The activation of growth factor receptors and receptors coupled to heterotrimeric guanine nucleotide-binding proteins (G-proteins) can increase mitogen-activated protein (MAP) kinase activity in many cells. Previously, we demonstrated that the activation of G-protein-coupled P_{2Y2} receptors by extracellular ATP and UTP stimulated MAP (p42 ERK2) kinase by a mechanism that was dependent on the elevation of [Ca^{2+}]_{i} and the activation of related adhesion focal tyrosine kinase (RAFTK) (also called PYK2, CAKβ, and CADTK) and protein kinase C (PKC). Here, we examine further the signaling cascade between the P_{2Y2} receptor and MAP kinase. MAP kinase was transiently activated by exposure of PC12 cells to UTP, UTP, ionomycin, and phorbol ester (phorbol 12-myristate 13-acetate) increased MAP kinase activity and also promoted the tyrosine phosphorylation of RAFTK, the epidermal growth factor (EGF) receptor, SHC, and p120^{cbl}. Down-regulation of PKC and inhibition of the elevation of [Ca^{2+}]_{i}, conditions that block the activation of MAP kinase, also blocked the increases in the tyrosine phosphorylation of RAFTK and the EGF receptor. AG1478, a tyrphostin selective for the EGF receptor, reduced the activation of MAP kinase, the tyrosine phosphorylation of SHC, the association of Grb2 with SHC, and the tyrosine phosphorylation of the EGF receptor and p120^{cbl} but did not block the tyrosine phosphorylation of RAFTK. The similar effects of UTP, ionomycin, and phorbol 12-myristate 13-acetate (PMA) on these signaling proteins demonstrate that the two signaling molecules from phosphatidylinositol 4,5-bisphosphate hydrolysis ([Ca^{2+}]_{i} from inositol 1,4,5-trisphosphate production, and diacylglycerol) can individually initiate the activation of MAP kinase in an EGF receptor-dependent manner. These results demonstrate that the P_{2Y2} receptor-mediated transactivation of the EGF receptor occurs at a point downstream of RAFTK and indicate that the EGF receptor is required for P_{2Y2} receptor-mediated MAP kinase activation. Although P_{2Y2} and EGF receptors may both activate a similar multiprotein signaling cascade immediately upstream of MAP kinase, the P_{2Y2} receptor appears to uniquely utilize [Ca^{2+}]_{i}, PKC, and, subsequently, RAFTK.

Recent studies have demonstrated that growth factor receptors and G-protein-coupled receptors (GPCRs) may both activate the same signal transduction molecules and utilize the same signaling cascades in cells. One of the most common signaling events mediated by both types of receptors is the activation of mitogen-activated protein (MAP) kinase, although portions of the signaling cascade between the receptors and MAP kinase can be different for growth factor receptors and GPCRs, particularly pertaining to the involvement of members of the PKC family of proteins. Previously, we examined the effects of extracellular nucleotides on PC12 cells (1). Extracellular nucleotides can bind to P_{2}Y-type purinoceptors, which constitute a large family of receptors that are either ion channels (P_{2}X subtypes) or else coupled to G-proteins (P_{2}Y subtypes) and which vary in their tissue distribution (2, 3). Previously, we observed that both extracellular ATP and UTP increased MAP kinase activity in a nucleotide concentration-dependent manner in PC12 cells, which also respond to EGF and NGF with increases in MAP kinase activity. For both ATP and UTP, the EC_{50} value was ~25 μM, and 100 μM promoted nearly a maximal effect (1). These results were consistent with the nucleotide-dependent activation of the P_{2Y2} receptor. This purinoceptor was previously designated P_{2U}, because it does not discriminate between ATP and UTP on the basis of potency. The P_{2Y2} receptor-mediated activation of MAP kinase involved the elevation of [Ca^{2+}]_{i}, the activation of PKC, and the tyrosine phosphorylation and activation of RAFTK (1). In contrast, these signaling events did not appear to play a major role in the mechanism of EGF-initiated increase in MAP kinase activity.

