0026-895X/92/050743-10$03.00/0

MOLECULAR PHARMACOLOGY, 42:743-752

Epidermal Growth Factor Activates Phospholipase C in Rat Hepatocytes via a Different Mechanism from that in A431 or Rat1hER Cells

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Received June 5, 1992; Accepted August 31, 1992

SUMMARY

Epidermal growth factor (EGF) can stimulate inositol lipid hydrolysis in rat hepatocytes and can accelerate GTP/GDP exchange in hepatic membranes. Both of these responses can be abolished by pretreatment with pertussis toxin, suggesting that EGF may regulate phospholipase C (PLC) activity via a guanine nucleotide-binding regulatory protein (G protein) in liver cells. In contrast, in A431 human epidermoid carcinoma cells EGF can induce a rapid phosphorylation of PLC-γ on tyrosine residues that increases the activity of immunoprecipitated PLC-γ, suggesting that tyrosine phosphorylation of PLC-γ may be the mechanism for EGF-stimulated inositol trisphosphate production in these cells. To determine the importance of the phosphorylation of PLC-γ on tyrosine residues in a system where the EGF receptor apparently couples to a G protein, the effect of EGF on tyrosine phosphorylation of PLC-γ was examined in rat hepatocytes. PLC-γ was immunoprecipitated from cell lysates with a PLC-γ antiserum and its tyrosine phosphorylation state was determined using both Western blot analysis with phosphotyrosine antibodies and direct measurement of phosphorylated amino acids. The results were compared with analogous experiments performed with A431 cells and another cultured cell line expressing high levels of human EGF receptors, Rat1hER fibroblasts. Although the amount of PLC-γ in rat hepatocytes is similar to that in A431 cells and slightly higher than that in Rat1hER cells, EGF causes a barely detectable increase in the phosphorylation of PLC-γ on tyrosine in hepatocytes, whereas it stimulates a significant degree of phosphorylation of PLC-γ on tyrosine in Rat1hER or A431 cells. Pretreatment of hepatocytes with pertussis toxin abolishes the ability of EGF to activate PLC, as determined by an increase in intracellular Ca^{2+}, but has no effect on the small amount of phosphate incorporated into tyrosine residues on the PLC-γ protein, demonstrating that this low level of PLC-γ phosphorylation does not correlate with changes in PLC activity. The data suggest that phosphorylation of PLC-γ on tyrosine is not important for EGF-enhanced PLC activity in hepatocytes. This conclusion implies that EGF may use a mechanism to regulate PLC activity in hepatocytes that is different from that used in cultured cells expressing high levels of EGF receptors.

Activation of the EGF receptor results in multiple signaling events, including inositol phosphate formation, Ca^{2+} influx, Na^+/H^+ exchange, c-*fos* or c-*myc* expression, activation of MAP kinase, and phosphorylation of ribosomal protein S6, which eventually lead to an increase in DNA synthesis and cell division (1, 2). The stimulation of the intrinsic tyrosine kinase activity of the receptor is thought to be essential for generating these signals, because kinase-deficient mutants of the receptor do not generate a signaling response and do not stimulate cell proliferation after EGF binding (1). One difficulty with this concept has been the inability to identify the enzymatic activities regulated by tyrosine phosphorylation. However, one relevant substrate for the receptor tyrosine kinase, PLC-γ, has been discovered recently (3, 4).

In A431 cells or other cultured cells that express high levels of human EGF receptors, it has been suggested that the phosphorylation of PLC-γ on tyrosine by the EGF receptor is the mechanism responsible for EGF-stimulated inositol phosphate production (3, 4). This hypothesis is supported by the observation that tyrphostins, selective inhibitors of the EGF receptor tyrosine kinase, blocked both the EGF-induced PLC-γ phosphorylation on tyrosine and the EGF-induced Ca^{2+} release in

This work was supported by National Institutes of Health Grant CA-40042 and an American Heart Association Virginia Affiliate Postdoctoral Fellowship to M.L.

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ABBREVIATIONS: EGF, epidermal growth factor; PLC, phospholipase C; $InsP_3$, *myo*-inositol trisphosphates (positional isomerism denoted in parentheses); PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; A431 cells, A431 human epidermoid carcinoma cells; HER14 cells, a NIH3T3 cell line expressing the human epidermal growth factor receptor at a high level; Rat1hER, a Rat-1 fibroblast cell line expressing high levels of the human epidermal growth factor receptor; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; G protein, guanine nucleotide-binding regulatory protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraecetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

A431 cells (5). Moreover, purified PLC-γ can be directly phosphorylated on tyrosine *in vitro* by the EGF receptor purified from A431 cells (6). Most importantly, it has been demonstrated *in vitro* that the activity of PLC-γ prepared by immunoprecipitation can be increased by tyrosine phosphorylation and decreased by removal of the phosphate on tyrosine with a phosphatase (7).

In contrast to the results obtained with A431 cells, an increasing amount of evidence suggests that EGF receptors can interact with G proteins to generate signals in other cell types, including rat hepatocytes (8–10), rat inner medullary collecting tubule cells (11, 12), and rat cardiac cells (13). In hepatocytes, the ability of EGF to increase Ins(1,4,5)P_3 production and intracellular Ca^{2+} levels can be blocked by pretreatment with pertussis toxin (8). Furthermore, EGF increases the initial rate of GTP/GDP exchange in hepatic membranes, and this effect is completely inhibited by pertussis toxin pretreatment (9). These observations suggest that the EGF receptor in hepatocytes may activate PLC via a pertussis toxin-sensitive G protein.

To investigate the relative importance of the phosphorylation of PLC-γ on tyrosine in a system such as hepatocytes where the EGF receptor apparently couples to a G protein, PLC-γ was immunoprecipitated using a PLC-γ antiserum and its tyrosine phosphorylation state was measured using two methods, Western blot analysis with phosphotyrosine antibodies and direct phosphoamino acid analysis. Analogous experiments were performed with A431 cells and a cultured cell line that expresses high levels of human EGF receptors, Rat1hER cells (14). The results demonstrate that, whereas EGF causes large increases in the phosphorylation of PLC-γ on tyrosine in A431 cells or Rat1hER cells, it causes a minimal increase in the phosphorylation of the protein in hepatocytes. Treatment of hepatocytes with pertussis toxin blocks the activation of PLC but does not alter the small effect of EGF on the phosphorylation of PLC-γ on tyrosine, suggesting that this phosphorylation event may not regulate PLC activity in hepatocytes. Overall, the results suggest that EGF may activate PLC in rat hepatocytes via a mechanism different from that used in A431 and Rat1hER cells.

