATP.

The biological effects of extracellular nucleotides are mediated by cell-surface, P_2 purinoceptors, which have been

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classified as ionotropic P_{2X} receptors and metabotropic P_{2Y} receptors (Abbracchio & Burnstock, 1994). Until now, seven P_{2X} and five P_{2Y} receptor subtypes have been cloned. The P_{2Y} receptor subtypes respond differently to a variety of naturally occurring agonists, including ATP, UTP and their diphosphate analogues (Ralevic & Burnstock, 1998). Some synthetic agonists, such as 2-methylthioadenosine triphosphate (2MeSATP), 2-methylthioadenosine diphosphate (2MeSADP) and adenosine 5′-O-(3-thiotriphosphate) (ATPγS) are relatively resistant to degradation by extonucleotidases and are somewhat more specific than the naturally occurring agonists (North & Barnard, 1997). The cloned receptors have been expressed in cell lines lacking endogenous P_{2Y} receptors and characterized pharmacologically. P_{2Y_1} receptors are equally responsive to 2MeSATP and ATP but unresponsive to uridine 5′-triphosphate (UTP). Recent studies have suggested that P_{2Y_1} receptors are ADP-preferring receptors with ATP and 2MeSATP as partial agonists or antagonists (Léon *et al*., 1997; Hechler *et al*., 1998), although the partial agonism and antagonism are more pronounced in cells with low rather than normal levels of receptors (Palmer *et al*., 1998). In contrast to P_{2Y_1} receptors, P_{2Y_2} and P_{2Y_4} receptors in rats are equally responsive to ATP and UTP but unresponsive to 2MeSATP. Another P_{2Y} receptor subtype, P_{2Y_6}, is preferentially stimulated by uridine nucleotides but to a lesser extent, or not at all, by ATP; similar properties are exhibited by human P_{2Y_4} receptors. By contrast, a $P_{2Y_{11}}$ receptor subtype recently identified in human placenta is activated by ATP but not by UTP (Communi *et al*., 1997). These differences in agonist affinities for nucleotides and related analogues have been used in attempts to identify receptor subtypes in various cells and tissues, but in some cases there are differences in agonist responses between cloned and native P_{2Y} receptors, thereby raising the possibility that there are P_{2Y} receptors not yet cloned (King *et al*., 1998).

P_{2Y} purinoceptors are linked to a variety of signal transduction mechanisms, such as phospholipase C and adenylyl cyclase (reviewed by Ralevic & Burnstock, 1998). In addition, occupancy of P_{2Y} receptors leads to activation of extracellular signal regulated kinase (Erk) (Neary & Zhu, 1994), a key component of a signalling cascade that plays a crucial role in cellular proliferation and differentiation (Seger & Krebs, 1995). The activation of Erk by nucleotide receptors is widely distributed over several cell types, including astrocytes (Neary & Zhu, 1994; King *et al*., 1996; Neary *et al*., 1999), PC12 cells (Soltoff *et al*., 1998), brain capillary and aortic endothelial cells (Patel *et al*., 1996; Graham *et al*., 1996; Albert *et al*., 1997), vascular smooth muscle cells (Harper *et al*., 1998; Wilden *et al*., 1998), kidney cells (Huwiler & Pfeilschifter, 1994; Gao *et al*., 1999) and cardiac myocytes (Post *et al*., 1996), thereby indicating the importance of the link between purinoceptor signalling and Erk pathways.

The Erk pathway is one of three distinct mitogen-activated protein kinase (MAPK) cascades identified in mammals; the others have been termed stress-activated protein kinase (SAPK, also known as Jnk for c-Jun N-terminal kinase) and $p38^{MAPK}$ (reviewed by Kyriakis & Avruch, 1996). The cascades are composed of a sequence of three cytosolic protein kinases which phosphorylate and activate the next kinase in the cascade. The first discovered and most studied cascade is the Erk cascade (reviewed by Seger & Krebs, 1995). This cascade involves Erk1/2, which can be activated by a MAPK kinase (MAPKK) termed MAPK/Erk kinase 1 (Mek1), which, in turn, can be activated by a MAPKK kinase (MAPKKK) identified as a member of the Raf family of serine/threonine kinases: cRaf-1 (Kyriakis *et al*., 1992). Among the Raf isoenzymes that specifically activate the Erk pathway, cRaf-1 is stimulated by a variety of growth factors and ligands of G protein-coupled receptors (reviewed by Avruch *et al*., 1994) whereas B-Raf seems to be the major Mek1 activator in nerve growth factor-treated PC12 cells (Jaiswal *et al*., 1994) and in the brain (Catling *et al*., 1994). There are also MAPKKK enzymes that can activate both Erk and SAPK/Jnk cascades. These enzymes, termed MekK, have different specificity for the Mek subtypes, leading to a different degree of activation of the Erk and SAPK/Jnk cascades.

In this report, we have focused on the types of P_{2Y} receptors expressed in primary cultures of rat cortical astrocytes and the nature of the Mek activators that are recruited by these P_{2Y} receptors to stimulate Erk signalling. Previously, we reported that ATP, UTP and 2MeSATP stimulate Erk activity in astrocytes (King *et al*., 1996). In other studies, we found that stimulation of Erk by extracellular ATP was not affected by activation of the cyclic AMP-dependent protein kinase (PKA) pathway (Neary & Zhu, 1994). Because PKA can inhibit the Erk cascade by interacting with cRaf-1 (Cook & McCormick, 1993; Graves *et al*., 1993; Sevetson *et al*., 1993; Wu *et al*., 1993), these findings suggested that P_{2Y} receptors may utilize a different Mek activator. To address this question directly, we have now measured the ability of P_{2Y} receptor agonists to activate cRaf-1, B-Raf and MekK. Surprisingly, we find that, while activation of P_{2Y} receptors by ATP does not recruit cRaf-1, activation of other P_{2Y} receptors by UTP, 2MeSATP and ADP does stimulate cRaf-1, thereby revealing for the first time that distinct P_{2Y} receptor subtypes recruit different Mek activators.

Methods

Reagents

All chemicals were reagent grade or better. Anti-B-Raf monoclonal antibody (sc-166-G), anti-MekK1 (sc-252), anti-MekK2 which also recognizes MekK3 (sc-1089), and protein A/G-agarose (sc-2003) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-cRaf-1 was produced as described (Kyriakis *et al*., 1992). Mek1 and Erk1 polypeptides were expressed as glutathione S-transferase (GST) fusion proteins; after purification by glutathione-Sepharose affinity chromatography, free Erk1 was obtained by thrombin cleavage (Luo *et al*., 1997). A polyhistidine-tagged Erk2 (sc-4024, Santa Cruz Biotechnology) was used in some experiments with similar results. ATP, ADP, UTP, myelin basic protein (MBP), aprotinin, leupeptin and pepstatin A were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [γ-^{32}P]-ATP was purchased from DuPont NEN (Boston, MA, U.S.A.) and Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). 2MeSATP was obtained from RBI (Natick, MA, U.S.A.). Human recombinant fibroblast growth factor (FGF) 2 was purchased from R&D Systems (Minneapolis, MN, U.S.A.). 4-(2-Aminoethyl)benzenesulphonylfluoride (AEBSF) was obtained from Calbiochem (San Diego, CA, U.S.A.). Protein assay kits were supplied by BioRad Laboratories (Hercules, CA, U.S.A.).

Cell culture and treatment

Primary astrocytes were obtained from neonatal Fischer rat cerebral cortices as previously described (Neary *et al*., 1994). At least 99% of the cell population was astrocytes, as determined by staining with cell-specific markers (Neary *et al*., 1994).

Experiments were conducted with 3–5 week-old cultures. Prior to treatment with nucleotides or FGF-2, cells that had been maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum were shifted to the quiescent phase by incubation in DMEM containing 0.5% horse serum for 48 h prior to MAPKKK, Mek and Erk studies. Stock solutions of nucleotides were divided into single-use aliquots and stored at −80°C.

