JOURNAL OF CELLULAR PHYSIOLOGY 187:196–208 (2001)

$P2Y_2$ Nucleotide Receptor Signaling in Human Monocytic Cells: Activation, Desensitization, and Coupling to Mitogen-Activated Protein Kinases

LAURA I. SANTIAGO-PÉREZ,[1] ROSA V. FLORES,[1] CYNTHIA SANTOS-BERRÍOS,[1] NATALIYA E. CHORNA,[1] BRENT KRUGH,[2] RICHARD C. GARRAD,[2] LAURIE ERB,[2] GARY A. WEISMAN,[2] AND FERNANDO A. GONZÁLEZ[1]*

[1]*Department of Chemistry, University of Puerto Rico, San Juan, Puerto Rico*
[2]*Department of Biochemistry, University of Missouri-Columbia, Columbia, Missouri*

Activation of $P2Y_2$ receptors by extracellular nucleotides has been shown to induce phenotypic differentiation of human promonocytic U937 cells that is associated with the inflammatory response. The $P2Y_2$ receptor agonist, UTP, induced the phosphorylation of the MAP kinases MEK1/2 and ERK1/2 in a sequential manner, since ERK1/2 phosphorylation was abolished by the MEK1/2 inhibitor PD 098059. Other results indicated that $P2Y_2$ receptors can couple to MAP kinases via phosphatidylinositol 3-kinase (PI3K) and c-src. Accordingly, ERK1/2 phosphorylation induced by UTP was inhibited by the PI3K inhibitors, wortmannin and LY294002, and the c-src inhibitors, radicicol and PP2, but not by inhibitors of protein kinase C (PKC). The phosphorylation of ERK1/2 was independent of the ability of $P2Y_2$ receptors to increase the concentration of intracellular free calcium, since chelation of intracellular calcium by BAPTA did not diminish the phosphorylation of ERK1/2 induced by UTP. A 5-minute treatment with UTP reduced U937 cell responsiveness to a subsequent UTP challenge. UTP-induced desensitization was characterized by an increase in the EC_{50} for receptor activation (from 0.44 to 9.3 μM) and a dramatic (~75%) decrease in the maximal calcium mobilization induced by a supramaximal dose of UTP. Phorbol ester treatment also caused $P2Y_2$ receptor desensitization ($EC_{50} = 12.3$ μM UTP and maximal calcium mobilization reduced by ~33%). The protein kinase C inhibitor GF 109203X failed to significantly inhibit the UTP-induced desensitization of the $P2Y_2$ receptor, whereas the protein phosphatase inhibitor okadaic acid blocked receptor resensitization. Recovery of receptor activity after UTP-induced desensitization was evident in cells treated with agonist for 5 or 30 min. However, $P2Y_2$ receptor activity remained partially desensitized 30 min after pretreatment of cells with UTP for 1 h or longer. This sustained desensitized state correlated with a decrease in $P2Y_2$ receptor mRNA levels. Desensitization of ERK1/2 phosphorylation was induced by a 5-minute pretreatment with UTP, and cell responsiveness did not return even after a 30-minute incubation of cells in the absence of an agonist. Results suggest that desensitization of the $P2Y_2$ receptor may involve covalent modifications (i.e., receptor phosphorylation) that functionally uncouple the receptor from the calcium signaling pathway, and that transcriptional regulation may play a role in long-term desensitization. Our results indicate that calcium mobilization and ERK1/2 phosphorylation induced by $P2Y_2$ receptor activation are independent events in U937 monocytes. J. Cell. Physiol. 187:196–208, 2001.

© 2001 Wiley-Liss, Inc.

Pro-inflammatory activation of monocytes is an important stage of myeloid differentiation. The human promonocytic U937 cell line is a progenitor cell model that has been extensively used for studies of myelomonocytic differentiation induced by a wide variety of physiological and pharmacological agents. For example,

© 2001 WILEY-LISS, INC.

Contract grant sponsor: NIH-NCRR; Contract grant number: G12 RR03641.

*Correspondence to: Fernando A. González, University of Puerto Rico, Department of Chemistry, P.O. Box 23346, San Juan, PR 00931-3346. E-mail: fgonzal@upracd.upr.clu.edu

Received 30 March 2000; Accepted 30 November 2000

Published online in Wiley InterScience, 7 March 2001.

phorbol esters, vitamin D, and cytokines have been shown to induce monocyte to macrophage patterns of maturation in U937 cells (Cowen et al., 1991). Extracellular ATP and UTP through activation of a G protein-coupled P2Y$_2$ receptor causes the mobilization of intracellular calcium stores in U937 cells by a phospholipase C (PLC)-dependent pathway leading to phenotypic changes characteristic of monocyte to macrophage differentiation, such as inhibition of proliferation, increased expression of complement receptors types 1 and 3 (CR1 and CR3), and increased responsiveness to chemotactic peptides (Cowen et al., 1989, 1991). Extracellular ATP also has been shown to activate phospholipase D (Kusner et al., 1993). The cellular mechanisms whereby P2Y$_2$ receptors modulate myelomonocytic differentiation remain to be elucidated.

A role for the mitogen-activated protein kinases (MAPKs) in myelomonocytic differentiation is recognized. MAPKs are a family of intracellular serine-threonine protein kinases that mediate signal transduction between plasma membrane receptors and components in the cytoplasm and nucleus that regulate cellular processes such as proliferation, differentiation, inflammation, and apoptosis (Seger and Krebs, 1995). The activation of MAPKs by covalent modification (i.e., phosphorylation of threonine and tyrosine residues found in a regulatory TXY motif present in all MAPKs) is mediated by threonine-tyrosine protein kinases known as MAPK kinases (Cobb and Goldsmith, 1995). Differentiation of U937 cells induced by phorbol esters and ceramide is mediated by MAPK (Ragg et al., 1998), and the phosphorylation by MAPK of the amino terminus of a TATA-binding protein has been observed (Biggs et al., 1998). Activation of MAPK also occurs during apoptosis induced by taxol and bryostatin 1 in U937 cells (Wang et al., 1998). Phorbol esters can activate the MAPK-specific phosphatase MKP-1 in U937 cells that regulate the inactivation of MAPKs (Franklin and Kraft, 1997). Recently, we found that the MEK1/2 inhibitor PD-098059 and the p38 MAPK inhibitor SB202190 blocked phorbol ester-induced differentiation of U937 cells (Weisman et al., 1998). Therefore, a role for MAPK activation in U937 cell differentiation is appreciated. Several studies have shown that P2Y$_2$ receptors can mediate the activation of MAPKs in various cell types (Neary et al., 1998, 1999; Soltoff, 1998; Soltoff et al., 1998).

The present study investigated the molecular/biochemical consequences of P2Y$_2$ receptor activation and its desensitization in human promonocytic U937 cells. Our results indicate that ERK1/2 activation is dependent on c-src, phosphatidylinositol 3′-kinase (PI3K), and MEK1/2, but not calcium-sensitive protein kinase C (PKC) isoforms or increases in the intracellular calcium concentration, $[Ca^{2+}]_i$. The mechanisms underlying desensitization and resensitization of P2Y$_2$ receptor activity (i.e., UTP-induced calcium mobilization and ERK1/2 activation) after acute and chronic agonist stimulation also were examined. Elucidation of the molecular basis for the effects of nucleotides on the U937 monocyte/macrophage lineage will better define the physiological role of extracellular nucleotides in the inflammation process and may provide novel strategies for regulating inflammation via pharmacotherapy.

MATERIALS AND METHODS

Materials

UTP and other nucleotides and nucleotide analogs were obtained from Boehringer-Mannheim. Kinase and phospho-kinase-specific antibodies were purchased from New England BioLabs. Protein kinase inhibitors were obtained from Calbiochem (La Jolla, CA). Fura-2, fura-2-AM, and BAPTA-AM were obtained from Molecular Probes. Taq polymerase and avian myeloblastosis virus reverse transcriptase were obtained from Promega (Madison, WI). Oligonucleotides were synthesized in a Beckman Oligo 1000 instrument. Culture media and sera were purchased from Invitrogen. All other reagents were obtained from Sigma or Fisher Scientific.

Cell culture

Human monocytic U937 cells were obtained from the American Type Culture Collection (ATCC CRL-1593). U937 cells were cultured in suspension in RPMI 1640 medium supplemented with 10% Cosmic calf serum (Invitrogen) and 1% antimycotic antibiotic solution (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Phosphorylation of MAP kinases

Assays to detect the phosphorylation of MAPKs were performed by a modification of published procedures (Nakashima et al., 1994; Patel et al., 1996). U937 cells in the exponential phase of growth were subcultured at 2×10^6 cells/ml in 35×10 mm sterile plates (Nunclon) and incubated for 18–24 h at 37°C in serum-free RPMI medium. For experiments, the cells were incubated at 37°C in the same medium containing nucleotides, activators or inhibitors for varying time periods. The cells were then placed on ice and harvested by centrifugation ($11,400 \times g$ for 30 sec at 25°C). The pelleted cells were rinsed with ice-cold phosphate-buffered saline (PBS), lysed with 250 μl of ice-cold lysis buffer (25 mM Tris–HCl, pH 7.4, 25 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4(P_2O_7)$, 25 mM β-glycerophosphate, 25 mM *p*-nitrophenylphosphate, 0.5 mM ethylenebis(oxyethylenenitrilo) tetraacetic acid (EGTA), 0.5% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μM okadaic acid) and centrifuged ($8,200 \times g$ for 10 min at 4°C) to remove insoluble material. Then, cells were extracted for 5 min at 96°C with 100 μl of 3x SDS-PAGE loading buffer (187.5 mM Tris–HCl, pH 6.8, 6% (w/v) sodium dodecylsulphate (SDS), 150 μM DL-dithiothreitol (DTT), and 0.3% (w/v) bromophenol blue).