The effects of a number of GPCRs have been reported to involve increases in tyrosine phosphorylation. Signaling proteins such as SHC (4, 5) and members of the Src kinase family may contribute to GPCR-mediated activation of signaling pathways, including MAP kinase (4–11). In addition, the G_{bg} subunit of heterotrimeric G-proteins can mediate MAP kinase activation (5, 12, 13). GPCRs also can transactivate growth factor receptors, including the EGF receptor and the PDGF receptor (4, 9–11). Several reports suggest that the EGF receptor and other proteins may serve as a scaffolding structure or as an adaptor protein to which other signaling proteins may be recruited in response to GPCR signaling (5, 14). Proteins serving in this capacity can localize proteins to a particular region

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*This work was supported in part by National Institutes of Health Grant DE10877. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: GPCR, G-protein-coupled receptor; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; DAG, diacylglycerol; EGF, epidermal growth factor; ERK, extracellular signal-regulated protein kinase; NGF, nerve growth factor; RAFTK, related adhesion focal tyrosine kinase; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, acetoxymethyl ester; MAP, mitogen-activated protein; PDGF, platelet-derived growth factor.
RAFTK and EGFR Mediate GPCR-promoted MAP Kinase Activation

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The following phosphatase and protease inhibitors: 10 mM vanadate, 1 mM ZnCl2, 4.5 mM sodium pyrophosphate, 2 mg/ml NaF, 2 mg/ml β-glycerophosphate, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml apropin, and 2 μg/ml 4-2-aminoethylbenzenesulfonyl fluoride. The lysates were vortexed thoroughly and centrifuged at 16,000 × g (Eppendorf 5414 microcentrifuge) for 5–10 min. The cleared supernatants were transferred to fresh microcentrifuge tubes. In some experiments, a portion (5–10% of the volume) of the lysate was removed and combined with an equal volume of 2× sample buffer (62.5 mM Tris, pH 6.8, 10% [v/v] glycerol, 6.25% [v/v] SDS, 0.72% β-mercaptoethanol; bromphenol blue for color). The remainder was incubated at 4 °C for 3 or 8 h overnight with 0.25 μg/ml anti-SH2 (1:2000 dilution) or anti-doing receptor (1.5 μg/ml), anti-RAFTK (5 μl), or anti-Tyr(P) antibody (1 μg/ml), plus protein A-Sepharose (4 mg/ml) with the anti-RAFTK antibody and protein A-Sepharose (4 mg/ml) with the other antibodies. At the end of the incubation, the immunoprecipitates were collected by centrifugation. The immunoprecipitates were washed two times in ice-cold phosphate-buffered saline (137 mM NaCl, 15.7 mM NaH2PO4, 1.47 mM KH2PO4, 2.68 mM KC1, 1% Nonidet P-40, pH 7.4) one time in 0.1 μM Tris (pH 7.5), 0.5 mM LiCl; and two times in TNE (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.5). All wash solutions contained 0.2 mM vanadate. The majority of the TNE was removed, the remaining volume was diluted with 70 μl of 2× sample buffer, and the samples were boiled for 5–10 min. The immunoprecipitated proteins and the lysate fractions were subjected to electrophoresis or stored at −80 °C prior to electrophoresis.

Samples were subjected to electrophoresis on a 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, 157 mM NaCl), 2% [w/v] BSA for 1 h. The filters were washed in TBS (TBS, 0.2% [w/v] Tween 20) three times. The nitrocellulose filters were exposed to blotting antibodies in TBS, 1% bovine serum albumin for ~16 h at 4 °C. The filters were washed three times in TBS and exposed to goat anti-rabbit or goat anti-mouse horseradish peroxidase (Boehringer Mannheim) at a 1:10,000 dilution in TBS plus 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 TBS and twice with TBS and were visualized on x-ray film (Eastman Kodak Co.) using a chemiluminescence system (Amerham Pharmacia Biotech 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 M β-mercaptoethanol, 2% [w/v] SDS at 70 °C for 40 min. The stripped filters were washed as described above and reimmunoblotted with TBS plus 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 twice with RIPA lysis 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 (200 μg/ml kinase buffer), and the kinase assay was initiated with the addition of 40 μM ATP plus [γ-32P]ATP (1 μCi). After 30 min, the supernatant was added to an equal volume of 2× sample buffer, boiled, and subjected to SDS-polyacrylamide gel electrophoresis using a 10% separating gel. The phosphorylated myelin basic protein was quantified using a molecular imager system (Bio-Rad GS-363). For each independent experiment, each condition was assayed in duplicate or triplicate. 