Materials and Methods

Culture of Rat1hER fibroblasts and A431 cells and preparation of rat hepatocytes. Rat1hER (14) and A431 cells were grown in DMEM containing 5% heat-inactivated fetal calf serum, at 37° in a humidified incubator supplied with 7.5% CO_2/92.5% air. Isolated hepatocytes were prepared from 200–250-g, fasted, male Wistar rats as described (15) and were resuspended in Kreb's Ringer bicarbonate buffer (103 mM NaCl, 4.8 mM KCl, 0.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 20 mg/ml bovine serum albumin, 20 mM lactate, 5 mM pyruvate, 5 mM glucose) for experiments designed to immunoprecipitate PLC-γ or EGF receptors. Before measurement of intracellular Ca^{2+} levels, hepatocytes were resuspended in Kreb's Ringer bicarbonate buffer supplemented with 20 mM HEPES, pH 7.4 (8). Before labeling with [^{32}P]orthophosphate for phosphoamino acid analysis, the cells were resuspended in phosphate-free Kreb's Ringer bicarbonate buffer (containing no KH_2PO_4).

Treatment of Rat1hER cells or intact rats with pertussis toxin. After Rat1hER cells reached confluency, the medium was changed to serum-free DMEM containing approximately 500 ng/ml pertussis toxin and the cultures were continued for another 18–24 hr. To treat intact rats, 50 μg of pertussis toxin/100 g of body weight were injected into the peritoneal cavity of 200–250-g, male, Wistar rats 72 hr before isolation of the hepatocytes. This protocol has been demonstrated to produce complete intoxication of the G_i in the membranes, as judged by the absence of an ADP-ribosylated protein band in membranes incubated with activated pertussis toxin and [^{32}P]NAD (9, 16).

Measurement of intracellular Ca^{2+} concentration. Intracellular Ca^{2+} was measured in hepatocytes using the Ca^{2+}-sensitive dye, indo-1, in a SLM 8000C spectrofluorometer. The excitation wavelength was 332 nm and emission fluorescence readings were obtained at 1-sec intervals by monitoring the wavelengths of 485 nm and 410 nm for free indo-1 and Ca^{2+}-bound indo-1, respectively. The Ca^{2+} concentration was calculated by the ratio of fluorescence of bound versus free indo-1. Total intracellular Ca^{2+} was estimated by using digitonin at 30 μg/ml to permeabilize the cells, and zero free Ca^{2+} was estimated by adding 5 mM EGTA after the digitonin treatment (9).

Accumulation of inositol phosphates in Rat1hER fibroblasts. Rat1hER cells were grown in 35-mm dishes for at least 48 hr, as described above, and were labeled with 5 μCi/ml *myo*-[1,2-^{3}H]-inositol in serum-free DMEM for 24 hr. After labeling, the cells were washed once with cold phosphate-buffered saline and incubated in DMEM containing 20 mM HEPES (pH 7.2) at 37° for 90 min and then with 100 mM LiCl for 30 min. The reaction was started by addition of 82 nM EGF (by dilution of a 200-fold concentrated stock in water), terminated with 0.4 ml of cold 0.5 M $HClO_4$ containing 0.5 mM EDTA and 0.1 mM diethylenetriaminepentaacetic acid, and then prepared and analyzed by high performance liquid chromatography as described (17). The cell pellets were dissolved in 1 ml of 0.1 N NaOH and 1% (w/v) sodium deoxycholate before determination of protein (18).

Production and characterization of rabbit antisera against a PLC-γ-TrpE fusion protein. A 1.1-kilobase *PstI* fragment of the PLC-γ cDNA inserted in the *PstI* site of the pATH II vector was given to the University of Virginia Cancer Center by Dr. T. Pawson (University of Toronto). The fragment includes nucleotides 3194–4299 of the bovine brain PLC-γ cDNA (19). After expression in *Escherichia coli* with induction by tryptophan starvation and indoleacrylic acid, this plasmid generates a PLC-γ-TrpE fusion protein with a total molecular mass of approximately 60 kDa, including about 23 kDa of the carboxyl terminus of the PLC-γ protein. The fusion protein was purified by SDS-PAGE (20) and the protein was eluted from the gel slice into a phosphate-buffered saline solution before injection into two rabbits. The rabbit antisera were tested for their ability to immunoprecipitate and/or identify PLC-γ via Western blot analysis (21). The antisera from the different rabbits were termed antisera plcA and plcB. Both antiserum plcA and antiserum plcB were able to immunoprecipitate PLC-γ, although antiserum plcB had a higher titer than did antiserum plcA. Only antiserum plcA was able to identify the PLC-γ protein on a Western blot. Therefore, antiserum plcA was used for Western blot analysis and antiserum plcB was used for immunoprecipitation in this study. Pilot experiments were performed to evaluate the specificity of the new PLC antisera used in this study. PLC-γ was immunoprecipitated from lysates of Rat1hER cells or hepatocytes with antiserum plcB or antiserum plcA, and Western blots were prepared from the immunoprecipitates and probed with monoclonal antibody mixtures specific for either PLC-γ or PLC-β. The results indicate that both antisera plcA and plcB can immunoprecipitate a 145-kDa protein that is recognized in Western blot analysis by antibodies against PLC-γ but not by antibodies against PLC-β. Based on these results, the new antisera plcA and plcB are specific for PLC-γ and show no cross-reactivity with PLC-β.

Immunoprecipitation of PLC-γ or the EGF receptor from hepatocyte, Rat1hER, or A431 cell lysates. Rat hepatocytes or cultured cells were treated with vehicle or 82 nM EGF at 37° for the times indicated in the figure legends. The cells were then solubilized with a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM sodium orthovanadate, 50 μg/ml leupeptin, and 0.1%

(w/v) aprotinin. Sodium deoxycholate and SDS were deleted from the solubilizing buffer when the experiment was designed to investigate whether the EGF receptor and PLC-γ could be coimmunoprecipitated. Immunoprecipitation of the EGF receptor or of PLC-γ from the solubilized cell lysates was performed as follows: a rabbit antiserum against the purified rat liver EGF receptor was added to the lysate at a 1/100 dilution or antiserum plcB against PLC-γ was added to the lysate at a 1/50 dilution and the mixture was incubated on ice for 90 min. Protein A-Sepharose (1/1 volume of Sepharose to water) was added and the protein-IgG complex was allowed to bind to Protein A-Sepharose on ice for another 40 min. The Sepharose was centrifuged in a microfuge and washed three times with solubilizing buffer and once with phosphate-buffered saline. The immunoprecipitated proteins were solubilized by boiling for 3 min in SDS sample buffer [final concentrations, 50 mM Tris, pH 6.8, 10% (v/v) glycerol, 1% (w/v) SDS, 2% (v/v) β-mercaptoethanol, and 0.0025% (w/v) bromophenol blue] and resolved on an 7% SDS-polyacrylamide slab gel (20). The proteins were transferred to nitrocellulose membranes (21). Proteins containing phosphotyrosine residues and/or PLC-γ were identified by Western blot analysis with antibodies raised against phosphotyrosine or PLC-γ, respectively, and the reaction was visualized using ^{125}I-Protein A. Autoradiographs were obtained by exposing the dried blots to Kodak XK-1 film for 1–14 days.