Cell extracts were then subjected to 12% SDS-PAGE, and after the run, the gels were incubated for 30 min in transfer buffer (48 mM Tris base, pH 9.0, 39 mM glycine, 20% (v/v) methanol, and 0.038% (w/v) SDS). The proteins in the gel were transferred to a nitrocellulose membrane using a semi-dry electroblotting transfer device (BioRad, Hercules, CA). The membranes were blocked with 5% (w/v) low fat milk in TBS-T solution (20 mM Tris base, pH 7.6, 137 mM NaCl, and 0.1% (w/v) Tween 20) for 1 h at room temperature with gentle agitation. Then, the membranes were incubated with either rabbit anti-phospho p42/p44 MAPK IgG or rabbit

anti-phospho MEK1/2 IgG (New England BioLabs) overnight at 4°C at 1:1,000 dilution in blocking buffer (5% (w/v) low fat milk in TBS-T solution). The membranes were then rinsed once for 15 min and twice for 5 min with TBS-T solution and incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG (at a dilution of 1:2,000) and anti-biotin IgG conjugated to HRP (1:2,000 dilution) (to detect the biotinylated protein molecular weight markers) in blocking buffer for 1 h at room temperature. The membranes were rinsed three times with TBS-T solution and the proteins were visualized using autoradiographic film and the LumiGlo chemiluminescence system (New England BioLabs). The chemiluminescence in the blots was quantitated using an imaging screen and a Molecular Imager GS-525 (BioRad) and MultiAnalyst software (BioRad) at a Macintosh workstation. For normalization of the signal, the blots were stripped of antibodies by a 30 min incubation at 60°C in stripping buffer (100 mM 2-mercaptoethanol, 2% (w/v) SDS, and 62.5 mM Tris–HCl, pH 6.7) followed by three washes with TBS-T solution, and then reprobed with either anti-p42/p44 MAPK IgG (New England BioLabs) or anti-MEK1/2 IgG (New England BioLabs) (1:1,000 dilution). These antibodies bind to the protein kinases independent of their state of phosphorylation. Detection of bound antibodies was performed as described above. Occasional variations between experiments in the basal level of phosphorylation in cells caused some variations in the relative fold-increase induced by agonists. However, this result did not affect the qualitative interpretation of the data.

ERK1/2 activity assay

ERK1/2 activity assays were performed in vitro using immunopurified protein kinase by a modification of published procedures (Nakashima et al., 1994; Patel et al., 1996). U937 cells in the exponential phase of growth were subcultured at 2×10^6 cells/ml in 35×10 mm sterile plates (Nunclon) and incubated for 18–24 h at 37°C in serum-free RPMI medium. For experiments, the cells were incubated at 37°C in the same medium containing the nucleotide agonist for the indicated period of time. The cells were then placed on ice and harvested by centrifugation (11,400 × g for 30 sec at 25°C). The pelleted cells were rinsed with ice-cold PBS, lysed with 500 μl of ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton X-100, 2.5 mM $Na_4(P_2O_7)$, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM PMSF) and centrifuged (8,200 × g for 10 min at 4°C) to remove nuclei and unbroken cells. An aliquot of the cell extract (200 μl) was incubated with mouse anti-phospho p42/p44 MAPK IgG (New England BioLabs) (1:100 dilution) overnight at 4°C with gentle rocking. Then, 20 μl of a protein A Sepharose bead slurry (Sigma) were added and incubated for 3 h at 4°C with gentle rocking. The beads were microcentrifuged and washed twice with ice-cold lysis buffer and twice with ice-cold kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$).

For the kinase reaction, the pellets were resuspended in kinase buffer supplemented with 200 μM ATP and 2 μg/ml Elk-1 (ERK1/2 specific substrate, (Marais et al., 1993)) (New England BioLabs). The kinase reactions were performed for 30 min at 30°C and terminated during the linear phase of the reaction by incubation with 100 μl of 3x SDS-PAGE loading buffer for 5 min at 96°C. After agitation and microcentrifugation of the termination mixture, the supernatants were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes as described above. The phosphorylation of Elk-1 was detected by Western blot analysis using rabbit anti-phospho Elk-1 IgG (1:1,000 dilution), as described above.

Measurement of intracellular free calcium concentration

We performed fluorescence measurements with fura-2-loaded cells following the protocol of Grynkiewicz et al. (1985), as previously described (Garrad et al., 1998; Otero et al., 2000; Velázquez et al., 2000). Briefly, 2×10^6 cells were incubated for 30 min with 3 μM acetoxymethyl ester fura-2 (Molecular Probes, Oregon) dissolved in DMSO. Cells were centrifuged for 3–4 sec and resuspended in HBS (15 mM HEPES, pH 7.4, 120 mM NaCl, 4 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1 mM $CaCl_2$, 0.1% (w/v) albumin, and 0.18% (w/v) glucose). The fura-2-loaded cells were immediately transferred to a thermostated cuvette (37°C) containing a magnetic stirrer to allow continuous sample agitation during fluorescence measurements. The cuvette was positioned in computer-controlled dual excitation DeltaScan spectrofluorimeter (Photon Technology International, Newark, NJ) and fluoresced at excitation wavelengths of 340 and 380 nm with emission detected at 505 nm. After a 5-minute equilibration, an aliquot of ligand was added, as indicated. The ratio of fluorescence intensities at 340 to 380 nm ($R = F_{340\ nm}/F_{380\ nm}$) as a function of time was determined. The maximum (R_{max}) and minimum (R_{min}) ratios of fluorescence were determined by addition of 100 μl of 20% Triton X-100 and 300 μl of 100 mM EGTA in 250 mM Tris–HCl (pH 7.4), respectively. R_{max} and R_{min} values were used to calculate the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) from the equation:

$$[Ca^{2+}]_i = K_D(R - R_{min}/R_{max} - R)(S_{f380}/S_{b380})$$

where K_D is the dissociation constant for the Ca^{2+}/fura-2 complex (224 nM) and S_{f380} and S_{b380} are the fluorescence intensities at 380 nm of Ca^{2+}-free fura-2 and Ca^{2+}-saturated fura-2, respectively.

Desensitization of $P2Y_2$ nucleotide receptors

U937 cells were incubated for 5 min–4 h with extracellular nucleotide (typically 100 μM) in serum-free DMEM/Waymouth medium (1:1). Then, cells were washed, incubated in HBS, and rechallenged with nucleotide. $P2Y_2$ receptor desensitization was defined as decreased cellular responsiveness to a second nucleotide treatment, as compared to the response in cells treated with a single dose of nucleotide. Nucleotide-treated cells were assayed for full responsiveness to bradykinin or the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin, as a measure of the selectivity of $P2Y_2$ receptor desensitization (Otero et al., 2000). The rate of recovery of $P2Y_2$ receptor activity from desensitization was monitored by washing nucleotide-treated cells to

remove the agonist and incubation of cells for varying times in the absence of nucleotide prior to agonist rechallenge.

Ecto-nucleotidase activity measurements

The rate of nucleotide hydrolysis by U937 cells was determined, as previously described (Magnusson et al., 1976), to evaluate whether significant decreases in the extracellular nucleotide concentration occurred over the course of the desensitization experiments. Briefly, UTP was added to cells in RPMI 1640 culture medium and the medium was sampled after varying lengths of time. UTP, UDP, UMP, and uridine in the medium were resolved by chromatography on polyethyleneimine (PEI)-coated cellulose columns. The anion exchange PEI-cellulose columns were equilibrated with 0.1 M HCl and washed with water. For separation of nucleotides, 2.0 ml aliquots of nucleotide-containing medium were applied to the top of each column, and uridine and UMP were eluted with four 1-ml fractions of 0.325 M LiCl. Then, UDP was eluted with four 1-ml fractions of 1.0 M LiCl, and UTP was eluted with four 1-ml fractions of 2.0 M LiCl. Known concentrations of nucleotide and uridine standards were used to confirm the elution pattern. The concentration of nucleotide/nucleoside in each fraction was determined by spectrophotometry at 260 nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR) of P2Y receptor mRNA

Since $P2Y_2$ receptor mRNA in U937 cells was not easily detected by Northern blot analysis, we monitored its expression by a semi-quantitative RT-PCR that has been previously used to measure the expression levels of $P2Y_2$ receptor mRNA in other cells types (Martin et al., 1997; Turner et al., 1997; Clarke et al., 1999). This procedure has been validated and optimized for the selective amplification of mRNA for different P2Y receptor subtypes (Koshiba et al., 1997; Clarke et al., 1999). Briefly, total RNA was isolated from U937 cells and human 1321N1 astrocytoma cell transfectants stably expressing the $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors using the RNeasy Mini Kit (Qiagen, Chatsworth, CA). Removal of deoxyribonucleic acids was accomplished through treatment with RNase-free DNase I (Promega) followed by a clean-up step using the RNeasy Mini Kit. cDNA was synthesized from the purified RNA using the First Strand cDNA Synthesis Kit for RT-PCR (AMV) (Boehringer Mannheim, Indianapolis, IN). Ten percent of the resulting cDNA was used as a template in the polymerase chain reaction (PCR) using the Expand High Fidelity PCR System (Boehringer Mannheim). Oligonucleotide primers were designed to amplify specific P2 receptor subtypes. The $P2Y_1$ receptor primers (upstream: 5′-AAGACCGGCTTC-CAGTTCTACTAC-3′ and downstream: 5′-CACATTT-CTGGGGTCTGGAAATCC-3′, representing nucleotides 136–159 and 887–864, respectively, of the human receptor), the $P2Y_2$ receptor primers (upstream: 5′-ATCTTCTTGTGCCGCCTCAAGACCT-3′ and downstream: 5′-AGCAGACCCAGCATGACTGAGCTGT-3′, representing nucleotides 169–193 and 617–593, respectively, of the human receptor), the $P2Y_4$ receptor primers (upstream: 5′-ATGAGGATTTCAAGTTCATC-CTGC-3′ and downstream: 5′-TAGACCACGTTGAC-AATGTTCAGT-3′ representing nucleotides 89–112 and 863–840, respectively, of the human receptor), and the $P2Y_6$ receptor primers (upstream: 5′-TGCCA-CCCACCTGTGTCTACCG-3′ and downstream: 5′-AG-TAGAAGAGGATGGGGTCCAGCAC-3′, representing nucleotides 38–62 and 907–883, respectively, of the human receptor) were designed from the coding regions of the respective receptor cDNA. Primers specific for amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clontech, Palo Alto, CA) were used as positive controls for the presence and integrity of the cDNA. Parallel aliquots of RNA samples were subjected to *mock* RT reactions as controls. These samples were treated as above, but no AMV-RT was added. Diluted aliquots from the experimental and *mock* RT reactions were then used as templates for the PCR. Reaction mixtures contained 20 pmol of each primer and 2 U of high fidelity polymerase in a 50 μl final reaction volume. The PCR was carried out for 30 cycles as follows: denaturation at 95°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min with an additional 5 min extension at 72°C following the final cycle. The resulting PCR products were resolved on a 1.0% (w/v) agarose gel containing 10 μg/ml ethidium bromide and photographed under UV illumination. The amplification products from each reaction were quantitated using Multi-Analyst software (Bio-Rad) after digitizing the gel photograph.