MAPKKK assays

Activation of cRaf-1 and other MAPKKKs was measured by means of a coupled assay in which inactive GST–Mek1 and inactive bacterially expressed Erk1 were added to immunoprecipitates of cRaf-1 or other MAPKKKs, as previously described (Kyriakis *et al*., 1993; Luo *et al*., 1997) with minor modifications. After treatment, cells grown on 60 mm plates were lysed in 1 ml lysis buffer (mM) Tris-HCl 20, EDTA 2, β-glycerophosphate 50, 1% w v^{-1} Triton X-100, 10% v v^{-1} glycerol, 1 mM sodium orthovanadate, 0.1 mg ml^{-1} AEBSF, 50 μg ml^{-1} aprotinin, 2 μM leupeptin, 2 μM pepstatin A; pH 8.0). The lysates were centrifuged (10,000 × *g* for 5 min at 4°C) and aliquots of supernatants were set aside for Erk assay. Supernatants containing equivalent amounts of protein (80–120 μg, as determined with the BioRad Coomassie dye method using bovine serum albumin as standard) were incubated with anti-cRaf-1, anti-B-Raf, anti-MekK1 or anti-MekK2 antibodies for 30 min at 4°C. Twenty microlitres of protein A/G agarose was added and incubation at 4°C was continued overnight, with agitation. The protein A/G agarose beads were washed twice with lithium wash buffer (mM) (Tris-HCl 100, LiCl 500, dithiothreitol (DTT) 1; pH 7.6) and twice with assay buffer (mM) β-glycerophosphate 50, EGTA 1.5, DTT 1, 0.03% Brij 35; pH 7.3). Beads were divided among assay tubes, residual liquid was absorbed with paper strips and 45 μl assay buffer was added. Samples were incubated with 0.2 μCi [γ-^{32}P]-ATP, 10 mM $MgCl_2$ and 0.1 mM ATP for 20 min, at 30°C, in the presence or absence of 0.2 μg recombinant inactive GST–Mek1. Following this, 0.5 μg inactive bacterially expressed Erk1 was added for 20 min, after which 13 μg MBP was added for an additional 20 min (total incubation time, 60 min). Reactions were stopped by adding sodium dodecyl sulphate (SDS) sample buffer (Laemmli, 1970) and proteins were separated by SDS polyacrylamide (11%) gel electrophoresis (PAGE). Incorporation of ^{32}P into MBP was quantified with an Instant Imager™ electronic autoradiography system (Packard Instruments, Meriden, CT, U.S.A.). To correct for Mek contamination in the MAPKKK immunoprecipitates, control assays were conducted without the addition of GST-Mek1 and the results from these samples were subtracted from those containing the complete reaction mix. MAPKKK activation in nucleotide- and FGF-2-treated cells was compared to basal levels in untreated cells and expressed as fold-stimulation.

Mek assay

Lysate supernatants containing equivalent amounts of protein (80–120 μg) were incubated with 0.2 μCi [γ-^{32}P]-ATP, 10 mM $MgCl_2$, 0.1 mM ATP and 0.5 μg inactive bacterially expressed Erk1 for 20 min at 30°C. Reactions were stopped and samples were subjected to SDS gel electrophoresis as described above. Incorporation of ^{32}P into Erk1 was quantified with a Packard InstantImager.

Erk activity and phosphorylation

Erk activity was assayed as described elsewhere (Neary & Zhu, 1994; Neary *et al*., 1999). For experiments in which coupled assays were performed, Erk assays were conducted using aliquots of the same samples. Erk phosphorylation was determined by probing immunoblots with an antibody that recognizes dually phosphorylated (Thr^{183}, Tyr^{185}) Erk1 and Erk2 (pTEpY; Promega Corporation, Madison, WI, U.S.A.). Samples containing equal amounts of protein were subjected to SDS–PAGE (Laemmli, 1970) using 11% polyacrylamide and transferred to nitrocellulose filters with a Genie™ electrophoretic blotter (Idea Scientific Inc., Minneapolis, MN, U.S.A.) for 1 h at 12 V in a transfer buffer containing 25 mM Tris, 192 mM glycine and 20% methanol. Filters were incubated with a blocking solution containing Tween Tris-buffered saline (mM) (TTBS: Tris 20, NaCl 137, Tween 20 v v^{-1} 0.1%; pH 7.7) and 5% fat-free dried milk for 1 h at room temperature, rinsed in TTBS and then incubated for 1 h at room temperature with anti-phosphoErk1/2 antibodies diluted 1 : 20,000 in TTBS. Following three rinses in TTBS, filters were incubated for 1 h at room temperature with peroxidase-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech) diluted in TTBS (1 : 20,000). Filters were washed three times in TTBS and proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). Identical blots were probed with anti-Erk1/2 antibodies which recognize total Erk1/Erk2 (1 : 500; Santa Cruz Biotechnology).

RT–PCR analysis

Total RNA was isolated by a modification of the method of Chomczynski & Sacchi (1987) using the Trizol™ reagent (GibcoBRL Life Technologies, Rockville, MD, U.S.A.) according to the manufacturer's recommendations. Prior to RT–PCR, the RNA was pre-treated with 1 unit ribonuclease-free deoxyribonuclease (Promega) per microgram RNA for 15 min at 37°C to remove any genomic DNA contaminants. One-step RT–PCR was conducted using the Titan RT–PCR system (Boehringer Mannheim, Indianapolis, IN, U.S.A.). Complementary DNA was synthesized from 0.6 μg total RNA in the presence of (mM) nucleotide triphosphates 0.2, DTT 5, $MgCl_2$ 1.5, 0.4 μM upstream and downstream primers and a mixture of avian myeloblastosis virus (AMV) reverse transcriptase, *Thermus aquaticus (Taq)* DNA polymerase and *Pyrococcus woesei (Pwo)* DNA polymerase in a total volume of 25 μl, according to the manufacturer's recommendations. Oligonucleotide amplification primers were designed from the published rat P_{2Y_1} (Tokuyama *et al*., 1995), P_{2Y_2} (Chen *et al*., 1996), P_{2Y_4} (Bogdanov *et al*., 1998) and P_{2Y_6} (Chang *et al*., 1995) sequences. P_{2Y_1} (nt 1097–1487): sense primer 5′-CTGGGCAGGCTCAAGAAGAAGAAT-3′, antisense primer 5′-AATCCAGCCGTGCCCTCAAAT-3′; $P2Y_2$ (nt 735–1337): sense primer 5′-CTCCCTGCCGCTGCTGGT-TTATTA-3′, antisense primer 5′-ATGTTGATGGCGTT-GAGGGTGTG-3′; $P2Y_4$ (nt 1739-2115): sense primer 5′-CACCGATACCTGGGTATCTGCCAC-3′, antisense primer 5′-CAGACAGCAAAGACAGTCAGCACC-3′; $P2Y_6$ (nt 689–1091): sense primer 5′-TATAACTACGCCA-GAGGGGACCAC-3′, antisense primer 5′-GGCGGGCC-ATGCGACAGTAG-3′. RT–PCR was conducted as follows. Samples were incubated for 30 min in a thermocycler at 50°C, after which denaturation was conducted at 94°C for 2 min. Ten cycles of denaturation

at 94°C for 30 s, annealing at 58°C (P_{2Y_1}), 61°C (P_{2Y_2}), 65°C (P_{2Y_4}) or 60°C (P_{2Y_6}) for 30 s and elongation at 68°C for 45 s was then conducted and followed by 25 cycles of the same denaturing and annealing conditions but with an additional 5 s of elongation for each cycle. The final elongation was conducted for 7 min at 68°C. Reactions were also conducted without the reverse transcriptase step as a control for genomic DNA contamination in the RNA sample. Amplification products were resolved by agarose (1.2%) gel electrophoresis; bands were excised and eluted from the gel, purified (Wizard DNA purification kit; Promega) and sequenced. Three RNA samples from independent culture seedings were analysed by RT–PCR with identical results.

Cyclic AMP determination

Cyclic AMP was assayed as previously described (Tasca *et al*., 1995). In brief, astrocyte cultures (60 mm plates) were maintained and treated in DMEM supplemented with 10% horse serum. After treatment, cells were lysed in 200 μl 50 mM Tris-HCl (pH 7.4) containing 4 mM EDTA. Lysates were centrifuged, protein was determined and 50 μl of the supernatant (or standard solution) was used in the cyclic AMP assay. [^{3}H]-cAMP (50 μl) and 100 μl binding protein (3 μg of protein kinase A, Sigma) were added and incubations were conducted for 4–16 h on ice. The reaction was stopped with 100 μl activated charcoal, solutions were centrifuged, and [^{3}H]-cAMP in the supernatant was determined by scintillation counting. Data were fitted to the standard curve and corrected for protein content; results were expressed as nmol mg^{-1} protein.