To determine whether the density corresponding to the bands on the autoradiographs made from the phosphotyrosine or PLC-γ blots increased linearly with the amount of PLC-γ immunoprecipitate loaded on the gels, eight different volumes (10–100 μl) of a PLC-γ immunoprecipitate from a hepatocyte lysate were loaded on a gel and Western blots were prepared and probed with phosphotyrosine antibodies or antiserum plcA as described above. The density information on the autoradiographs was digitized and quantified as described (9). The density of the PLC-γ band was plotted versus the volume of PLC-γ immunoprecipitate loaded on the gel. A straight line was obtained with a correlation coefficient of 0.9970 for antiserum plcA and 0.9887 for the phosphotyrosine antibodies. In subsequent experiments, 40–50 μl of immunoprecipitate were loaded on the gels, to remain within the linear range of the assay.

The efficiency of the immunoprecipitation of PLC-γ from hepatocyte lysates with antiserum plcB was determined using a repetitive immunoprecipitation protocol. PLC-γ was immunoprecipitated from a hepatocyte lysate and the immunoprecipitation procedure was repeated five more times with the supernatant obtained from each previous immunoprecipitation. These experiments demonstrated that >95% of the tyrosine-phosphorylated PLC-γ in the lysate from 10^7 hepatocytes was immunoprecipitated using 6 μl of antiserum plcB and 60 μl of Protein A-Sepharose. To ensure complete immunoprecipitation, an excess of antiserum plcB (12 μl) and Protein A-Sepharose (120 μl) was used throughout this study. The titer of antiserum plcA was determined in pilot experiments and a 1/10 dilution of antiserum plcA was chosen for probing the Western blots.

Determination of the proportion of PLC-γ phosphorylated on tyrosine in hepatocytes and A431 cells. PLC-γ was immunoprecipitated as described above and released from the Protein A-Sepharose by boiling for 3 min in 100 μl of buffer containing 50 mM Tris, pH 6.8, 10% (v/v) glycerol, 1% (w/v) SDS, and 2% (v/v) β-mercaptoethanol. The solution was diluted with a buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, and 1 mM sodium orthovanadate, to maintain the SDS concentration at 0.1% (w/v). The rabbit IgG released by boiling was removed by incubation with excess Protein A-Sepharose (addition of 60 μl of Protein A-Sepharose/ml of supernatant followed by centrifugation), leaving the phosphorylated and dephosphorylated forms of PLC-γ in solution. The tyrosine-phosphorylated PLC-γ was immunoprecipitated with phosphotyrosine antibodies (the ratio of antibodies to protein was 1:50, w/w) as described above. The efficiency of the immunoprecipitation of heat-denatured tyrosine-phosphorylated PLC-γ was determined using antiserum plcB as follows. All of the PLC-γ remaining in the supernatant after immunoprecipitation with the phosphotyrosine antibodies was immunoprecipitated with antiserum plcB, the immunoprecipitate was resolved on a 7% polyacrylamide gel, and the amount of tyrosine-phosphorylated PLC-γ was quantitated using Western blot analysis with phosphotyrosine antibodies as described above. The total amount of tyrosine-phosphorylated PLC-γ in the sample was determined using an aliquot of the original sample by the same methodology. Dividing the amount of PLC-γ in the supernatant by the total gave the percentage of tyrosine-phosphorylated PLC-γ remaining in the supernatant. The percentage was typically about 40%, indicating that about 60% of the tyrosine-phosphorylated PLC-γ was immunoprecipitated by the phosphotyrosine antibodies. This value was used to correct the raw densities obtained for tyrosine-phosphorylated PLC-γ determined from the autoradiographs. The corrected values are presented in Fig. 6, C and D.

Phosphoamino acid analysis. Rat hepatocytes at a concentration of 10^7 cells/ml were incubated at 37° in a shaking water bath for 45 min in 800 μl of phosphate-free Kreb's Ringer bicarbonate buffer containing 1.25 mCi of [^{32}P]orthophosphate. After stimulation with 82 nM EGF for 30 sec, the cells were centrifuged, and the cell pellet was solubilized with 400 μl of solubilizing buffer. Rat1hER and A431 cells were grown to confluency on 100-mm dishes and rinsed once with phosphate-free medium 199 supplemented with 18 mM $NaHCO_3$, 0.5% (v/v) phosphate-free dialyzed fetal calf serum, 4 mM glutamine, and 0.1% (w/v) bovine serum albumin, pH 7.2. To label the cells, they were incubated in the aforementioned medium containing 0.5 mCi/ml [^{32}P] orthophosphate for 16–24 hr. After stimulation with 82 nM EGF for 2 min, the medium was aspirated and the cells were rinsed once with 5 ml of cold phosphate-buffered saline and lysed with 700 μl of solubilizing buffer. The lysates were centrifuged at 14,000 × *g* for 10 min at 4° and PLC-γ was immunoprecipitated from the lysates using antiserum plcB, as described above, and electrophoresed on a 7% polyacrylamide gel. The dried gel was exposed to Kodak XAR-5 film. The ^{32}P-labeled band corresponding to PLC-γ was cut from the gel, the protein was extracted, and the phosphoamino acids were obtained by acid hydrolysis of the protein as described (22). The hydrolyzed samples were taken to dryness in a SpeedVac (Savant) for 1 hr and suspended in 4 μl of a mixture containing 1 mg/ml each of phosphorylated serine, phosphorylated threonine, and phosphorylated tyrosine in 2.2% (v/v) formic acid, 7.8% (v/v) acetic acid, pH 1.9. The phosphoamino acids were resolved by one-dimensional thin layer electrophoresis on a cellulose-coated thin layer plate using 0.5% (v/v) pyridine, 5% (v/v) acetic acid, pH 3.5, to develop the chromatogram. The plate was exposed to Kodak XAR-5 film or sprayed with ninhydrin.

Materials. The reagents used were obtained from the following sources: human recombinant EGF and monoclonal antibody mixtures against PLC-γ and PLC-β, Upstate Biotechnology, Inc. (Lake Placid, NY); affinity-purified rabbit anti-mouse IgG, Jackson ImmunoResearch Laboratories Inc. (West Grove, PA); Protein A-Sepharose, Pharmacia (Piscataway, NJ); [^{32}P]orthophosphate, New England Nuclear (Boston, MA); cellulose-coated thin layer plates, EM Science (Gibbstown, NJ); DMEM, fetal calf serum, tryptophan, phosphate-free medium 199, and phosphate-free dialyzed fetal calf serum, GIBCO (Grand Island, NY); bovine serum albumin (fraction V for Western blotting), Boehringer Mannheim (Indianapolis, IN); crystalized bovine serum albumin for all other uses, Schwarz/Mann Biotech (Cleveland, OH); indo-1/acetoxymethyl ester, Molecular Probes (Eugene, OR); SDS, bromophenol blue, acrylamide, and bisacrylamide, Bio-Rad (New York, NY); ^{125}I-Protein A, Amersham (Arlington Heights, IL); *myo*-[1,2-^{3}H]inositol (30–50 Ci/mmol), American Radiolabeled Chemicals Inc. (St. Louis, MO); and nitrocellulose paper, Schleicher & Schuell (Keene, NH). The source of other biochemical reagents was Sigma Chemical Company (St. Louis, MO). Reagent grade chemicals were from Fisher Scientific (Columbia, MO). Wistar rats were purchased from Hill Top Laboratories (Scottdale, PA) and A431 cells were from the American Type Culture Collection (Rockville, MD). The following reagents were kindly provided: pertussis toxin, Dr. E. L. Hewlett, University of Virginia; phosphotyrosine antibodies, Dr. J. T. Parsons,

