Adenosine Nucleotides Acting at the Human P2Y1 Receptor Stimulate Mitogen-activated Protein Kinases and Induce Apoptosis*

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For the widely distributed P2Y receptors for nucleotides, the transductional and functional responses downstream of their coupling to G proteins are poorly characterized. Here we describe apoptotic induction and the associated differential stimulation of mitogen-activated protein (MAP) kinase family members by the human P2Y1 receptor. The potent P2Y1 receptor agonist, 2-methylthio-ADP (2-MeSADP), stimulated the extracellular-signal regulated kinases (ERK1/2) (EC50 ~5 nM) as well as several, but not all isoforms detected, of the stress-activated protein kinase (SAPK) family. Phosphoisoforms of p38 were unaffected. The induced kinase activity was blocked by the P2Y1 receptor-selective antagonist, adenosine-2’-phosphate-5’-phosphate, but unaffected by pertussis toxin. In addition, the endogenous ligand ADP, and significantly also 2-MeSATP, induced concentration-dependent phosphorylation changes in the same MAP kinase family members. The sustained activation of ERK1/2 was associated with Elk-1 phosphorylation that was abolished by the MEK1 inhibitor, PD 98059. However, the concomitant transient activation of the SAPKs was not sufficient to induce c-Jun or ATF-2 phosphorylation. The transient phase of the ERK activity was partially inhibited either by the phosphatidylinositol 3-kinase inhibitor, LY 294002, or the PKC inhibitor, Gö 6976. In addition, the Src inhibitor, PP1, or expression of dominant negative Ras also attenuated the transient phase of ERK phosphorylation. In contrast, inhibition of Ras or Src had no effect on the sustained ERK activity, which was critically dependent on phosphatidylinositol 3-kinase. The transient SAPK activity was suppressed by expression of a dominant negative form of MKK4. Furthermore, this kinase-deficient mutant inhibited 2-MeSADP-induced caspase-3 stimulation and the associated decrease in cell number. In conclusion, adenosine di- and triphosphate stimulation of the human P2Y1 receptor can transiently activate the Ras-ERK cascade via the cooperative effects of phosphatidylinositol 3-kinase, Src and PKC. The sustained ERK stimulation, via a Ras-insensitive pathway, culminates in Elk-1 activation without inducing a proliferation effect. The transient SAPK activity did not evoke transcription factor phosphorylation but was required for the P2Y1 receptor-mediated apoptotic function.

Extracellular nucleotides can interact with cell surface P2 receptors both in the central nervous system and in peripheral tissues to produce a broad range of physiological effects. The P2 family is divided into two main types as follows: the P2X receptors are ligand-gated ion channels, and the P2Y receptors are G protein-coupled (1, 2). Part of the present study describes the signaling pathways of the P2Y1 receptor, the first member of the P2Y family to be identified (3). The P2Y1 receptor is widely distributed and has been described in mammalian heart, vascular, liver, kidney, prostate, gastrointestinal, pulmonary, connective, and immune tissues (4, 5). It has also been identified in skeletal muscle and appears to be the most abundant P2 receptor in the nervous system (5–7). ADP and more potently 2-methylthio-ADP (2-MeSADP)1 are agonists at the P2Y1 receptor, but its activation by ATP and 2-MeSATP has been controversial. Due to the ready conversion of triphosphates by ectonucleotidases to the aforementioned agonistic diphosphates, some earlier findings are in doubt. Thus, it is important to maintain totally the triphosphate integrity by a constant regenerating system (8, 9), which has been included in the experimental design of the present study. With such precautions in place, the recombinant P2Y1 receptor can be activated by ATP and 2-MeSATP (10, 11), whereas others (9) have found these ligands to be antagonists in other cell systems. This difference has been proposed to depend critically on the degree of P2Y1 receptor reserve (10, 11).

The second messengers generated by the P2Y1 receptor are due to the activation of phospholipase Cβ leading to the formation of diacylglycerol as well as inositol trisphosphate (12, 13) and mobilization of intracellular Ca2+ (9, 10), suggesting that the subsequent activation of protein kinase C (PKC) is likely. These responses are insensitive to pertussis toxin, and the G protein involved has been identified as G11, in the case of the turkey erythrocyte (14), but G11 can also act at the P2Y1 receptor in some systems including the human platelet (15). Furthermore, a primary signaling action of the P2Y1 receptor in neurons is the closing of an N-type Ca2+ channel (11), and in platelets there is some suggestion that the P2Y1 receptor is coupled to the RhoA-ROCK pathway (16). It is known that activation of PKC isoforms by G protein-coupled receptors can stimulate the extracellular signal-regulated kinases (ERKs), which are members of the mitogen-activated protein (MAP) kinase family (17). For the P2Y1 receptors, activation of the

1 The abbreviations used are: 2-MeSATP, 2-methylthio-ATP; 2-MeSADP, 2-methylthio-ADP; MAP, mitogen-activated protein; SAPK, stress-activated protein kinase; ERK, extracellular-signal regulated kinase; MEK, MAP kinase/ERK kinase; PKC, protein kinase C; A2P5P, adenosine-2’-phosphate-5’-phosphate; FITC, fluorescein isothiocyanate; PI 3-K, phosphatidylinositol 3-kinase; TBS, Tris-buffered saline; PBS, phosphate-buffered saline.

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ERK cascade has been shown in several cell types including astrocytes (18, 19), endothelial cells (20, 21), vascular smooth muscle cells (22), and renal mesangial cells (23). However, since the cells studied co-express several types of P2Y receptors or have an unknown complement of purinoceptors, the downstream events observed have not identified those associated with a molecule, specifically, defined P2Y receptor.

The MAP kinases are proline-directed serine/threonine kinases that have been classified into at least four subfamilies as follows: ERKs, stress-activated protein kinases (SAPKs), p38 kinases, and BMK1/ERK5 (17). Whereas ERKs are implicated in cell growth as well as differentiation, SAPKs and p38 appear to play a role in regulating the cell death machinery (24). Whereas the pathway linking cell surface receptors to ERKs has been partially elucidated, the mechanism of activation of p38 and SAPKs is poorly understood. This is particularly so for members of the G protein-coupled receptor family, which have only recently been shown to utilize these alternative MAP kinase cascades for transduction purposes. Activation of p38 and SAPKs has been demonstrated following stimulation of the Gα11-coupled m3 and Gα-coupled m2 muscarinic acetylcholine receptors (25, 26). More recent studies have also shown p38 activation in rat glomerular mesangial cells following stimulation with UTP and ATP, possibly mediated through the P2Y2 receptor (27). In addition, stimulation of P2Y2 receptors in C6 gloma cells (28) and/or P2Y4 receptors in rat glomerular mesangial cells can induce proliferation (29). At present apoptosis initiated by nucleotides is known only for a P2X receptor, being a prominent consequence of P2X receptor activation in human macrophages and leukocytes as well as in mesangial, dendritic, and microglial cells (30, 31). With most examples of apoptosis, the P2X receptor-initiated cascade, which includes the coupling of the ion channel to the SAPK-signaling cascade (32), involves a defined sequence of phenotypic changes that culminate in death only several hours after the exposure to ATP.