Statistical analyses

All experiments were conducted a minimum of three times, each time with cultures from different seedings. Data were analysed by Student *t*-tests for two groups or by ANOVA followed by Bonferroni *post hoc* comparisons for multiple groups using the InStat® software package (GraphPad™ Software, San Diego, CA, U.S.A.).

Results

Erk phosphorylation and Mek activation by P_{2Y} receptor agonists in rat cortical astrocytes

Erk isoforms 1 and 2 (p44 and p42, respectively) are activated by phosphorylation of Thr^{183} and Tyr^{185}; this dual phosphorylation reaction is catalysed by Mek. To examine the ability of P_{2Y} receptor agonists to stimulate phosphorylation of Erk, we conducted immunoblot studies with an antibody that recognizes phosphorylated Thr^{183} and Tyr^{185} residues of Erk1 and Erk2. We found that the P_{2Y} receptor agonists ATP, UTP and 2MeSATP stimulated Erk phosphorylation (Figure 1). ATP and UTP (both at 100 μM) were approximately equivalent while 2MeSATP (10 μM) was slightly less effective. The extent of Erk phosphorylation stimulated by these P_{2Y} agonists is similar to that obtained with FGF-2 (Figure 1). These findings confirm and extend our previous observations that ATP, UTP and 2MeSATP increase Erk activity (King *et al*., 1996). The concentrations of nucleotides used in these studies stimulate maximal or near maximal activation of Erk. We also investigated the ability of P_{2Y} agonists to stimulate Mek. ATP and UTP (both at 100 μM) activated Mek; 2MeSATP (10 μM) appeared to be slightly less effective but this difference was not statistically significant (Figure 2). FGF-2 also activated Mek (Figure 2).

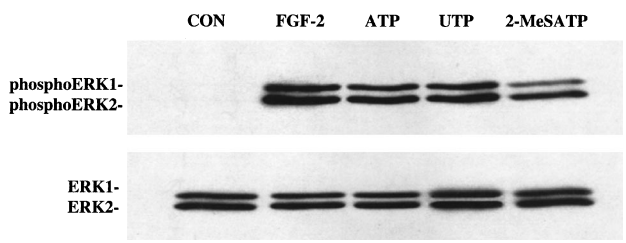

Figure 1 P_{2Y} receptor agonists stimulate phosphorylation and activation of Erk. Primary cultures of rat cortical astrocytes were treated for 10 min with FGF-2 (25 ng ml^{-1}), ATP (100 μM), UTP (100 μM), or 2-methylthioATP (2MeSATP, 10 μM). Cells were lysed, and lysates containing equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis. Immunoblots were probed with antibodies which recognize dual-phosphorylated Erk1/Erk2 (upper panel) or total Erk1/Erk2 (lower panel). All three P_{2Y} receptor agonists stimulated Erk phosphorylation to an extent similar to that obtained with FGF-2.

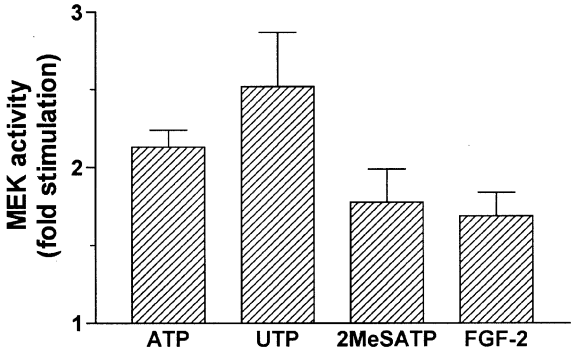

Figure 2 Mek activation by P_{2Y} agonists and FGF-2. Primary astrocyte cultures were treated for 2 min with ATP (100 μM), UTP (100 μM), 2MeSATP (10 μM) or FGF-2 (25 ng ml^{-1}); Mek activity was determined as described in Methods. Data (mean ± s.e.mean) were obtained from a minimum of six experiments. ATP, UTP, 2MeSATP and FGF-2 stimulated Mek activity ($P<0.05$).

Differences in coupling of P_{2Y} receptor subtypes to the Erk pathway

To further explore the coupling of native P_{2Y} receptors in rat cortical astrocytes to the Erk signalling pathway, we investigated whether P_{2Y} receptor agonists recruit cRaf-1, an activator of Mek (Kyriakis *et al*., 1992). For comparison, experiments were also conducted with FGF-2, a known activator of cRaf-1 in astrocytes (Kurino *et al*., 1996). Previously, we found that activation of the cyclic AMP/PKA pathway, which can inhibit the Erk cascade by interacting with cRaf-1 (Cook & McCormick, 1993; Wu *et al*., 1993; Graves *et al*., 1993; Sevetson *et al*., 1993), did not affect extracellular ATP-evoked stimulation of Erk (Neary & Zhu, 1994); this suggested that one or more P_{2Y} receptor subtypes may utilize a Mek activator other than cRaf-1. Consistent with this, we found that, although FGF-2 did activate cRaf-1, ATP did not, even though ATP and FGF-2 were equally effective in activating Erk (Figure 3). Time course studies were then conducted to determine whether ATP might activate cRaf-1 earlier than the 5 min point described in the preceding

experiment. However, ATP did not activate cRaf-1 at 1 and 2 min (Figure 4A); subsequent experiments indicated that ATP was also ineffective at 30 s (data not shown). Surprisingly, when other P_{2Y} receptor agonists were examined in these studies, we found that cRaf-1 was activated by 2MeSATP and UTP at 2 and 5 min (Figure 4A). It should be noted that, even though cRaf-1 was not activated by ATP, Erk activity was stimulated by ATP, UTP and 2MeSATP with a similar time course (Figure 4B). The effect of ADP on components of the Erk cascade was also examined (Table 1). A similar, but not greater, increase in cRaf-1 activity was observed with ADP as compared to 2MeSATP and UTP. ADP also activated Mek to a similar extent to ATP, UTP and 2MeSATP. At the peak time of Erk activation (10 min), the ADP response was approximately 30% less than that of ATP (data not shown), a finding that confirms our previous observation that ADP was less effective than ATP in stimulating Erk activity (King *et al*., 1996).

In an effort to determine the Mek activator recruited by ATP, we measured the ability of ATP to activate B-Raf, the main form of Raf found in the brain (Catling *et al*., 1994), as well as MekK1 and MekK2/3. However, ATP did not stimulate any of these Mek activators (Figure 5). The effects of 2MeSATP and UTP on B-Raf, MekK1, and MekK2/3 were also examined. We found that 2MeSATP was also ineffective in stimulating these Mek activators, whereas UTP significantly, but moderately (50%), stimulated MekK1 (Figure 5). UTP also appeared to stimulate MekK2/3 but the results were not significant owing to variability in the four experiments. FGF-2 stimulated MekK1 and MekK2/3 but not B-Raf, although B-Raf was detected in immunoprecipitates by Western blot analysis (data not shown).

Table 1 Effect of ADP on components of the ERK cascade

ERK cascade component	*Activation* (fold stimulation; mean ± s.e.mean)	n	P
cRaf-1	1.44 ± 0.12	4	0.04
MEK	2.10 ± 0.27	4	0.03
ERK	2.03 ± 0.19	5	0.005

Primary astrocyte cultures were treated for 2 min with ADP (100 μM). cRaf-1, Mek and ERK activities were determined as described in Methods.

