Activation of P2Y2 Receptors by UTP and ATP Stimulates Mitogen-activated Kinase Activity through a Pathway That Involves Related Adhesion Focal Tyrosine Kinase and Protein Kinase C*

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We examined downstream signaling events that followed the exposure of PC12 cells to extracellular ATP and UTP, and we compared the effects of these P2 receptor agonists with those of growth factors and other stimuli. Based on early findings, we focused particular attention on the mitogen-activated protein (MAP) kinase pathway. ATP and/or UTP produced increases in tyrosine phosphorylation of multiple proteins, including p42 MAP (ERK2) kinase, related adhesion focal tyrosine kinase (RAFTK) (PYK2, CAKβ), focal adhesion kinase (FAK), Shc, and protein kinase C6 (PKCδ). MAP (ERK2) kinase activity (quantified by substrate phosphorylation) was increased by UTP, ATP, phorbol 12-myristate 13-acetate, ionomycin, and growth factors. UTP and ATP were equipotent (EC50 = 25 μM) in stimulating MAP kinase activity, suggesting that these effects were mediated via the G1-linked P2Y2 (P2Y) receptor. Consistent with this, the UTP- and ATP-promoted activation of MAP kinase was diminished in p21ras or Shc-null-transformed cells. Treatment of cells with pertussis toxin also reduced the activation of MAP kinase by UTP and ionomycin was blocked, and the tyrosine phosphorylation of RAFTK was reduced. The UTP-promoted increase in MAP kinase activity was partially reduced in cells in which PKC was down-regulated, suggesting that both PKC-dependent and PKC-independent pathways were involved. PKCδ, which increases MAP kinase activity in some systems, became tyrosine-phosphorylated within 15 s of exposure of cells to ATP or UTP; but epidermal growth factor, nerve growth factor, and insulin had little effect. UTP also promoted the association of Shc with Grb2. These results suggest that the P2Y2 receptor-initiated activation of MAP kinase was dependent on the elevation of [Ca2+]i, involved the recruitment of Shc and Grb2, and was mediated by RAFTK and PKC.

P2-type purinoceptors constitute a diverse set of proteins that are linked by their common ability to bind extracellular ATP and elicit an increase in intracellular Ca2+ and other ions.

ATP is a neurotransmitter or co-transmitter in some systems (1–3), and this has been the proposed rationale for the existence of P2 receptors on some cells. Previously, classifications of receptor subtypes were based on pharmacological hierarchies of the responses to ATP analogs (4, 5). The P2 subclassifications have been reorganized based on recent molecular biology approaches and now encompass more than a dozen types of P2 receptors into a family of two main receptor types: P2X and P2Y purinoceptors. P2X receptors are ligand-gated ion channels, and P2Y receptors are seven transmembrane proteins that are GTP-dependent protein (G-protein)-coupled receptors (6, 7).

In preliminary studies we observed that ATP and UTP produced alterations in the tyrosine phosphorylation of multiple proteins in PC12 cells, including one similar in mass to mitogen-activated protein (MAP) (p42 ERK) kinase, suggesting the involvement of the P2Y2 receptor in various signal transduction events. This receptor is a 53-kDa protein (8) that is linked by a heterotrimeric G-protein to phospholipase C, and UTP promotes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacyglycerol and inositol 1,4,5-trisphosphate, which activate protein kinase C (PKC) and elevate the intracellular calcium ion concentration ([Ca2+]i), respectively. The P2Y2 receptor has been postulated to be activated by the physiological release of intracellular ATP from cells, and thus ATP may act as an autocrine or paracrine factor (9–11). However, signaling molecules that are downstream of the P2Y2 and other P2 receptors have not been characterized as well as those activated by growth factors.

In the present study we focused attention on several proteins, particularly those involved in the MAP kinase cascade, to determine which signaling molecules were stimulated by activation of the P2Y2 receptor by ATP and UTP. In addition, we compared the effects of extracellular nucleotides and growth factors on PKCδ tyrosine phosphorylation. PKCδ, a Ca2+-in-sensitive member of the PKC family of proteins, is phosphorylated on tyrosine residues in response to various stimuli. These include the activation of platelet-derived growth factor receptors in NIH3T3 and 32D cells (12), muscarinic and substance P receptors in freshly isolated parotid acinar cells (13), and the transformation of cells with the oncogenic Ras protein (14). In 32D cells, a murine myeloid progenitor cell line, the tyrosine phosphorylation of PKCδ was associated with cell differentiation.