University of Virginia; Rat1hER cells, Drs. W. J. Wasilenko and M. J. Weber, University of Virginia; PLC-γ cDNA inserted into pATH II vector, Dr. T. Pawson, University of Toronto; and antiserum raised against purified hepatic EGF receptor, Dr. H. S. Earp, University of North Carolina.

Results

Phosphorylation of PLC-γ on tyrosine has been suggested as the mechanism for activation of PLC by the EGF receptor in A431 cells and other cultured cells expressing high levels of the human EGF receptor (3, 4). However, in systems such as rat hepatocytes where the EGF receptor may activate PLC via a G protein (8, 9), the effect of EGF on the tyrosine phosphorylation of PLC-γ has not been extensively investigated. To begin comparing the effect of EGF on the tyrosine phosphorylation of PLC-γ in hepatocytes and Rat1hER cells, the kinetics of the response were examined in cells treated with vehicle or EGF for 15 sec, 1 min, or 5 min, and PLC-γ was immunoprecipitated from the cell lysates. The tyrosine phosphorylation state and the amount of PLC-γ were determined by Western blot analysis with phosphotyrosine antibodies and antiserum plcA, respectively. The results obtained with hepatocytes are shown in Fig. 1A. In the absence of EGF, a minimal basal tyrosine phosphorylation of PLC-γ was detected at each time point (Fig. 1A, *PTyr, lanes 1–3*). EGF was able to stimulate the phosphorylation of PLC-γ on tyrosine at all time points (Fig. 1A, *PTyr, lanes 4–6*). The density of the phosphotyrosine band on the autoradiograph was quantitated and the increase in tyrosine phosphorylation was plotted against the time of EGF treatment, with the results shown in Fig. 1B. The effect of EGF was evident within 15 sec and continued for at least 5 min, although the magnitude of the stimulation decreased 30–40% after the first minute of EGF treatment. The amount of PLC-γ loaded on the gel, as indicated by the density of the PLC-γ band on the blot probed with antiserum plcA, was quantitated and varied <10% (Fig. 1A, *PLCγ*). A very similar time course was obtained with Rat1hER cells, although the peak occurred at 1 min and did not decline as much as in hepatocytes (data not shown). To ensure a maximum response throughout this study, hepatocytes were treated with EGF for 30–60 sec and Rat1hER cells were treated for 2 min.

To determine whether the increase in the phosphorylation of PLC-γ on tyrosine in hepatocytes was as large as in cultured cells, experiments similar to those shown in Fig. 1 were performed with A431 cells, Rat1hER cells, and hepatocytes. The blots containing PLC-γ from all three types of cells were probed with phosphotyrosine antibodies and antiserum plcA and an estimate of the increase in the tyrosine phosphorylation of PLC-γ was obtained by normalizing the increase in the phosphotyrosine signal to the amount of PLC-γ in the cell. In every type of cell, a minimal basal phosphorylation of PLC-γ on tyrosine was detected (Fig. 2A, *lanes 1, 2, 5, 6, 9,* and *10*). EGF strongly stimulated the phosphorylation of PLC-γ on tyrosine in both A431 (Fig. 2A, *lanes 3* and *4*) and Rat1hER cells (Fig. 2A, *lanes 7* and *8*), whereas it caused a much smaller increase in the phosphorylation of PLC-γ in hepatocytes (Fig. 2A, *lanes 11* and *12*). The prominent bands at approximately 170 kDa in EGF-treated A431 and Rat1hER cells (Fig. 2A) are probably EGF receptors, based on the molecular mass and the observation that EGF receptors are coimmunoprecipitated with PLC-γ in A431 (23) and Rat1hER cells (14). Fig. 2B indicates the

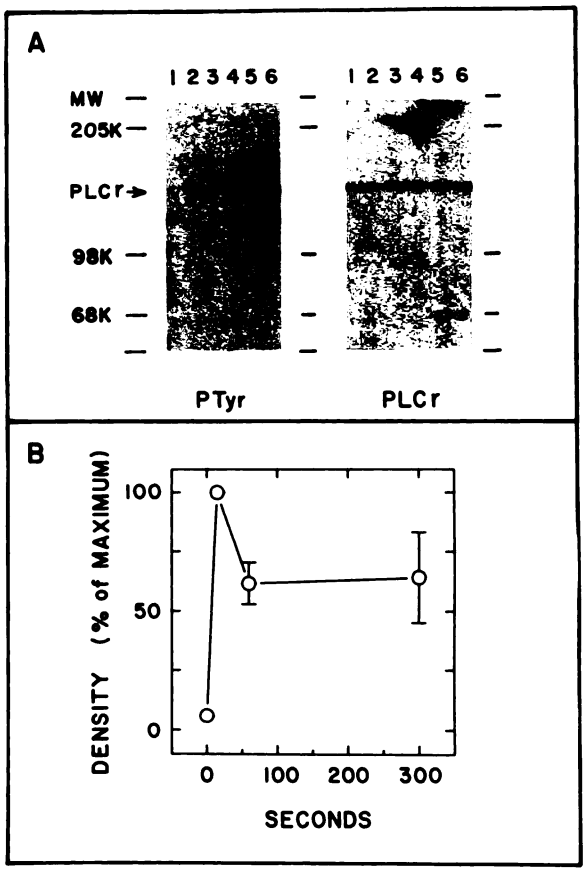

Fig. 1. Time course of phosphorylation of PLC-γ on tyrosine stimulated by EGF in rat hepatocytes. A, Autoradiographs of immunoblots probed with phosphotyrosine antibodies (*PTyr*) or antiserum plcA (*PLCγ*). Rat hepatocytes were treated with vehicle for 15 sec (*lane 1*), 1 min (*lane 2*), or 5 min (*lane 3*) or with 82 nM EGF for 15 sec (*lane 4*), 1 min (*lane 5*), or 5 min (*lane 6*). The cells were solubilized, PLC-γ in the lysates was immunoprecipitated, and the proteins were resolved on SDS gels and transferred to nitrocellulose as described in Materials and Methods. The tyrosine phosphorylation state and the amount of PLC-γ in the immunoprecipitates were determined using immunoblot analysis with phosphotyrosine antibodies or antiserum plcA, respectively, as described in Materials and Methods. The data are representative of five experiments. B, Quantitative analysis of the time course of tyrosine phosphorylation of PLC-γ stimulated by EGF. The amount of tyrosine in the PLC-γ band in the autoradiographs in A was quantified as described in Materials and Methods. The densities of the PLC-γ bands in the phosphotyrosine blots from five experiments were averaged and the percentage of maximum was plotted as a function of the time of treatment with EGF. The measured densities of the PLC-γ band of the control and after 15 sec or 5 min of EGF treatment are 0.266 ± 0.046, 3.685 ± 0.564, and 2.202 ± 0.44 OD × mm², respectively. The data are presented as mean ± standard error (five experiments). The densities of the PLC-γ bands in the PLC-γ blot in A were also measured and varied <10%.