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 below.

RESULTS

Time Dependence of UTP on MAP Kinase Activity—An established paradigm in PC12 cells is that agents that cause proliferation (e.g., EGF) produce short lived increases in MAP kinase activity, whereas agents that cause differentiation (e.g., NGF) produce much longer lived increases that are sustained for hours (17, 18). Previously, we observed that UTP did not produce a measurable increase in MAP kinase activity at 1 min but produced a substantial increase after a 5-min exposure (1). Therefore, we examined the degree to which the UTP-promoted increase in MAP kinase activity was sustained as a function of...
consistent with the desensitization of the P2Y2 receptor in the presence of UTP for a 5-min period between 55 and 60 min after a single exposure at time 0, there was an increase in MAP kinase activity. These results are presented by upward and downward arrows, respectively. After reaching a peak at 5 min, the MAP activity returned to basal levels after 60 min regardless of whether cells were exposed to UTP by a single exposure to UTP-containing media or by multiple exposures to UTP-containing media. However, if cells were exposed to fresh UTP-containing media for various periods of time. Since many cells have ectonucleotidases and ecto-ATPases that can hydrolyze extracellular nucleotides, it was possible that the lack of a sustained MAP kinase activation was due to the consumption of ligand, rather than to an intrinsic deactivation of MAP kinase. Therefore, in order to maintain the UTP concentration and study the time-dependent alterations in MAP kinase activity, we modified the protocol to include multiple solution switches during a 60-min time course. In these experiments, the cells were exposed to a single UTP-containing solution for an additional 5 min, there was an increase in MAP kinase activity (Fig. 1B). These results suggest that UTP is degraded when it is exposed to cells for an extended period of time and that the P2Y<sub>2</sub> receptor can reseenseitize to UTP under these conditions. In addition, the activation of MAP kinase is not maintained even under conditions designed to maintain the UTP concentration.

**Time Course of RAFTK Tyrosine Phosphorylation**—The tyrosine phosphorylation and activation of RAFTK (PYK2) is upstream of MAP kinase (7, 8, 15). Therefore, the time course of RAFTK tyrosine phosphorylation was examined in PC12 cells exposed to UTP and other stimuli (Fig. 2A). The tyrosine phosphorylation of RAFTK increased after a 1- and 5-min exposure. EGF produced increases in RAFTK tyrosine phosphorylation, but these increases were much smaller than those produced by UTP and ionomycin. This is in agreement with the fact that RAFTK can be stimulated by PKC activation/DAG production as well as by an elevation of [Ca<sup>2+</sup>], (15). In addition, since the activation of exposure to a single UTP-containing solution for 60 min. However, if cells were exposed continuously to a single UTP-containing solution for 55 min followed by exposure to a fresh solution of UTP for an additional 5 min, there was an increase in MAP kinase activity (Fig. 1B). These results suggest that UTP is degraded when it is exposed to cells for an extended period of time and that the P2Y<sub>2</sub> receptor can reseenseitize to UTP under these conditions. In addition, the activation of MAP kinase is not maintained even under conditions designed to maintain the UTP concentration.

**PKC Down-regulation Reduces the UTP-dependent Tyrosine Phosphorylation of RAFTK**—To determine whether PKC was involved in the UTP-initiated tyrosine phosphorylation of RAFTK, cells were treated overnight with PKC inhibitor (200 nM) to down-regulate protein kinases C. This treatment substantially reduced the tyrosine phosphorylation of RAFTK by UTP (100 µM, 1 min) and by acute exposure to PMA (200 nM, 1 min) (Fig. 2B). This is in agreement with the fact that RAFTK can be stimulated by PKC activation/DAG production as well as by an elevation of [Ca<sup>2+</sup>], (15). In addition, since the activation of...
RAFTK and EGFR Mediate GPCR-promoted MAP Kinase Activation

MAP kinase by UTP is dependent on PKC (1), it is consistent with the involvement of RAFTK in the UTP-promoted activation of MAP kinase.