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§ The abbreviations used are: G-protein, GTP-dependent protein; MAP, mitogen-activated protein; [Ca2+]i, intracellular calcium ion concentration; PKC, protein kinase C; NGF, nerve growth factor; RAFTK, related adhesion focal tyrosine kinase; FMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; P-Tyr, phosphotyrosine; PTX, pertussis toxin; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; MPB, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; FAK, focal adhesion kinase; BzATP, 3-O-(4’-benzoyl)-benzoyl-ATP.
tion (13). However, as reported here, we find that nerve growth factor (NGF), which promotes differentiation (neurite outgrowth) in PC12 cells, did not promote the tyrosine phosphorylation of PKCδ; in contrast, the activation of the P2Y2 receptor was much more effective in promoting this phosphorylation.

Our results indicate that MAP (p42 ERK) kinase activity was increased by UTP by a mechanism distinct from that of growth factors. MAP kinase activation by UTP was correlated with the tyrosine phosphorylation of related adhesion focal tyrosine kinase (RAFTK), a protein that is upstream of MAP kinase, and conditions that reduced the tyrosine phosphorylation of RAFTK also reduced the activation of MAP kinase by UTP. The tyrosine phosphorylation of p42 MAP kinase, PKCδ, RAFTK, and other proteins in cells treated with ATP and UTP indicates that stimulation of the P2Y2 receptor promotes the activation of multiple signaling molecules and demonstrates that tyrosine phosphorylation is involved in mediating signal transduction events initiated by the activation of this G-protein-coupled receptor.

MATERIALS AND METHODS

Reagents—All chemicals were reagent grade or better. Dulbecco’s modified Eagle’s medium and phorbol 12-myristate 13-acetate (PMA) were obtained from Life Technologies, Inc. Calf serum, horse serum, UTP, and ATP were purchased from Sigma. EGF was from Upstate Biotechnology Co. (Lake Placid, NY), insulin from Collaborative Biomedical Products (Bedford, MA), and NGF (2.5 S) from Boehringer Mannheim. [32P]ATP (specific activity, 3,000 Ci/mol) was purchased from NEN Life Science Products. Anti-phosphotyrosine (anti-P-Tyr) antibody was a generous gift of Dr. Tom Roberts (Dana Farber, Boston). Polyclonal anti-RAFTK antibody was produced as described previously (15). Polyclonal anti-Sc (S14630) and monoclonal anti-Sc (S14620) antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-PKCδ (SC-213) and anti-ERK2 (SC-154) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Pertussis toxin (PTX; product 1803) was purchased from List Biological Laboratories, Inc. (Campbell, CA).

Cell Culture—PC12 cells were grown in 100-mm-diameter dishes at 37 °C in a mixture of 95% air and 5% CO2 in Dulbecco’s modified Eagle’s medium with 5% calf serum and 5% horse serum. Cells were used at or near confluence. In all experiments, cells were maintained overnight in low serum medium (Dulbecco’s modified Eagle’s medium plus 0.05% calf serum, 0.05% horse serum). Cells were exposed to stimuli by changing the medium with stimuli or vehicle. Cells were lysed in lysis buffer (137 mM NaCl, 20 mM Tris base, pH 7.5, 1 mM EGTA, 1 mM EDTA, 0.2 mM vanadate, pH 7.5). All wash solutions contained 0.2 mM vanadate. The majority of the TNE was removed, the remaining volume was diluted with an equal volume of 2 × sample buffer, and the samples were boiled for 5–10 min. Phosphorylated proteins and the lysate fractions were subjected to electrophoresis or stored at −80 °C before electrophoresis.

Samples were subjected to electrophoresis on an SDS-polyacrylamide separating gel with a 3% stacking gel. The separating gel was 12% for anti-Shc immunoprecipitates, and 7% for all others. Proteins were transferred to 0.2-μm pore size nitrocellulose filters, and the filters were blocked with TBS (20 mM Tris, pH 7.6, 137 mM NaCl) and 2% (w/v) bovine serum albumin for 1 h. The filters were washed in TTBS (TBS and 0.2% (w/v) Tween 20) three times. The nitrocellulose filters were exposed to blotting antibodies in TTBS and 1% bovine serum albumin for ~16 h at 4 °C. The filters were washed three times in TTBS and exposed to anti-rabbit or anti-mouse horseradish peroxidase (Boehringer Mannheim) at a 1:10,000 dilution in TTBS and 1% bovine serum albumin for 1 h. All washes and exposure to the secondary antibody were performed at room temperature. Filters were washed three times with TTBS and twice with TBS and were visualized on x-ray film (Kodak) using a chemiluminescence system (Amersham or NEN Life Science Products). In some experiments, the filters were stripped of antibodies by exposing them to 62.5 mM Tris, pH 6.8, 0.1% β-mercaptoethanol, and 2% (w/v) SDS at 70 °C for 40 min. The stripped filters were washed several times in TTBS, once in TBS, blocked with TBS and 2% bovine serum albumin for 1 h, and reprobed with antibody overnight. Blots were then treated as described above.