relative amount of PLC-γ in every lane shown in Fig. 2A. Although PLC-γ was immunoprecipitated from the cell lysates of similar numbers of cells in each case, the amount of PLC-γ in each cell type varied. The amount of PLC-γ was similar in A431 cells (Fig. 2B, *lanes 1–4*) and hepatocytes (Fig. 2B, *lanes 9–12*) and was lower in Rat1hER cells (Fig. 2B, *lanes 5–8*). The density of the PLC-γ bands in the autoradiographs shown in Fig. 2, A and B, was quantitated and the increase in phosphorylation in the three cell types was normalized to the amount of PLC-γ detected on the blots. The results are presented as the density ratio in Fig. 2C. Note that in the absence of EGF there was a very low basal phosphorylation of PLC-γ on tyrosine in

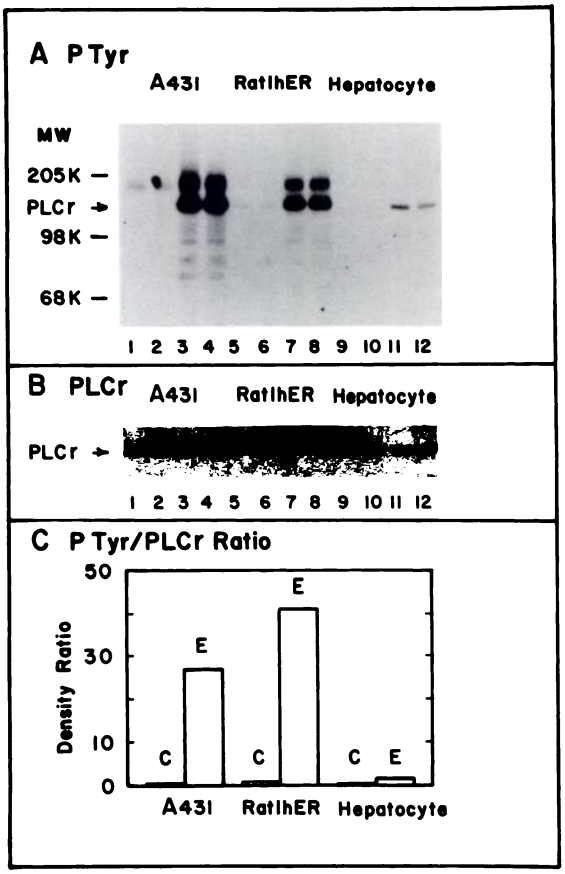

Fig. 2. Comparison of the degree of tyrosine phosphorylation of PLC-γ stimulated by EGF in A431 cells, Rat1hER cells, and hepatocytes. Autoradiographs of immunoblots were made with phosphotyrosine antibodies (A) or antiserum plcA (B). A431 cells were treated with vehicle for 1 min (*lane 1*) or 5 min (*lane 2*) or with 82 nM EGF for 1 min (*lane 3*) or 5 min (*lane 4*). Rat1hER cells (*lanes 5–8*) and hepatocytes (*lanes 9–12*) were also treated with vehicle or 82 nM EGF for 1 or 5 min. The cells were solubilized, the PLC-γ in the lysates was immunoprecipitated, and the proteins were resolved on SDS gels and transferred to nitrocellulose, as described in Materials and Methods. A, The tyrosine phosphorylation state of PLC-γ was determined using immunoblot analysis with phosphotyrosine antibodies. B, The amount of PLC-γ was determined using immunoblot analysis with antiserum plcA. C, The degree of phosphorylation of PLC-γ on tyrosine with vehicle (*C*) or 1 min of EGF treatment (*E*). The amount of tyrosine phosphorylation in the PLC-γ band from the autoradiographs was quantified as described in Materials and Methods and normalized using the densities of the PLC-γ bands in the PLC-γ blot. The ratio is shown in the bar graph. The data are representative of two experiments for A431 cells and more than four experiments for Rat1hER cells and hepatocytes.

all three types of cells (density ratio = 0.3 ± 0.2). EGF enhanced the tyrosine phosphorylation of PLC-γ in all three cell types, but the increase in phosphorylation was 25–40-fold less in hepatocytes than in A431 and Rat1hER cells. It is important to stress that the low level of tyrosine phosphorylation of PLC-γ observed in hepatocytes in response to EGF is not due to an inactive receptor kinase (or very active tyrosine phosphatases). In hepatic membranes, activation of the EGF receptor causes a large and rapid autophosphorylation of the EGF receptor on tyrosine, as measured by probing Western blots with phosphotyrosine antibodies (9). A similar large response is obtained if intact hepatocytes are treated with EGF.

The immunoblots in Fig. 2 suggest that EGF stimulates a minimal (but detectable) amount of tyrosine phosphorylation of PLC-γ in hepatocytes. To confirm this result, phosphoamino acid analysis was used to measure the amount of phosphotyrosine in PLC-γ directly. These data are shown in Fig. 3, B–D. In the absence of EGF, there is a minimal basal level of tyrosine phosphorylation of PLC-γ in A431 cells, Rat1hER cells, and hepatocytes. The basal serine phosphorylation is very obvious. In keeping with the result shown in Fig. 2C, the increase in tyrosine phosphorylation of PLC-γ after EGF treatment was large in A431 (Fig. 3B) and Rat1hER cells (Fig. 3D) but barely detectable in hepatocytes (Fig. 3C). Overall, the results presented in Figs. 2 and 3 demonstrate that EGF stimulates the phosphorylation of PLC-γ on tyrosine to a far greater extent in A431 cells or Rat1hER cells than in hepatocytes. The data also imply that the increase in tyrosine phosphorylation is greatly amplified by Western blot analysis with phosphotyrosine antibodies.