**UTP and Other Stimuli Increase the Tyrosine Phosphorylation of the EGF Receptor**—Previously, we observed that UTP, ATP, and other stimuli produced an increase in the tyrosine phosphorylation of a ~160-kDa protein (1) the size of the EGF receptor. Since ligands of some G-protein-coupled receptors, Gβγ, and increases in [Ca^{2+}]_i (5, 19, 20) can activate the EGF receptor, we examined whether UTP and other stimuli promoted the tyrosine phosphorylation of the EGF receptor, which was immunoprecipitated using an anti-EGF receptor antibody. UTP, ATP, and ionomycin produced significant increases in the tyrosine phosphorylation of the EGF receptor after 5 min of exposure to the cells (Fig. 3A). PMA also increased the level of EGF receptor tyrosine phosphorylation (Fig. 3B), although generally it was somewhat less effective than the increases produced by UTP or ionomycin. Not surprisingly, the effects of all of these stimuli on EGF receptor tyrosine phosphorylation were much less than those produced by maximum concentrations (100 ng/ml) of EGF. These studies suggest that ionomycin, which increases [Ca^{2+}]_i; PMA, which mimics DAG; and UTP, a ligand that increases both [Ca^{2+}]_i and DAG production, all promote an increase in the tyrosine phosphorylation of the EGF receptor. These results are consistent with the transactivation of the EGF receptor by these stimuli (see below).

Since the activation of MAP (ERK2) kinase by UTP and PMA was diminished in cells in which PKC was down-regulated by treatment of the cells with PMA overnight (1), the effect of this treatment on the tyrosine phosphorylation of the EGF receptor was examined. Down-regulation of PKC reduced the ability of both UTP and PMA to promote increases in the tyrosine phosphorylation of the EGF receptor (Fig. 3B). The activation of MAP kinase by UTP and ionomycin also was decreased in cells exposed to conditions that block the elevation of [Ca^{2+}]_i (1). In a similar manner, the UTP- and ionomycin-promoted increases in the EGF receptor tyrosine phosphorylation were blocked in BAPTA-AM-loaded EGTA-treated cells (Fig. 3C). Thus, increases in the tyrosine phosphorylation of the EGF receptor are blocked by conditions that diminish the activation of MAP kinase by UTP, increases in [Ca^{2+}]_i, and the activation of PKC. Increases in the tyrosine phosphorylation of RAFTK also are reduced by down-regulation of PKC (Fig. 2B) and treatment of cells with BAPTA-AM and EGTA (1). These results indicate that transactivation of the EGF receptor and the activation of MAP kinase are both downstream of the activation of the G-protein-coupled P_2Y2 receptor, but they do not demonstrate whether these downstream events occur in parallel or in a linear and dependent manner.

**Inhibition of the EGF Receptor Blocks MAP Kinase Activation by UTP and Other Stimuli**—To determine whether the EGF receptor was involved in the stimulation of MAP kinase by extracellular nucleotides and other stimuli, the EGF receptor tyrosine kinase activity was blocked by treating cells with AG1478, a tyrphostin that is selective for the EGF receptor (21). The concentration dependence of AG1478 was examined using cell lysates immunoblotted with anti-Tyr(P) antibody. The EGF-dependent tyrosine phosphorylation of the EGF receptor was reduced by AG1478 in a concentration-dependent manner between 10 and 300 nM (Fig. 4A). Ionomycin and UTP increased the tyrosine phosphorylation of a band that co-migrated with the EGF receptor. This was observable only faintly for ionomycin- and UTP-treated cells at this length of exposure of the immunoblot to enhanced chemiluminescence solutions but was more detectable at longer times of exposure (not shown). The phosphorylation of this band was decreased in cells exposed to AG1478.

EGF, UTP, and ionomycin also produced an increase in the tyrosine phosphorylation of a ~42-kDa band that co-migrated with ERK2, and this phosphorylation was nearly completely reduced by 100 nM AG1478 (Fig. 4A). AG1478 did not block the NGF-stimulated tyrosine phosphorylation of this 42-kDa protein (not shown), consistent with the lack of effect of AG1478 on p140^trk, the receptor for NGF. These results suggest that increases in MAP kinase by UTP, ionomycin, and PMA are mediated by the EGF receptor. To examine this in a more quantitative and definitive manner, the effects of AG1478 on ERK2 activity also were measured using an in vitro kinase (substrate phosphorylation) assay of anti-ERK2 immunoprecipitates. AG1478 did not significantly reduce the basal (unstimulated) ERK2 activity (Fig. 4B). However, the stimulations of ERK2 by UTP, ionomycin, and PMA, were blocked to the same degree (60–80%) as was that of EGF (Fig. 4B), suggesting that the effects of these three agents were mediated by the activation of the EGF receptor at a point upstream of MAP kinase. This is consistent with the stimulation of EGF receptor tyrosine phosphorylation by UTP, ionomycin, and PMA (Fig. 3).