The goal of this study was to examine the ability of the human recombinant P2Y1 receptor, heterologously expressed in human astrocytoma cells (1321N1, containing no endogenous P2Y receptors) to stimulate the MAP kinase transduction cascades. A specific antagonist of the P2Y1 receptor was applied to confirm the authenticity of the responses observed. In addition, the time course of the MAP kinase activity was also determined. There is much evidence to suggest that the duration of ERK activity is critically important for determining functional outcome (33, 34), and in every case examined thus far, only sustained ERK activation induces cytoplasmic nuclear migration (35, 36). Prolonged stimulation of ERK will therefore have very different consequences for gene expression compared with that of transient activation. Part of this study was thus to determine if the duration of the other MAP kinase cascades is similarly important for controlling transcriptional events. In addition, it was determined whether the P2Y1 receptor-mediated MAP kinase activities could be correlated with either a proliferative or an apoptotic functional response.

**EXPERIMENTAL PROCEDURES**

**Materials**—The 1321N1 astrocytoma cell line heterologously expressing the human P2Y1 receptor (5) was a generous gift from Dr. S. P. Kunapuli (Temple University, Philadelphia). All tissue culture and reagents were purchased from Life Technologies, Inc., and plasticware was from Costar. Carbamylcholine chloride (carbachol), creatine phosphokinase, creatine phosphate, 2-MeSADP, 2-MeSATP, and adenine-2′-phosphate-5′-phosphosphate (Ap2S) were purchased from Sigma. ADP and hexokinase were from Roche Molecular Biochemicals. Antibodies specific to ERK1 and ERK2 were obtained from Santa Cruz Biotechnology. Monoclonal antibodies specific for the dually phosphorylated and hence active forms of ERK1 and ERK2 (at Thr183 and Tyr185), α, β, and δ isoforms of p38 (at Thr180 and Tyr182), and SAPK family members (at Thr183 and Tyr183), together with antibodies to p38 and SAPKs with a specificity for the kinases independent of their phosphorylation state, were all obtained from New England Biolabs. Antibodies to the transcription factors c-Jun, Elk-1, and ATF-2 as well as those to the phosphorylated forms of these proteins were also supplied by New England Biolabs. The PKC inhibitors, Go 6976 and the phorbol ester, 12-O-tetradecanoyl phorbol 13-acetate (TPA) were purchased from Sigma. The MEK1 inhibitor, PD 98059, the Src inhibitor, PP1, and the phosphatidylinositol 3-kinase (PI3-K) inhibitor, LY 294002, were all from Calbiochem. Antibodies to the G protein α subunits were from Santa Cruz Biotechnology. An anti-MKK4 (SEK1) antibody was obtained from New England Biolabs. Antibodies specific for Ras (L2 region) and human Hs-RAS cDNA (dominant negative mutant, tagged) in pUSExpress, together with the vector, were supplied by Upstate Biotechnology, Inc. Staurosporin, FITC-conjugated annexin V, and a caspase-3 substrate that is cleaved to release a colorimetric product were all from CLONTECH.

**Enzymatic Conversion of Tri- or Diphosphate Contamination—**ATP and ADP analogues are metabolically unstable and can be degraded by various ectonucleotidases present on cells. In addition, commercially available nucleotides often contain other nucleotides as by-products. Therefore, enzymatic systems that regenerate degraded nucleotides were routinely used in all experimental procedures carried out in this study. To eliminate the diphosphate contamination of 2-MeSADP, the creatine phosphokinase-regenerating system was used according to a method described previously (9). Thus, 1 mM stock solutions of 2-MeSADP were treated with 2 units ml−1 creatine kinase at 37 °C to make 10 mM creatine phosphate for 90 min at 37 °C. In order to ensure the purity of ADP and 2-MeSADP, 1 mM stock solutions were treated with 10 units ml−1 hexokinase, 0.1 mM glucose for 60 min at room temperature to convert the triphosphate contamination to diphosphates (37). The appropriate enzymes (with their substrates) were also included (at 1 unit ml−1) in the culture medium during all experiments. A 1:1 mixture of ADP and 2-MeSADP was also included (at 1 unit ml−1) in the culture medium during all experiments. In order to substantiate the consistency of protein content across experimental treatments, the wells were rinsed with 100 μl of distilled H2O. Equivalent amounts of protein were electrophoretically resolved on 10% polyacrylamide gels. Following electrophoretic transfer onto nitrocellulose (0.22 μm) using a semi-dry blotter, the membrane was washed briefly in Tris-buffered saline (TBS) and saturated overnight in TBS supplemented with 0.1% Tween 20 and 5% dried milk. For detection of the phosphorylated forms of the kinases, the nitrocellulose membrane was incubated with a 1:800 dilution of the anti-phosphospecific antibodies. Primary incubations were for 1 h at 22 °C in TBS containing 0.1% Tween 20 (TBST) followed by washing five times for 10 min each in TBST. Membranes were incubated for 1 h at 22 °C with a 1:1,000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody in TBST containing 5% dried milk. Excess antibody was removed by washing as above, and immunocomplexes were visualized using enhanced chemiluminescence detection, according to the manufacturer’s instructions (Amersham Pharmacia Biotech). In order to substantiate the consistency of protein content between treatment groups, the membranes were re-probed with phosphorylation state-independent antibodies to ERK1 and ERK2 (1:1,000 dilution) or SAPK/p38 (1:500 dilution) for 1 h at 22 °C and processed as above. The Western blots shown are representative of three separate experiments, and each panel is taken from a single immunoblot.

**Phosphorylation of Transcription Factors**—Samples obtained from the 2-MeSADP time course experiments were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membrane as described above. The membrane was incubated with primary antibodies directed against p38, Elk-1, and c-Jun (1:400 dilution) for 1 h at 22 °C. The phosphorylation of c-Jun was regulated by phosphorylation at Ser63 and Ser73 by SAPKs. Antibodies specific to both these phosphorylation sites were used. Elk-1 is phosphorylated by ERK1 and ERK2 at a cluster of Ser/Thr motifs at its COOH terminus, and phosphorylation of Ser207 (to which the antibody was raised) has been shown to be critical for tran-
scopulation activation. Activation of ATF-2 requires phosphorylation of Thr^32 and Thr^37, and these sites are both substrates for the p38 kinase as well as for the SAPKs. The antibody used was raised to a synthetic phospho-Thr^37 peptide. For detection of total protein, phospho-indepen
dependent antibodies to the transcription factors were used at a 1:500 dilution. Western blots shown are representative experiments, and each panel is taken from a single immunoblot.

Detection of the P2Y Receptor and G Protein Complement—Whole cell extracts from 1321N1 human astrocytoma cells expressing the human P2Y1 receptor were separated on 10% polyacrylamide gels and probed with antibodies specific for some relevant subtypes of the G protein subunits. The Western blots shown are representative of three separate experiments, and each panel is taken from a single immunoblot.

Immunocytochemistry—Astrocytoma cells (1321N1) stably expressing the recombinant P2Y1 receptor were grown on poly-L-lysine (100 µg/ml)–treated glass coverslips in 12-well plates until they reached ~70% confluence. The media were removed from the wells, and the coverslips were rinsed for 5 min (times three) with phosphate-buffered saline (PBS), fixed with 2% formaldehyde in PBS for 30 min, and washed as above. The cells were incubated for 30 min in 500 µl of blocking solution (PBS containing 3% goat serum, 1% bovine serum albumin, and 0.1% Triton X-100) followed by overnight incubation at 4 °C with the primary antibody recognizing the P2Y1 receptor (1:200 dilution in blocking solution). Control coverslips were incubated with blocking solution alone. After three washes for 5 min each in PBS, the cells were incubated with 500 µl of the secondary antibody conjugated to cyanine 3 (at 1:1,000 dilution, Sigma) in blocking solution for 1 h at 22 °C. After three washes for 5 min each in PBS, the coverslips were removed from the wells, dipped in distilled water, and dried before being mounted onto slides using an antifade agent (DAKO). The fluores-
cence was visualized using a Nikon Optiphot-2 microscope.