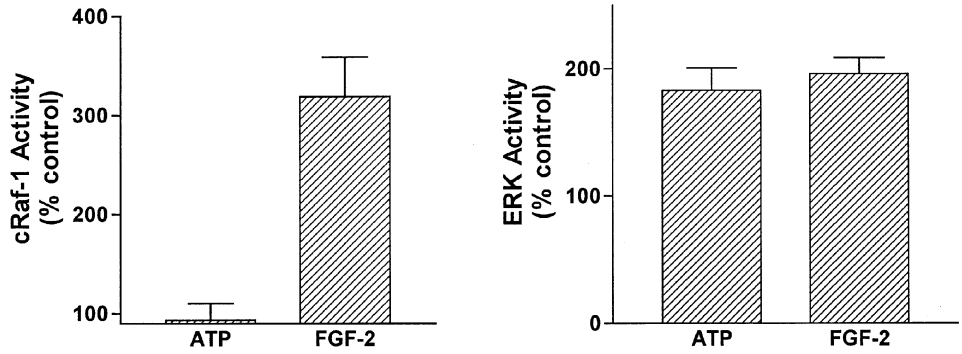

Figure 3 Inability of extracellular ATP to activate cRaf-1. Primary astrocyte cultures were treated with ATP (100 μM) or FGF-2 (25 ng ml^{-1}) for 5 min. cRaf-1 was immunoprecipitated from lysates, and cRaf-1 activity was determined by the coupled assay described in Methods. Erk assays were conducted using aliquots of the same samples. Data (mean ± s.e.mean) were obtained from seven independent experiments. FGF-2 activated Raf-1 but ATP did not (left panel), even though both FGF-2 and ATP stimulated Erk to the same extent (right panel).

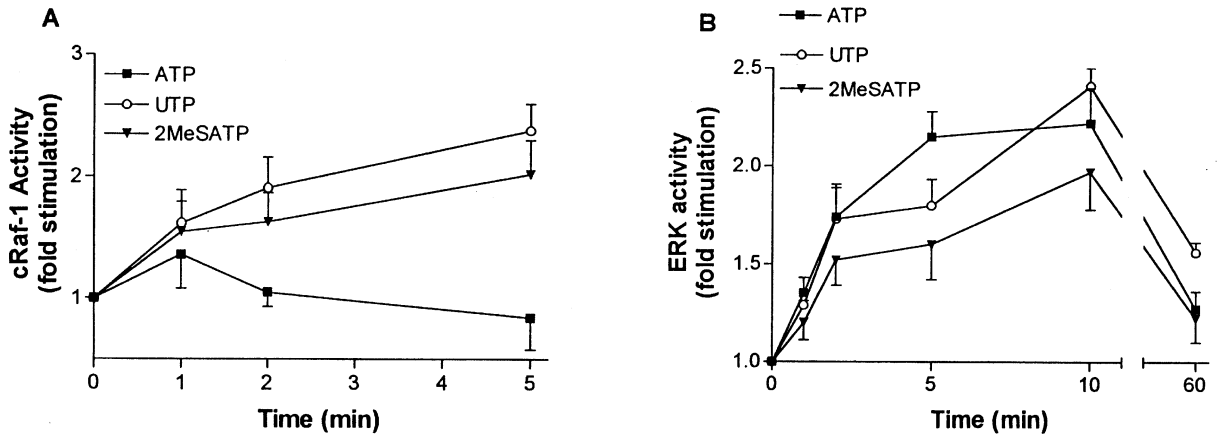

Figure 4 Time course studies: cRaf-1 activation by UTP and 2MeSATP. (A) Primary astrocyte cultures were treated with ATP (100 μM), UTP (100 μM), 2MeSATP (10 μM) or FGF-2 (25 ng ml^{-1}) for the indicated times. cRaf-1 was immunoprecipitated from lysates, and cRaf-1 activity was determined by the coupled assay described in Methods. Data (mean ± s.e.mean) were obtained from at least three independent experiments. The data for the 1 min time points obtained with P_{2Y} agonists and all ATP time points were not significantly different from control values ($P > 0.05$). All other data were significantly different from control values ($P < 0.05$). UTP and 2MeSATP, but not ATP, activated cRaf-1. (B) Aliquots of samples from A were assayed for Erk activation. All P_{2Y} agonists tested stimulated Erk activity from 2 to 10 min; by 60 min Erk activity in ATP- and 2MeSATP-treated cells had returned to near basal levels, whereas Erk activity in UTP- and particularly in FGF-2 (data not shown)-treated cells remained elevated.

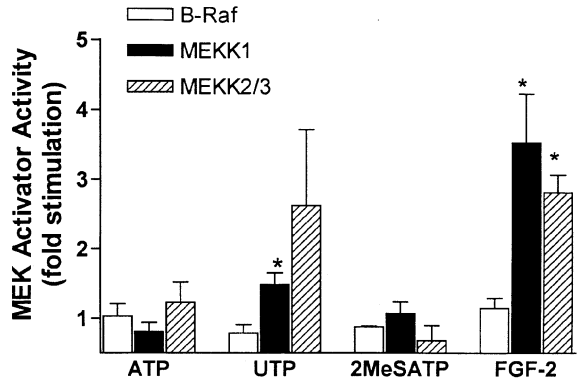

Figure 5 Effect of the P_{2Y} agonists and FGF-2 on different Mek activators. Primary astrocyte cultures were treated for 2 min with ATP (100 μM), UTP (100 μM), 2MeSATP (10 μM) or FGF-2 (25 ng ml^{-1}). B-Raf, MekK1 and MekK2/3 were immunoprecipitated from lysates, and B-Raf, MekK1 and MekK2/3 activities were determined by the coupled assay described in Methods. Data (mean $\pm$ s.e.mean) were obtained from a minimum of three independent experiments. ATP and 2MeSATP did not activate any of the Mek activators tested ($P>0.05$). UTP and FGF-2 activated MekK1 and MekK2/3 (*$P<0.05$).

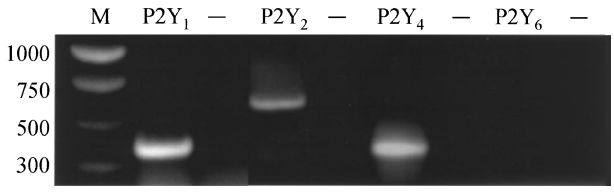

Figure 6 RT–PCR analysis of P_{2Y} receptor subtype expression in rat neonatal cortical astrocytes. Primer pairs specific for the amplification of rat P_{2Y_1}, P_{2Y_2}, P_{2Y_4}, and P_{2Y_6} receptor subtypes were used in these experiments. Amplification products were resolved by agarose gel electrophoresis. M, size markers as indicated; –, PCR reactions conducted without the reverse transcriptase step to control for amplification of genomic DNA in the RNA samples. Results are representative of experiments conducted with RNA extracted from at least three independent culture seedings. P_{2Y_1}, P_{2Y_2} and P_{2Y_4}, but not P_{2Y_6}, receptor subtypes were expressed in rat neonatal cortical astrocytes.

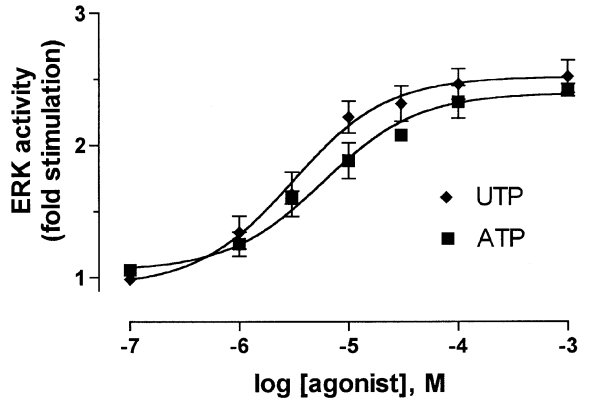

Figure 7 Concentration dependence of ATP and UTP stimulation of Erk activity. Primary cultures of rat cortical astrocytes were treated for 10 min with the indicated concentrations of ATP or UTP, and Erk activity was determined. Data (mean $\pm$ s.e.mean) are from four independent experiments and were fitted in a sigmoid log concentration-response curve by Prism® v2.0 (GraphPad™). The concentration-response relationships revealed that ATP and UTP have similar potencies in terms of Erk activation.

