The Epidermal Growth Factor Receptor Is Coupled to a Pertussis Toxin-sensitive Guanine Nucleotide Regulatory Protein in Rat Hepatocytes

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Activation of epidermal growth factor (EGF) receptors stimulates inositol phosphate production in rat hepatocytes via a pertussis toxin-sensitive mechanism, suggesting the involvement of a G protein in the process. Since the first event after receptor-G protein interaction is exchange of GTP for GDP on the G protein, the effect of EGF was measured on the initial rates of guanosine 5'-O-(3-[35S]thiotriphosphate) (GTPyS) association and [32P]GDP dissociation in rat hepatocyte membranes. The initial rate of [35S]GTPyS binding was stimulated by EGF, with a maximal effect observed at 8 nM EGF. EGF also increased the initial rate of [32P]GDP dissociation. The effect of EGF on [35S]GTPyS association was blocked by boiling the peptide for 5 min in 5 mM dithiothreitol or by incubation in the membranes with guanosine 5'-O-(2-thiodiphosphate) (GDPβS). EGF-stimulated [35S]GTPyS binding was completely abolished in hepatocyte membranes prepared from pertussis toxin-treated rats and was inhibited in hepatocyte membranes that were treated directly with the resolved A-subunit of pertussis toxin. The amount of guanine nucleotide binding affected by occupation of the EGF receptor was ~6 pmol/mg of membrane protein. Occupation of angiotensin II receptors, which are known to couple to G proteins in hepatic membranes, also stimulated [35S]GTPyS association with [32P]GDP dissociation from the membranes. The effect of angiotensin II on [32P]GDP dissociation was blocked by the angiotensin II receptor antagonist [Sar1,Ile8]angiotensin II, demonstrating that the guanine nucleotide binding was receptor-mediated. In A431 human epidermoid carcinoma cells, EGF stimulates inositol lipid breakdown, but the effect is not blocked by treatment of the cells with pertussis toxin. In these cells, EGF had no effect on [35S]GTPyS binding. Occupation of the β-adrenergic receptor in A431 cell membranes with isoproterenol did stimulate [35S]GTPyS binding, and the effect could be completely blocked by l-propranolol. These results support the concept that in hepatocyte membranes, EGF receptors interact with a pertussis toxin-sensitive G protein via a mechanism similar to other hormone receptor-G protein interactions, but that in A431 human epidermoid carcinoma cells, EGF may activate phospholipase C via different mechanisms.

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Activation of EGF receptors elicits multiple intracellular signals that ultimately lead to DNA synthesis and cell division. One primary signaling event following receptor activation is thought to be the phosphorylation of certain cellular proteins on tyrosine residues (1). Stimulation of EGF receptors can also lead to activation of phospholipase C and generation of two second messengers, Ins(1,4,5)P3 and diacylglycerol, with diacylglycerol activating protein kinase C and Ins(1,4,5)P3 releasing Ca2+ from intracellular stores. It has also been suggested that EGF may directly stimulate Ca2+ influx in hepatocytes (2), Rat-1 (3), and Swiss 3T3 (4) fibroblasts. There is still no definitive evidence, however, to indicate which of the multiple pathways activated by the EGF receptor is most important in regulating cell division.

Recently, a number of substrates for the EGF receptor tyrosine kinase have been discovered, including microtubule-associated protein kinase and phospholipase C-γ. Microtubule-associated protein kinase is a serine (threonine) protein kinase whose phosphorylation is increased upon EGF receptor activation (5). Phospholipase C-γ has been demonstrated to be a substrate for both the EGF and platelet-derived growth factor receptor tyrosine kinases (6-12). In A431 and HER14 cells, both of which overexpress the human EGF receptor, activation of phospholipase C-γ via phosphorylation on tyrosine has been shown to be a mechanism through which EGF receptor activation causes production of inositol phosphates (6-9, 12).

Whereas a large body of data exists pointing to tyrosine phosphorylation as an important mechanism of EGF receptor action, there is also an increasing amount of evidence that EGF receptors can interact with G proteins to generate signals (13-17). However, the G protein-coupled hormone receptors that have been cloned all exhibit seven transmembrane-spanning domains and lack intrinsic tyrosine kinase activity, raising the interesting question of how a receptor with intrinsic tyrosine kinase activity can interact with G proteins.