ERK2/MAP Kinase Activity—ERK2 was immunoprecipitated from PC12 cells by incubating cleared lysates with anti-ERK2 antibody and protein A-Sepharose beads for 3–4 h (see above). The immunoprecipitates were washed two times with RIPA buffer containing 1 mM vanadate and two times with kinase buffer (50 mM Tris, pH 7.5, 5 mM MgCl2). Immunoprecipitates were resuspended in a final volume of 50 μl of kinase buffer containing myelin basic protein (MBP) (200 μg/ml kinase buffer), and the kinase assay was initiated with the addition of 40 mM ATP plus [γ-32P]ATP (1 μCi). After 30 min, in some experiments 20 μl of the supernatant were removed and spotted onto p81 Whatman paper. An identical volume of supernatant was collected and spotted from a kinase mixture prepared in the absence of immunoprecipitated proteins as a control, and this background value of radioactivity was subtracted from all samples. Unreacted [γ-32P]ATP was removed by rinsing the p81 papers five times with 0.22 μl phosphoric acid, and the phosphorimaged paper was quantified by scanning. In other experiments the supernatant was added to an equal volume of 2 × sample buffer, boiled, and subjected to SDS-PAGE using a 15% separating gel. The phosphorylated MBP was quantified using a molecular imager system (Bio-Rad GS-363). Similar results were obtained using the two methods of MAP kinase quantification.

[Ca2+]/Ca2+—PC12 cells were suspended in a physiological salt solution containing 5 μM fura-2-acetoxymethylester and 10 μM probenecid in a physiological salt solution for 50 min, as reported previously for other cells (16). The cells were washed twice, and [Ca2+]i was monitored at room temperature in a SPEX FluoroMax-2 spectrofluorometer. [Ca2+]i was quantified from the fluorometric ratio emitted at 510 nm from dual wavelength excitation at 340 and 380 nm.

Data—Immunoblots similar to those shown in the figures were obtained in two or more independent experiments. The numbers (n) of independent assays of ERK2 activity were as noted in the figures or in the text.

RESULTS

UTP, ATP, and PMA Promote the Tyrosine Phosphorylation of Multiple Proteins, Including MAP Kinase—Analysis of the tyrosine phosphorylation pattern of cells treated with UTP, ATP, and PMA indicated that multiple proteins were phosphorylated after various lengths of exposure to ATP and UTP (Fig. 1). These included proteins in the following apparent molecular mass region: the time- and peak-immunoreactive phosphorylated 159 kDa (UTP or ATP for 1 min), ~101 and 90 kDa (ATP or UTP for 1 or 5 min), ~76 kDa (UTP or PMA for 5 min), ~63 and ~67 kDa (UTP or ATP for 1 min), ~49 and ~49 kDa (ATP or ATP for 1 min), ~40 and ~44 kDa (UTP and PMA for 5 min). Thus, there were distinct proteins that exhibited different peak times in tyrosine phosphorylation when exposed to P2Y2 agonists or...
MAP Kinase Stimulation by P₂Y₂ Receptors

Fig. 1. UTP, ATP, and PMA increase the tyrosine phosphorylation of multiple proteins in PC12 cells. Cells were exposed to UTP (100 μM) or ATP (100 μM) for 0.2–15 min or to PMA (200 μM) for 5 min. Cells were lysed, and a fraction of the lysate was subjected to SDS-PAGE and was immunoblotted using anti-P-Tyr antibody (IB Ab; 1 μg/ml). Molecular mass markers are shown on the left, and the arrows on the right indicate the location of some of the proteins that displayed an increase in tyrosine phosphorylation. For additional information, see "Results."

PM. Several proteins were identified by reprobing the blots with antibodies to known proteins of similar size. The ~40- and ~44-kDa proteins co-localized with the MAP kinases ERK2 and ERK1 (not shown), respectively, which are proteins of 42 and 44 kDa. This suggested that activation of the P₂Y₂ (P₂U) receptor activated MAP kinase and affected other signaling proteins in these cells.