Expression Plasmids—Dominant negative human MKK4 (K95R) was constructed as described previously (38), and the full-length cDNA was cloned into the mammalian expression vector, pCMV. Human Ha-RAS (S17N) cDNA was inserted as an EcoRI fragment into pCMV-
amp also under the control of the cytomegalovirus promoter. Transfec-
tions were performed using the LipofectAMINE™ reagent according to the protocol suggested by the manufacturer (Life Technologies, Inc.). Briefly, astrocytoma cells (1321N1) stably expressing the recombinant P2Y1 receptor at 50% confluence were transfected in serum-free media with 2 µg of DNA following complex formation with LipofectAMINE™ reagent. Transfected containing media were removed following overnight transfection for 3 h at 37 °C, and the cells were incubated with complete medium. Gene expression using immunoblot analysis as described above was determined immediately prior to drug addictions, ~48 h post-transfec
tion using a primary antibody concentration of 1:1,000.

Annexin V Binding—In normal, non-apoptotic cells, phosphatidyli-
sine is segregated to the inner leaflet of the plasma membrane. During apoptosis, this asymmetry collapses and phos-
phatidylinositol becomes expressed on the outer surface of cells (39). An-
nein V is a protein that preferentially binds to phosphatidylinositol in a Ca^2+–
dependent manner. Binding of annexin V in conjunction with propidium iodide exclusion to establish membrane integrity was used to identify apoptotic cells. Astrocytoma cells heterologously expressing the recombinant P2Y1 receptor were grown on poly-L-lysine (100 µg/ml)–treated glass coverslips in 12-well plates until ~40% confluence was reached. The cells were serum-starved for 1 h before a 5-h incubation in the presence of various agents. The media were removed from the wells, and the coverslips were rinsed in ice-cold PBS. The coverslips were incubated for 15 min in the dark at 22 °C with 200 µl of binding buffer (150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 10 mM NaHEPES, pH 7.4) containing 0.5 µg/ml annexin V-FITC and 10 µg/ml propidium iodide. The coverslips were washed twice in binding buffer, dried, and mounted onto microscope slides as described above. Annexin V-FITC binding and propidium iodide incorporation were detected using either a Nikon Optiphot-2 microscope or a confocal laser scanning microscope (Zeiss LSM 510) with FITC excitation at 488 nm, emission 505–550 nm, and propidium iodide excitation at 543 nm, emission >600 nm. Maximum projection images of z series are shown.

Results

Detection of the Human Recombinant P2Y1 Receptor and the G Protein α Subunit Complement of Astrocytoma (1321N1) Cells—The heterologous expression of the human P2Y1 receptor in astrocytoma cells (1321N1) was confirmed by immuncytochemistry as well as by Western analysis using an anti-peptide antibody directed against the COOH-terminal tail of the human P2Y1 receptor. This antibody is selective for the P2Y1 receptor subtype and recognizes both the human and rat orthologues. Fixed cells incubated without the primary anti-
body showed no detectable staining, whereas those incubated with the anti-P2Y1 receptor antibody showed marked fluores-
cence that was localized to the membrane surface (Fig. 1A). Whole cell protein extracts prepared from the selected clonal line showed concentration-dependent immunoreactivity following Western analysis with the P2Y1 receptor-specific antibody (Fig. 1B). The electrophoretic mobility of the broad band de-
tected had an apparent molecular mass of 45–55 kDa. No detectable staining was observed following Western analysis of protein from wild-type 1321N1 astrocytoma cells (data not shown). Whole cell extracts from astrocytomas expressing hu-
man P2Y1 receptors were also analyzed using antibodies speci-
cific for some relevant subtypes of the α subunits of G proteins (Fig. 1C). Immunoreactive bands of the predicted molecular masses were identified for Go_{13}, Go_{i3}, and the short form of Go_{s}. A double band was detected using an antibody that cross-reacts with both Go_{q} and Go_{q,14}. No detectable immunoreactivity was observed with an antibody specific for Go_{s,11}, but a strong band was observed following analysis with an antibody that recog-
nizes all three forms of Go_{s}, Go_{q,11}, and Go_{q,14}.

P2Y1 Receptor-mediated Effects on the Phosphorylation Sta-
tus of MAP Kinases—To determine changes in the phosphory-
lation status of the different MAP kinases upon activation of the human P2Y1 receptor, whole cell protein extracts were ana-
yzed by Western blotting using antibodies specific for the dually phosphorylated kinases and hence active forms. In se-
rum-starved astrocytoma cells expressing the P2Y1 receptor, the immunoreactivity detected with antibodies selective for ERK1, ERK2, p38, and the SAPK isoforms, independent of their phosphorylation status, showed the expression of these proteins to be unaffected over the time course studied (up to 4 h) and by the application of the potent P2Y1 receptor agonist, 2-MeSADP (300 µM) (Fig. 2). A single species could be detected with the antibody to p38, and ERK1 and ERK2 had the pre-
directed molecular mass of 44 and 42 kDa, respectively. However, several distinct entities could be observed following detection with the anti-SAPK antibody. The p54 and p46 members of this kinase family were apparent. Other immunoreactive bands were also detected with mobilities corresponding to 45 and 48 kDa (Fig. 2B).

Under basal conditions over the time course studied, a slight phosphorylation of ERK1 and ERK2 was detected that peaked 10 min following application of the vehicle control and which had declined to undetectable levels by 1 h (Fig. 2A). Application of 2-MeSADP (300 nM) induced a marked and rapid phosphorylation of both ERK kinases that was detectable after 5 min and had reached a maximal response at 15 min and returning to basal levels by 1 h (Fig. 2B). In contrast, the activity status of the 48-kDa isoform remained unaffected by 2-MeSADP (300 nM) over the time course studied. The level of immunoreactivity using phospho-independent antibodies was comparable for p38 and the p45 and p54 forms of the SAPKs. The level detected for the remaining SAPK isoforms, however, was much lower, particularly that with a molecular mass of 45 kDa was much more pronounced than that for the other SAPK isoforms present (46, 48, and 54 kDa). Following application of vehicle alone, none of the isoforms showed any change in their activity status over the 4-h period investigated (Fig. 2B). A transient increase in the phosphorylation of the 45-, 46-, and 54-kDa forms was observed upon application of 2-MeSADP (300 nM), reaching a maximum by 15 min and returning to basal levels by 1 h (Fig. 2B). In contrast, the activity status of the 48-kDa isoform remained unaffected by 2-MeSADP (300 nM) over the time course studied. The level of immunoreactivity using phospho-independent antibodies was comparable for p38 and the p45 and p54 forms of the SAPKs. The level detected for the remaining SAPK isoforms, however, was much lower, particularly that with a molecular mass of 46 kDa. The levels of the p38 and the SAPK proteins were essentially constant across the time course studied and between basal and 2-MeSADP-treated groups (Fig. 2B).