RESULTS

Calcium mobilization mediated by $P2Y_2$ nucleotide receptors in U937 cells

$P2Y_2$ receptor activation causes inositol 1,4,5 trisphosphate (IP_3)-dependent calcium mobilization from intracellular stores in a variety of mammalian cell types (Boarder et al., 1995). Addition of 10 μM UTP to fura-2-loaded monocyte-like U937 cells produced a transient increase in $[Ca^{2+}]_i$ (Fig. 1A). The agonist potency profile of the calcium response in U937 cells typifies the $P2Y_2$ receptor subtype (Fig. 1B), that has been described for other endogenous or heterologously expressed $P2Y_2$ receptors (Cowen et al., 1991; Lustig et al., 1993; Parr et al., 1994). The expression in U937 cells of mRNA for both $P2Y_2$ and $P2Y_6$ receptor subtypes, but not for $P2Y_1$ and $P2Y_4$, was confirmed by RT-PCR (data not shown). This result is consistent with the P2Y receptor expression profile previously reported for U937 cells (Jin et al., 1998). The $P2Y_2$ receptor is pharmacologically distinguished by the equi-potency and -efficacy of the agonists ATP and UTP (Parr et al., 1994) whereas the human $P2Y_6$ receptor is uridine nucleotide-selective with UDP being the most potent agonist (Communi et al., 1996). The lack of a demonstrable response to UDP (Fig. 1B) suggests that U937 cells used in the present study lack a functional $P2Y_6$ receptor.

Extracellular nucleotides activate MAPKs in U937 cells

Activation of MAPKs in U937 cells can mediate inflammatory responses (Franklin and Kraft, 1997; Biggs et al., 1998; Ragg et al., 1998; Wang et al., 1998; Weisman et al., 1998), and $P2Y_2$ receptors have been shown to activate MAPKs in a variety of cell types

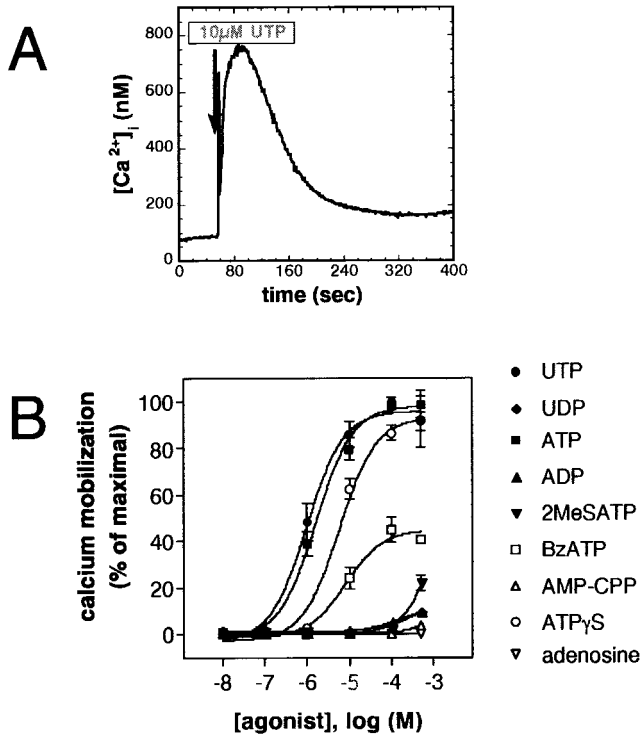

Fig. 1. Calcium mobilization mediated by $P2Y_2$ nucleotide receptors in U937 cells. **A:** The concentration of intracellular free calcium ($[Ca^{2+}]_i$) was measured in fura-2-loaded cells as described in Materials and Methods. Addition of 10 μM UTP was made at the time indicated (arrow). **B:** $[Ca^{2+}]_i$ was measured in fura-2-loaded U937 cells incubated with the indicated concentration of nucleotides and analogs for 1 min at 37°C. UDP and ADP stock solutions were pretreated with hexokinase (10 units/ml) and 25 mM glucose for 1 h at 37°C to convert potentially contaminating UTP and ATP to UDP and ADP, respectively. The data presented are expressed as a percentage of the maximal response to 100 μM UTP ($[Ca^{2+}]_i = 1.7 \pm 0.2 \times 10^{-6}$ M, n = 56) and are the average ± SEM of results from three independent experiments. 2MeSATP: 2-methyl-S-adenosine 5′-triphosphate; BzATP: 2′- & 3′-(4-benzoylbenzoyl)-adenosine 5′-triphosphate; AMP-CPP: α,β-methyleneadenosine 5′-triphosphate; ATPγS: adenosine-5′-O′(-3-thiotriphosphate). The lines shown were obtained using the Prism 2.0b software (GraphPad) from a least squares protocol designed to satisfy the equation $Y = Bottom + \frac{(Top-Bottom)}{1+10^{Log(EC_{50})-X}}$; where Y is the response, X is the logarithm of agonist concentration, *Bottom* is the Y value at the bottom plateau, *Top* is the Y value at the top plateau, and $Log(EC_{50})$ is the logarithm of the EC_{50}, the concentration of agonist that gives a half-maximal response. The iterations stopped when two iterations in a row changed the sum-of-squares by less than 0.01%.

(Neary et al., 1998, 1999; Soltoff, 1998; Soltoff et al., 1998). Accordingly, we investigated whether $P2Y_2$ receptors were coupled to MAPKs in U937 cells. Addition of 100 μM UTP to U937 cells caused a transient increase in the phosphorylation of ERK1/2 that was detected using Western analysis with anti-phosphoMAPK antibodies that bind selectively to the phosphorylated regulatory TXY motif of ERK1/2 (Fig. 2A–C). Reprobing of the blot with anti-ERK1/2 antibody that binds ERK1/2 independent of phosphorylation state, confirmed that the apparent increase in ERK1/2 phosphorylation induced by UTP was not due to changes in MAPK protein levels (Fig. 2B). The EC_{50} for UTP-induced ERK1/2 phosphorylation (Fig. 2C Inset) was 2.7 μM (log (EC_{50}) = -5.5 ± 0.2), a value typical for $P2Y_2$ receptor-mediated responses (Turner et al., 1998). To confirm that the increase in ERK1/2 phosphorylation caused by UTP correlated with an increase in ERK1/2 activity, we immunoprecipitated ERK1/2 from cytoplasmic extracts of UTP-treated U937 cells and used it to phosphorylate exogenous Elk-1, a transcription factor whose activity is known to be regulated by ERK1/2-dependent phosphorylation of Ser383 (Marais et al., 1993). Results indicated that the time dependence (Fig. 2D) and EC_{50} (not shown) for UTP-induced Elk-1 phosphorylation by immunoprecipitated ERK1/2 was virtually identical for UTP-induced ERK1/2 phosphorylation in U937 cells (Fig. 2A–C). The relative potencies of a variety of nucleotides and analogs for phosphorylation of ERK1/2 in U937 cells (Fig. 3) strongly suggest that the $P2Y_2$ receptor subtype mediate this response.

Signal transduction pathway for ERK1/2 activation by the $P2Y_2$ receptor

The activation by phosphorylation of ERK1/2 is catalyzed by the upstream dual threonine-tyrosine protein MAPK kinases, MEK1/2 (Zheng and Guan, 1993). The activation of MEK1/2 is regulated by phosphorylation on serine residues 217 and 221 catalyzed by a family of MAPK kinase kinases (MAPKKKs) of which Raf is the best characterized (Dent et al., 1992; Yan and Templeton, 1994). Consistent with this pathway for activation of ERK1/2, we detected the rapid phosphorylation of MEK1/2 upon $P2Y_2$ receptor activation in U937 cells (Fig. 4).

Since several protein kinases have been shown to activate the MEK–ERK pathway, we tested the effects of various kinase inhibitors on UTP-induced ERK1/2 phosphorylation. PD 098059 (30 μM), an inhibitor of MEK1/2 (Dudley et al., 1995), and wortmannin (100 nM) and LY294002 (50 μM), inhibitors of PI3K (Arcaro and Wymann, 1993; Vlahos et al., 1994) inhibited ERK1/2 phosphorylation induced by 100 μM UTP (Fig. 5). However, GF 109203X (1 μM), a potent inhibitor of various PKC isoforms (Toullec et al., 1991), had no significant effect on UTP-induced phosphorylation of ERK1/2. The ability of GF 109203X to inhibit PKC was confirmed (see Fig. 10). These results suggest that the $P2Y_2$ receptor couples to PI3K, but not PKC, to modulate the activity of the MEK–ERK pathway.

Although PKC does not appear to directly mediate the activation of ERK1/2 by the $P2Y_2$ receptor, it is plausible that intracellular calcium mobilization induced by extracellular UTP (Fig. 1) could modulate ERK1/2 phosphorylation. However, the addition to U937 cells of 10 μM BAPTA-AM (to generate the intracellular calcium chelator BAPTA) and 5 mM EGTA (to chelate extracellular calcium) did not inhibit UTP-induced ERK1/2 phosphorylation (Fig. 6). BAPTA-AM treatment decreased the 100 μM UTP-induced increase in the concentration of intracellular calcium to 15.5 ± 1.2% of the response in the absence of BAPTA-AM (data not shown).