PTX Reduces the P₂Y₂ Receptor-mediated Increase in MAP Kinase Activity—Additional experiments were performed to confirm that MAP kinase activity was increased by activation of the P₂Y₂ receptor. In vitro phosphorylation assays were performed on ERK2 kinase immunoprecipitates using MBP as a substrate (see "Materials and Methods"). MAP kinase activity was increased in cells exposed to UTP (100 μM) or ATP (100 μM) for 5 min (Fig. 2A). This period of time (5 min) was one at which there appeared to be a readily detectable increase in tyrosine phosphorylation of MAP kinase promoted by these stimuli (Fig. 1). In experiments in which the effects of UTP (100 μM) and ATP (100 μM) were both measured after a 5-min exposure, the activation of MAP kinase by UTP was 67.0 ± 9.0% (10) as large as that produced by ATP, consistent with the ability of either UTP or ATP to bind to and activate the P₂Y₂ receptor. There were negligible increases in MAP kinase activity in cells treated with UTP (100 μM) for 1 min. PMA, EGF, NGF, and the calcium ionophore ionomycin also produced large increases in MAP kinase activity after a 5-min exposure (Fig. 2A), as reported by others.

The concentration dependences of ATP and UTP on MAP kinase activity also were examined. Although the absolute magnitudes of MAP kinase activities were different for the two nucleotides at each concentration, the relative effects of different concentrations of each nucleotide were very similar, and the EC₅₀ value was ~25 μM for both ATP and UTP (Fig. 2B). This is consistent with the stimulatory effects of ATP and UTP on MAP kinase being caused by the activation of a P₂Y₂ receptor.

The activation of MAP kinase by UTP and ATP was reduced by about 40% in PTX-treated cells (Fig. 3). In contrast, the basal MAP kinase activity and the activation of MAP kinase by EGF and ionomycin were not significantly affected by PTX treatment. These results are consistent with the P₂Y₂ receptor being coupled to Gᵢ, a PTX-sensitive G-protein, and suggest that the activation of MAP kinase by UTP and ATP is initiated by the activation of this receptor.

Activation of MAP Kinase by UTP Requires the Elevation of [Ca²⁺]i—Because UTP elevates [Ca²⁺]i, in PC12 (17) and other cells (for review, see Ref. 5) and calcium ionophores activate MAP kinase in PC12 (18) and other cells, we examined whether the UTP-dependent activation of MAP kinase was dependent on an elevation of [Ca²⁺]i. To block the elevation of [Ca²⁺]i, PC12 cells were loaded with the calcium chelator BAPTA, and extracellular calcium was depleted by the addition of EGTA to the medium. These conditions reduced the activation of MAP kinase by UTP and ionomycin to near basal levels, but the activation of MAP kinase by EGF was not affected significantly (Fig. 4). Treatment of cells with BAPTA/EGTA also blocked the tyrosine phosphorylation of ERK2 and ERK1 by UTP and ionomycin but not by EGF (not shown). These results indicated that the elevation of [Ca²⁺]i, mediated the activation of MAP kinase by UTP and ionomycin but not by EGF.

We also examined the effects of PTX on alterations of [Ca²⁺]i, by different stimuli. The elevation of [Ca²⁺]i, by 100 μM UTP was reduced by 50% in PTX-treated PC12 cells compared with control cells (not shown), consistent with the coupling of Gᵢ between the P₂Y₂ receptor and phospholipase C. In contrast, PTX did not affect the increase in [Ca²⁺]i, which was produced by 10⁻⁸ M bradykinin (not shown). Along with the BAPTA/EGTA data presented in Fig. 4, these results suggest that an increase in [Ca²⁺]i, is upstream of the the P₂Y₂ receptor-initiated activation of MAP kinase.

RAFTK Is Involved in Mediating the Activation of MAP Kinase by UTP—RAFTK (also called PYK2 and CAKb), is a protein tyrosine kinase that can couple G-protein-linked receptors to the MAP kinase activation cascade (19–21). PYK2/RAFTK is phosphorylated on tyrosine residues when [Ca²⁺]i, is elevated or PKC is activated (20). Because the effects of UTP on MAP kinase activation require elevations in [Ca²⁺]i, we determined if it might be involved in mediating the MAP kinase activation by UTP. In anti-RAFTK immunoprecipitations, RAFTK was phosphorylated on tyrosine residues in cells exposed to UTP, ionomycin, and EGF (Fig. 5A), indicating that all three stimuli activated this kinase. In most experiments, the tyrosine phosphorylations initiated by UTP and ionomycin were much greater than those produced by EGF. The tyrosine phosphorylation of focal adhesion kinase (FAK), a tyrosine kinase that has substantial sequence homology to RAFTK (hence the name, Related Adhesion Focal Tyrosine Kinase) was also increased by UTP and EGF (Fig. 5B). EGF produced a greater increase in FAK tyrosine phosphorylation than did UTP, and ionomycin decreased the phosphorylation to levels below that found under basal conditions.