In rat hepatocytes, EGF stimulates phospholipase C to generate Ins(1,4,5)P3 and diacylglycerol in addition to activating the tyrosine kinase activity of the receptor. The effect of EGF on inositol phosphate production can be completely blocked by pretreatment of the hepatocytes with pertussis toxin.
toxin (17). These observations suggest that the EGF receptor in hepatocytes may interact with a pertussis toxin-sensitive G protein, although such a direct interaction between the EGF receptor and a G protein has not been rigorously established. Therefore, it is important to determine whether the EGF receptor can interact with a G protein in isolated membrane preparations. This study demonstrates an EGF-dependent increase in the initial rate of GDP/GTP exchange in hepatic membranes and a complete inhibition of the effect by pertussis toxin pretreatment. These observations support the hypothesis that the EGF receptor directly interacts with a pertussis toxin-sensitive G protein in rat hepatocytes. Similar studies using membranes prepared from A431 cells demonstrate that EGF has no effect on GTP binding, suggesting that the EGF receptor may generate signals via different mechanisms in A431 cells and hepatocytes.

**Experimental Procedures**

**Preparation of Membranes from Rat Liver and A431 Cells—** Rat liver membranes were prepared from 200- to 300-g male Wistar rats. The livers were removed immediately after the rats were anesthetized with an overdose of sodium pentobarbital (3 g/kg of body weight), and liver membranes were purified by sucrose gradient centrifugation according to the procedure of Pohl (18) with the modifications described by Campanile et al. (19). The membranes were resuspended in 20 mM Tris, pH 8.5, 5 mM EDTA to a protein concentration of 15 mg/ml in a buffer devoid of sodium azide.

A431 cells were cultured in DMEM containing 10% fetal calf serum at 37°C under 10% CO₂. Confluent cell monolayers were rinsed with hypotonic lysis buffer (10 mM HEPES, pH 7.4, 5 mM EDTA) and lysed in this buffer at 4°C for 30 min. The cells were scraped; and the lysates were collected, homogenized with a glass Dounce homogenizer, and centrifuged at 480 X g at 2°C for 10 min to remove intact cells and nuclei. The supernatant was centrifuged at 14,600 X g for 20 min; and the crude membrane pellet washed once in hypotonic lysis buffer, resuspended in the same buffer to a protein concentration of 10 mg/ml, and stored in liquid nitrogen.

**Treatment of Intact Rats or Rat Liver Membranes with Pertussis Toxin—** Pertussis toxin-treated rat liver membranes were obtained using two different procedures: by preparing membranes from the livers of pertussis toxin-treated rats or by preparing membranes from normal rats and treating them with the resolved A-subunit of pertussis toxin in vitro. Intact 150-200-g male Wistar rats were treated with 80 nM EGF or inactivated EGF in fresh DMEM with 2% serum and penicillin/streptomycin at 37°C in a humidified incubator supplied with 7.5% CO₂ and 92.5% air. After approaching confluence, the cells were transferred to a cuvette maintained at 37°C; stirred gently, and gassed with a steady supply of 96% O₂, 4% CO₂. Emission fluorescence readings were obtained at 1-s intervals from a SLM 8200C spectrofluorometer monitoring at wavelengths of 485 and 410 nm for free Indo-1 and Ca2+ bound Indo-1, respectively. The Ca2+ concentration was calculated by the ratio of fluorescence of bound versus free Indo-1 as described (24). Total intracellular Ca2+ was estimated by using digitonin at 30 µg/ml to permeabilize the cells, and zero free Ca2+ was estimated by the addition of 5 mM EGTA following the digitonin treatment (25).