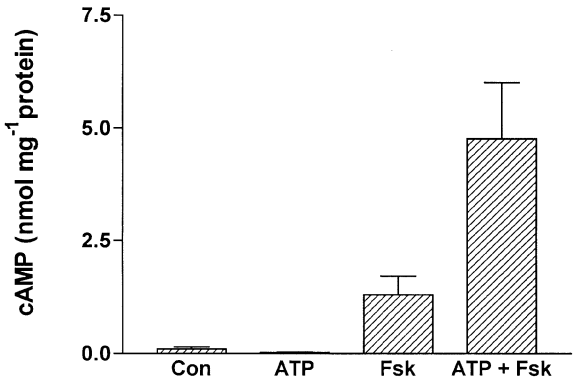

Figure 8 Effect of ATP and forskolin on cyclic AMP synthesis. Primary astrocyte cultures were treated for 10 min with ATP (100 μM), forskolin (Fsk, 10 μM) or ATP + forskolin, and cyclic AMP synthesis was determined as described in Methods. Data (mean $\pm$ s.e.mean) were obtained from five experiments. ATP did not stimulate cyclic AMP formation but it significantly potentiated forskolin-induced cyclic AMP synthesis ($P<0.01$, ANOVA).

Expression of P_{2Y} receptors in rat cortical astrocytes

To identify P_{2Y} receptor subtypes expressed in rat cortical astrocytes, we conducted RT–PCR analysis using primer pairs designed to amplify regions of the cDNA encoding the four cloned rat P_{2Y} receptor subtypes, P_{2Y_1}, P_{2Y_2}, P_{2Y_4}, and P_{2Y_6}. PCR analysis revealed that three receptor sequences could be amplified from rat cortical astrocytes: P_{2Y_1}, P_{2Y_2} and P_{2Y_4} (Figure 6). All three amplification products were of the expected sizes (P_{2Y_1}, 391 base pairs; P_{2Y_2}, 603 base pairs; P_{2Y_4}, 377 base pairs) and their identities were confirmed by sequencing. An amplification product for P_{2Y_6} was not found in rat astrocytes, although P_{2Y_6} expression was detected using the same primers with cDNA templates from rat heart tissue (data not shown). ATP and UTP are equipotent agonists at recombinant rat P_{2Y_2} and P_{2Y_4} in terms of stimulating increases in intracellular calcium (Chen *et al*., 1996) or membrane currents ($I_{Cl,Ca}$) (Bogdanov *et al*., 1998), respectively. To examine the potency of ATP and UTP on native P_{2Y} receptors in rat cortical astrocytes, concentration-response studies were conducted. We found that although ATP and UTP recruit different Mek activators, these nucleotides have similar potencies (EC_{50} values: ATP, 5.4 μM; UTP, 3.0 μM) in terms of stimulating Erk activity (Figure 7).

Effect of extracellular ATP on cyclic AMP synthesis

Because of the differences in P_{2Y} agonist stimulation of cRaf-1 and Erk activities, we tested for the presence of $P_{2Y_{11}}$, an ATP-preferring receptor recently found in human placenta (Communi *et al*., 1997). This P_{2Y} subtype has not been cloned from rat tissue but functional studies demonstrated that it is linked positively to adenylyl cyclase (Communi *et al*., 1997). To assess whether $P_{2Y_{11}}$ is involved in signal transduction in cortical astrocyte cultures, cells were treated with ATP (100 μM) for 10 min and cyclic AMP assays were performed. Extracellular ATP did not increase cyclic AMP levels, although treatment with forskolin (10 μM) did stimulate cyclic AMP production (Figure 8). ATP treatment resulted in a decrease in cyclic AMP synthesis that was significant ($P<0.01$) when normalized to basal levels within each experiment. Although extracellular ATP did not stimulate basal cyclic AMP production, it potentiated the forskolin-induced increase

in cyclic AMP levels (Figure 8), an effect reported previously in endothelial cells (Albert *et al*., 1997).

Discussion

The role of purines and pyrimidines as transmitters and trophic factors in a greater variety of cells and organs has seen a rapid growth in the last 5 years. Seven subtypes of the ionotropic purinoceptors (P_{2X}) and five subtypes of the metabotropic purinoceptors (P_{2Y}) have been cloned from mammalian tissues (Ralevic & Burnstock, 1998). Although eleven P_{2Y} receptor subtypes have been classified, doubts have been raised about the real identity of some of these receptors. The receptor cloned as being a purinoceptor and named P_{2Y_7}, actually was shown to be a leukotriene B_4 receptor (Yokomizo *et al*., 1997), and Janssens *et al*. (1997) did not find any effect of purinergic agonists on cells transfected with a plasmid containing a receptor closely related to P_{2Y_5}. This observation is strengthened by the lack of four positively charged amino acids conserved in all other P_{2Y} subtypes and not present in P_{2Y_5} and the related P_{2Y_9} and $P_{2Y_{10}}$ subtypes (Janssens *et al*., 1997). P_{2Y_8} has been described in *Xenopus* embryos but its presence in mammals is uncertain (Bogdanov *et al*., 1997).

The results presented here demonstrate that native P_{2Y} receptors in astrocytes recruit different Mek activators to stimulate Erk. UTP, 2MeSATP and ADP utilize cRaf-1, whereas ATP-preferring receptors recruit a different Mek activator. ATP was reported to activate Raf-1 in vascular smooth muscle cells (Yu *et al*., 1996) but this paper has been retracted (Wu *et al*., 1998). The identity of the Mek activator recruited by ATP-preferring P_{2Y} receptors in astrocytes remains unknown. Two major families of Mek activators have been described. The Raf family includes cRaf-1 and B-Raf, the predominant form in the brain; these serine/threonine kinases activate Mek1. A-Raf, another member of the Raf family, is not expressed in rat brain (Morice *et al*., 1999). The second Mek activator family comprises MekK1-5. Mek1 and Sek1 are targets of MekK1, 2 and 3, while Sek1 is stimulated by MekK4 and 5. Besides these two families, there are other proteins which function as Mek activators such as Mos (Nebreda *et al*., 1993), which activates specifically Mek1, and Tpl2, which activates both Mek1 and Sek1 (Salmerón *et al*., 1996). The mixed lineage kinases SPRK and ASK1 do not activate Mek1 but activate the other MAPK cascades (Rana *et al*., 1996; Ichijo *et al*., 1997). Besides these MAPKK activators, an atypical, non-phorbol ester-stimulated PKC (PKCζ), which is structurally related to Raf, can activate Mek1 (Liao *et al*., 1997; van Dijk *et al*., 1997; Schönwasser *et al*., 1998) but it does so in an indirect manner, acting through an as-yet unidentified factor. The Erk pathway stimulated by ATP in astrocytes is dependent on PKC but this pathway involves PKCδ, a calcium-independent, phorbol ester-stimulated PKC isoform, rather than an atypical PKC (Neary *et al*., 1999). It is not known whether calcium-independent, phorbol ester-stimulated PKC isoforms can bypass Raf but overexpression of a constitutively active mutant of PKCδ is sufficient to activate Mek and Erk (Ueda *et al*., 1996). In the studies presented here, ATP activated Erk without using cRaf-1, B-Raf or the known activators of Mek1, namely, MekK1, 2 or 3. Although the nature of the Mek activator recruited by extracellular ATP remains to be identified, we have found that UTP, 2MeSATP and ADP recruit cRaf-1 whereas ATP does not, thereby indicating that distinct P_{2Y} receptors are coupled to different Mek activators in rat cortical astrocytes.