In cells loaded with BAPTA and exposed to EGTA, the tyrosine phosphorylation of RAFTK was decreased significantly in UTP- and ionomycin-treated cells but not in EGF-treated cells (Fig. 5C). In multiple experiments, exposure of cells to BAPTA/EGTA blocked the ionomycin-promoted phosphorylation of RAFTK more completely than this treatment blocked the UTP-promoted phosphorylation. This was probably because both the elevation of [Ca²⁺]i, and the activation of PKC contributed to the UTP-promoted phosphorylation of RAFTK, and BAPTA/EGTA blocked the former. These results are consistent with the involvement of RAFTK in the activation of MAP kinase by UTP and suggest that at least part of the response is caused by the UTP-promoted increase in [Ca²⁺]i.

The UTP-promoted tyrosine phosphorylation of RAFTK was also decreased substantially in PTX-treated cells (see Fig. 7D), consistent with the inhibitory effect of PTX on the elevation of [Ca²⁺]i (see above) and a role for [Ca²⁺]i, upstream of RAFTK activation. These results suggest the involvement of RAFTK in...
the activation of MAP kinase by UTP and indicate that a large part of the MAP kinase activation is caused by the UTP-promoted increase in $[\text{Ca}^{2+}]_i$.

MAP Kinase Activation by UTP Involves PKC—To determine whether PKC was involved in the activation of MAP kinase by UTP, the response of cells to UTP was measured in cells in which PKC was down-regulated by treating the cells overnight with PMA. The exposure of PKC-down-regulated cells to UTP for 5 min produced a reduced but significant increase in activity (Fig. 6A). In contrast, the basal MAP kinase activity was not affected significantly in PKC-down-regulated cells, and the effect of an acute addition of PMA was completely ineffective in stimulating MAP kinase activity. These findings suggest that MAP kinase activation via stimulation of the P$_{2Y2}$ receptor involves both PKC-dependent and PKC-independent pathways.

The effects of PKC down-regulation on the activation of MAP
MAP Kinase Stimulation by P$_{2Y2}$ Receptors

PKC down-regulation blocks the activation of MAP kinase by UTP, ATP, and PMA, and reduces the shift to a hyperphosphorylated form of p42 ERK. PC12 cells were treated overnight with PMA (1 $\mu$m) or dimethyl sulfoxide (control). Cells were exposed to UTP (100 $\mu$m), ATP (100 $\mu$m), PMA (200 nm), or vehicle (dimethyl sulfoxide or water) for 5 min, lysed, and p42 ERK2 was immunoprecipitated. Panel A, anti-ERK2 immunoprecipitates were used to assay MAP (ERK2) kinase activity by measuring the phosphorylation of MBP (see "Materials and Methods"). All measurements of MAP kinase activity were normalized to that measured under control basal conditions (no agonist, no PMA overnight). Pretreatment with PMA completely blocked the response to the acute addition of PMA and blocked the majority of the response to UTP. The values are means ± S.E. from four separate experiments. Panel B, the anti-ERK2 immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose filters, and immunoblotted using anti-ERK2 antibody (0.2 $\mu$g/ml) and subsequently reprobed with anti-P-Tyr antibody (1 $\mu$g/ml). The arrows on the right designate the upper form of p42 ERK2, which is phosphorylated on multiple amino acid residues, including tyrosine. In cells pretreated with PMA overnight, the shift to a hyperphosphorylated form was blocked completely in cells treated acutely (5 min) with PMA but was present at a reduced level in cells treated with UTP (5 min).
PKCδ is phosphorylated on tyrosine residues in cells exposed to UTP, ATP, and PMA. PC12 cells were exposed to stimuli, lysed, and PKCδ was immunoprecipitated using anti-PKCδ antibody. Proteins were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with anti-P-Tyr antibody (1 µg/ml) unless otherwise indicated. Proteins were visualized on X-ray film using enhanced chemiluminescence techniques. Panel A, cells were treated with PMA (200 nM, 5 min), EGF (100 ng/ml), insulin (100 nM, 1 min), nucleotide analogs (100 µM, 1 min), or vehicle (dimethyl sulfoxide or water, 1 min). A molecular mass marker is shown on the left, and the arrow on the right indicates the location of the tyrosine-phosphorylated form of PKCδ. α,β-ATP, α,β-methylene ATP, 2-M-SATP, 2-methyl-thio-ATP, ATP, UTP, and PMA increased the tyrosine phosphorylation of PKCδ. Panel B, cells were exposed to ATP (100 µM) or UTP/100 µM for 0.2–15 min or PMA (200 nM) for 5 min. A molecular mass marker is shown on the left, and the arrow on the right indicates the location of the tyrosine-phosphorylated form of PKCδ. ATP and UTP produced rapid increases in the tyrosine phosphorylation of PKCδ. Panel C, cells were exposed to UTP (100 µM), NGF (100 ng/ml), or EGF (100 ng/ml) for 0.2–5 min or PMA (200 nM) for 5 min. A molecular mass marker is shown on the left, and the arrow on the right indicates the location of the tyrosine-phosphorylated form of PKCδ. ATP and UTP produced rapid increases in the tyrosine phosphorylation of PKCδ. Panel D, cells were treated with vehicle (-) or 1 µM PMA (+PMA) overnight to down-regulate PKC, and subsequently they were exposed to an acute addition of PMA (200 nM) for 5 min. Anti-PKCδ immunoprecipitates were immunoblotted using anti-PKCδ antibody (0.2 µg/ml) and were reprobed subsequently with anti-P-Tyr antibody (0.2 µg/ml). The arrows on the right designate the location of the tyrosine-phosphorylated form of PKCδ. The amount of immunoprecipitable PKCδ was reduced significantly in cells pretreated with PMA (upper panel). Control cells, but not cells in which PKCδ was down-regulated, responded to the acute addition of PMA with an increase in PKCδ tyrosine phosphorylation (lower panel).