Recent reports indicate that cytoplasmic tyrosine kinases, such as c-src, may function as transducers between G proteins and Ras-dependent activation of MAPKs (Hawes et al., 1996; Luttrell et al., 1996; Yamauchi et al., 1997). Therefore, we investigated the

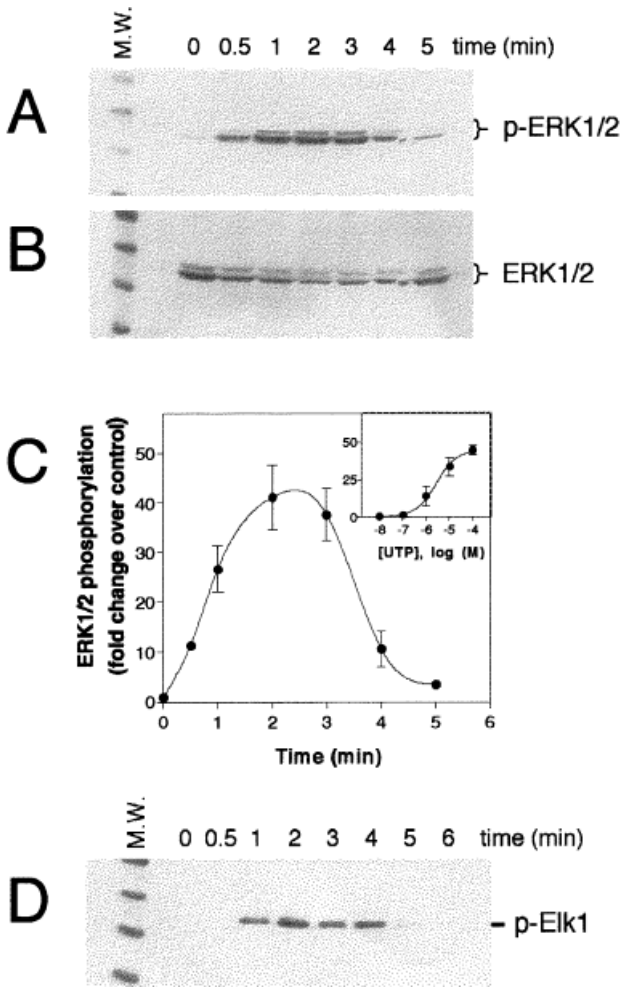

Fig. 2. Activation of ERK1/2 by extracellular UTP in U937 cells. **A:** The phosphorylation of ERK1/2 was detected by Western analysis of cell extracts from U937 cells treated with 100 μM UTP for the indicated times, as described in Materials and Methods. **B:** Total ERK1/2 protein in cell extracts was detected by reprobing the blot with anti-p42/p44(ERK1/2) MAPK IgG, as described in Materials and Methods. **C:** The time course for UTP-stimulated ERK1/2 phosphorylation was determined by normalizing the density of phospho-ERK1/2 (shown in Fig. 2A) to the density of total ERK1/2 protein (shown in Fig. 2B), as described in Materials and Methods. The data are expressed as the fold change over ERK1/2 phosphorylation in untreated cells and are the average ± SEM of results from four experiments. **Inset:** Dose dependence of UTP-induced ERK1/2 phosphorylation. U937 cells were stimulated with the indicated concentrations of UTP for 2 min. Quantitations were made as described for Figure 2C, the line was generated as in Figure 1B and data represent the mean ± SEM of results from four experiments. **D:** The time course for Elk-1 phosphorylation was determined with ERK1/2 immunoprecipitated from U937 cells that were treated with 100 μM UTP for the indicated period of time. ERK1/2 was immunoprecipitated with anti-phospho p42/p44 MAPK IgG, and in vitro kinase reactions using 2 μg of Elk-1 protein as substrate were performed, as described in Materials and Methods. Data are representative of results from four independent experiments.

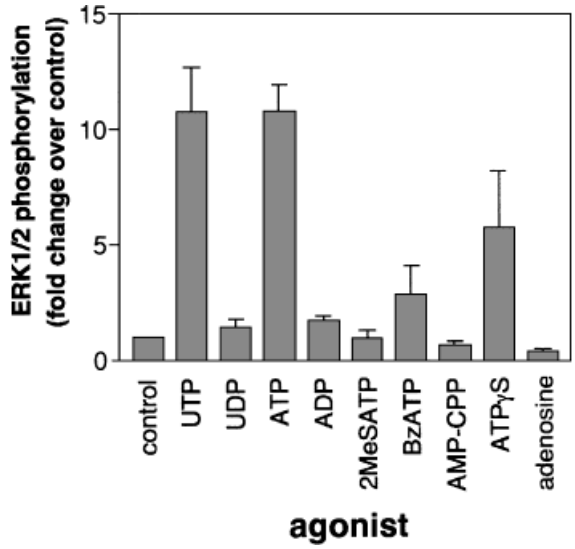

Fig. 3. Activation of ERK1/2 by P2 receptor agonists. U937 cells were treated with the indicated agonist (100 μM) for 2 min. UDP and ADP stock solutions were pretreated with hexokinase and glucose, as in Figure 1B. Cell extracts were analyzed by Western analysis with anti-phospho p42/p44 MAPK antibody and blots were reprobed for normalization to total ERK1/2 protein with anti-p42/p44 MAPK antibody, as described in Materials and Methods. Data are expressed as the fold change over ERK1/2 phosphorylation in untreated cells and are the average ± SEM of results from four independent experiments.

effect of the c-src inhibitors, PP2 (Hanke et al., 1996), and radicicol (Kwon et al., 1992), on P2Y$_2$ receptor-mediated ERK1/2 phosphorylation. Figure 7 shows that a 30-minute incubation with either 10 μM PP2 or radicicol caused a dramatic inhibition of ERK1/2 phosphorylation induced by UTP. These data suggest that c-src can mediate the activation of ERK1/2 by P2Y$_2$ receptors in U937 cells.

Desensitization of the P2Y$_2$ nucleotide receptor in U937 cells

Since chronic exposure to nucleotide agonists is required to induce the differentiation of monocytic U937 cells to the macrophage-like phenotype, we investigated whether prolonged exposure to UTP-induced desensitization of P2Y$_2$ nucleotide receptor-mediated responses. A 5-minute exposure of U937 cells to UTP decreased the responsiveness of cells to a second challenge with agonist (Fig. 8A). The IC$_{50}$ (0.30 μM) for UTP-induced receptor desensitization was comparable to the EC$_{50}$ (0.44 μM) for receptor activation (Fig. 8A). We determined that UTP-induced desensitization increased both the EC$_{50}$ for P2Y$_2$ receptor activation and the capacity of the desensitized receptor to mobilize intracellular free calcium in response to UTP (Table 1; Fig. 8B).

Activation of PKC with phorbol 12,13-dibutyrate (PdBu) caused desensitization of the P2Y$_2$ receptor in U937 cells (IC$_{50}$ for PdBu ~26 nM; Fig. 9A). PdBu-induced desensitization was characterized by an increase in the EC$_{50}$ for UTP but, in contrast to agonist-induced desensitization (Fig. 8B), PdBu only partially reduced the capacity of the receptor to mobilize intracellular calcium (Fig. 9B, Table 1). We further investigated whether PdBu-sensitive PKC isoforms mediate agonist-induced desensitization of the P2Y$_2$ receptor in U937 cells. Pretreatment of U937 cells with the non-selective PKC inhibitor GF 109203X (Toullec et al., 1991) almost completely blocked the desensitiza-

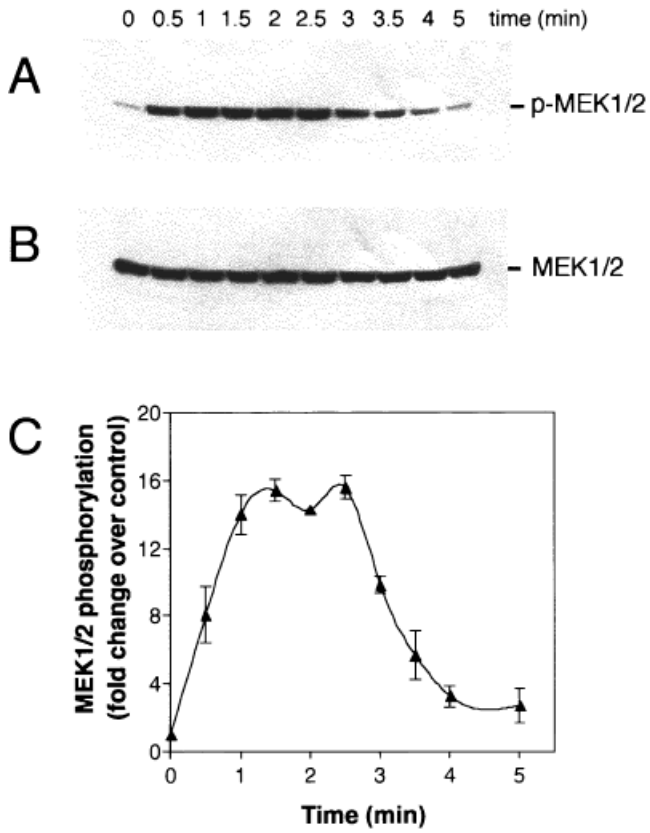

Fig. 4. Activation of MEK1/2 by extracellular UTP in U937 cells. **A:** The phosphorylation of MEK1/2 was detected with anti-phospho MEK1/2 IgG by Western analysis of cell extracts from U937 cells treated with 100 μM UTP for the indicated times, as described in Materials and Methods. **B:** Total MEK1/2 protein in the cell extracts was determined by reprobing the blot with anti-MEK1/2 IgG, as described in Materials and Methods. **C:** The time course for UTP-stimulated MEK1/2 phosphorylation was determined by normalizing the density of phospho-MEK1/2 (shown in Fig. 4A) to the density of total MEK1/2 protein (shown in Fig. 4B), as described in Materials and Methods. The data are expressed as the fold change over MEK1/2 phosphorylation in untreated cells and are the average ± SEM of results from four experiments.