The phosphorylation of p38 in serum-starved 1321N1 cells was only just detectable using the phosphospecific antibody, and the level of this activity remained unchanged throughout the time course investigated following application of either vehicle control (basal) or 2-MeSADP (300 nM) (Fig. 2B). Differential levels of phosphorylation were detected for the SAPKs after serum starvation and that observed for the isoform with apparent molecular mass of 45 kDa was much more pronounced than that for the other SAPK isoforms present (46, 48, and 54 kDa). Following application of vehicle alone, none of the isoforms showed any change in their activity status over the 4-h period investigated (Fig. 2B). A transient increase in the phosphorylation of the 45-, 46-, and 54-kDa forms was observed upon application of 2-MeSADP (300 nM), reaching a maximum by 15 min and returning to basal levels by 1 h (Fig. 2B). In contrast, the activity status of the 48-kDa isoform remained unaffected by 2-MeSADP (300 nM) over the time course studied. The level of immunoreactivity using phospho-independent antibodies was comparable for p38 and the p45 and p54 forms of the SAPKs. The level detected for the remaining SAPK isoforms, however, was much lower, particularly that with a molecular mass of 46 kDa. The levels of the p38 and the SAPK proteins were essentially constant across the time course studied and between basal and 2-MeSADP-treated groups (Fig. 2B). Effects of an Adenosine Triphosphate and ADP—Activation of the human recombinant P2Y<sub>1</sub> receptor by 2-MeSATP or by ADP (0.1–10 μM) induced concentration-dependent phosphorylation changes in ERK1 and ERK2 (Fig. 3A). However, in contrast to that evoked by 2-MeSADP (300 nM), the enhanced immunoreactivity was transient in duration, with increased phosphorylation detected at 15 min but not following 120 min of exposure to either ADP or 2-MeSATP, even at the highest concentration tested (Fig. 3A). The level of ERK1 and ERK2

**Fig. 1.** Detection of the heterologously expressed human recombinant P2Y<sub>1</sub> receptor and the complement of G protein α subunits in 1321N1 astrocytoma cells. A, immunocytochemical localization of the human P2Y<sub>1</sub> receptor expressed in astrocytoma cells was determined using the COOH-terminally directed anti-P2Y<sub>1</sub> receptor antibody (1:200 dilution) in the presence of Triton X-100. Control cells were incubated without primary antibody. Scale bar, 50 μm. B and C, whole cell protein extracts prepared from 1321N1 cells were electrophoretically separated on 10% polyacrylamide gels. Following transfer onto nitrocellulose, the membranes were probed either with serial dilutions of the anti-P2Y<sub>1</sub> receptor antibody (B) or with antibodies directed against the α subunits of G proteins (at 1:250 dilution except for s<sub>1</sub> that was at a 1:100 dilution) (C). The electrophoretic mobilities of marker proteins are also shown (arrows). The data are representative of at least three separate experiments.

**Fig. 2.** Effect of 2-MeSADP on the phosphorylation status of ERK1 and ERK2 (A) or p38 and SAPKs (B) in astrocytoma cells heterologously expressing P2Y<sub>1</sub> receptors. Whole cell extracts were prepared from cells that had been serum-starved for 4 h (T<sub>b</sub>) before incubation with incomplete media (Basal) or 2-MeSADP (300 nM) for the times shown in minutes. Samples were analyzed by Western detection following separation on 10% polyacrylamide gels. Consistency of protein loading was substantiated by determining the immunoreactivity of samples with phosphorylation state independent antibodies (top panels). Phosphorylation changes were demonstrated by detection with an antibody to ERK1 and ERK2 that recognizes only the dually phosphorylated (at Thr<sup>202</sup> and Tyr<sup>204</sup>) and hence active forms (ERK-P). Similarly, SAPK and p38 activations were assessed using antibodies specific for the doubly phosphorylated forms of all isoforms at residues Thr<sup>180</sup> and Tyr<sup>182</sup> (p46-P and p54-P shown) or Thr<sup>185</sup> and Tyr<sup>187</sup> (p38-P), respectively.
2-MeSADP in cells stably expressing the recombinant P2Y<sub>1</sub> receptor, the enhanced immunoreactivity was observed at both 15 and 120 min. No change could be detected at either time point examined in the phosphorylation status of any of the other SAPK isoforms detected or of p38 (Fig. 3C). Similar immunoreactive changes were observed following carbachol treatment in the astrocytoma cells expressing the recombinant P2Y<sub>1</sub> receptor (data not shown). The level of the MAP kinase protein content was consistent across the different treatment groups in the wild-type astrocytoma cells and showed an expression pattern that was identical to that observed in the transfected line (Fig. 3).

2-MeSADP Concentration-Response Relationship and Antagonism of the P2Y<sub>1</sub> Receptor-mediated Phosphorylation of MAP Kinases—The concentration dependence of the phosphorylation changes induced by 2-MeSADP at the human recombinant P2Y<sub>1</sub> receptor was determined at the peak of the transient response (15 min) and also during the sustained phase of the MAP kinase activity profiles (2 h). Enhanced immunoreactivity was observed using the anti-phosphospecific ERK antibody, in samples from 1321N1 cells incubated for 15 min with increasing concentrations of 2-MeSADP, until a maximal response was obtained at 30 nM (Fig. 4A). At 120 min (sustained phase), the phosphorylation of ERK1 and ERK2 also increased in a concentration-dependent manner, with a maximal response at and above 100 nM 2-MeSADP (Fig. 4B). In contrast, the phosphorylation of the SAPK isoforms at 15 min continued to increase over the entire concentration range of 2-MeSADP used (1–1000 nM) (Fig. 5A). Phosphorylation of p38 and the 48-kDa isoform of SAPK remained unchanged over basal levels following incubation for 15 min with increasing concentrations of 2-MeSADP (up to 1 μM). In addition, no changes were observed in the phosphorylation states of p38 or any of the SAPK isoforms at 120 min over the concentration range of 2-MeSADP examined (Fig. 5B). At all 2-MeSADP concentrations used in this study the level of expression of the MAP kinases was unaffected (Figs. 4 and 5).
To confirm that changes in the phosphorylation state of the MAP kinases observed upon application of 2-MeSADP were due to the activation of the heterologously expressed P2Y1 receptor, the transfected cells were preincubated with the P2Y1 receptor-specific antagonist, A2P5P (10 μM), for 30 min before 15-min (A) or 120-min (B) incubation with increasing concentrations of 2-MeSADP (nM). Consistency of protein loading was substantiated by determining the immunoreactivity of samples with phosphorylation state independent antibodies to p38 and the SAPKs (top panels). Phosphorylation changes were demonstrated by detection with an antibody to p38 that recognizes only the dually phosphorylated (at Thr180 and Tyr182) and hence active forms (p38-P). Similarly, SAPK activation was assessed using an antibody specific for the doubly phosphorylated forms of all SAPK isoforms at residues Thr183 and Tyr185 within the TPY sequence.

To confirm that changes in the phosphorylation state of the MAP kinases observed upon application of 2-MeSADP were due to the activation of the heterologously expressed P2Y1 receptor, the transfected cells were preincubated with the P2Y1 receptor-specific antagonist, A2P5P (10 μM), for 30 min. Phosphorylation of ERK1 and ERK2 induced by a 15-min incubation with 2-MeSADP was abolished over the agonist concentration range 1–100 nM in cells pretreated with the antagonist and partially inhibited when using the agonist at concentrations of 300 nM or greater (Fig. 4A). The activation of the SAPK isoforms induced by 2-MeSADP at 15 min was similarly abolished by pretreatment with A2P5P (10 μM) at all concentrations of agonist tested (Fig. 5A). The activity of the 48-kDa SAPK isoform and of p38 was unaffected by treatment with the P2Y1 receptor-selective antagonist. Phosphorylation of ERK1 and ERK2 induced by incubation with 2-MeSADP (1–1000 nM) for 120 min was also greatly attenuated in cells pretreated with A2P5P (Fig. 4B), whereas basal phosphorylation of p38 and all the SAPK isoforms observed at this time point remained unchanged (Fig. 5B). The antagonist had no effect on the expression levels of the MAP kinases or on their basal phosphorylation status at the times investigated (Fig. 6).