It was important to determine whether the minor increase in

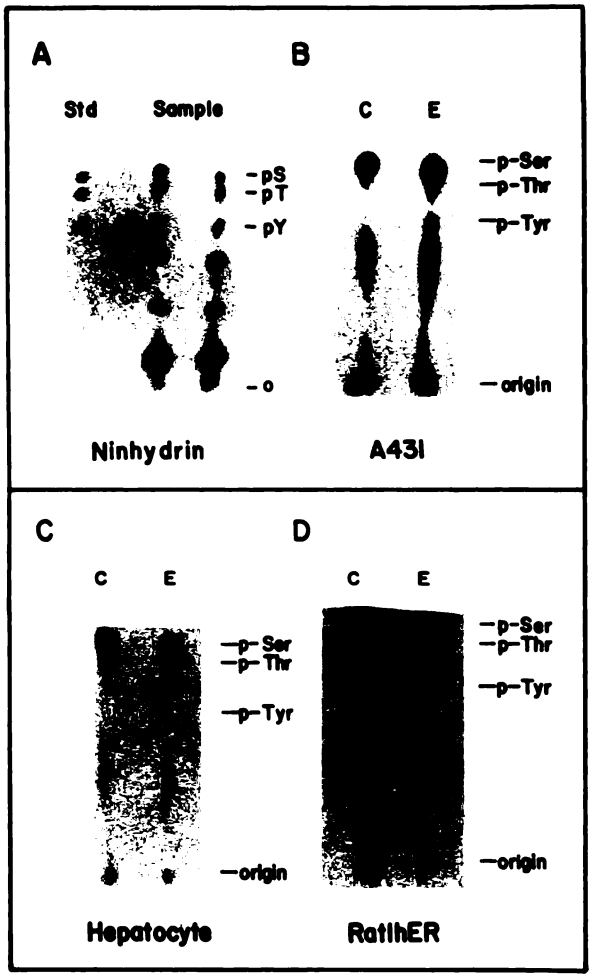

Fig. 3. Phosphoamino acid analysis of PLC-γ immunoprecipitated from ^{32}P-labeled A431 cells, Rat1hER cells, and hepatocytes. Cells were labeled with $^{32}PO_4^{3-}$ and stimulated with vehicle (*C*) or 82 nM EGF (*E*) for 1 min (hepatocytes) or 2 min (cultured cells), as described in Materials and Methods. PLC-γ was immunoprecipitated and the phosphoamino acid content was analyzed as described in Materials and Methods. The locations of the origin, phosphoserine (*p-Ser*), phosphothreonine (*p-Thr*), and phosphotyrosine (*p-Tyr*) on the thin layer plates were identified by ninhydrin staining of a standard composed of the three phosphoamino acids. A, Ninhydrin-stained thin layer plate showing migration of phosphoamino acid standards; B, autoradiographs from A431 cells; C, autoradiographs from hepatocytes; D, autoradiographs from Rat1hER cells. The data are representative of three experiments.

phosphorylation of hepatic PLC-γ shown in Figs. 1–3 was correlated with activation of PLC. Because pertussis toxin can abolish EGF-stimulated PLC activity in hepatocytes, it was used as a probe to determine whether the change in phosphorylation of PLC-γ was correlated with the stimulation of inositol lipid breakdown and the resultant increase in intracellular Ca^{2+} levels. Earlier studies have shown that a 10–30-sec stimulation of hepatocytes with EGF causes a 1.6–1.8-fold increase in $InsP_3$ levels, which results in a 4-fold change in intracellular Ca^{2+} levels (8). Because the Ca^{2+} signal is the most sensitive measurement of PLC activity, it was used as an indirect measure of PLC activity in these experiments. The effect of EGF on the intracellular Ca^{2+} concentration and on the tyrosine phosphorylation of PLC-γ were measured in the same preparations of control and pertussis toxin-pretreated hepatocytes, with the results shown in Figs. 4 and 5. In control hepatocytes, EGF increased the intracellular Ca^{2+} concentration (Fig. 4A). The kinetics and magnitude of the change are consistent with the rapid increase in $InsP_3$ levels observed previously (8). Vasopressin also increased the intracellular Ca^{2+} concentration but with a faster rate of Ca^{2+} rise, compared with EGF, presumably due to a much larger increase in $InsP_3$ production. After pertussis toxin pretreatment, the effect of EGF was completely abolished, whereas the effect of vasopressin was unchanged (Fig. 4B). Again, this result is consistent with the ability of pertussis toxin to block the EGF-stimulated increase in $InsP_3$ levels (8).

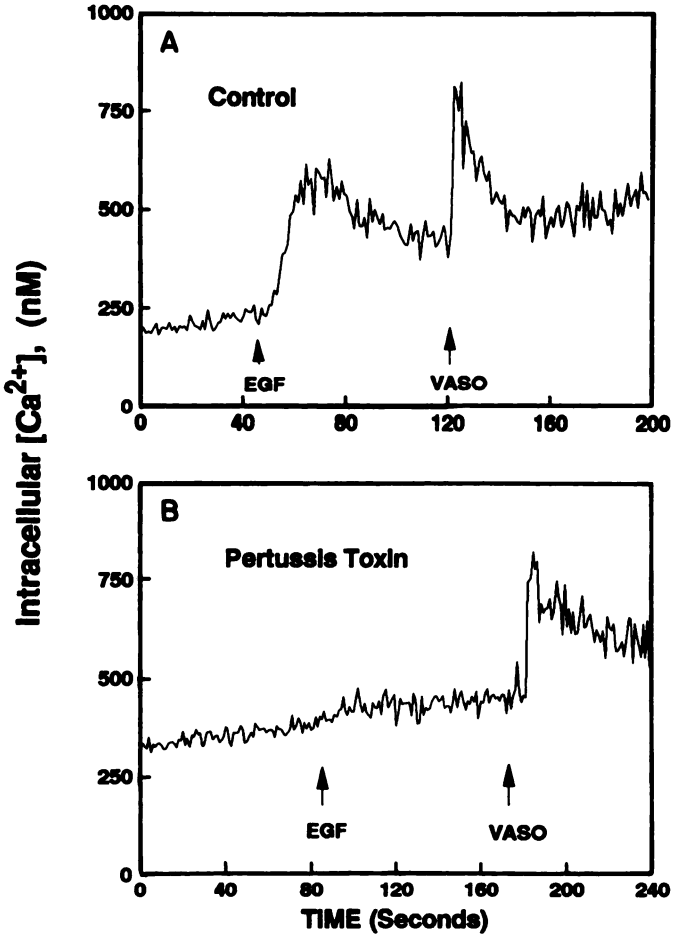

Fig. 4. Effect of pertussis toxin pretreatment on EGF-enhanced intracellular Ca^{2+} concentration in rat hepatocytes. Intracellular Ca^{2+} concentration was measured in indo-1/acetoxymethyl ester-loaded hepatocytes in the presence of 82 nM EGF or 24 nM vasopressin (*VASO*), as described in Materials and Methods. A, Control hepatocytes. B, Hepatocytes from pertussis toxin-treated rats. The data are representative of five experiments in each case.