**Measurement of Intracellular Ca2+ Concentration—** Intracellular Ca2+ was measured using the Ca2+-sensitive dye Indo-1 (24, 25). Isolated hepatocytes were prepared from 200-250-g fasted male Wistar rats and resuspended to 3 x 10⁶ cells/ml in buffer containing 103 mM NaCl, 4.8 mM KCl, 0.5 mM CaCl₂, 1.2 mM MgSO₄, 1.3 mM H₂PO₄, 24.9 mM NaHCO₃, 20 mM HEPES, pH 7.4, 20 µg/ml BSA, 250 mM NaCl, 5 mM EDTA, 5 mM NaF, 5 µM Indo-1 acetoxymethyl ester at 37°C for 20 min. After washing out free dye, the cells were transfected to a cuvette maintained at 37°C; stirred gently, and gassed with a steady supply of 96% O₂, 4% CO₂. Emission fluorescence readings were obtained at 1-s intervals from a SLM 8200C spectrofluorometer monitoring at wavelengths of 485 and 410 nm for free Indo-1 and Ca2+ bound Indo-1, respectively. The Ca2+ concentration was calculated by the ratio of fluorescence of bound versus free Indo-1 as described (24). Total intracellular Ca2+ was estimated by using digitonin at 30 µg/ml to permeabilize the cells, and zero free Ca2+ was estimated by the addition of 5 mM EGTA following the digitonin treatment (25).

**Culture of Rat-1 hER Fibroblasts—** Routine culture of Rat-1 hER cells was performed as described (26). Cells were seeded into 35-mm culture dishes in DMEM containing 10% heat-inactivated fetal calf serum and penicillin/streptomycin at 37°C in a humidified incubator supplied with 7.5% CO₂ and 92.5% air. After approaching confluence, the cells were treated with 8 nM EGF or inactivated EGF in fresh DMEM with 2% serum. The progressive morphological transformation was observed with a microscope at 12, 24, and 36 h and photographed at 36 h.

**Phosphorylation of EGF Receptors in Rat Hepatic Membranes Prepared from Control or Pertussis Toxin-treated Rats—** Sucrose gradient-purified hepatic membranes from control or pertussis toxin-treated rats were suspended in 20 mM HEPES, pH 7.4 (at 4°C), 2 mM MnCl₂, 0.1 mM NaVO₃ to ~15 mg/ml of protein and were equilibrated with or without GTP at 4°C. The phosphorylation reaction was started by addition of ATP to a final concentration of 2 mM and incubated on ice for 2 min. The reaction...
was terminated by addition of an equal volume of 2-fold concentrated SDS sample buffer (final concentrations: 50 mM Tris, pH 6.8, 10% glycerol, 1% SDS, 2% β-mercaptoethanol, 0.0025% bromophenol blue) and boiled for 5 min, and the proteins were resolved on a 7% SDS-polyacrylamide slab gel (27). The proteins were transferred to nitrocellulose paper (28), and proteins phosphorylated on tyrosine were identified by immunoblotting with affinity-purified anti-phosphotyrosine antibodies and 125I-protein A as described (29) with the following modifications. After blocking for 2 h at 37 °C in buffer containing 50 mM Tris, pH 7.5, 0.15 M NaCl, 10 μM NaVO₄, 0.01% (w/v) sodium azide, 0.05% (v/v) Tween 20, 3% (v/v) BSA, the blots were incubated with 20 μg of affinity-purified anti-phosphotyrosine antibodies in 10 ml of blocking buffer at 37 °C for 2 h. After washing (four times, 5 min each) with blocking buffer at room temperature, the blots were incubated with 125I-protein A (1 μCi/ml) in blocking buffer at 37 °C for 1 h and then washed as described above six times. Autoradiographs were obtained by exposing the dried blots to Kodak XAR-5 film overnight at -90 °C with intensifying screens. The density information of the autoradiographs was digitized by scanning the autoradiographs with a Bio Image Visage 4000 image analysis system and quantified using the Whole Band analysis software.