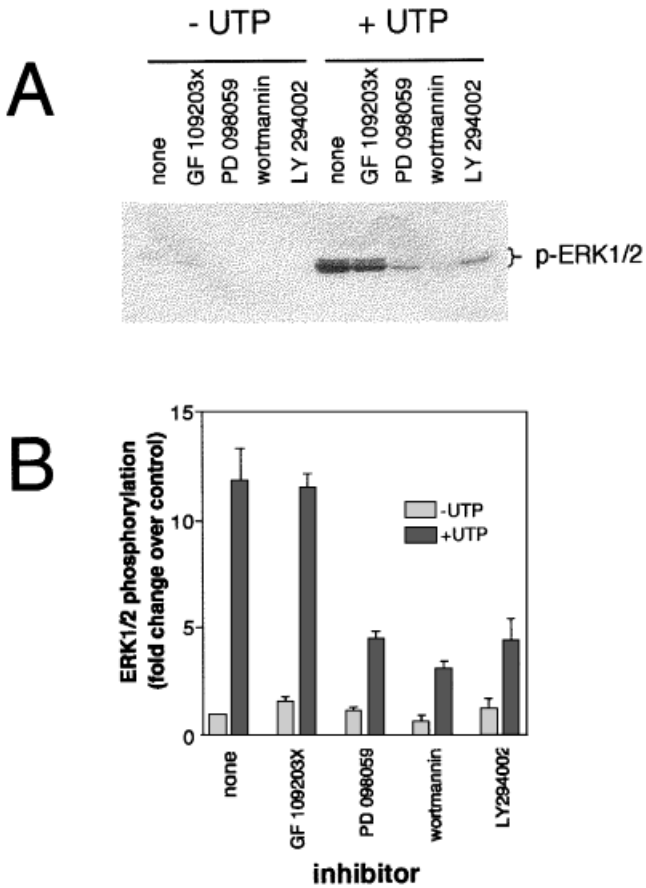

Fig. 5. Effect of protein kinase inhibitors on $P2Y_2$ receptor-mediated ERK1/2 phosphorylation. **A:** The phosphorylation of ERK1/2 was detected by Western analysis of cell extracts from U937 cells treated in the presence or absence of 1 μM GF 109203X, 30 μM PD 098059, 100 nM wortmannin, or 50 μM LY294002 for 30 min followed by incubation in the presence or absence of 100 μM UTP for 2 min. **B:** ERK1/2 phosphorylation was determined by normalizing the density of phospho-ERK1/2 (shown in Fig. 5A) to the density of total ERK1/2 protein (not shown), as described in Materials and Methods. The data are expressed as the fold change over ERK1/2 phosphorylation in cells treated in the absence of UTP and are the average ± SEM of results from four experiments.

tion of the $P2Y_2$ receptor induced by PdBu (Fig. 10), but only partially inhibited UTP-induced desensitization. These results suggest a role for both PKC-dependent and -independent pathways in agonist-induced receptor desensitization, similar to studies with a recombinant $P2Y_2$ receptor expressed in human 1321N1 astrocytoma cells (Otero et al., 2000).

We also investigated whether protein phosphatase activity plays a role in the recovery of $P2Y_2$ receptor function after agonist-induced desensitization. Addition to UTP-desensitized U937 cells of 1 μM okadaic acid, an inhibitor of protein phosphatases-1 and -2A (Bialojan and Takai, 1988; Haystead et al., 1990), prevented recovery of receptor activity that occurs within 90 min of the initial UTP challenge (Fig. 11). These results implicate protein phosphorylation and dephosphorylation in agonist-induced desensitization and recovery of receptor activity (resensitization), respectively.

Prolonged treatment (>30 min) of U937 cells with 100 μM UTP caused persistent desensitization from which the cells did not recover upon removal of agonist (Fig. 12). Treatment of cells with 100 μM UTP for >30 min also caused a significant decrease in levels of $P2Y_2$ receptor mRNA (Fig. 13).

Agonist-induced desensitization was also observed for $P2Y_2$ receptor-mediated ERK1/2 activation (Fig. 14A). In contrast, desensitization of PdBu-induced ERK1/2 phosphorylation was not detected in cells pretreated with 100 μM UTP for 30 min (Fig. 14A). Furthermore, desensitization of $P2Y_2$ receptor-induced ERK1/2 phosphorylation induced by a 30-minute pretreatment with UTP was not reversed by a 20-minute incubation in UTP-free medium (Fig. 14B), in contrast to recovery from agonist-induced desensitization of $P2Y_2$ receptor-mediated calcium mobilization under similar conditions (Fig. 12). These results provide further evidence that

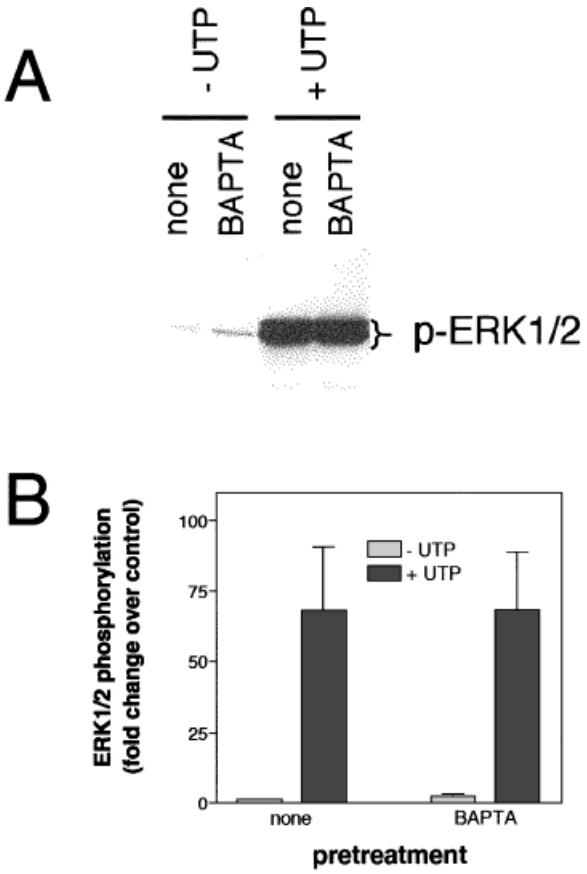

Fig. 6. P2Y$_2$ receptor-mediated ERK1/2 activation is not dependent on intracellular calcium mobilization. **A:** The phosphorylation of ERK1/2 was detected by Western analysis of cell extracts from U937 cells treated in the presence or absence of 10 μM BAPTA-AM and 5 mM EGTA for 30 min followed by incubation in the presence or absence of 100 μM UTP for 2 min. **B:** ERK1/2 phosphorylation was determined by normalizing the density of phospho-ERK1/2 (shown in Fig. 6A) to the density of total ERK1/2 protein (not shown), as described in Materials and Methods. The data are expressed as the fold change over ERK1/2 phosphorylation in cells treated in the absence of UTP and are the average ± SEM of results from three experiments.

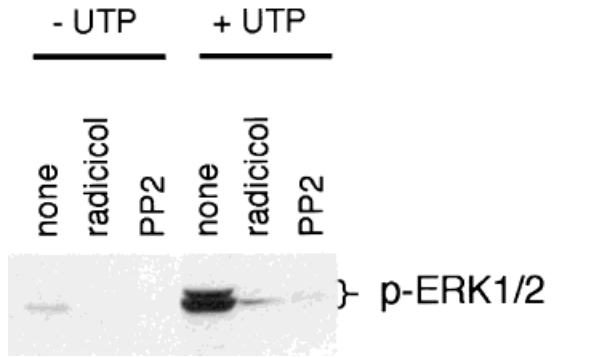

Fig. 7. Effect of c-src inhibitors on P2Y$_2$ receptor-mediated ERK1/2 phosphorylation. The phosphorylation of ERK1/2 was detected by Western analysis of cell extracts from U937 cells treated in the presence or absence of 10 μM radicicol or 10 μM PP2 for 30 min followed by incubation in the presence or absence of 100 μM UTP for 2 min. The data shown are representative of results from two similar experiments.

calcium mobilization and ERK1/2 activation are distinct signaling pathways coupled to P2Y$_2$ receptors in U937 cells, and may be independently regulated.

DISCUSSION

Persistent activation of P2Y$_2$ receptors in U937 cells causes phenotypic changes characteristic of monocyte-to-macrophage differentiation, including increased responsiveness to chemotactic peptides, reduced rates of proliferation, and increased expression of complement receptors type 1 (CR1) and 3 (CR3) (Cowen et al., 1991). Although an important role for nucleotides in modulating myelomonocytic differentiation is appreciated, the molecular/biochemical basis for the effects remains unclear and little is known about the regulation of P2 nucleotide receptors that mediate these effects in human monocytes.

In this study, we investigated signal transduction pathways for a P2Y$_2$ receptor subtype that is expressed in undifferentiated U937 cells. Experiments with inhibitors of certain protein kinases and other pharmacological agents demonstrated that the activation of ERK1/2 is independent of the well-known P2Y$_2$ receptor/G$_{q/11}$ signaling pathway that mediates the IP$_3$-dependent mobilization of intracellular calcium and the activation of calcium/diacylglycerol-dependent PKC isoforms (Cowen et al., 1989, 1991; Weisman et al., 1998). Our results implicate PI3K, c-src, and MEK1/2 in the coupling of P2Y$_2$ receptors to ERK1/2 activation, and rule out the involvement of PKC isoforms and the mobilization of intracellular calcium in this pathway. A caveat to our interpretations is the potential for non-specific effects of the inhibitors tested. Whenever possible, more than one inhibitor for the same enzymatic activity was used to confirm the results.

Since P2Y$_2$ receptors in U937 cells are known to activate PLC to generate diacylglycerol (DAG) as well as IP$_3$ (Cowen et al., 1989, 1991; Weisman et al., 1998), it was surprising to find no role for DAG-activated PKC in ERK1/2 phosphorylation. However, it is possible that the amount of DAG produced by P2Y$_2$ receptor activation is insufficient to activate PKC. Conversely, the main source of DAG for PKC activation in U937 cells might be a phospholipid other than phosphatidylinositol 4,5-bisphosphate, the substrate for IP$_3$ generation. It has been reported previously that extracellular nucleotides induced calcium mobilization without a concomitant activation of PKC in murine 3T6 fibroblasts (Gonzalez et al., 1989).