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**Fig. 3.** UTP and other stimuli increase the tyrosine phosphorylation of the EGF receptor in a [Ca^{2+}]_i, and PKC-dependent manner. Cells were exposed to UTP (100 μM), ATP (100 μM), ionomycin (10^-6 M), UTP (1 or 2 min), and ionomycin (1 min) for 5 min, each in the presence or absence of PMA (100 ng/ml), and then exposed to vehicle (5 min), UTP (5 min), ionomycin (5 min), or EGF (1 min). The immunoprecipitated proteins were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted using anti-Tyr(P) (α-Tyr) antibody. A, UTP (1 or 2 min, as indicated), ATP (1 min), and ionomycin (1 min) promoted significant increases in the tyrosine phosphorylation of the EGF receptor. EGF (1 min) produced much a larger increase in tyrosine phosphorylation than any of the other stimuli. Each lane is an immunoprecipitate from a separate dish of cells treated as indicated. B, cells were treated with 1 μM PMA (+) or MeSO_4 (-) overnight, and then exposed to vehicle (-), UTP (5 min), PMA (5 min), or EGTA (1 min). Each set of lanes (upper and lower) are the results from a separate experiment conducted on different days. The responses to UTP and PMA were reduced in cells in which PKC was down-regulated by long term exposure to PMA. C, cells were pretreated with either MeSO_4 (-) or BAPTA-AM (+) for 30 min and then exposed to vehicle (5 min), UTP (5 min), ionomycin (5 min), or EGTA (1 min). Cells exposed to BAPTA-AM were also exposed to EGTA during their exposure to stimuli. The effects of UTP and ionomycin on the tyrosine phosphorylation of the EGF receptor were reduced in BAPTA-AM/EGTA-treated cells.
AG1478 Blocks the Tyrosine Phosphorylation of p120<sup>cbl</sup> by UTP and Other Stimuli—p120<sup>cbl</sup> is a 120-kDa protein that is phosphorylated on tyrosine in EGF-treated PC12 cells. Exposure of PC12 cells to UTP promoted the tyrosine phosphorylation of p120<sup>cbl</sup> in a time-dependent manner. Phosphorylation was observable after 1 and 5 min of UTP treatment (Fig. 5A). The phosphorylation was not readily detectable after a 15-s exposure to UTP, although there is a large increase in the tyrosine phosphorylation of p120<sup>cbl</sup> in PC12 cells treated for 15 s with EGF (22). Ionomycin also promoted an increase in the tyrosine phosphorylation of p120<sup>cbl</sup> (Fig. 5B). The degree of p120<sup>cbl</sup> phosphorylation initiated by UTP and ionomycin were significantly less than that produced by EGF, and the effects of all three stimuli were reduced by AG1478 (Fig. 5B).

AG1478 Does Not Block the UTP-stimulated Tyrosine Phosphorylation of RAFTK—To determine whether the UTP-dependent activation of RAFTK was upstream or downstream of the EGF receptor, the effect of AG1478 on RAFTK tyrosine phosphorylation was examined. The UTP-initiated phosphorylation of RAFTK was not affected by pretreatment of the cells with either 100 nM AG1478 (Fig. 5C) or 300 nM AG1478 (not shown). These results suggest that RAFTK is upstream of the activation of the EGF receptor. Along with the inhibitory effects of AG1478 on MAP kinase (Fig. 4), these results suggest that the activation of the EGF receptor is between RAFTK and EGFR.

AG1478 Blocks the Tyrosine Phosphorylation of SHC by UTP, Ionomycin, PMA, and EGF—Previously, we demonstrated that UTP and other stimuli produced an increase in the tyrosine phosphorylation of SHC, and this was observable after exposure of cells to UTP for 1–5 min. Grb2 was co-immunoprecipitated in the anti-SHC immunoprecipitate of PC12 cells exposed to UTP and other stimuli, and the relative amount of immunoprecipitable Grb2 was proportional to the level of tyrosine phosphorylation of SHC produced by UTP, ionomycin, PMA, NGF, and EGF (1). This was consistent with Grb2 binding via its Src homology 2 domain to tyrosine residues on SHC. To determine if the tyrosine phosphorylation of SHC was dependent on the intrinsic tyrosine kinase activity of the EGF receptor, the effects of various stimuli on SHC phosphorylation were examined in the presence and absence of AG1478.