**Measurement of Adenylate Cyclase Activity**—Eighty μg of A431 cell membranes from Dr. J. T. Parsons (University of Virginia) and 50 μg of protein from Dr. E. L. Hewlett (University of Virginia) were resuspended at 30 °C for 0, 5, 10, and 20 min in buffer containing 50 mM Tris, pH 8.0, 5 mM MgCl₂, 1 mM EGTA, 0.5 mM ATP, 0.1 mM GTP, 0.1 mg/ml BSA, 0.4 mM 3-isobutyl-1-methylxanthine, 10 mM phosphocreatine, 10 units/ml creatine phosphokinase (30) with or without agonist. At the end of each incubation, the reaction was terminated with 4 volumes of 0.125 N HCl. The cAMP formed in the reaction was measured using an automated radioimmunoassay (31).

**Statistical Analysis of GTPyS and GDP Binding Kinetics**—Statistical analysis was performed by a computer program kindly written by Dr. M. L. Johnson (University of Virginia). The statistical difference between binding kinetics obtained with or without agonist treatment was evaluated by calculating the difference in the amount of labeled guanine nucleotide bound at each time point and plotting the differences versus time. The resulting data from each set of experiments were fitted to an orthogonal polynomial straight line, and the slope and intercept of each fitted line were compared with zero using Student’s t test. If either the slope or the intercept of the fitted line was significantly different from zero with 95% confidence, the effect of agonist was considered to be statistically significant.

**Materials**—The reagents used were obtained from the following sources: pertussis toxin and pertussis toxin A-subunit, gift from Dr. E. L. Hewlett (University of Virginia); anti-phosphotyrosine antibodies, a gift from Dr. M. L. Johnson (University of Virginia). The statistical difference between binding kinetics obtained with or without agonist treatment was evaluated by calculating the difference in the amount of labeled guanine nucleotide bound at each time point and plotting the differences versus time. The resulting data from each set of experiments were fitted to an orthogonal polynomial straight line, and the slope and intercept of each fitted line were compared with zero using Student’s t test. If either the slope or the intercept of the fitted line was significantly different from zero with 95% confidence, the effect of agonist was considered to be statistically significant.

**Results**

In an attempt to identify an interaction between the EGF receptor and putative G proteins, the effect of EGF on [35S]GTPyS binding was measured in hepatic membranes using the assay conditions optimized as described under “Experimental Procedures.” The time courses of [35S]GTPyS binding to hepatic membranes in the presence and absence of 82 nM EGF are shown in Fig. 1A. The stimulation was observed within 30 s and continued for at least 10 min. [35S]GTPyS binding at equilibrium was not changed by EGF (data not shown). The increase in [35S]GTPyS binding caused by EGF in hepatic membranes was statistically significant within the first 10 min (p < 0.05). The magnitude of the difference in EGF-stimulated GTPyS binding was ~6 pmol/mg protein, an amount similar to the endogenous levels of pertussis toxin substrates in liver membranes (20). Other experiments demonstrated that the effect of EGF on [35S]GTPyS binding was dose-dependent. A submaximal effect was observed at 0.8 nM EGF with maximal effects observed near 8 nM (data not shown). To ensure a maximal effect in subsequent experiments, a supermaximal dose (82 nM) was employed.