P2 receptors are present in monocytes and their expression is modulated by inflammatory cytokines and agents that induce cell differentiation (Di Virgilio et al., 1996). The P2Y$_2$ receptor, through the transient activation of MAPK, may modulate the differentiation process in U937 cells. The transient activation of MAPKs by lipopolysaccharides (LPS) has been shown to modulate differentiation and the production of proinflammatory cytokines in U937 cells (Mander et al., 1997). In PC12 cells, EGF causes transient MAPK activation that induces neuronal differentiation, a response that is

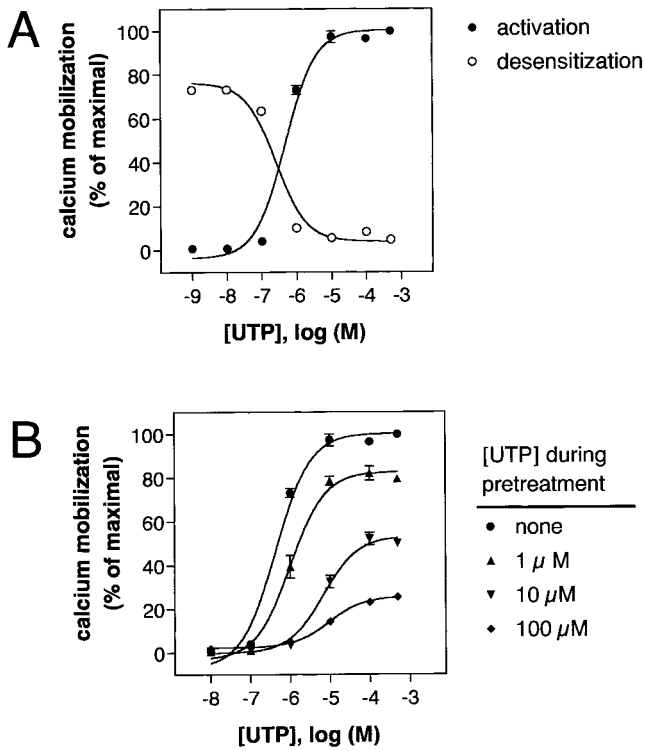

Fig. 8. UTP-induced desensitization of $P2Y_2$ receptors in U937 cells. **A:** $P2Y_2$ receptor-mediated changes in $[Ca^{2+}]_i$ were measured in fura-2-loaded cells in response to various concentrations of UTP (closed circles), as described in Materials and Methods. Each value shown is the maximal UTP-induced increase in $[Ca^{2+}]_i$ expressed as a percentage of the maximal response to 500 μM UTP. The data presented are the average ± SEM of results from four independent experiments. $P2Y_2$ receptor desensitization was measured in fura-2-loaded U937 cells that were preincubated with the indicated concentration of UTP for 5 min at 37°C. Then, cells were washed, resuspended in assay buffer, and changes in $[Ca^{2+}]_i$ in response to 1 μM UTP were monitored (open circles) as described in Materials and Methods. Each value shown is the maximal UTP-induced increase in $[Ca^{2+}]_i$ expressed as a percentage of the response to 500 μM UTP in cells not pretreated with UTP. The data presented are the average ± SEM of results from four independent experiments. The lines shown were generated as described in Figure 1B. **B:** Fura-2-loaded U937 cells were pretreated with the indicated concentrations of UTP for 5 min at 37°C. Then, cells were washed, resuspended in assay buffer, incubated for 1 min with the indicated concentration of UTP, and changes in $[Ca^{2+}]_i$ were monitored (see Materials and Methods). Each value shown is the maximal UTP-induced increase in $[Ca^{2+}]_i$ expressed as a percentage of the maximal response to 500 μM UTP in cells not pretreated with UTP. The data presented are the average ± SEM of results from four independent experiments. The lines shown were generated as described in Figure 1B.

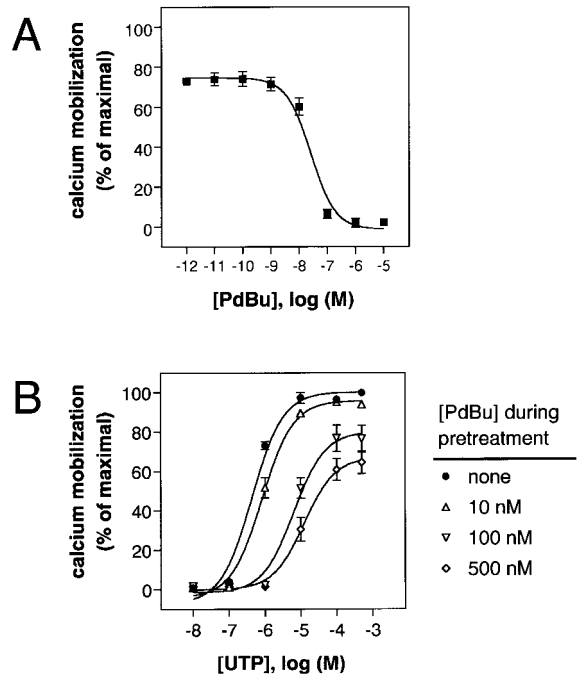

Fig. 9. Phorbol ester-induced desensitization of $P2Y_2$ receptors in U937. **A:** Fura-2-loaded cells were pretreated with the indicated concentration of phorbol 12,13-dibutyrate (PdBu) for 5 min at 37°C. Then, the cells were washed, resuspended in assay buffer, and changes in $[Ca^{2+}]_i$ in response to 1 μM UTP were monitored (see Materials and Methods). Each value shown is the maximal UTP-induced increase in $[Ca^{2+}]_i$ expressed as a percentage of the maximal response to 100 μM UTP in cells not pretreated with UTP. The data presented are the average ± SEM of results from four independent experiments. The lines shown were generated as described in Figure 1B. **B:** Fura-2-loaded U937 cells were pretreated with the indicated concentrations of PdBu for 5 min at 37°C. Then, cells were washed, resuspended in assay buffer, incubated for 1 min with the indicated concentration of UTP, and changes in $[Ca^{2+}]_i$ were monitored (see Materials and Methods). Each value shown is the maximal UTP-induced increase in $[Ca^{2+}]_i$ expressed as a percentage of the maximal response to 500 μM UTP in cells not pretreated with UTP. The data presented are the average ± SEM of results from four independent experiments. The lines shown were generated as described in Figure 1B.

sustained by NGF treatment (Morooka and Nishida, 1998). Importantly, phorbol ester-induced differentiation of U937 cells is blocked by the MEK1/2 inhibitor PD 098059 (Weisman et al., 1998), providing further evidence for the role of ERK1/2 in the differentiation of monocytic U937 cells. Although chronic treatment of U937 cells with extracellular ATP and UTP failed to

TABLE 1. $P2Y_2$ receptor desensitization in U937 cells

Pretreatment	EC_{50} (μM)	log EC_{50}	Maximal response (% of control)
Control	0.44	−6.28 ± 0.15	100.3 ± 0.15
UTP 1 μM	1.0	−5.99 ± 0.09	82.7 ± 2.5
10 μM	6.8	−5.16 ± 0.11	53.2 ± 2.3
100 μM	9.3	−5.03 ± 0.04	25.6 ± 0.4
PdBu 10 nM	0.83	−6.08 ± 0.08	95.9 ± 2.5
100 nM	6.36	−5.19 ± 0.14	80.1 ± 4.3
500 nM	12.3	−4.91 ± 0.07	67.2 ± 2.1

PdBu, phorbol 12,13-dibutyrate. The EC_{50}, log EC_{50}, maximal calcium response, and SEM values were determined from data shown in Figures 8 and 9 using Prism 2.0b software (GraphPad, San Diego, CA) as described in the legend of Figure 1B.

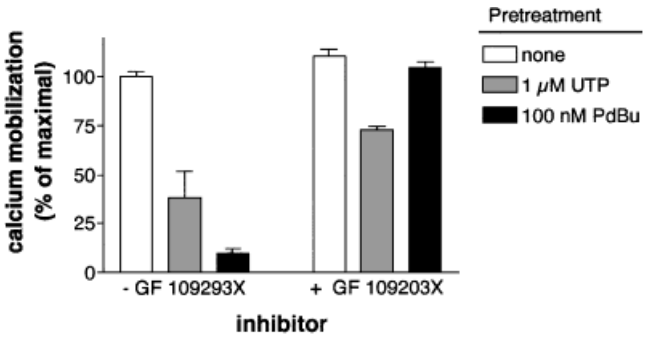

Fig. 10. Effect of the protein kinase C inhibitor, GF 109203X, on P2Y$_2$ receptor desensitization in U937 cells. Fura-2-loaded cells were pretreated with 1 μM UTP or 100 nM phorbol 12,13-dibutyrate (PdBu) for 5 min in the presence or absence of 1 μM GF 109203X. Then, cells were washed, resuspended in assay buffer, incubated for 1 min with 1 μM UTP, and changes in $[Ca^{2+}]_i$ were monitored (see Materials and Methods). Each value shown is the maximal UTP-induced increase in $[Ca^{2+}]_i$ expressed as a percentage of the maximal response to 100 μM UTP in cells not pretreated with UTP. The data presented are the average ± SEM of results from four independent experiments.

induce the *morphological* differentiation associated with the macrophage phenotype, presumably due to nucleotide degradation, the slowly hydrolyzable analog and P2Y$_2$ receptor agonist, ATPγS, induced the morphological differentiation of U937 cells (Cowen et al., 1989, 1991) and activated ERK1/2 (Fig. 3).

Our data indicate that the P2Y$_2$ receptor is rapidly desensitized by exposure to agonists. The desensitization resulted in a decrease in the potency of receptor agonists (increased EC_{50} values), and a decrease in the maximal response obtained after receptor activation (reduced receptor capacity for signaling). Although the molecular basis for those two effects remains unclear, we speculate that a post-translational modification (e.g., phosphorylation) of the receptor may decrease the affinity of the receptor for its ligands, whereas a reduction in receptor number on the cell surface (i.e., receptor internalization) may be responsible for the decrease in

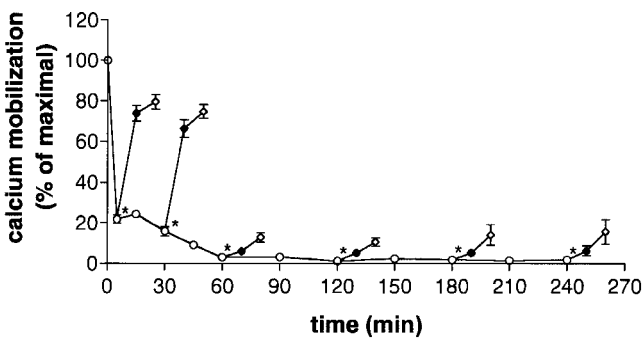

Fig. 12. Recovery from desensitization induced by prolonged treatment with UTP. UTP-induced calcium mobilization was measured in fura-2-loaded U937 cells that were preincubated with 100 μM UTP for the indicated times (open circles). For some cells, UTP was removed (*) and the cells were incubated in UTP-free medium for 10 (closed circles) or 20 (diamonds) min followed by the addition of 100 μM UTP for 2 min. The maximal UTP-induced increase in $[Ca^{2+}]_i$ was determined and expressed as a percentage of the maximal response to 100 μM UTP in cells not pretreated with UTP. The data presented are the average ± SEM of results from four independent experiments.