**Effect of MEK1, PKC, Src, and PI 3-K Inhibitors on the Induced Phosphorylation of ERK1 and ERK2 by P2Y1 Receptors Expressed in 1321N1 Cells**—The effect of selective inhibitors of MEK1 (PD 98059, 20 μM), Src (PP1, 200 nM), PI 3-K (LY 294002, 100 μM), or PKC (Go 6976 for Ca2+-dependent isoforms, 10 nM, and Ro 32–1432 for all isoforms, 50 nM) on the phosphorylation of ERK1 and ERK2 induced by 2-MeSADP (50 nM) was examined. Preincubation of the cells with these inhibitors for 10 min had no observable effect on basal levels of ERK phosphorylation obtained at 15 min (Fig. 7A). The 2-MeSADP-induced phosphorylation at 15 min was greatly inhibited by pretreatment with the PI 3-K or Src inhibitors as well as by either of the PKC inhibitors. PD 98059 at the concentration used was less effective in reducing the 2-MeSADP-induced
phosphorylation (Fig. 7A). Basal levels of ERK phosphorylation observed at 120 min were unaffected by preincubation with LY 294002, PP1, or PD 98059 (Fig. 7B), whereas that induced by 2-MeSADP was abolished by LY 294002 and PD 98059 but unaffected by PP1 (Fig. 7B). In contrast, both protein kinase C inhibitors increased basal levels of ERK phosphorylation, comparable to that observed following activation by 2-MeSADP (Fig. 7B). This enhanced basal activity of ERK1 and ERK2 after a 2-h period in the presence of the PKC inhibitors was also evident in non-transfected astrocytoma cells (data not shown). The expression levels of ERK1 and ERK2 were unaffected by the kinase inhibitors at both time points examined (Fig. 7).

**Effect of a Dominant Negative Mutant of Ras or Pertussis Toxin Pretreatment on P2Y₁ Receptor-mediated ERK Phosphorylation**—To evaluate the involvement of Ras in mediating the activation of ERK by P2Y₁ receptors, transient expression of the dominant negative mutant of Ras (RasNS-17) was performed. This Ras mutant, in which amino acid 17 (serine) is changed to asparagine, is thought to function by inhibiting guanine nucleotide exchange factors (42). The increase in RasNS-17 levels following transfection was evaluated by immunoblotting cell extracts immediately prior to drug addition with a polyclonal antibody to Ras. The inset in Fig. 7B shows that in mock-transfected cells the immunoreactivity with the anti-Ras antibody was almost undetectable, compared with the intense reactivity obtained from the same number of cells transfected with pCMV-MKK4(K95R) (inset, Fig. 7D). The pattern of 2-MeSADP-induced SAPK phosphorylation was similar in cells containing the empty plasmid compared with those that had not been transiently transfected (Fig. 7D). Transfection with either plasmid showed no apparent effect on basal levels of SAPK phosphorylation observed at 15 min (Fig. 7D).

**Regulation of Transcription Factors by the Human P2Y₁ Receptor**—Phosphorylation of transcription factors is a prerequisite for their activation (43). By using phospho-specific antibodies, the regulation of c-Jun, activating transcription factor-2 (ATF-2) and Elk-1 by the recombinant P2Y₁ receptor, was determined using Western analysis of whole cell extracts from the P2Y₁ receptor-expressing cell line. Phosphorylation of the transcription factors c-Jun and Elk-1 was detectable under basal conditions following incubation with incomplete media and remained unchanged over the time course investigated (Fig. 9A). There was only faint immunoreactivity detectable at all time points examined under basal conditions using antibodies to phosphorylated ATF-2. Incubation with 2-MeSADP (300 nM) had no effect on the basal immunoreactivity detected for c-Jun or ATF-2 over the time course investigated (Fig. 9A). However, 2-MeSADP increased the phosphorylation of Elk-1 over time, reaching a maximal response following 60 min of agonist application and which was maintained throughout the duration of the time course (Fig. 9A). The level of expression of the transcription factors was unaffected by the application of 2-MeSADP and unchanged over the period investigated (data not shown). The phosphorylation of Elk-1 induced by 2-MeSADP at 60 min was inhibited by a 10-min preincubation with PD 98059 (20 μM). Basal levels of Elk-1 phosphorylation at this time point were also slightly inhibited by the MEK1 inhibitor (Fig. 9B). The expression levels of Elk-1 were unaffected by all treatments (Fig. 9B).

**Induction of Apoptosis but Not Cell Proliferation by the Human Recombinant P2Y₁ Receptor and Effect of Ras and MKK4 Dominant Negative Mutants**—Following treatment for 5 h with either 2-MeSADP (300 nM) (Fig. 10A) or staurosporin (300 nM) (data not shown), some astrocytoma cells fluoresced brightly in the presence of annexin V-FITC which were not counterstained by propidium iodide. In these cells FITC green fluorescence patches could be observed by confocal microscopy that were located on the cell surface (Fig. 10, B and C). None of the control cells incubated in media alone showed any detectable annexin V binding at this time point (data not shown). Activation of phosphorylation (Fig. 8A). The level of expression of ERK1 and ERK2 protein was unchanged by pertussis toxin pretreatment (Fig. 8C).

**Effect of a Dominant Negative Mutant of MKK4 on P2Y₁ Receptor-mediated SAPK Phosphorylation**—SAPKs are activated by phosphorylation on threonine and tyrosine within the activation motif by one of two cloned dual specificity kinases, MKK4 and MKK7. These kinases are in turn activated by an MKKK, of which several examples have been identified. Transient expression (54% efficiency) of a dominant negative mutant of MKK4 in astrocytoma cells was found to decrease the levels of SAPK phosphorylation induced by a 15-min application of 2-MeSADP (300 nM), compared with mock-transfected controls (Fig. 8D). However, the suppression of the activity status of the higher molecular weight SAPK species appeared to be greater than for the isoform with apparent molecular mass of 45 kDa (Fig. 8C). Expression of the mutant MKK4 protein was determined by immunoblotting the transient transfected cell extracts immediately prior to 2-MeSADP addition. The immunoreactivity with the anti-MKK4 antibody was weak in mock-transfected cells compared with the intense reactivity obtained from the same number of cells transfected with pCMV-MKK4(K95R) (inset, Fig. 8D). The pattern of 2-MeSADP-induced SAPK phosphorylation was similar in cells containing the empty plasmid with those that had not been transiently transfected (Fig. 8D). Transfection with either plasmid showed no apparent effect on basal levels of SAPK phosphorylation observed at 15 min (Fig. 8D).
P2Y1 receptors. Inhibition negative MKK4 on SAPK activation in cells expressing the phosphorylation of ERK1 and ERK2 and the effect of dominant negative Ha-RAS expression also had no effect on the caspase-3 activity induced by 2-MeSADP, whereas the kinase-deficient MKK4 mutant partially blocked this latter response (Fig. 10E). Transfection of cells (48 h prior to the addition of 2-MeSADP) with dominant negative forms of either Ras or MKK4 had no significant effect on basal levels of caspase-3 activity compared with mock-transfected cells (data not shown) and were similar to the values obtained for non-transfected cells expressing the P2Y1 receptor (Fig. 10E). Dominant negative Ras expression also had no effect on the caspase-3 activity induced by 2-MeSADP, whereas the kinase-deficient MKK4 mutant partially blocked this latter response (Fig. 10E).