The available data suggest that the angiotensin II receptor in rat hepatic membranes interacts with phospholipase C and adenylate cyclase via G proteins in a manner typical for G protein-coupled receptors, e.g. β-adrenergic or muscarinic receptors. Therefore, as a positive control, the effect of angiotensin II on [35S]GTPyS binding kinetics was measured in rat hepatic membranes. Angiotensin II at a concentration of 100 nM stimulated the initial rate of [35S]GTPyS binding kinetics with kinetics similar to those observed with EGF (Fig. 1B). To demonstrate that the effect of EGF on [35S]GTPyS binding association is due to binding of EGF to its receptor, EGF was inactivated, and its effects were compared to those of native EGF in hepatic membranes. Boiling in 5 mM DTT for 5 min completely abolished the effects of EGF in this assay (Fig. 2A). The data in Fig. 2A are derived from time courses similar to those shown in Fig. 1. However, the change in GTP yS binding was calculated as the difference between the GTP yS binding in the presence or absence of agonist or agonist and the indicated treatment at each time point and was replotted. The inactivation of EGF was verified independently by measuring the loss of two biological actions of EGF: its ability to increase intracellular Ca²⁺ levels in isolated rat hepatocytes and its ability to cause morphological transformation of Rat-1 hER cells. After boiling in 5 mM DTT for 5 min, EGF completely lost its ability to increase intracellular Ca²⁺ levels in Indo-1-loaded rat hepatocytes or to morphologically transform Rat-1 hER cells (data not shown). The inactivated EGF was shown to remain in solution by the observation that there
FIG. 2. Manipulations that block effects of EGF on \[^{35}S\]GTP\(\gamma\)S association with rat liver membranes. \[^{35}S\]GTP\(\gamma\)S binding was measured as described under “Experimental Procedures” using rat hepatic membranes. The initial data, which appeared as shown in Fig. 1, were recalculated to yield the difference between the GTP\(\gamma\)S bound in the presence of 82 nM EGF or EGF and the inhibitory manipulation at each time point. Data were then expressed as the time course of change in GTP\(\gamma\)S binding in the presence of the indicated treatment A, \[^{35}S\]GTP\(\gamma\)S binding measured in the presence of native EGF and inactive EGF. EGF was inactivated by boiling in 5 mM DTT for 5 min. The effect of native EGF versus control (○) is statistically different (\(p < 0.05\)), whereas the effect of inactive EGF versus control (■) is not statistically different (\(p > 0.05\)). B, effect of EGF on \[^{35}S\]GTP\(\gamma\)S binding measured in the presence and absence of 100 \(\mu\)M GDP\(\beta\)S. GTP\(\gamma\)S binding stimulated by 82 nM EGF is statistically different from control (\(p < 0.05\)) in the absence of GDP\(\beta\)S (○) and is not statistically different from control (\(p > 0.05\)) in the presence of GDP\(\beta\)S (■). C, \[^{35}S\]GTP\(\gamma\)S binding measured in membranes prepared from control and pertussis toxin-treated rats. In the control membranes (○), EGF-regulated GTP\(\gamma\)S binding is significantly different from control (\(p < 0.05\)). In the pertussis toxin (PT) treated membranes (■), EGF-regulated GTP\(\gamma\)S binding is not significantly different from control (\(p > 0.05\)). Data are representative of three similar experiments in A–C. A similar result was obtained when rat liver membranes were treated directly with the pertussis toxin A-subunit.

is no change of the absorbance at 280 nm of an EGF solution before and after boiling in DTT followed by centrifugation at 14,000 \(\times\) g.

A nonhydrolyzable GDP analogue (GDP\(\beta\)S) was used as a competitive inhibitor of \[^{35}S\]GTP\(\gamma\)S for binding to G proteins. The EGF effect on \[^{35}S\]GTP\(\gamma\)S binding was completely abolished by including 100 \(\mu\)M GDP\(\beta\)S in the binding assay (Fig. 2B). To directly test the hypothesis that the EGF receptor in rat hepatocytes interacts with a pertussis toxin-sensitive G protein, the effect of EGF on \[^{35}S\]GTP\(\gamma\)S binding was measured in hepatic membranes prepared from pertussis toxin-treated rats. The effect of EGF was abolished by pertussis toxin pretreatment (Fig. 2C) using a protocol demonstrated to completely ADP-ribosylate the pertussis toxin-sensitive 41-kDa G protein of hepatic membranes (20). Although pertussis toxin has been widely used to modify G proteins, some biological actions of the toxin other than G protein modification have also been observed (32–34). To avoid a possible complication of effects due to the B-subunit, the A-subunit of pertussis toxin was used to ADP-ribosylate the hepatic toxin-sensitive G proteins in vitro, and the membranes were used immediately in the \[^{35}S\]GTP\(\gamma\)S binding assay. This protocol produces results very similar to those shown in Fig. 2C (\(n = 3\); data not shown). In these experiments, the control membranes were incubated in the ADP-ribosylation buffer without the toxin A-subunit. EGF stimulated GTP\(\gamma\)S binding to the same extent in these membranes as in native membranes. All the data in Figs. 1 and 2 are consistent with the hypothesis that the EGF receptor in rat hepatocytes directly interacts with a pertussis toxin-sensitive G protein.