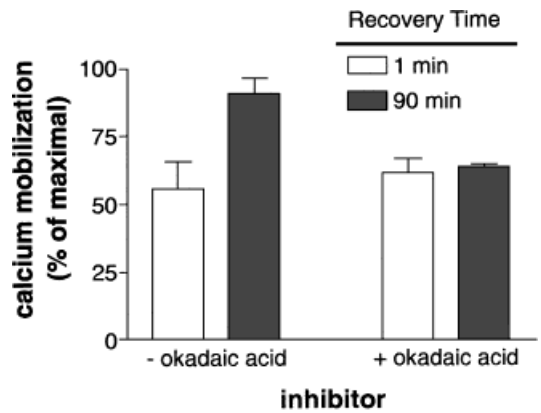

Fig. 11. Effect of okadaic acid on recovery from UTP-induced P2Y$_2$ receptor desensitization. P2Y$_2$ receptor desensitization was induced in fura-2-loaded U937 cells by incubation for 30 min with 100 μM UTP. Then, cells were washed, incubated in assay buffer for 1 min (unshaded bars) or 90 min (shaded bars) in the presence or absence of 1 μM okadaic acid and changes in $[Ca^{2+}]_i$ in response to a 1 min incubation with 100 μM UTP were monitored (see Materials and Methods). Each value shown is the maximal UTP-induced increase in $[Ca^{2+}]_i$ expressed as a percentage of the maximal response to 100 μM UTP in cells not pretreated with UTP. The data presented are the average ± SEM of results from four independent experiments.

signaling capacity. Studies by Garrad et al. (1998) have shown that the C-terminus of the recombinant P2Y$_2$ receptor, which contains potential phosphorylation sites for protein kinases, mediates both agonist-induced desensitization and sequestration. Other evidence of a role for P2Y$_2$ receptor phosphorylation in desensitiza-

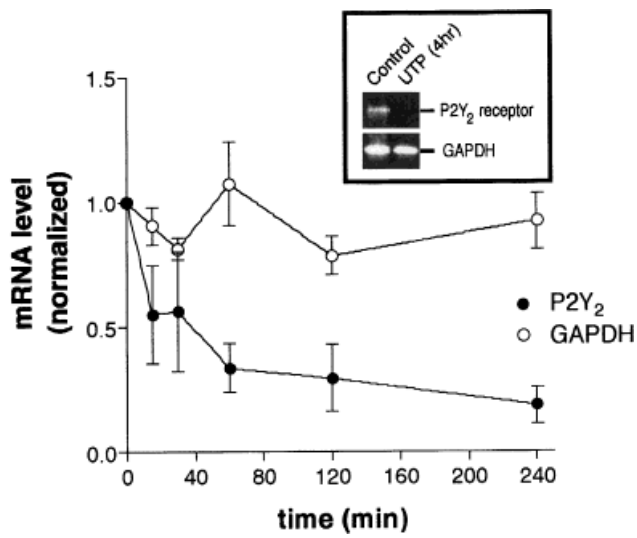

Fig. 13. Effect of prolonged UTP exposure on P2Y$_2$ receptor mRNA levels in U937 cells. P2Y$_2$ receptor and GAPDH mRNA levels were measured by semi-quantitative RT-PCR with cDNA derived from U937 cells treated with 100 μM UTP for the indicated times, as described in Materials and Methods. Values were normalized to the densities of P2Y$_2$ receptor mRNA from cells incubated in the absence of UTP and are the average of results from three independent experiments. **Inset:** Results from a typical experiment showing the RT-PCR amplification product obtained with P2Y$_2$ receptor-specific primers and cDNA derived from U937 cells that were incubated in the presence or absence of 100 μM UTP for 4 h. Amplification of GAPDH with GAPDH specific primers was used as a control.

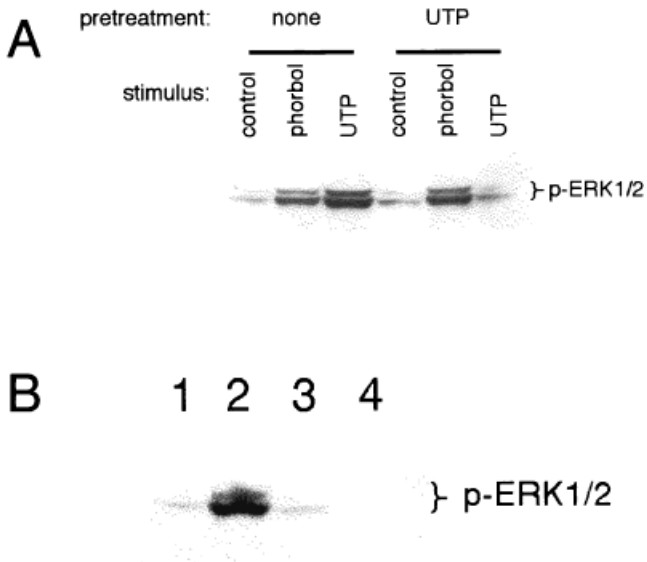

Fig. 14. Desensitization and recovery of $P2Y_2$ receptor-induced ERK1/2 activity. The phosphorylation of ERK1/2 was detected by Western analysis as described in Materials and Methods. **A:** Cell extracts were obtained from U937 cells that were incubated for 30 min in the presence or absence of 100 μM UTP, washed and treated with 100 μM UTP or 500 nM phorbol myristate acetate for 2 min. **B:** For recovery experiments, ERK1/2 phosphorylation was measured, as in Figure 14A, in: untreated cells (lane 1); cells pretreated with 100 μM UTP for 2 min (lane 2); cells pretreated with 100 μM UTP for 30 min, washed, and retreated with 100 μM UTP for 2 min (lane 3); or cells pretreated with 100 μM UTP for 30 min, washed, incubated for 20 min in UTP-free medium and then 2 min with 100 μM UTP (lane 4). These results are representative of two additional experiments.

tion is the ability of the protein phosphatase inhibitor, okadaic acid, to inhibit recovery of receptor activity from the desensitized state (Fig. 11).

Activation of protein kinase C by phorbol esters can cause desensitization of the $P2Y_2$ receptor (Fig. 10) and this pathway presumably mediates heterologous desensitization (Otero et al., 2000). However, PKC does not appear to be involved in agonist-induced desensitization, which was nearly insensitive to potent and specific inhibitors of PKC (Fig. 10). It is interesting that treatment of cells with either UTP or phorbol esters caused a decrease in the potency of a subsequent dose of UTP for induction of receptor-mediated calcium mobilization, suggesting that these compounds activate convergent pathways that attenuate receptor responsiveness.

The internalization of recombinant $P2Y_2$ receptors expressed in a heterologous cell system can be induced by nucleotide agonists but not phorbol esters (Garrad et al., 1998). In U937 cells, we observed a marked reduction in receptor signaling capacity induced by a supramaximal dose of UTP, but not by direct activation of PKC with phorbol esters (Table 1). Further research is needed to determine the relationship of $P2Y_2$ receptor phosphorylation to homologous and heterologous desensitization and receptor internalization.

The agonist-induced desensitization of $P2Y_2$ receptor-mediated calcium mobilization in U937 cells caused by acute (short-term) UTP treatments was reversible (Fig. 10–12). In contrast, chronic (long-term) treatments produced a sustained desensitized state in the U937 monocytic cell (Fig. 12) that correlated with a decrease in $P2Y_2$ receptor mRNA levels, suggesting a role for gene transcription or mRNA degradation in the regulation of receptor activity in U937 cells (Fig. 13). It was surprising to discover that desensitization of $P2Y_2$ receptor-mediated ERK1/2 activation induced by acute exposure of U937 cells to UTP was not readily reversible (Fig. 14). It is possible that increased activity of intracellular protein phosphatases in response to calcium mobilization masked recovery of ERK1/2 activity. The protein phosphatase MKP-1 has been reported to mediate ERK1/2 and p38 MAPK dephosphorylation in U937 cells (Franklin and Kraft, 1997), although the regulation of MKP-1 activity is poorly understood. Further studies are required to assess the independent regulation of calcium signaling and MAPK cascades by $P2Y_2$ receptors in U937 cells.

The role of $P2Y_2$ receptor desensitization in the induction of phenotypic changes in U937 cells caused by chronic treatment with UTP remains to be determined. Recently, it has been determined that the desensitized β-adrenergic receptor forms a complex with c-src protein kinase that functions as a critical structural component of a mitogenic signal (Luttrell et al., 1999). It is possible that a similar complex is formed between the desensitized $P2Y_2$ receptor and c-src, since both ERK1/2 activation and receptor desensitization occur within 2–5 min of UTP addition to U937 cells (compare Figs. 2C and 12), and c-src is implicated in $P2Y_2$ receptor-mediated ERK1/2 activation (Fig. 7). It is noteworthy that $P2Y_2$ receptor activation for only several minutes is mitogenic for several cell types (Huang et al., 1989; Wang et al., 1990, 1992), suggesting that nucleotides act as a "competence factor" in mitogenesis (Huang et al., 1989; Wang et al., 1992). It is plausible that extracellular nucleotides also make cells competent for monocytic differentiation, and that once $P2Y_2$ receptors are activated, other secondary factors (i.e., inflammatory cytokines) promote progression of the differentiation process. Phorbol esters, considered to be second phase inflammatory regulators, can down-regulate the expression of $P2Y_2$ receptor mRNA in monocytes (Martin et al., 1997). A role for $P2Y_2$ receptors in the activation and differentiation of monocytes is proposed, which presents novel pharmacotherapeutic approaches for the treatment of promonocytic leukemia and inflammation.

ACKNOWLEDGMENTS

This study was supported by grants from the National Institutes of Health and the Cystic Fibrosis Foundation to F.A.G. and G.A.W. R.V.F. is a NASA-EPSCoR Predoctoral Fellow. R.C.G. is a Cystic Fibrosis Foundation Fellow.

LITERATURE CITED

Arcaro A, Wymann MP. 1993. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem J 296:297–301.

Bialojan C, Takai A. 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Biochem J 256:283–290.

Biggs JR, Ahn NG, Kraft AS. 1998. Activation of the mitogen-activated protein kinase pathway in U937 cells induces the phosphorylation of the amino terminus of the TATA-binding protein. Cell Growth Differ 9:667–676.

Boarder MR, Weisman GA, Turner JT, Wilkinson GF. 1995. G protein-coupled P2 purinoceptors: from molecular biology to functional responses. Trends Pharmacol Sci 16:133–139.