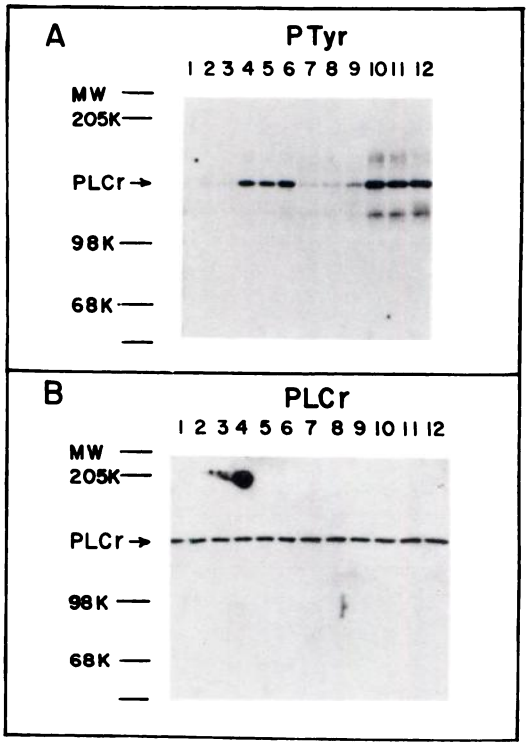

Fig. 5. Effect of pertussis toxin pretreatment on EGF-stimulated phosphorylation of PLC-γ on tyrosine in hepatocytes. Autoradiographs of Western blots probed with phosphotyrosine antibodies (*PTyr*) (A) or antiserum plcA (*PLCγ*) (B). Control hepatocytes were treated with vehicle for 15 sec (*lane 1*), 1 min (*lane 2*), or 5 min (*lane 3*) or with 82 nM EGF for 15 sec (*lane 4*), 1 min (*lane 5*), or 5 min (*lane 6*). Hepatocytes from pertussis toxin-treated rats were treated with vehicle for 15 sec (*lane 7*), 1 min (*lane 8*), or 5 min (*lane 9*) or with 82 nM EGF for 15 sec (*lane 10*), 1 min (*lane 11*), or 5 min (*lane 12*). The cells were solubilized, the PLC-γ in the lysates was immunoprecipitated, and the proteins were resolved on SDS gels and transferred to nitrocellulose, as described in Materials and Methods. The tyrosine phosphorylation state and the amount of PLC-γ were determined by probing the blots with phosphotyrosine antibodies (A) and antiserum plcA (B), respectively. The data are representative of three experiments.

The effect of EGF on the tyrosine phosphorylation of PLC-γ in the same preparations of hepatocytes used for the experiments in Fig. 4 is shown in Fig. 5. Hepatocytes were treated with vehicle or EGF for 15 sec, 1 min, or 5 min and PLC-γ was immunoprecipitated with antiserum plcB. The amount of phosphate incorporated into tyrosine on the PLC-γ protein and the total amount of PLC-γ were determined by immunoblotting. In the absence of EGF, there was a small basal tyrosine phosphorylation of PLC-γ in both control and pertussis toxin-treated hepatocytes at 15 sec (Fig. 5A, *lanes 1* and *7*), 1 min (Fig. 5A, *lanes 2* and *8*), and 5 min (Fig. 5A, *lanes 3* and *9*). EGF stimulated tyrosine phosphorylation of PLC-γ at all time points in control cells (Fig. 5A, *lanes 4–6*). Pretreatment with pertussis toxin had no effect on the ability of the EGF receptor to increase tyrosine phosphorylation of PLC-γ (Fig. 5A, *lanes 10–12*), implying that the coupling of the receptor to a G_i-like protein does not regulate this response. Analogous results were obtained if the experiment was performed with ^{32}P-labeled hepatocytes and the phosphoamino acids were analyzed directly

according to the methods used in Fig. 3 (data not shown). Note from Fig. 5B that the amount of PLC-γ loaded on the gel (based on quantitation of the immunoblot) varied <10% among the different samples. The data demonstrate that pertussis toxin did not affect the low level of tyrosine phosphorylation of PLC-γ induced by EGF in hepatocytes, whereas it completely abolished the EGF-induced increase in intracellular Ca^{2+} concentration. Overall, these results suggest that the EGF-induced tyrosine phosphorylation of PLC-γ is not correlated with EGF-stimulated production of $InsP_3$ and the resultant increase of intracellular Ca^{2+} concentration in hepatocytes.

To compare the effect of pertussis toxin on EGF-stimulated PLC activity in a cultured cell line with that in hepatocytes, the ability of EGF to stimulate $InsP_3$ production was measured in control and pertussis toxin-pretreated Rat1hER cells (Table 1). Note that EGF stimulated $InsP_3$ accumulation about 6-fold in this cultured cell line, but that neither the basal levels of $InsP_3$ nor the magnitude of the EGF effect were significantly altered by pretreatment with pertussis toxin. PLC-γ was also immunoprecipitated from the cell lysate of control and toxin-pretreated Rat1hER cells and the tyrosine phosphorylation state of PLC-γ was determined with phosphotyrosine antibodies. Without EGF treatment, the basal tyrosine phosphorylation of PLC-γ was minimal at 15 sec, 1 min, or 5 min. EGF stimulated the phosphorylation of PLC-γ on tyrosine in control cells at all time points to the same extent as shown in Fig. 2. As might be expected, treatment of the cells with pertussis toxin did not significantly change the EGF-stimulated phosphorylation of PLC-γ on tyrosine in Rat1hER cells at any time (data not shown). These experiments demonstrate that pertussis toxin has no effect on either EGF-stimulated $InsP_3$ production or EGF-induced phosphorylation of PLC-γ on tyrosine in cultured Rat1hER cells. Overall, the data described above and those in Fig. 5 suggest that the effects of EGF on the tyrosine phosphorylation of PLC-γ are not altered by ADP-ribosylation of the G_i-like proteins in either hepatocytes or Rat1hER cells.

In cultured cells such as A431 cells (23), HER14 cells (3), or WB rat hepatic epithelial cells (24, 25), the EGF receptor coimmunoprecipitates with PLC-γ, suggesting that there may be a direct interaction between EGF receptors and PLC-γ. To determine whether coimmunoprecipitation of EGF receptors and PLC-γ could be observed in rat hepatocytes, EGF receptors were immunoprecipitated from lysates of EGF-treated hepatocytes with a EGF receptor antiserum, using the same conditions described for coimmunoprecipitation of the two proteins from A431 or HER14 cells (23). The proteins were resolved via SDS-PAGE, transferred to nitrocellulose, and probed with both phosphotyrosine antibodies and antiserum plcA. No PLC-γ was detected in the EGF receptor immunoprecipitates, suggesting that PLC-γ may not directly interact with the EGF receptor in rat hepatocytes, at least at this sensitivity of detection (data not shown).