The kinetics of GTP\(\gamma\)S binding to membranes can be complicated by the requirement for prior GDP dissociation from the proteins and the possibility that GTP\(\gamma\)S can bind to other proteins in the membranes that are not involved in signal transduction. Therefore, hormone-regulated GDP dissociation may correlate more closely with the initial receptor-G protein interaction, a possibility exploited to examine the interaction of the \(\beta\)-adrenergic receptor and \(G_{\beta}\) (22). For these reasons, the effect of EGF on \([\alpha-^{32}P]\)GDP dissociation kinetics was examined in rat hepatic membranes. The time course of \([\alpha-^{32}P]\)GDP remaining bound to the membranes after dilution with a 1000-fold excess of unlabeled GDP in the presence and absence of 82 nM EGF is shown in Fig. 3A. EGF stimulated the initial rate of GDP dissociation within 30 s, and the effect was maintained for 8–10 min. Occupation of the EGF receptor decreased the amount of GDP bound to the membrane by ~6.7 pmol/mg of membrane protein, a value consistent with the hypothesis that the EGF receptor in rat hepatocytes directly interacts with a pertussis toxin-sensitive G protein.

The kinetics of GDP dissociation from rat liver membranes is stimulated by occupation of EGF or angiotensin II receptor. \([\alpha-^{32}P]\)GDP dissociation was measured as described under “Experimental Procedures” using rat hepatic membranes. Data are expressed as a time course of percent GDP bound remaining on the membranes after initiating \([\alpha-^{32}P]\)GDP release and are representative of three similar experiments. A, 82 nM EGF used as agonist. EGF-stimulated GDP dissociation (○) is significantly different from control (■) (\(p < 0.05\)). B, GDP release initiated by angiotensin II. ■, control time course; ○, time course in the presence of 100 nM angiotensin II (Ang II); □, time course in the presence of both 100 nM angiotensin II and 100 \(\mu\)M [Sar\(^1\),Ile\(^2\)]angiotensin II. Angiotensin II-stimulated GDP dissociation is significantly different from control in the absence of [Sar\(^1\),Ile\(^2\)]angiotensin II (\(p < 0.05\)) and is not significantly different from control in the presence of [Sar\(^1\),Ile\(^2\)]angiotensin II (\(p > 0.05\)).
similar to that seen in the [35S]GTPγS association experiments represented in Fig. 1. The interaction of the angiotensin II receptors and G proteins was used again as a positive control. As shown in Fig. 3B, 100 nM angiotensin I1 also stimulates [35S]GDP dissociation in hepatic membranes. The effect of angiotensin II was completely reversed by inclusion of 100 μM [Sar1,Ile6]angiotensin II, an angiotensin II receptor antagonist (Fig. 3B).

There are two potential mechanisms through which the EGFR could interact with a G protein: via a mechanism similar to other receptor-G protein interactions or perhaps via phosphorylation of the G protein on tyrosine. The first mechanism is consistent with the observation that EGF regulates GDP/GTP exchange in a manner similar to angiotensin II (Figs. 1 and 3), putatively a typical G protein-coupled hormone receptor. There are two ways to approach the second mechanism. The direct approach, to determine whether a specific G protein is phosphorylated on tyrosine by activation of the EGFR, is not viable since such a G protein has not yet been identified. An indirect approach is to determine whether tyrosine phosphorylation is required for the EGF receptor-G protein interaction suggested by Figs. 1–3. Two types of experiments were performed. First, since the effect of EGF on [35S]GTPγS binding presented in Figs. 1–3 was observed in the absence of ATP and Mn2+, the ability of the EGFR to act as a tyrosine kinase under these conditions was determined by the autophosphorylation of the EGFR receptor itself. Hepatic membranes were treated with EGF in the presence or absence of ATP, and EGFR receptor autophosphorylation was measured as described under “Experimental Procedures.” No phosphotyrosine was observed in the EGFR receptor in the absence of ATP, suggesting that this event is not required for the interaction of the EGFR receptor and a G protein. Second, since pertussis toxin blocked the effect of EGF on [35S]GTPγS binding (Fig. 2C), the effect of pertussis toxin on the tyrosine phosphorylation of the EGF receptor was measured to determine whether the intrinsic tyrosine kinase activity of the EGFR receptor was affected by treatment with pertussis toxin. Hepatic membranes prepared from normal and toxin-treated rats were incubated under conditions optimized for EGF-stimulated receptor autophosphorylation, and the membrane proteins were resolved on SDS-polyacrylamide gels followed by Western blot analysis with anti-phosphotyrosine antibodies. The results of a typical experiment of this type are shown in Fig. 4. The density information in the autoradiographs from four similar experiments was quantitated as described under “Experimental Procedures.” The average densities for the EGF receptor bands on the films were 8.04 ± 1.72 and 7.08 ± 1.64 OD × mm2 for the normal and pertussis toxin-treated rat hepatic membranes, respectively (Fig. 4). These results suggested that there is no difference in the ability of EGF to stimulate the phosphorylation of its receptor in the two membrane preparations (p > 0.1 by paired t test). Clearly, the tyrosine kinase activity of the EGFR is not affected by pertussis toxin treatment. However, the possibility that pertussis toxin inhibits the phosphorylation of a novel G protein and thus blocks receptor-G protein coupling cannot be excluded.