Clarke LL, Harline MC, Otero MA, Glover GG, Garrad RC, Krugh B, Walker NM, González FA, Turner JT, Weisman GA. 1999. Desensitization of $P2Y_2$ receptor-activated transepithelial anion secretion. Am J Physiol 276:C777–C787.

Cobb MH, Goldsmith EJ. 1995. How MAP kinases are regulated. J Biol Chem 270:14843–14846.

Communi D, Parmentier M, Boeynaems JM. 1996. Cloning, functional expression and tissue distribution of the human $P2Y_6$ receptor. Biochem Biophys Res Commun 222:303–308.

Cowen DS, Berger M, Nuttle L, Dubyak GR. 1991. Chronic treatment with P2-purinergic receptor agonists induces phenotypic modulation of the HL-60 and U937 human myelogenous leukemia cell lines. J Leukoc Biol 50:109–122.

Cowen DS, Lazarus HM, Shurin SB, Stoll SE, Dubyak GR. 1989. Extracellular adenosine triphosphate activates calcium mobilization in human phagocytic leukocytes and neutrophil/monocyte progenitor cells. J Clin Invest 83:1651–1660.

Dent P, Haser W, Haystead TA, Vincent LA, Roberts TM, Sturgill TW. 1992. Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science 257:1404–1407.

Di Virgilio F, Ferrari D, Chiozzi P, Falzoni S, Sanz JM, dal Susino M, Mutini C, Hanua S, Baricord OR. 1996. Purinoceptor function in the immune system. Drug Dev Res 39:319–329.

Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR. 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 92:7686–7689.

Franklin CC, Kraft AS. 1997. Conditional expression of the mitogen-activated protein kinase (MAPK). Phosphatase MKP-1 preferentially inhibits p38 MAPK and stress-activated protein kinase in U937 cells. J Biol Chem 272:16917–16923.

Garrad RC, Otero M, Erb L, Theiss PM, Clarke LL, Gonzalez FA, Turner JT, Weisman GA. 1998. Structural basis of agonist-induced desensitization and sequestration of the $P2Y_2$ nucleotide receptor: Consequences of truncation of the C-terminus. J Biol Chem 273: 29437–29444.

Gonzalez FA, Rozengurt E, Heppel LA. 1989. Extracellular ATP induces the release of calcium from intracellular stores without the activation of protein kinase C in Swiss 3T6 mouse fibroblasts. Proc Natl Acad Sci USA 86:4530–4534.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA. 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J Biol Chem 271:695–701.

Hawes BE, Luttrell LM, van Biesen T, Lefkowitz RJ. 1996. Phosphatidylinositol 3-kinase is an early intermediate in the $G\beta\gamma$-mediated mitogen-activated protein kinase signaling pathway. J Biol Chem 271:12133–12136.

Haystead TAJ, Weiel JE, Litchfield DW, Tsukitani Y, Fischer EH, Krebs EG. 1990. Okadaic acid mimics the action of insulin in stimulating protein kinase activity in isolated adipocytes. J Biol Chem 265:16571–16580.

Huang N, Wang D, Heppel LA. 1989. Extracellular ATP is a mitogen for 3T3, 3T6, and A431 cells and acts synergistically with other growth factors. Proc Natl Acad Sci USA 86:7904–7908.

Jin J, Dasari VR, Sistare FD, Kunapuli SP. 1998. Distribution of P2Y receptor subtypes on haematopoietic cells. Br J Pharmacol 123:789–794.

Koshiba M, Apasov S, Sverdlov P, Chen P, Erb L, Turner JT, Weisman GA, Sitkovsky MV. 1997. Transient up-regulation of P2Y2 nucleotide receptor mRNA expression is an immediate early gene response in activated thymocytes. Proc Natl Acad Sci USA 94:831–836.

Kusner DJ, Schomisch SJ, Dubyak GR. 1993. ATP-induced potentiation of G protein-dependent phospholipase D activity in a cell-free system from U937 promonocytic leukocytes. J Biol Chem 268: 19973–19982.

Kwon HJ, Yoshida M, Fukui Y, Horinouchi S, Beppu T. 1992. Potent and specific inhibition of p60v-src protein kinase both in vivo and in vitro by radicicol. Cancer Res 52:6926–6930.

Lustig KD, Shiau AK, Brake AJ, Julius D. 1993. Expression cloning of an ATP receptor from mouse neuroblastoma cells. Proc Natl Acad Sci USA 90:5113–5117.

Luttrell LM, Ferguson SSG, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F-T, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. 1999. Beta-arrestin-dependent formation of beta-2 adrenergic receptor-Src protein kinase complexes. Science 283:655–661.

Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ. 1996. Role of c-Src tyrosine kinase in G protein-coupled receptor- and $G\beta\gamma$ subunit-mediated activation of mitogen-activated protein kinases. J Biol Chem 271:19443–19450.

Magnusson RP, Portis Jr AR, McCarty RE. 1976. Quantitative, analytical separation of adenine nucleotides by column chromatography on polyethylene-coated cellulose. Anal Biochem 72:653–657.

Mander T, Hill S, Hughes A, Rawlins P, Clark C, Gammon G, Foxwell B, Moore M. 1997. Differential effects on TNF alpha production by pharmacological agents with varying molecular sites of action. Int J Immunopharmacol 19:451–462.

Marais R, Wynne J, Treisman R. 1993. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell 73:381–393.

Martin KA, Kertsy SB, Dubyak GR. 1997. Down-regulation of P_{2u}-purinergic nucleotide receptor messenger RNA expression during in vitro differentiation of human myeloid leukocytes by phorbol esters or inflammatory activators. Mol Pharmacol 51:97–108.

Morooka T, Nishida E. 1998. Requirement of p38 mitogen-activated protein kinase for neuronal differentiation in PC-12 cells. J Biol Chem 273:24285–24288.

Nakashima S, Chata Y, Nakamura M, Miyoshi N, Kohno M, Nozawa Y. 1994. Tyrosine phosphorylation and activation of mitogen-activated protein kinases by thrombin in human platelets: possible involvement in late arachidonic acid release. Biochem Biophys Res Commun 198:497–503.

Neary JT, Kang Y, Bu Y, Yu E, Akong K, Peters CM. 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 JT, McCarthy M, Kang Y, Zuniga S. 1998. Mitogenic signaling from P1 and P2 purinergic receptors to mitogen-activated protein kinase in human fetal astrocyte cultures. Neurosci Lett 242:159–162.

Otero M, Garrad RC, Velázquez B, Camdem JM, Hernández-Pérez M, Erb L, Clarke LL, Turner JT, Weisman GA, González FA. 2000. Mechanisms of agonist-dependent and -independent desensitization of a recombinant $P2Y_2$ nucleotide receptor. Mol Cell Biochem 205:115–123.

Parr CE, Sullivan DM, Paradiso AM, Lazarowski ER, Burch LH, Olsen JC, Erb L, Weisman GA, Boucher RC, Turner JT. 1994. Cloning and expression of a human P_{2U} nucleotide receptor: a target for cystic fibrosis pharmacotherapy. Proc Natl Acad Sci USA 91:3275–3279.

Patel V, Brown C, Goodwin A, Wilkie N, Boarder MR. 1996. Phosphorylation and activation of p42 and p44 mitogen-activated protein kinases are required for the P2 purinoceptor stimulation of endothelial prostacyclin production. Biochem J 320:221–226.

Ragg SJ, Kaga S, Berg KA, Ochi A. 1998. The mitogen activated protein kinase pathway inhibits ceramide-induced terminal differentiation of a human monoblastic leukemia cell line, U937. J Immunol 161:1390–1398.

Seger R, Krebs E. 1995. The MAPK signaling cascade. FASEB J 9:726–735.

Soltoff SP. 1998. Related adhesion focal tyrosine kinase and the epidermal growth factor receptor mediate the stimulation of mitogen-activated protein kinase by the G-protein-coupled $P2Y_2$ receptor. Phorbol ester or $[Ca^{2+}]i$ elevation can substitute for receptor activation. J Biol Chem 273:23110–23117.

Soltoff SP, Avraham H, Avraham S, Cantley LC. 1998. Activation of $P2Y_2$ 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.

Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F, Duhamel L, Charon D, Kirilovsky J. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 266:15771–15781.

Turner JT, Weisman GA, Camdem JM. 1997. Upregulation of $P2Y_2$ nucleotide receptors in rat salivary gland cells during short-term culture. Am J Physiol 273:C1100–C1107.

Turner JT, Weisman GA, Fedan JS. 1998. The P2 nucleotide receptors. Totowa, NJ: Humana Press.

Velázquez B, Garrad RC, Weisman GA, González FA. 2000. Differential agonist-induced desensitization of $P2Y_2$ nucleotide receptors by ATP and UTP. Mol Cell Biochem 206:75–89.

Vlahos CJ, Matter WF, Hui KY, Brown RF. 1994. A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem 269:5241–5248.

Wang D, Huang N, Heppel LA. 1990. Extracellular ATP shows synergistic enhancement of DNA synthesis when combined with agents that are active in wound healing or as neurotransmitters. Biochim Biophys Res Commun 166:251–256.

Wang D, Huang N, Heppel LA. 1992. Extracellular ATP and ADP stimulate proliferation of porcine aortic smooth muscle cells. J Cell Physiol 153:221–233.

Wang S, Guo C, Castillo A, Dent P, Grant S. 1998. Effect of bryostatin 1 on taxol-induced apoptosis and cytotoxicity in human leukemia cells (U937). Biochem Pharmacol 56:634–644.

Weisman GA, Erb L, Garrad RC, Theiss PM, Santiago-Pérez LI, Flores RV, Santos-Berríos C, Méndez Y, González FA. 1998. P2Y nucleotide receptors in the immune system: signaling by a $P2Y_2$ receptor in U937 monocytes. Drug Dev Res 45:222–228.

Yamauchi J, Nagao M, Kaziro Y, Itoh H. 1997. Activation of p38 mitogen-activated protein kinase by signaling through G protein-coupled receptors. Involvement of $G\beta\gamma$ and $G\alpha q/11$ subunits. J Biol Chem 272:27771–27777.

Yan M, Templeton DJ. 1994. Identification of 2 serine residues of MEK-1 that are differentially phosphorylated during activation by raf and MEK kinase. J Biol Chem 269:19067–19073.

Zheng CF, Guan KL. 1993. Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. J Biol Chem 268:23933–23939.