The ability of 2-MeSADP to modulate the proliferative outcome of astrocytoma cells expressing the recombinant P2Y1 receptor was also assessed. Application of 2-MeSADP (300 nM) incubated for 15 min with incomplete media (CON) or 2-MeSADP (MeS, 50 nM) following pertussis toxin pretreatment (PTX, 18 h at 100 ng ml\(^{-1}\)) and analyzed by Western blotting using phospho-specific anti-ERK antibodies (ERK-P). D, whole cell extracts were also prepared from cells that had been transiently transfected with the empty plasmid (Mock) or that incorporating dominant negative MKK4 (MeS, 300 nM). The effect of these agents on the phosphorylation status of the SAPKs and p38 is shown together with data from non-transiently transfected cells. The Western blot is a representative from two separate transfections, and each panel has been taken from a single immunoblot. The inset shows the level of expression of MKK4 immediately prior to drug addition.

The ability of 2-MeSADP to modulate the proliferative outcome of astrocytoma cells expressing the recombinant P2Y1 receptor was also assessed. Application of 2-MeSADP (300 nM) incubated for 15 min with incomplete media (CON) or 2-MeSADP (MeS, 50 nM) following pertussis toxin pretreatment (PTX, 18 h at 100 ng ml\(^{-1}\)) and analyzed by Western blotting using phospho-specific anti-ERK antibodies (ERK-P). D, whole cell extracts were also prepared from cells that had been transiently transfected with the empty plasmid (Mock) or that incorporating dominant negative MKK4 (MeS, 300 nM). The effect of these agents on the phosphorylation status of the SAPKs and p38 is shown together with data from non-transiently transfected cells. The Western blot is a representative from two separate transfections. The inset shows the level of expression of MKK4 immediately prior to drug addition.
in the absence of other exogenously administered mitogenic factors showed a small but significant decrease in the number of cells counted 24 h later, as compared with basal, which was not observed in cells transiently transfected with the MKK4 mutant (Fig. 10F). In contrast, fetal calf serum (1%) caused a significant increase in cell number (Fig. 10G) that was unaffected by the addition of 2-MeSADP at concentrations of 0.3 or 3 μM (Fig. 10G). A marked increase in cell number was also obtained following application of carbachol (10 mM) that was attenuated by the presence of 2-MeSADP (300 nM) (Fig. 10G).

**DISCUSSION**

In this study, we have examined the ability of the human P2Y₁ receptor to stimulate the MAP kinase transduction cascades and to determine if this activity could be correlated with transcription factor phosphorylation or the proliferative outcome of the host cell. Whereas a stably transfected cell line was employed for these studies, the host cell was derived from a human type (astrocytic), which in its original parent state contained the appropriate native transductional components for expressing functional P2Y₁ receptors (18) and hence should come of the host cell. Whereas a stably transfected cell line was employed for these studies, the host cell was derived from a human type (astrocytic), which in its original parent state contained the appropriate native transductional components for expressing functional P2Y₁ receptors (18) and hence should...

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**Fig. 10.** Effect of 2-MeSADP on annexin V binding, caspase-3 activation, and the proliferation of astrocytoma cells heterologously expressing P2Y₁ receptors. Annexin V binding to astrocytoma cells expressing human P2Y₁ receptors following incubation for 5 h with 2-MeSADP (300 nM) as detected by low power fluorescence microscopy (scale bar, 50 μm) (A) or confocal microscopy (B and C) which shows a cluster of annexin V-positive cells (green) in the center of the field of the propidium iodide-stained cells (red), where B is a single channel overlay confocal image (scale, 146.2 × 146.2 μm), and C is a single overlay of a group of apoptotic cells taken from z series (scale, 59 μm × 59 μm). D, in vitro caspase-3-like protease activity was determined using a colorimetric caspase-3 substrate peptide. D, cell lysates were prepared at the indicated times after incubation of astrocytoma cells in incomplete media (Basal, open histograms), 2-MeSADP (300 nM, closed histograms), or 2-MeSADP (300 nM) following a 30-min preincubation with A2P5P (10 μM, hatched histograms). Values are expressed in arbitrary units as the mean ± S.E. (n = 3). Groups labeled * are significantly different from basal (p < 0.001) and groups labeled # are significantly different from those incubated with 2-MeSADP alone. E, caspase-3 activity was determined 5 h following incubation in incomplete media (Basal, open histograms) or 2-MeSADP (300 nM, closed histograms) with or without a 10-min preincubation with inhibitors of Src (PP1, 200 nM), PI 3-K (LY 294002, 100 μM), MEK1 (PD 98059, 20 μM), or the classical PKC isoforms (Go 6976, 10 nM). Data are also shown for cells transfected 48 h prior to drug addition with either dominant negative Ras or MKK4 mutants. Groups labeled * are significantly different from that without inhibitors present (p < 0.01), and the group labeled # is significantly different from the same treatment but in non-transiently transfected cells. F, the mean number of cells harvested from a single well, 24 h following application of incomplete media (Basal, open histogram) or 2-MeSADP (300 nM, closed histogram). Groups labeled mock had been transfected with the empty vector, and those labeled MKK4 had been transfected with the vector containing the kinase-deficient mutant of MKK4. Values are expressed as the mean cell number ± S.E. (n = 3, four replicates). The group labeled * is significantly different from basal (p < 0.01), and that labeled # is significantly different (p < 0.01) from that incubated in the presence of carbachol alone.
the recombinant receptor. The presence of P2Y<sub>1</sub> as the sole P2Y receptor type has enabled the transduction cascades of an identified receptor type to be characterized. Definitive receptor specificity was also confirmed by the suppression of the stimulated responses by a P2Y<sub>1</sub> receptor-selective antagonist. Expression of the P2Y<sub>1</sub> receptor protein at the cell membrane of the transfected cells was demonstrated by immunocytochemistry, using an anti-peptide antibody specific for the human P2Y<sub>1</sub> receptor subtype. The size of the immunoreactive band detected in Western blots of the astrocytoma cells is consistent with the known polypeptide size of the P2Y<sub>1</sub> receptor (373 amino acids, 42 kDa) and its glycosylation (1).