The time course of PKCδ tyrosine phosphorylation was examined using ATP and UTP. The largest increases in tyrosine phosphorylation occurred in cells exposed to ATP or UTP for −15 s or 1 min, and the phosphorylation returned to near basal levels after 5 min or more (Fig. 7B). These results indicate that activation of the P2Y2 receptor produces very rapid increases in the tyrosine phosphorylation of PKCδ. This event temporally precedes the peak activation of MAP kinase (Fig. 7A) by UTP, consistent with the possibility that the stimulatory effect of UTP on MAP kinase could involve PKCδ (see “Discussion”).

The effects of UTP, NGF, and EGF on PKCδ tyrosine phosphorylation were compared over an expanded time course. At times of 0.2, 1, and 5 min of exposure, neither NGF nor EGF produced increases in PKCδ tyrosine phosphorylation which were of a magnitude similar to those produced by a 0.2- or 1-min exposure to UTP or by a 5-min exposure to PMA (Fig. 7C). Slight increases in PKCδ tyrosine phosphorylation were sometimes evident for EGF- or NGF-treated cells during longer exposures of the immunoblot using enhanced chemiluminescence techniques. In contrast, NGF and EGF produced large increases in tyrosine phosphorylation of other proteins, observed on anti-P-Tyr immunoblots of cell lysates (not shown). There was no detectable increase in the tyrosine phosphorylation of PKCδ produced by the exposure of PC12 cells to the calcium ionophore ionomycin (10−6 M) (not shown). These results indicate that the phosphorylation of PKCδ in response to UTP at these early times (≤1 min) is not mimicked by the activation of growth factor receptor tyrosine kinases or the elevation of [Ca2+]i.

The amount of PKCδ in anti-PKCδ immunoprecipitates from PC12 cells treated overnight with PMA (1 µM) was reduced greatly compared with cells in which PKCδ was not down-regulated (Fig. 7D, upper panel). Unlike the control cells, the cells in which PKCδ was down-regulated did not respond to the acute addition of PMA (200 nM, 5 min) with a detectable increase in tyrosine phosphorylation (Fig. 7D, lower panel). These data parallel the diminished effect of PMA and UTP on the activation of MAP kinase in PKCδ-down-regulated cells (Fig. 8A) and are consistent with the potential involvement of PKCδ in the activation of MAP kinase (see “Discussion”).