The tyrosine phosphorylation of SHC by UTP, ionomycin, PMA, and EGF, but not by NGF, was blocked by pretreatment of cells with AG1478 (Fig. 6, middle panel). The association of
EGF receptor, and other proteins. The tyrosine-phosphorylated protein except NGF, the treatment of cells with AG1478 reduced the association of p66 SHC were observable at this exposure. For all stimuli p46 and p52 forms of SHC, small increases in the tyrosine phosphorylation of SHC (Fig. 6), and the association of Grb2 with SHC were also blocked by AG1478, except for that promoted by NGF (Fig. 6, lower panel). These results suggest that the tyrosine phosphorylation of SHC by UTP, ionomycin, and PMA is downstream of the activation of the EGF receptor. Consistent with this, these stimuli promoted an increase in the tyrosine phosphorylation of a protein that was co-immunoprecipitated with SHC and that co-migrated with the EGF receptor (Fig. 6, upper panel), and this was reduced in cells pretreated with AG1478. These results, along with the effects of AG1478 on MAP kinase activity (Fig. 4), suggest that the EGF receptor is in a signaling cascade that is upstream of MAP kinase and downstream of the activation of the P2Y2 receptor, the elevation of \([Ca^{2+}]_i\), and the production of DAG.

**DISCUSSION**

The results of these studies demonstrate that activation of the P2Y2 receptor by UTP stimulates MAP kinase in PC12 cells via a cascade of signaling proteins that include RAFTK and the EGF receptor. UTP, which increases \([Ca^{2+}]_i\), in these cells (23, 24),2 promotes the tyrosine phosphorylation of RAFTK (Fig. 2A). This is reduced by down-regulating PKC (Fig. 2B) as well as by blocking the elevation of \([Ca^{2+}]_i\) (1), and both of these alterations reduce the UTP-promoted activation of MAP kinase (1). UTP, ionomycin, and PMA promote the tyrosine phosphorylation of the EGF receptor (Fig. 3). The effects of these stimuli on MAP kinase activity (Fig. 4), the tyrosine phosphorylation of SHC (Fig. 6), and the association of Grb2 with SHC (Fig. 6) were reduced by blocking the EGF receptor tyrosine kinase with the tyrphostin AG1478.

A model for the signaling cascade between the P2Y2 receptor and MAP kinase is shown in Fig. 7. In this model, RAFTK is upstream of the EGF receptor and downstream of the elevation of \([Ca^{2+}]_i\), and PKC, and the activation of both the EGF receptor and RAFTK is critical to the P2Y2 receptor-dependent stimulation of MAP kinase. The results are also consistent with the critical involvement of RAFTK and the EGF receptor in the effects of both ionomycin and PMA on MAP kinase activation in PC12 cells. The EGF receptor may act as a scaffolding or adaptor protein to help to coordinate the signaling molecules involved in the MAP kinase activation scheme outlined in the model (Fig. 7). In this way, the EGF receptor and other proteins (reviewed in Ref. 14) may provide an organizational role beyond their intrinsic biochemical function. In this model, the active tyrosine kinase activity of the EGF receptor is also required, since AG1478 blocks the stimulation of MAP kinase and SHC tyrosine phosphorylation.