Functional responses of the P2Y<sub>1</sub> receptor have been shown to be mediated through G<sub>q</sub> and/or G<sub>q</sub>-mediated pathways, which were shown to be expressed by the astrocytoma host cells used in this study. Upon agonist stimulation, G protein-coupled receptors transduce their effects through both the GTP-bound G<sub>q</sub> and the dissociated G<sub>q</sub> component of the heterotrimeric G protein, regulating directly downstream effectors (44) including adenylyl cyclases, phospholipase C isoforms, ion channels, PI 3-K (45), and Tec family tyrosine kinases (46). Several G protein-coupled receptors have been shown to stimulate the ERK pathway through a variety of G protein subunits. In the case of the G<sub>q11</sub>-coupled m<sub>3</sub>-muscarinic acetylcholine and α<sub>q</sub>-adrenergic receptors, the activation of ERK is mediated mainly by G<sub>q</sub><sub>q11</sub>. In contrast, G<sub>q</sub>-coupled m<sub>3</m>uscarinic acetylcholine, α<sub>q</sub>-adrenergic, somatostatin sst<sub>2</sub>, and the G<sub>q</sub>-coupled β-adrenergic receptors, all induce ERK activation through G<sub>q</sub> release and the subsequent stimulation of tyrosine kinases such as Src (47, 48). The pathways downstream of G protein coupling have not previously been established for any molecularly defined P2Y receptor subtype. In the present report, the potential actions of nucleotides at the P2Y<sub>1</sub> receptor in stimulating the MAP kinase cascades were investigated. The P2Y<sub>1</sub> receptor had no effect on the activity status of p38 kinase but caused a marked phosphorylation of the SAPK and the ERK cascades. For the ERK cascade, this was shown to be via a pertussis toxin-insensitive pathway through a number of transduction mediators including PKC, PI 3-K, and Src, with apparent cooperative effects.

The mechanisms by which G<sub>q</sub>- and G<sub>q</sub>-coupled receptors typically activate ERK are through Ras-dependent or protein kinase C-dependent pathways, respectively. However, several exceptions to this rule have been reported for G<sub>q</sub>-coupled receptors, in that ERK can be activated through a pertussis toxin-insensitive and PKC-independent pathway (49). The recombinant P2Y<sub>1</sub> receptor utilizes transduction cascades to activate ERKs that are thus commonly associated with G<sub>q</sub>-linked receptors (PKC activation) in addition to the recruitment of Src implying some Ras dependence, which is more typical of G<sub>q</sub>-coupled receptors. An involvement of Ras in the mediation of the P2Y<sub>1</sub> receptor-induced ERK activity was demonstrated in this study by overexpression of a dominant negative Ras mutant. However, although inhibition of Src or Ras attenuated the transient phase of the ERK activity profile, these transduction effectors did not seem necessary for the sustained activation of ERK. This differential requirement for transduction mediators during the time course of ERK activation has also recently been demonstrated for the G<sub>q</sub>-coupled somatostatin sst<sub>2</sub> receptor in which the acute phase is both Src- and Ras-dependent, but the prolonged ERK response is mediated by protein kinase C (50). The mobilization of Ca<sup>2+</sup> through the P2Y<sub>1</sub> receptor could account for the stimulation of the Ca<sup>2+</sup>-dependent PKC isoforms that have been shown to activate the ERK cascade at the point of Raf (51) and thus through a Ras-independent mechanism.

The PI 3-K pathway is also important for regulating ERK activity by a number of mechanisms that have been shown to occur both upstream and downstream of Ras (52, 53). The sustained phase of ERK phosphorylation mediated through the P2Y<sub>1</sub> receptor was abolished following application of a PI 3-K inhibitor, whereas the transient phase was only partially dependent on this kinase activity. It thus appears that the transient activation of ERK by the P2Y<sub>1</sub> receptor is mediated through the cooperative effects of Src, Ras, Ca<sup>2+</sup>-dependent PKC isoforms as well as PI 3-K, whereas the sustained phase requires only PI 3-K activity (and possibly PKC). This mechanism may be similar to that reported for G<sub>i</sub>-protein-coupled receptors that have been shown to activate ERK via a Ras-independent pathway through PI 3-K and PKC<sub>e</sub> (54). Stimulation of PKC isoforms in addition to their requirement for allosteric activators has recently been shown to be critical on subsequent phosphorylation, possibly through the PI 3-K-dependent kinase, PDK1 (55). In accord with this concept is the finding that ERK phosphorylation by the P2Y<sub>1</sub> receptor requires PI 3-K for both the acute and sustained phases. We could not conclude if the sustained phase of ERK activation is additionally mediated via PKC due to the marked increase in basal ERK activity observed in the presence of various PKC inhibitors. The cause of this ERK stimulation following prolonged PKC down-regulation has not been determined but could also be observed in non-transfected astrocytoma cells. The ineffectiveness of the MEK1 inhibitor at blocking the P2Y<sub>1</sub> receptor-mediated transient activation of ERK1 and ERK2 may reflect the inability of PD 98059 to abolish high intensity signals (56). However, PD 98059 is a MEK1-selective inhibitor, and it is possible that ERK activation through P2Y<sub>1</sub> receptors is primarily mediated through MEK2 in the early transductional events and via MEK1 for the sustained phase of stimulation.

The rapid and sustained phosphorylation of both ERK1 and ERK2 induced by the potent P2Y<sub>1</sub> receptor agonist, 2-MeSADP, was abolished by the P2Y<sub>1</sub> receptor-specific antagonist, A2P5P. The concentration dependence of this ERK activity was similar to that observed with 2-MeSADP for other functional responses mediated by this receptor type (EC<sub>50</sub> values of the order of 10 nM) (9–11, 57). The natural ligand ADP, although less potent than 2-MeSADP, induced phosphorylation of ERK1 and ERK2 that was comparable to that evoked by 2-MeSADP for other functional responses mediated by this receptor type (EC<sub>50</sub> values of the order of 10 nM) (9–11, 57). The natural ligand ADP, although less potent than 2-MeSADP, induced phosphorylation of ERK1 and ERK2 that was comparable to that evoked by 2-MeSADP, inducing phosphorylation of ERK1 and ERK2 that was comparable to that evoked by 2-MsATP, supporting recent evidence that uncontaminated adenosine triphosphates can serve as agonists at the recombinant P2Y<sub>1</sub> receptor (10, 11). Release of ATP by cells in culture occurs very readily (1) and can also give rise to misleading results; this might have contributed to the transient and low degree of ERK1 and ERK2 stimulation observed in this study following incubation of the astrocytoma cells with incomplete media. However, the P2Y<sub>1</sub> receptor-selective antagonist, A2P5P, showed no effect on the basal levels of ERK phosphorylation, suggesting that this activity may result directly from mechanical stimulation (34).

Although the Ras-ERK cascade is well documented for several G protein-coupled receptors, very little is known of any activation of the other MAP kinases, p38 and the SAPKs, by this receptor class. Activation of p38 has been shown in rat glomerular mesangial cells following stimulation with UTP and ATP, suggesting that this may be mediated through the P2Y<sub>2</sub> receptor (27). In addition, a very recent report on native P2Y receptors in the endothelial cell line, EAhy926, has shown an unusual inhibition of a pre-stimulated SAPK and p38 activity mediated by UTP and suggested to be via P2Y<sub>2</sub> or P2Y<sub>4</sub> receptors (58). In the present study, we have shown differential activation of members of the SAPK family through the P2Y<sub>1</sub> receptor but not of p38. The kinetic profiles of the SAPK iso-
forms activated, those with apparent molecular masses of 45, 46, and 54 kDa, were similar and transient, although a fourth immunoreactive band of 48 kDa that was identified by both the phospho-dependent and -independent SAPK antibodies remained unaffected by 2-MeSADP, ADP, or 2-MeSATP. Phosphorylation of the p54, p46, and p45 SAPK isoforms was inhibited by the P2Y<sub>1</sub> receptor-specific antagonist, A2P5P. At least 10 SAPK isoforms have been identified that correspond to alternatively spliced isoforms derived from the JNK1, JNK2, and JNK3 genes (59). The consequence of the differential activation of the SAPK isoforms identified in the astrocytoma cells by the P2Y<sub>1</sub> receptor remains to be determined, but possibly provides fine-tuning of various cellular events including apoptosis (see below). In addition, this differential activation suggests that discrete mechanisms are in place upstream of the individual SAPK family members. Stimulation of only the p45 form of the SAPKs by the muscarinic receptor agonist, carbachol, and the finding that this displayed a different kinetic profile to that induced by the P2Y<sub>1</sub> receptor provide additional evidence that members of this MAP kinase family are regulated via distinct mechanisms in the same host cell.