Activation of the P2Y2 Receptor Affects Shc and Grb2—Because RAFTK/PYK2 appears to activate MAP kinase by engaging Shc and Grb2 (20), we also examined whether UTP promotes the formation of a Grb2-Shc complex. There was an increase in the tyrosine phosphorylation of two forms of Shc (46 and 52 kDa) after treatment of PC12 cells with UTP for 1 and 5 min (Fig. 8A). EGF, NGF, ionomycin, and PMA also produced substantial increases in the tyrosine phosphorylation of these proteins.
forms of Shc. EGF and NGF also increased the tyrosine phosphorylation of the 66-kDa form of Shc (Fig. 8A). Grb2 was co-immunoprecipitated with Shc in cells treated with all of these stimuli, and the amount of immunoprecipitated Grb2 (Fig. 8B) was directly proportional to the tyrosine phosphorylation of Shc (Fig. 8A), consistent with the recruitment of Grb2 to Shc via its SH2 domain. In cells treated with UTP for various times, the peak increase in the tyrosine phosphorylation of Shc (Fig. 8A) and the co-immunoprecipitation of Grb2 (Fig. 8B) was at 5 min, similar to the time of the peak increase in MAP kinase activity (Figs. 1 and 2A). These results suggest that Shc and Grb2 are signaling molecules involved in the downstream effects of the activation of the P2Y2 receptor in PC12 cells, and they place RAFTK between the P2Y2 receptor and these molecules in the signaling cascade involved in the activation of MAP kinase by UTP.

**DISCUSSION**

In this study it is demonstrated that multiple signaling proteins, including p42 ERK2/MAP kinase, PKCα, Shc, FAK, and RAFTK are phosphorylated on tyrosine residues in PC12 cells exposed to ATP and/or UTP. UTP and ATP had similar potencies in activating MAP kinase (Fig. 2B), and PTX blocked the activation of MAP kinase by UTP and ATP to a similar degree (Fig. 3), indicating that the effects were mediated by a G-protein-coupled P2Y2/P2U receptor. The involvement of RAFTK/PYK2/CAKβ in the activation of MAP kinase by some stimuli was indicated by previous findings demonstrating that the overexpression of PYK2/RAFTK activated MAP kinase in a PYK2/RAFTK concentration-dependent manner, and the expression of kinase-dead PYK2/RAFTK reduced the activation of MAP kinase (20). PYK2/RAFTK is activated by multiple stimuli, including the elevation of [Ca2+]i, and the activation of PKC. In the present studies of PC12 cells, the involvement of RAFTK in the activation of MAP kinase by UTP was suggested by the following: (a) the similar [Ca2+]i dependence of UTP-stimulated RAFTK tyrosine phosphorylation (Fig. 5C) and MAP kinase activation (Fig. 4); (b) the similar PTX sensitivity of the UTP-stimulated RAFTK tyrosine phosphorylation (Fig. 5D) and MAP kinase activation (Fig. 3); and (c) the ability of the calcium ionophore ionomycin to mimic the effects of UTP on both RAFTK tyrosine phosphorylation (Fig. 5A) and MAP kinase activation (Fig. 2A). The PTX sensitivity of the effects of UTP on [Ca2+]i, RAFTK tyrosine phosphorylation, and MAP kinase activation are consistent with the coupling of the P2Y2 receptor to a G1-type G-protein, and the Ca2+ sensitivities of RAFTK and MAK kinase indicate that the UTP-promoted elevation of [Ca2+]i is an important signal between P2Y2 receptor activation and the activation of MAP kinase. Other P2Y2 Receptor-promoted effects were also blocked by PTX in different cells, including IL-60 (25), smooth muscle (26), and human airway epithelial (27) cells.

UTP, ionomycin, PMA, EGF, and NGF all stimulated the activation of MAP kinase (Fig. 2A), the tyrosine phosphorylation of Shc (Fig. 8A), and the association of Grb2 and Shc (Fig. 8B). Thus, these results are consistent with the convergence of serpine receptor-mediated and growth factor receptor-mediated activation of MAP kinase occurring at the level of Shc/Grb2 in the MAP kinase activation cascade (28, 29). However, because our results indicate that EGF also stimulated the tyrosine phosphorylation of RAFTK, albeit to a much less degree than UTP or ionomycin, and because neither this phosphorylation nor the activation of MAP kinase by EGF was sensitive to decreases in calcium, we cannot rule out a contribution of RAFTK in the EGF-dependent activation of MAP kinase. The activation of MAP kinase by EGF may involve the stimulation of RAFTK/PYK2/CAKβ primarily via a PKC-dependent pathway.