EGF also stimulates inositol lipid metabolism in A431 cells, where the effect is not blocked by treatment of the cells with pertussis toxin (35). Indeed, the data obtained from A431 cells are consistent with the suggestion that the EGFR receptor in A431 cells stimulates phospholipase C through tyrosine phosphorylation of the enzyme and not via a G protein (6–9, 12, 35). Therefore, it was important to compare the effects of EGF on [35S]GTPγS binding kinetics in A431 cells membranes with those in hepatic membranes. EGF has no effect on [35S]GTPγS binding to membranes prepared from A431 cells (Fig. 5A). In these membranes, the positive control is provided by the β-adrenergic receptor, which is coupled to adenylate cyclase via Gs. Basal adenylate cyclase activity in the membranes is 13.52 ± 0.14 pmol/min/mg of protein and
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can be stimulated to 69.91 ± 8.11 pmol/min/mg of protein by 10 μM isoproterenol and reduced to basal levels (9.08 ± 0.93 pmol/min/mg of protein) by addition of excess l-propranolol. Using conditions identical to those used in the experiments with EGF, 10 μM isoproterenol stimulated [35S]GTPγS binding to A431 cell membranes, and the effect was completely blocked by inclusion of 1 mM l-propranolol in the assay (Fig. 5B). Taken together, the results presented in Figs. 1, 3, and 5 suggest that EGF receptors in rat hepatocytes and A431 cells may mediate their responses through different mechanisms.

DISCUSSION

EGF has been demonstrated to stimulate phospholipase C activity, generating Ins(1,4,5)P3, in a number of cell types, including A431 cells (35–37), rat hepatocytes (17), MDA-468 human breast cancer cells (37), and WB rat hepatic epithelial cells (38, 39). Although different mechanisms for phospholipase C activation by EGF have been proposed in different cell types, the exact pathways used are still unclear. In rat hepatocytes, the effect of EGF can be completely abolished by pertussis toxin pretreatment (17), suggesting that the EGF receptor may interact with a pertussis toxin-sensitive G protein. However, this does not exclude the possibility that the EGF receptor may interact with a G protein indirectly or that the effect of pertussis toxin may be due to an effect other than ADP-ribosylation of a G protein. Therefore, a more direct approach to demonstrate the interaction of the EGF receptor with a G protein would be to measure the G protein activation by the EGF receptor in a membrane preparation (22, 23, 40).

Receptors activate G proteins by increasing the rate of GDP dissociation from the inactive protein and stimulating GTP association to the protein (41). Hydrolysis of bound GTP to GDP by the intrinsic GTPase activity of the G protein α-subunit returns the protein to the basal state (41). Therefore, the interaction of receptor and G protein can be followed using GTPase activity or GDP/GTP exchange. Hormonestimulated GTPyS binding has been used successfully as an assay for determining the interaction between receptors and G proteins using purified G proteins and β-adrenergic receptors (22) or muscarinic receptors (23) in phospholipid vesicles and the transforming growth factor β1 receptor in membranes (40).