Comparison of the pharmacological profile for the activation of cRaf-1 by P_{2Y} agonists with the subtypes of P_{2Y} receptors expressed in rat cortical astrocytes makes it difficult to identify the subtypes of P_{2Y} receptors coupled to the Erk cascade because the profile does not fit the properties of recombinant P_{2Y} receptors. Studies on the activation of Erk by ATP in rat cortical astrocytes had demonstrated previously that signalling occurs *via* P_{2Y} receptors rather than adenosine/P_1 receptors, the latter of which could be stimulated by breakdown of ATP to adenosine catalyzed by ectonucleotidases (Neary & Zhu, 1994; King *et al*., 1996). These studies also showed that 2MeSATP, ATP and UTP activated Erk to a similar extent, whereas ADP was less effective than ATP. This suggested the presence of endogenous ATP-, ATP/UTP- and perhaps UTP-preferring receptors. Previous RT–PCR studies demonstrated the presence of P_{2Y_1} receptors (Webb *et al*., 1996) and this has been confirmed in the results presented here. P_{2Y_1} receptors respond preferentially to ATP and 2MeSATP, without any response to UTP (Simon *et al*., 1995; Tokuyama *et al*., 1995; Henderson *et al*., 1995), and 2MeSADP and ADP have a high selectivity for this receptor subtype (Léon *et al*., 1997; Hechler *et al*., 1998; Palmer *et al*., 1998). However, we found that 2MeSATP and ADP recruit a different Mek activator than ATP in rat cortical astrocytes, a finding that is inconsistent with the P_{2Y_1} pharmacological profile. It has been suggested that 2MeSATP and ATP are partial agonists or antagonists of P_{2Y_1} receptors (Léon *et al*., 1997; Hechler *et al*., 1998), and thus the lack of effect of ATP on cRaf-1 activation could be interpreted to suggest that ATP is acting as partial agonist or antagonist in astrocytes. However, recent studies have shown that maximal effects of ATP and 2MeSATP on P_{2Y_1} receptors are very similar to ADP and 2MeSADP under conditions of normal receptor reserve; the partial agonism or antagonism of ATP and 2MeSATP may be the result of low receptor reserve (Palmer *et al*., 1998). Because both ATP and 2MeSATP stimulated similar responses in term of Erk activation while ATP did not recruit cRaf-1, low receptor reserve cannot explain the difference in activation of cRaf-1 by these nucleotides; thus it is unlikely that ATP and 2MeSATP would be partial agonists or antagonists of P_{2Y_1} receptors under the conditions used in our studies. Moreover, our findings that (1) a 10 fold higher concentration of ADP did not cause a greater activation of cRaf-1 than 2MeSATP and (2) the maximal effect of ATP on Erk activity was greater than that of ADP are inconsistent with an effect on P_{2Y_1} receptors because ADP is more active than 2MeSATP on P_{2Y_1} receptors (Léon *et al*., 1997; Hechler *et al*., 1998; Palmer *et al*., 1998). Furthermore, adenosine 3′-phosphate 5′-phosphosulphate, a selective competitive antagonist of P_{2Y_1} receptors (Boyer *et al*., 1996), was not effective in blocking activation of Erk by ATP or 2MeSATP (Y. Kang and J.T. Neary, unpublished observations). Collectively, these observations indicate that, if P_{2Y_1} receptors are coupled to Erk in rat cortical astrocytes, their pharmacological properties differ from those of the cloned receptors.

The RT–PCR data presented here indicate that rat cortical astrocytes also express P_{2Y_2} and P_{2Y_4} receptors. However, the pharmacological profile for recombinant P_{2Y_2} and P_{2Y_4} receptors is also inconsistent with our findings on the activation of cRaf-1 by P_{2Y} agonists. For example, ATP and UTP would be expected to activate rat P_{2Y_2} and P_{2Y_4} receptors but our results show that UTP recruits cRaf-1 while ATP does not, thereby excluding receptors activated by both adenine and uridine nucleotides. If P_{2Y_2} and P_{2Y_4} receptors in astrocytes are not coupled to Erk, then what is the nature of the endogenous P_{2Y} receptor which is responsible for the activation of Erk by

UTP? Among the cloned P_{2Y} subtypes from rat tissues, P_{2Y_6} receptors preferentially recognize uridine nucleotides when compared to adenosine nucleotides (Chang *et al*., 1995). However, this subtype can be excluded because RT–PCR analysis failed to reveal an amplification product for this receptor in rat cortical astrocytes. Recently, we reported that P_{2Y} receptors in rat cortical astrocytes are coupled independently to Erk and phosphatidylinositol-specific phospholipase C/calcium pathways (Neary *et al*., 1999). Astrocyte P_{2Y_1}, P_{2Y_2} and P_{2Y_4} receptors may be coupled to the phospholipase C/calcium pathway but the Raf and Erk data presented here suggest that either the properties of the native P_{2Y} receptors differ from those of the recombinant P_{2Y_1}, P_{2Y_2} and P_{2Y_4} receptors, or rat cortical astrocytes express novel purine-preferring and pyrimidine-preferring receptors coupled to the Erk signalling pathway.

It has been reported that P_{2Y_2} and P_{2Y_4} receptors are coupled to Erk in endothelial (Graham *et al*., 1996; Albert *et al*., 1997), smooth muscle (Harper *et al*., 1998; Wilden *et al*., 1998) and PC12 cells (Soltoff *et al*., 1998). In another study, stimulation of a P_{2Y_2}-like receptor in human embryonic kidney-293 cells activated Erk in a Ras-dependent manner (Gao *et al*., 1999). Collectively, these observations suggest that the coupling of ATP/UTP-preferring receptors to the Erk cascade in these cells differs from rat cortical astrocytes, although cRaf-1 recruitment by different P_{2Y} agonists was not examined in the previous studies.

Another subtype, $P_{2Y_{11}}$, has been recently cloned from human placenta. This receptor is positively coupled to phosphoinositide and cyclic AMP pathways (Communi *et al*., 1997). Because the rat homologue of this receptor has not been reported, we have not been able to investigate its expression in rat astrocytes by RT–PCR analysis. However, we have conducted experiments to measure cyclic AMP levels in response to extracellular ATP. In these studies, extracellular ATP did not evoke an increase in cyclic AMP synthesis, suggesting that $P_{2Y_{11}}$ receptors are not present in rat cortical astrocytes or are not coupled to adenylyl cyclase in these cells. Interestingly, forskolin-induced cyclic AMP production was potentiated by ATP; this phenomenon has also been observed in endothelial cells (Albert *et al*., 1997). Similarly, forskolin-induced cyclic AMP formation is also potentiated by metabotropic glutamate receptor agonists in cortical astrocytes (Balazs *et al*., 1998).

In summary, our data suggest that purine-preferring P_{2Y} receptors and pyrimidine-preferring P_{2Y} receptors are functionally linked to the activation of the Erk/MAPK cascade in primary cultures of rat cortical astrocytes. Rat P_{2Y} receptors activated by both adenine and uridine nucleotides, such as P_{2Y_2} and P_{2Y_4}, are expressed in these cells but they do not appear to be coupled to the Erk cascade because ATP and UTP do not recruit the same MEK activator. The ability of native P_{2Y} receptors to signal to Erk *via* different pathways provides opportunities to regulate the trophic actions of extracellular nucleotides by enhancing or interrupting signalling from specific P_{2Y} receptor subtypes. In addition, P_{2Y} receptors are coupled to phospholipase C/calcium and adenylyl cyclase/cyclic AMP signalling pathways. This suggests that a set of receptor subtypes linked to different signal transduction mechanisms will be able to produce very different responses according to the set of signalling pathways activated.

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References

ABBRACCHIO, M.P. & BURNSTOCK, G. (1994). Purinoceptors: are there families of P_{2X} and P_{2Y} purinoceptors? *Pharmacol. Ther.,* **64,** 445–475.

ALBERT, J.L., BOYLE, J.P., ROBERTS, J.A., CHALLISS, R.A.J., GUBBY, S.E. & BOARDER, M.R. (1997). Regulation of brain capillary endothelial cells by P2Y receptors coupled to Ca^{2+}, phospholipase C and mitogen-activated protein kinase. *Br. J. Pharmacol.,* **122,** 935–941.

AVRUCH, J., ZHANG, X.-F. & KYRIAKIS, J.M. (1994). Raf meets Ras: completing the framework of a signal transduction pathway. *Trends Biochem. Sci.,* **19,** 279–283.

BALAZS, R., MILLER, S., CHUN, Y., O'TOOLE, J. & COTMAN, C.W. (1998). Metabotropic glutamate receptor agonists potentiate cyclic AMP formation induced by forskolin or beta-adrenergic receptor activation in cerebral cortical astrocytes in culture. *J. Neurochem.,* **70,** 2446–2458.

BEAN, B.P. (1992). Pharmacology and electrophysiology of ATP-activated ion channels. *Trends Pharmacol. Sci.,* **13,** 87–90.

BOGDANOV, Y.D., DALE, L., KING, B.F., WHITTOCK, N. & BURNSTOCK, G. (1997). Early expression of a novel nucleotide receptor in the neural plate of *Xenopus* embryos. *J. Biol. Chem.,* **272,** 12583–12590.

BOGDANOV, Y.D., WILDMAN, S.S., CLEMENTS, M.P., KING, B.F. & BURNSTOCK, G. (1998). Molecular cloning and characterization of rat $P2Y_4$ nucleotide receptor. *Br. J. Pharmacol.,* **124,** 428–430.