An increasing number of studies have demonstrated the involvement of the EGF receptor and various signaling proteins, including SHC and Src, in the activation of MAP (ERK) kinase by GPCRs. The EGF receptor can be activated by signaling events initiated by \(G\beta\gamma\) subunit (5), ionomycin (19, 25), and agonists for lysophosphatidic acid, thrombin, endothelin 1, and angiotensin II receptors (5, 9–11). In neuronal cell types, including PC12 cells, \([Ca^{2+}]_i\), increases produced by GPCR ligands, calcium ionophores, or membrane depolarization, have been shown to promote neurite outgrowth and activate the EGF receptor, MAP kinase, and other signaling proteins (19, 20, 25, 26). Similar to results presented here, the tyrphostin AG1478 blocked MAP kinase activation and the tyrosine phosphorylation of SHC and other signaling proteins promoted by ligands to GPCRs in other studies (9–11, 19). Although there are a number of similarities in the signaling cascades downstream of the GPCRs in different cellular systems, there are also differences. GPCR- and \(G\beta\gamma\)-mediated tyrosine phosphorylation of the EGF receptor and SHC were also found to be dependent on Src kinase, and these events were upstream of MAP kinase activation (5). Since an autophosphorylation-specific antibody did not detect an increase in phosphorylation, these increases in MAP kinase activity did not appear to require the intrinsic kinase activity of the EGF receptor (5). This conclusion is different from that reached in the present study and one that used both AG1478 and the expression of dominant negative EGF receptor (10). It is therefore of interest that growth hormone, which binds to a receptor that is a member of the cytokine receptor family, promoted an increase in MAP kinase that was dependent on an increase in EGF receptor tyrosine phosphorylation, but the intrinsic tyrosine kinase activity of the EGF receptor was not required for the activation of MAP kinase (27). Thus, the EGF receptor plays an important role in MAP kinase activity in different cell types, but in some cases its kinase activity may not be required.

A number of studies indicate that Src is involved in mediating the activation of MAP kinase by GPCRs. Src can co-immunoprecipitate with the EGF receptor in cells exposed to ligands to GPCRs, including lysophosphatidic acid (5) and angiotensin II (11). In PC12 cells, the activation of GPCRs promoted the association of PYK2 (RAFTK) and activated Src, and this coupling was involved in the activation of MAP kinase (7). Through the use of dominant negative PYK2 and Src proteins, it was demonstrated that PYK2 and Src were involved in mediating the activation of MAP kinase by the \(G\alpha\)-coupled \(\alpha_2A\)-adrenergic receptor and the \(G\alpha\)-coupled \(\alpha_1B\)-adrenergic receptor at a point upstream of SHC in nonneuronal cells (8). An inhibitor (PP1) of Src-like kinases blocked the effects of both GPCR- and EGF receptor-promoted activation of MAP kinase and tyrosine phosphorylation of signaling proteins (including SHC) downstream of EGF receptor activation but produced only a modest reduction in the lysophosphatidic acid-promoted tyrosine phosphorylation of the EGF receptor (10). This suggests that Src or other kinases may play a regulatory role immediately distal to GPCR-mediated EGF receptor tyrosine kinase activation. A similar conclusion was reached concerning

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*S. P. Soltoff, unpublished results.*
The involvement of Src and the activation of the PDGFRα receptor by PDGF. Src was required for efficient PDGF-dependent tyrosine phosphorylation of SHC but not for tyrosine phosphorylation of the receptor itself or for other signaling proteins (phospholipase C-γ1, SHP2) that are recruited to this receptor (28).

The model for the P₂Y₂ receptor-mediated activation of MAP kinase is generally compatible with many of the studies cited above. Src and/or Src-like kinases may play regulatory roles at multiple points in the signaling cascade between growth factor/G-protein-coupled receptors and MAP kinase, and this may vary with different kinds of cells or GPCRs. In addition, although it is not shown explicitly in the model, the UTP- and PMA-dependent activation of MAP kinase may also involve PKC-mediated effects that are independent of RAFTK and the EGF receptor, since PKC can increase MAP kinase by directly phosphorylating Raf-1 (29). Based on the degree of inhibition of MAP kinase activity by AG1478, the major P₂Y₂-mediated pathway is that outlined in Fig. 7.

Several observations suggest that the acute activation of PKC by PMA can produce a positive effect on the EGF receptor, including (a) the PMA-promoted increase in the tyrosine phosphorylation of the EGF receptor (Figs. 3B and 6) and (b) the inhibition by AG1478 of the PMA-promoted MAP kinase activation (Fig. 4) and SHC tyrosine phosphorylation (Fig. 6). In other reports, topical treatment of mice with PMA increased the tyrosine phosphorylation of the EGF receptor in epidermal tissue (30). Of related interest, PMA and PKC activation also promoted the tyrosine phosphorylation and activation of ErbB2 and ErbB3, other members of the EGF receptor family, in a rat hepatoma cell line (31). However, many studies have demonstrated that PMA and ligands to PLC-linked GPCRs, including P₂ receptors (32), reduce the basal and/or EGFR-dependent tyrosine kinase activity of the EGF receptor and reduce signaling by the EGF receptor (Ref. 33; for a review, see Ref. 34). Down-regulation of the EGF receptor tyrosine kinase activity may involve phosphorylation of Thr⁵⁴⁴ and Thr⁶⁰⁹ by PKC and MAP kinase, respectively, as well as the phosphorylation of additional sites (35, 36). Thus, it appears that the EGF receptor tyrosine kinase can be either activated (as in our studies) or inhibited by ligands to GPCRs and other stimuli.