This study demonstrates that EGF stimulates [35S]GTPγS association with [α-32P]GDP dissociation from rat hepatic membranes (Figs. 1A and 3A) and that the effects of EGF are completely abolished in membranes prepared from pertussis toxin-treated rats (Fig. 2C). These data further support the concept that the EGF receptor in rat hepatocytes can interact directly with a pertussis toxin-sensitive G protein. This observation is not unique as EGF receptors can interact directly with a pertussis toxin-sensitive G protein (48). However, no correlation between tyrosine phosphorylation of a G protein and its function in intact cells has been reported. In rat hepatocytes, two pieces of evidence suggest that tyrosine phosphorylation may not be required for the interaction of the EGF receptor and a G protein. First, the EGF effect on [35S]GTPγS binding was detected under the conditions in which tyrosine phosphorylation does not occur, i.e., in the absence of ATP and Mn2+ (Figs. 1–3). Second, pertussis toxin had no effect on the intrinsic tyrosine kinase activity of the EGF receptor, measured by receptor autophosphorylation (Fig. 4), even though the toxin completely abolished the effect of EGF on GDP/GTP exchange (Fig. 2C). However, it must be stressed that these data are indirect, and the possibility that the EGF receptor may activate a specific G protein via tyrosine phosphorylation can only be examined when the specific G protein is identified.

Pilot experiments explored the possibility of measuring an EGF-stimulated GTPase activity. However, hepatic membranes have a high ATPase activity, contributing to a high background in the GTPase assay. Perhaps because of the high background, angiotensin II, glucagon, and EGF were not observed to increase GTPase activity in these membranes.
pertussis toxin has no effect on EGF-stimulated Ins(1,4,5)P$_3$ formation in A431 cells (33), whereas it completely abolishes EGF-stimulated Ins(1,4,5)P$_3$ formation in rat hepatocytes (17). In this study, EGF was shown to stimulate the exchange of GDP/GTP in hepatic membranes (Figs. 1A and 3A), whereas it had no effect on [32P]GTPyS binding to A431 cell membranes (Fig. 5). These results are consistent with the hypothesis that the EGF receptor may stimulate phospholipase C through different mechanisms in rat hepatocytes and A431 cells. Although the reasons for these multiple signaling mechanisms for the EGF receptor are unclear, these differences could be explained by the possible existence of subtypes of EGF receptor that couple to the different effector systems.

Another potential explanation for the multiple mechanisms of hormone-stimulated phospholipase C activity is the involvement of different phospholipase C isoforms that may be activated via different mechanisms. There are at least four phospholipase C isoforms (α, β, γ, and δ), identified by their cloned cDNAs (51). Different receptors may interact with different phospholipase C isoforms to stimulate production of Ins(1,4,5)P$_3$, via pertussis toxin-sensitive G proteins (52–60), via toxin-insensitive G proteins (61–65), or via phosphorylation of phospholipase C-γ (6–12, 66–68). Recently, both platelet-derived growth factor and EGF receptors have been proposed to stimulate phospholipase C activity via phosphorylation of certain tyrosine residues in phospholipase C-γ (6–12, 66–68). Most of the data supporting this hypothesis have been obtained using A431 cells and other cell types that overexpress human EGF receptors. For example, EGF enhanced the phospholipase C activity in a fraction immunopurified from A431 cell extracts by anti-phosphotyrosine chromatography (6). EGF also increases phosphorylation of phospholipase C-γ on tyrosine and serine in 32P-labeled A431 cells (7). Phospholipase C-γ purified from bovine brain membranes (Fig. 5). These results are consistent with the hypothesis that the EGF receptor may activate phospholipase C through different mechanisms in different cells. It will be interesting to determine if similar mechanisms of EGF receptor signal transduction are used in other normal cells, the nature of the putative G protein coupled to the EGF receptor, and whether different phospholipase C isoforms interact with different EGF receptors in different cells.

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