BOYER, J.L., ROMERO-AVILA, T., SCHACHTER, J.B. & HARDEN, T.K. (1996). Identification of competitive antagonists of the $P2Y_1$ receptor. *Mol. Pharmacol.,* **50,** 1323–1329.

BRUNER, G. & MURPHY, S. (1993). UTP activates multiple second messenger systems in cultured rat astrocytes. *Neurosci. Lett.,* **162,** 105–108.

BURNSTOCK, G. (1990). Overview: purinergic mechanisms. *Ann. N.Y. Acad. Sci.,* **603,** 1–18.

BURNSTOCK, G. (1997). The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology,* **36,** 1127–1139.

CATLING, A.D., REUTER, C.W.M., COX, M.E., PARSONS, S.J. & WEBER, M.J. (1994). Partial purification of a mitogen-activated protein kinase kinase activator from bovine brain: identification as B-Raf or a B-Raf associated activity. *J. Biol. Chem.,* **269,** 30014–30021.

CHANG, K., HANAOKA, K., KUMADA, M. & TAKUWA, Y. (1995). Molecular cloning and functional analysis of a novel P_2 nucleotide receptor. *J. Biol. Chem.,* **270,** 26152–26158.

CHEN, Z.P., KRULL, N., XU, S., LEVY, A. & LIGHTMAN, S.I. (1996). Molecular cloning and functional characterization of a rat pituitary G protein-coupled adenosine triphosphate (ATP) receptor. *Endocrinology,* **137,** 1833–1840.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method for RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.,* **162,** 156–159.

COMMUNI, D., GOVAERTS, C., PARMENTIER, M. & BOEYNAEMS, J.-M. (1997). Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. *J. Biol. Chem.,* **272,** 31969–31973.

COOK, S.J. & MCCORMICK, F. (1993). Inhibition by cAMP of Ras-dependent activation of Raf. *Science,* **262,** 1069–1072.

EDWARDS, F.A., GIBB, A.J. & COLQUHOUN, D. (1992). ATP receptor-mediated synaptic currents in the central nervous system. *Nature,* **359,** 144–147.

GAO, Z., CHEN, T., WEBER, M.J. & LINDEN, J. (1999). A_{2B} adenosine and $P2Y_2$ receptors stimulate mitogen-activated protein kinase in human embryonic kidney-293 cells: cross-talk between cyclic AMP and protein kinase C pathways. *J. Biol. Chem.,* **274,** 5972–5980.

GRAHAM, A., MCLEES, A., KENNEDY, C., GOULD, G.W. & PLEVIN, R. (1996). Stimulation by the nucleotides ATP and UTP of mitogen-activated protein kinase in EAhy 926 endothelial cells. *Br. J. Pharmacol.,* **117,** 1341 – 1347.

GRAVES, L.M., BORNFELDT, K.E., RAINES, E.W., POTTS, B.C., MACDONALD, S.G., ROSS, R. & KREBS, E.G. (1993). Protein kinase A antagonizes platelet-derived growth factor-induced signaling by mitogen-activated protein kinase in human arterial smooth muscle cells. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 10300 – 10304.

HARPER, S., WEBB, T.E., CHARLTON, S.J., NG, L.L. & BOARDER, M.R. (1998). Evidence that $P2Y_4$ nucleotide receptors are involved in the regulation of rat aortic smooth muscle cells by UTP and ATP. *Br. J. Pharmacol.,* **124,** 703 – 710.

HECHLER, B., VIGNE, P., LÉON, C., BREITTMAYER, J.-P., GACHET, C. & FRELIN, C. (1998). ATP derivatives are antagonists of the $P2Y_1$ receptor: similarities to the platelet ADP receptor. *Mol. Pharmacol.,* **53,** 727 – 733.

HENDERSON, D.J., ELLIOT, D.G., SMITH, G.M., WEBB, T.E. & DAINTY, I.A. (1995). Cloning and characterization of a bovine P_{2Y} receptor. *Biochem. Biophys. Res. Commun.,* **212,** 648 – 656.

HUWILER, A. & PFEILSCHIFTER, J. (1994). Stimulation by extracellular ATP and UTP of the mitogen-activated protein kinase cascade and proliferation of rat renal mesangial cells. *Br. J. Pharmacol.,* **113,** 1455 – 1463.

ICHIJO, H., NISHIDA, E., IRIE, K., TEN DIJKE, P., SAITOH, M., MORIGUCHI, T., TAKAGI, M., MATSUMOTO, K., MIYAZONO, K. & GOTOH, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science,* **275,** 90 – 94.

JAISWAL, R.K.M.S.A., WOLFMAN, A. & LANDRETH, G.E. (1994). The mitogen-activated protein kinase cascade is activated by B-Raf in response to NGF through interaction with $p21^{ras}$. *Mol. Cell. Biol.,* **14,** 6944 – 6953.

JANSSENS, R., BOEYNAEMS, J.-M., GODART, M. & COMMUNI, D. (1997). Cloning of a human heptahelical receptor closely related to the P2Y5 receptor. *Biochem. Biophys. Res. Commun.,* **236,** 106 – 112.

KASTRITSIS, C.H., SALM, A.K. & MCCARTHY, K. (1992). Stimulation of the P2Y purinergic receptor on type 1 astroglia results in inositol phosphate formation and calcium mobilization. *J. Neurochem.,* **58,** 1277 – 1284.

KING, B.F., NEARY, J.T., ZHU, Q., WANG, S., NORENBERG, M.D. & BURNSTOCK, G. (1996). P2 purinoceptors in rat cortical astrocytes: expression, calcium-imaging and signalling studies. *Neuroscience,* **74,** 1187 – 1196.

KING, B.F., TOWNSEND-NICHOLSON, A. & BURNSTOCK, G. (1998). Metabotropic receptors for ATP and UTP: exploring the correspondence between native and recombinant nucleotide receptors. *Trends Pharmacol. Sci.,* **19,** 506 – 514.

KURINO, M., FUKUNAGA, K., USHIO, Y. & MIYAMOTO, E. (1996). Cyclic AMP inhibits activation of mitogen-activated protein kinase and cell proliferation in response to growth factors in cultured rat cortical astrocytes. *J. Neurochem.,* **67,** 2246 – 2255.

KYRIAKIS, J.M. & AVRUCH, J. (1996). Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.,* **271,** 24313 – 24316.

KYRIAKIS, J.M., APP, H., ZHANG, X.-F., BANERJEE, P., BRAUTIGAN, D.L., RAPP, U.R. & AVRUCH, J. (1992). Raf-1 activates MAP kinase-kinase. *Nature,* **358,** 417 – 421.

KYRIAKIS, J.M., FORCE, T.L., RAPP, U.R., BONVENTRE, J.V. & AVRUCH, J. (1993). Mitogen regulation of c-Raf-1 protein kinase activity toward mitogen-activated protein kinase-kinase. *J. Biol. Chem.,* **268,** 16009 – 16019.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **227,** 680 – 685.

LÉON, C., HECHLER, B., VAIL, C., LERAY, C., CAZENAVE, J.-P. & GACHET, C. (1997). The $P2Y_1$ receptor is an ADP receptor antagonized by ATP and expressed in platelets and megakaryoblastic cells. *FEBS Lett.,* **403,** 26 – 30.

LIAO, D., MONIA, B., DEAN, N. & BERK, B.C. (1997). PKC-ζ mediates angiotensin II activation of ERK1/2 in vascular smooth muscle cells. *J. Biol. Chem.,* **272,** 6146 – 6150.

LUO, Z., DIAZ, B., MARSHALL, M.S. & AVRUCH, J. (1997). An intact Raf zinc finger is required for optimal binding to processed Ras and for Ras-dependent Raf activation in situ. *Mol. Cell. Biol.,* **17,** 46 – 53.

LYONS, S.A., MORELL, P. & MCCARTHY, K.D. (1994). Schwann cells exhibit P2Y purinergic receptors that regulate intracellular calcium and are up-regulated by cyclic AMP analogues. *J. Neurochem.,* **63,** 552 – 560.

MAGOSKI, N.S. & WALZ, W. (1992). Ionic dependence of a P2-purinoceptor mediated depolarization of cultured astrocytes. *J. Neurosci. Res.,* **32,** 530 – 538.