One may speculate that Src and/or RAFTK, which can be activated by either an elevation of [Ca²⁺], or PMA, may contribute to whether there is either GPCR-mediated activation or inhibition of the EGF receptor, but this will require a direct examination. Interestingly, ATP also produced an increase in MAP kinase activity in another PC12 cell line via the activation of an ionotropic P₂X-type receptor (which is not coupled to a G-protein) and not via a metabotropic P₂Y-type receptor (37). Although K⁺-stimulated depolarization and ATP both produced extracellular Ca²⁺-dependent increases in RAFTK (PYK2) tyrosine phosphorylation and MAP kinase activity in these cells, neither stimulus was found to increase the tyrosine phosphorylation of the EGF receptor.

NGF produced a small increase in the tyrosine phosphorylation of the EGF receptor in anti-EGF immunoprecipitates in some experiments (not shown) and in a protein (presumably the EGF receptor) that co-migrated with the EGF receptor in anti-SHC immunoprecipitates (Fig. 6). This is likely to reflect changes in the tyrosine phosphorylation of the EGF receptor by the elevation in [Ca²⁺], or the production of DAG in NGF-treated cells. The lack of effect of a significant inhibitory effect of AG1478 on the NGF-promoted tyrosine phosphorylation of SHC (Fig. 6), association of Grb2 with SHC (Fig. 6), and the activation of MAP kinase (not shown) are consistent with the p140⁵⁴⁴-mediated activation of MAP kinase by NGF (38). However, the effects of NGF on the EGF receptor tyrosine phosphorylation suggest that there is cross-talk between these two growth factor receptors as well as between GPCRs and growth factor receptors. Transmodulation of the EGF receptor by the PDGF receptor has been reported (reviewed in Ref. 39). In addition, recent studies indicate that the EGF receptor and the PDGFβ receptor can interact directly with each other, perhaps by heterodimerization or oligomerization (40).

P₂Y₂ receptors are present on many different types of tissues and cells in culture (reviewed in Refs. 2–4). These receptors may play a role in autocrine or paracrine signaling due to the release of intracellular ATP by physiological and pathophysiological conditions. Platelet secondary granules contain high concentrations of ADP and ATP, and platelets have P₂ receptors. ATP is co-stored and co-released with neurotransmitters and can act as a neurotransmitter itself (42–44). P₂ receptor-mediated effects of extracellular nucleotides on neuronal cells include MAP kinase activation, the formation of AP-1 complexes, and trophic effects (45). Cytosolic ATP may be lost from some epithelial cells by efflux through the CFTR channel, which may...
act as both a chloride channel and ATP channel (46, 47). In this role, cytosolic ATP has been suggested to leave the cells and subsequently activate P2Y2 receptors and thereby activate other chloride channels.

MAP kinase is activated by many growth factors and by various GPCRs in different cells. The effects of UTP and EGF on signaling molecules that are upstream of MAP kinase are representative of both similarities and differences in GPCR-mediated and receptor tyrosine kinase-mediated signaling in PC12 cells. Both stimuli, as well as ionomycin and PMA, increased the tyrosine phosphorylation of the EGF receptor and thereby activated SHC. The transactivation of the EGF receptor was required for the full activation of MAP kinase by the P2Y2 receptor-mediated stimulus as well as for MAP kinase activation promoted by ionomycin and PMA. As such, these studies illustrate the interactions that may occur between different types of receptors and demonstrate how Ca\(^{2+}\) and DAG can initiate signaling cascades that reproduce the downstream effects of GPCR-mediated signaling.

Acknowledgment—I thank Michelle Bradford for excellent technical assistance.

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