Proximally, SAPKs are activated by a cascade of kinases (17), although the upstream regulators in this pathway are incompletely characterized. Tumor necrosis factor-α-stimulated SAPK activation is perhaps best described and involves recruitment of the adapter protein TRAF2 to the cytosolic portion of the ligated tumor necrosis factor-α receptor (60). Other intermediates have been proposed to play a role in different models of SAPK activation including oxidative stress, DNA damage, altered ion fluxes, and caspase proteases. However, the present study suggests that caspase-3 activation is not an obligatory step in the signaling pathway coupling P2Y<sub>1</sub> receptors to SAPK activation, as this MAP kinase activity preceded the accumulation of active caspase-3 by several hours. It is possible that changes in intracellular Ca<sup>2+</sup> (61) may be important for the P2Y<sub>1</sub> receptor-mediated SAPK activation. SAPKs are activated by phosphorylation on threonine and tyrosine residues by one of two cloned dual specificity kinases, MKK4 and MKK7. The dependence on MKK4 for the activation of the p54 and p46 SAPK isoforms mediated by the P2Y<sub>1</sub> receptor was substantiated by showing a decrease in their phosphorylation status following expression of a kinase-deficient MKK4 mutant. The induced phosphorylation of the p45 isoform by 2-MoSADP was only slightly affected by the MKK4 mutant, which may possibly reflect the achieved transient transfection efficiency or that MKK7 may be the preferred upstream regulator of this kinase.

The apparent lack of phosphorylation of c-Jun and of ATF-2 transcription factors, both known substrates for the SAPKs, suggests that the observed transient activation of these kinases, induced via the recombinant P2Y<sub>1</sub> receptor, is insufficient for their cytoplasmic nuclear translocation. In every case studied so far, sustained ERK activation is required for nuclear-targeted transcription factor phosphorylation, although similar observations for the SAPK family have not as yet been reported. Consistent with this hypothesis is the demonstration that sustained ERK activity induced through the P2Y<sub>1</sub> receptor produced Elk-1 phosphorylation. These data, taken together, thus suggest that the strength and duration of the stimulus to other MAP kinase family members can also be important determinants that govern the biological response to a particular receptor activation.

The functional responses mediated by events downstream from the activation of P2Y receptors are poorly understood. It has been suggested that prostacyclin production following activation via P2Y receptors on endothelial cells is via ERK activation (20), consistent with the mechanism utilized by endothelial cells to regulate vascular smooth muscle cell proliferation. Proliferative activity has been reported using UTP in C<sub>6</sub> glioma cells through P2Y<sub>2</sub> receptor-mediated stimulation of the Ras-ERK pathway (28). P2Y<sub>4</sub> receptors in rat glomerular mesangial cells have been shown to induce proliferation (29), whereas stimulation via the P2X<sub>7</sub> receptor induces apoptosis (32). ERK is almost universally stimulated by mitogens and cell survival factors such as growth factors, hormones, and cytokines and is intimately connected with the regulation of cell growth as well as differentiation. SAPK and p38 on the other hand are activated by various stressors such as chemical agents and ultraviolet irradiation, tumor necrosis factor, and interleukin-1, which appear to play a decisive role in the control of cell death. A necessary role of SAPK in apoptotic induction by UV irradiation, but not Fas receptor ligation, was demonstrated in a recent study using embryonic fibroblasts derived from double knockout mice that lack the expression of both the JNK1 and JNK2 genes (62). It has been suggested that the ability of a cell to die or survive and proliferate may be dictated by a critical balance between the signaling pathways involving the various MAP kinase family members.

In the present study, we have shown that despite the sustained activation of ERK1 and ERK2 by the P2Y<sub>1</sub> receptor, a proliferative activity was not apparent. This is analogous to the situation with the somatostatin sst<sub>2(A)</sub> receptor, which mediates a strong and sustained activity of both ERK1 and ERK2 but that is associated with a concomitant anti-proliferative effect (48). However, activation of the endogenous muscarinic receptors in the astrocytoma cells induced a sustained ERK activation and an associated increase in cell number, suggesting that the proliferative outcome of this cell type is regulated by a complex interplay of transduction cascades. A role for the SAPK family members activated by the P2Y<sub>1</sub> receptor in the induction of an apoptotic event was confirmed by showing that the 2-MoSADP-stimulated caspase-3 activity and the associated decrease in cell number was inhibited by the presence of the dominant negative MKK4 mutant. The partial nature of the decrease in both cases could reflect the degree of transient transfection efficiency obtained (∼50%). However, it would seem that the p54 and p46 isoforms are much more important for apoptotic function than the p45 SAPK member, as the activity of this latter isoform was relatively unaffected by the MKK4 mutant and was strongly increased by the application of carbachol. The P2Y<sub>1</sub> receptor-induced caspase-3 activity, which could be blocked by the selective antagonist, A2P5P, appeared to be unaffected by inhibitors of the Ras-ERK cascade (including the Src inhibitor and a dominant negative Ras mutant). This suggests that there is little cross-talk between the ERK and SAPK cascades activated by the P2Y<sub>1</sub> receptor. The role of PI 3-K in regulating the apoptotic function of 2-MoSADP is less clear, as LY 294002 enhanced the basal rate of caspase-3 activity as well as that mediated by the P2Y<sub>1</sub> receptor. It is possible that the PI 3-K cascade is having an additional effect on maintaining cell survival that is independent of SAPK inhibition and presumably of its ability to induce ERK phosphorylation. Interestingly, the P2Y<sub>1</sub> receptor-mediated apoptotic activity was not sufficient to inhibit cell proliferation induced by 1% serum, despite high concentrations of 2-MoSADP being used to circumvent any problems associated with ligand depletion. It is possible that the transient activation of the SAPK isoforms through P2Y<sub>1</sub> receptor activation may be to regulate apoptotic events rather than to induce cell death as shown for the P2X<sub>7</sub> receptor that mediates sustained activation of the SAPKs in the presence of serum (32). However, the proliferative activity induced by carbachol was attenuated by the pres-
ence of 2-MeSADP, further suggesting that its ability to induce apoptosis is dependent on the net activity of transductional cascades.

In summary, we have demonstrated that the P2Y receptor can stimulate the prolonged activation of the ERK cascade leading to the phosphorylation of the transcription factor, Elk-1. This sustained ERK activation is critically dependent on PI 3-K, whereas the transient phase is mediated through Ras with an additional input from both PI 3-K and PKC isoforms. Transient activation of the SAPKs in this system is insufficient for transcription factor phosphorylation but appears to regulate apoptosis through a caspase-3-dependent mechanism. Hence, an identified member of the P2Y receptor family can activate SAPKs (but spares p38 kinase) and can evoke caspase proteolytic activity. This duality suggests that cell proliferation by extracellular nucleotides may be regulated by a critical balance of the activities of those receptor types, which mediate mitogenic or apoptotic processes.

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