MORICE, C., NOTHIAS, F., KONIG, S., VERNIER, P., BACCARINI, M., VINCENT, J.D. & BARNIER, J.V. (1999). Raf-1 and B-Raf proteins have similar regional distributions but different subcellular localization in adult rat brain. *Eur. J. Neurosci.,* **11,** 1995 – 2006.

NEARY, J.T., KANG, Y., BU, Y., YU, E., AKONG, K. & PETERS, C.M. (1999). Mitogenic signaling by ATP/P2Y purinergic receptors in astrocytes: involvement of a calcium-independent protein kinase C, extracellular signal regulated protein kinase pathway distinct from the phosphatidylinositol-specific phospholipase C, calcium pathway. *J. Neurosci.,* **19,** 4211 – 4220.

NEARY, J.T., LASKEY, R., VAN BREEMEN, C., BLICHARSKA, J., NORENBERG, L.O.B. & NORENBERG, M.D. (1991). ATP-evoked calcium signal stimulates protein phosphorylation/dephosphorylation in astrocytes. *Brain Res.,* **566,** 89 – 94.

NEARY, J.T., RATHBONE, M.P., CATTABENI, F., ABBRACCHIO, M.P. & BURNSTOCK, G. (1996). Tropic actions of extracellular nucleotides and nucleosides on glial and neuronal cells. *Trends Neurosci.,* **19,** 13 – 18.

NEARY, J.T., WHITTEMORE, S.R., ZHU, Q. & NORENBERG, M.D. (1994). Synergistic activation of DNA synthesis in astrocytes by fibroblast growth factor and extracellular ATP. *J. Neurochem.,* **63,** 490 – 494.

NEARY, J.T. & ZHU, Q. (1994). Signaling by ATP receptors in astrocytes. *NeuroReport,* **5,** 1617 – 1620.

NEBREDA, A.R., HILL, C., GOMEZ, N., COHEN, P. & HUNT, T. (1993). The protein kinase mos activates MAP kinase kinase in vitro and stimulates the MAP kinase pathway in mammalian somatic cell in vivo. *FEBS Lett.,* **333,** 183 – 187.

NORTH, R.A. & BARNARD, E.A. (1997). Nucleotide receptors. *Curr. Opin. Neurobiol.,* **7,** 346 – 357.

PALMER, R.K., BOYER, J.L., SCHACHTER, J.B., NICHOLAS, R.A. & HARDEN, T.K. (1998). Agonist action of adenosine triphosphates at the human $P2Y_1$ receptor. *Mol. Pharmacol.,* **54,** 1118 – 1123.

PATEL, V., BROWN, C., GOODWIN, A., WILKIE, N. & BOARDER, M.R. (1996). Phosphorylation and activation of p42 and p44 mitogen-activated protein kinase are required for the P2 purinoceptor stimulation of endothelial prostacyclin production. *Biochem. J.,* **320,** 221 – 226.

POST, G.R., GOLDSTEIN, D., THUERAUF, D.J., GLEMBOTSKI, C.C. & BROWN, J.H. (1996). Dissociation of p44 and p42 mitogen-activated protein kinase activation from receptor-induced hypertrophy in rat ventricular myocytes. *J. Biol. Chem.,* **271,** 8452 – 8457.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413 – 492.

RANA, A., GALLO, K., GODOWSKI, P., HIRAI, S., OHNO, S., ZON, L., KYRIAKIS, J.M. & AVRUCH, J. (1996). The mixed lineage kinase SPRK phosphorylates and activates the SAPK activator, SEK-1. *J. Biol. Chem.,* **271,** 19025 – 19028.

SALMERÓN, A., AHNAM, T.B., CARLILE, G.W., PAPPIN, D., NARSIMHAN, R.P. & LEY, S.C. (1996). Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. *EMBO J.,* **15,** 817 – 826.

SALTER, M.W. & HICKS, J.L. (1994). ATP-evoked increases in intracellular calcium in neurones and glia from the dorsal spinal cord. *J. Neurosci.,* **14,** 1563 – 1575.

SCHÖNWASSER, D.C., MARAIS, R.M., MARSHALL, C.J. & PARKER, P.J. (1998). Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol. Cell. Biol.,* **18,** 790 – 798.

SEGER, R. & KREBS, E.G. (1995). The MAPK signaling cascade. *FASEB J.,* **9,** 726 – 735.

SEVETSON, B.R., KONG, X. & LAWRENCE, Jr, J.C. (1993). Increasing cAMP attenuates activation of mitogen-activated protein kinase. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 10305 – 10309.

SIMON, J., WEBB, T.E., KING, B.F., BURNSTOCK, G. & BARNARD, E.A. (1995). Characterization of a recombinant P_{2Y} purinoceptor. *Eur. J. Pharmacol.,* **291,** 281 – 289.

SOLTOFF, S.P., AVRAHAM, H., AVRAHAM, S. & CANTLEY, L.C. (1998). Activation of P_{2Y2} receptors by UTP and ATP stimulates mitogen-activated kinase activity through a pathway that involves related adhesion focal tyrosine kinase and protein kinase C. *J. Biol. Chem.,* **273,** 2653–2660.

TASCA, C.I., VENDITE, D., GARCIA, K.L. & SOUZA, S.O. (1995). Effects of adenosine on cAMP production during early development in the optic tectum of chicks. *Int. J. Dev. Neurosci.,* **13,** 545–553.

TOKUYAMA, Y., HARA, M., JONES, E.M.C., FAN, Z. & BELL, G.I. (1995). Cloning of rat and mouse P2Y purinoceptors. *Biochem. Biophys. Res. Commun.,* **211,** 211–218.

UEDA, Y., HIRAI, S., OSADA, S. & SUZUKI, A. (1996). Protein Kinase C δ activates the MEK–ERK pathway in a manner independent of Ras and dependent on Raf. *J. Biol. Chem.,* **271,** 23512–23519.

VAN DIJK, M.C.M., MURIANA, F.J.G., VAN DER HOEVEN, P.D., DE WIDT, J., SCHAAP, D., MOOLENAAR, W.H. & VAN BLITTERSWIJK, W.J. (1997). Diacylglycerol generated by exogenous phospholipase C activates the MAPK pathway independent of Ras- and phorbol ester-sensitive protein kinase C: dependence on protein kinase C-ζ. *Biochem. J.,* **323,** 693–699.

WEBB, T.E., FEOLDE, E., VIGNE, P., NEARY, J.T., RUNBERT, A., FRELIN, C. & BARNARD, E.A. (1996). The P_{2Y} purinoceptor in rat brain microvascular endothelial cells couples to inhibition of adenylate cyclase. *Br. J. Pharmacol.,* **119,** 1385–1392.

WIERASZKO, A., GOLDSMITH, G. & SEYFRIED, T.N. (1989). Stimulation-dependent release of adenosine triphosphate from hippocampal slices. *Brain Res.,* **485,** 244–250.

WILDEN, P.A., AGAZIE, Y.M., KAUFMAN, R. & HALENDA, S.P. (1998). ATP-stimulated smooth muscle cell proliferation requires independent ERK and PI3K signaling pathways. *Am. J. Physiol.,* **275,** H1209–H1215.

WU, D., YANG, C.-M., LAU, Y.-T. & CHEN, J.-C. (1998). Retraction of publication. *Mol. Pharmacol.,* **53,** 346.

WU, J., DENT, P., JELINEK, T., WOLFMAN, A., WEBER, M.J. & STURGILL, T.W. (1993). Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3′,5′-monophosphate. *Science,* **262,** 1065–1069.

WU, P.H. & PHILLIS, J.W. (1978). Distribution and release of adenosine triphosphate in rat brain. *Neurochem. Res.,* **3,** 563–571.

YOKOMIZO, T., IZUMI, T., CHANG, K., TAKUWA, Y. & SHIMIZU, T. (1997). A G protein-coupled receptor for leukotriene B_4 that mediates chemotaxis. *Nature,* **387,** 620–624.

YU, S.M., CHEN, S.F., LAU, Y.T., YANG, C.M. & CHEN, J.C. (1996). Mechanism of extracellular ATP-induced proliferation of vascular smooth muscle cells. *Mol. Pharmacol.,* **50,** 1000–1009.

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