A number of studies have characterized effects of ATP and other P2 analogs on PC12 cells (30–35). The results of these and other studies indicated that PC12 cells have multiple P2 subtypes. A P2X receptor was cloned from PC12 cells (24). Various studies observed effects of UTP on PC12 cells. ATP and UTP had similar potencies in promoting intracellular calcium mobilization, indicating the presence of a P2Y2 receptor (17). However, other studies reported that UTP was ineffective in elevating [Ca2+]i (36), and one study concluded that the UTP-sensitive nucleotide receptor in PC12 cells was not the 53-kDa UTP receptor (P2Y2 receptor). Part of the reason for the differences in reports from various laboratories using PC12 cells may result from variations in PC12 cell lines in different laboratories.

In the present studies we show that UTP is more effective than the growth factors NGF, EGF, or insulin in stimulating the tyrosine phosphorylation of PKCα, although these growth factors promote the tyrosine phosphorylation of a number of other signaling molecules. The maximum increase in the UTP-dependent tyrosine phosphorylation of PKCα occurred in cells exposed to UTP for ≤ 1 min (Fig. 7B), a time earlier than the UTP-dependent increase in MAP kinase activity, which did not occur until well after 1 min of UTP exposure (Fig. 2A). Overnight treatment of PC12 cells with PMA resulted in the down-regulation of PKCα (Fig. 5D) as well as a substantial decrease in the PMA- and UTP-dependent activation of MAP kinase (Fig. 6A). However, these studies alone are insufficient to support a conclusion that PKCα is involved in the activation of MAP kinase by extracellular nucleotides because PC12 cells express other members of the PKC family of proteins, including the isoforms α, β, ε, η, and ι (37, 38). In addition, MAP kinase can be activated by both PKC-dependent and PKC-independent pathways (Fig. 6, A and B). The transfection of constitutively active PKCα into COS1 cells resulted in the activation of MAP/ERK kinase (MEK) and MAP kinase in a Raf-dependent fashion, and this was not produced by transfection of constitutively active PKCa and PKCe, suggesting that activation of PKCα does lead to MAP kinase activation in some systems (22). It remains to be determined whether PKCα and/or other PKC family members are involved in the UTP-promoted activation of MAP kinase in PC12 cells. In addition, there are contrasting reports of whether the tyrosine phosphorylation of PKCα increases (12, 39) or reduces (14, 40) the activation of the enzyme. However, a more important consideration may be that PKCα translocates from a cytosolic location to a cellular membrane. This may be more relevant than measurements demonstrating a fractional reduction in its activity because the translocation of PKC presumably will be to the sites of their physiological substrates.

The P2Y2 receptor is found on a wide variety of cell types, including freshly isolated cells as well as multiple cell lines (41; for review, see Ref. 5). In some cells extracellular ATP has mitogenic effects (42, 43), but in others ATP acts as an anti-proliferative agent (44). In primary cultures of dog tracheal epithelial cells, UTP promoted the activation and phosphorylation of the Na-K-Cl cotransport protein (45). UTP and/or ATP activates Cl− secretion in many epithelial cells. Recently it was suggested that the P2Y2/P2X1 receptor is involved in modulating the cystic fibrosis transmembrane conductance regulator (CFTR). This model suggests that intracellular ATP released through the CFTR activates P2Y2 receptors, which, in turn, stimulate chloride secretion via the activation of outwardly rectifying chloride channels by a G-protein-coupled mechanism (9, 43). The precise mechanism of activation was not defined, but other channels, notably the cardiac K+ channel, are acti-
vated by Gβγ.

A number of studies have shown that growth factors and G-protein-coupled receptors activate certain signaling proteins in common and that tyrosine phosphorylation also plays a role in signaling pathways activated by G-proteins (for review, see Ref. 46). Gβγ subunits also activate MAP kinase in a Ras-dependent manner in response to activation of the tyrosine kinase insulin-like growth factor receptor (29). This activation was sensitive to PTX, and the effect of G-protein-linked receptors was dependent on Ras activation and G-proteins (29). Subsequently, the Gi-protein-coupled receptors activate certain signaling proteins by ATP and UTP, demonstrating that G-protein-coupled P2Y2 receptors and growth factor receptors, including those for EGF and NGF, activate some intracellular signaling proteins and pathways in common. Other signaling proteins, notably PKCδ, were uniquely activated by UTP and ATP. These results add to the expanding list of the involvement of tyrosine phosphorylation in events promoted by G-protein-linked receptors.

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Activation of P2Y2 Receptors by UTP and ATP Stimulates Mitogen-activated Kinase Activity through a Pathway That Involves Related Adhesion Focal Tyrosine Kinase and Protein Kinase C

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