Figs. 2 and 3 suggest that only a very small fraction of the total PLC-γ may be phosphorylated on tyrosine after EGF treatment in hepatocytes. Therefore, it was important to determine the percentage of the total PLC-γ molecules that are phosphorylated on tyrosine after EGF treatment in hepatocytes and to compare it with the value found in A431 cells. This analysis was performed using antiserum plcB and phosphotyrosine antibodies to immunoprecipitate PLC-γ and tyrosine-phosphorylated PLC-γ, respectively, from control and EGF-treated cells. Fig. 6, A and B, shows the PLC-γ band in sections of immunoblots probed with a monoclonal antibody mixture to PLC-γ. The amount of PLC-γ precipitated from each cell type with antiserum plcB is indicated in Fig. 6, *lanes P*, and the

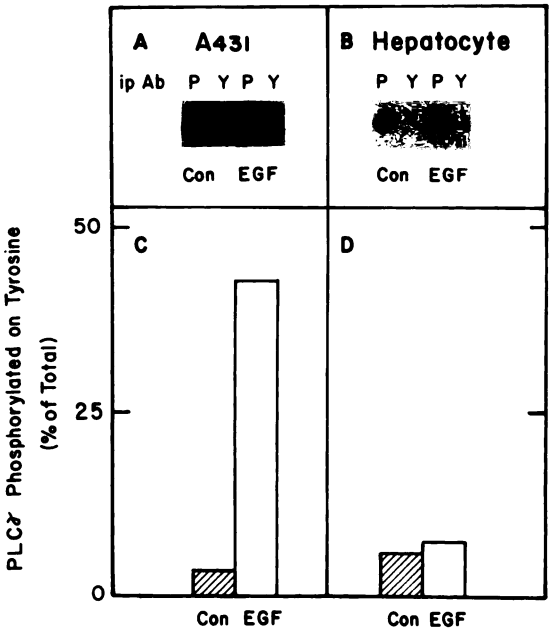

Fig. 6. Comparison of the amount of tyrosine-phosphorylated PLC-γ stimulated by EGF in rat hepatocytes and A431 cells. A and B, Autoradiographs of Western blots probed with monoclonal antibodies against PLC-γ. A431 cells and hepatocytes were treated with or without 82 nM EGF for 1 min and 30 sec, respectively, and cell lysates were prepared as described in Materials and Methods. All of the PLC-γ in the lysates was immunoprecipitated with antiserum plcB and the protein was released from the IgG by boiling. The immunoprecipitated PLC-γ samples were divided into two aliquots for further immunoprecipitation with two different antibodies (*ip Ab*). The tyrosine-phosphorylated PLC-γ was immunoprecipitated from one aliquot with phosphotyrosine antibodies (*Y*) and the other aliquot was used for immunoprecipitating the total PLC-γ with antiserum plcB (*P*). In each case (*P* and *Y*), the amount of PLC-γ protein present in the immunoprecipitates was determined by probing the Western blots with monoclonal antibodies against PLC-γ and was visualized with rabbit anti-mouse IgG and ^{125}I-Protein A. C and D, The amount of tyrosine-phosphorylated PLC-γ in each sample was quantitated as described in Materials and Methods, and the data are expressed as a percentage of the total amount of PLC-γ present (▨, control; □, EGF-treated). The values for the fraction of PLC-γ phosphorylated on tyrosine are as follows: control A431 cells, 3.2 ± 1.8%; EGF-treated A431 cells, 41.5 ± 16.4%; control hepatocytes, 5.6 ± 2.1%; EGF-treated hepatocytes, 7.1 ± 4.9%. Data are means ± standard deviations (two experiments).

TABLE 1

Effect of pertussis toxin pretreatment on EGF-stimulated $InsP_3$ accumulation in cultured Rat1hER cells

Control and pertussis toxin-pretreated Rat1hER cells were labeled with *myo*-[^{3}H] inositol and stimulated with vehicle or 82 nM EGF for 5 min in the presence of 100 mM LiCl and the levels of inositol phosphates were measured, as described in Materials and Methods. To obtain a large signal from the cultured cells, both Ins(1,4,5)P_3 and its principal metabolite, Ins(1,3,4)P_3, were measured in these experiments. The Ins(1,4,5)P_3 and Ins(1,3,4)P_3 produced over the 5-min period were added together and are presented as total $InsP_3$ below. Data are presented as the average ± standard error of four experiments.

Pretreatment	Total $InsP_3$	
	Control	82 nM EGF
	cpm/mg of protein	
None	1328 ± 329	7864 ± 653
Pertussis toxin	1203 ± 469	6631 ± 437

amount of tyrosine-phosphorylated PLC-γ in the band is shown in Fig. 6, *lanes Y*. Note that there is a minimal amount of tyrosine-phosphorylated PLC-γ in control A431 cells and hepatocytes and that EGF causes an increase in the phosphorylation state of a significant fraction of PLC-γ in A431 cells. In contrast, EGF has little effect on the amount of PLC-γ phosphorylated on tyrosine in hepatocytes. The percentage of PLC-γ recovered in the tyrosine-phosphorylated form was quantified and the results are shown in Fig. 6, C and D. In control A431 cells, only 3.2 ± 1.8% (mean ± standard deviation) of PLC-γ was phosphorylated on tyrosine. Treatment with EGF increased the amount of PLC-γ phosphorylated on tyrosine to 41.5 ± 16.4%. In rat hepatocytes, the basal phosphorylation of PLC-γ on tyrosine was 5.6 ± 2.1%, a value similar to that in A431 cells; however, EGF did not greatly increase the amount of tyrosine-phosphorylated PLC-γ (7.1 ± 4.9%). The data demonstrate that EGF does not stimulate tyrosine phosphorylation of a significant number of the total PLC-γ molecules in hepatocytes. Therefore, tyrosine phosphorylation of PLC-γ is not likely to be the mechanism for PLC activation by EGF in hepatocytes.

Discussion

Activation of EGF receptors stimulates the intrinsic tyrosine kinase activity of the receptor and triggers multiple intracellular signals that eventually lead to mitogenesis (1). Among the signals generated, the mechanism for EGF-stimulated production of $InsP_3$ and, in turn, increased intracellular Ca^{2+} concentration is understood most completely. EGF can stimulate PLC activity, generating $Ins(1,4,5)P_3$, in a number of cell types, including A431 cells (26, 27), rat hepatocytes (8), MDA-468 human breast cancer cells (28), and WB rat hepatic epithelial cells (24). Both EGF and PDGF can stimulate the phosphorylation of PLC-γ on tyrosine *in vivo* and *in vitro* (3, 4), but do not affect PLC-β or PLC-δ (3). PLC-γ has also been found to associate with EGF and PDGF receptors (3, 4). These results suggest that phosphorylation of PLC-γ on tyrosine may play an important role in EGF- and PDGF-stimulated PLC activity. Significant biochemical evidence also supports these results. For example, EGF enhanced the PLC activity in a fraction immunopurified from A431 cell extracts by antiphosphotyrosine chromatography (29). EGF also increases the phosphorylation of PLC-γ on tyrosine and serine in ^{32}P-labeled A431 cells (30), and PLC-γ purified from bovine brain can be directly phosphorylated on tyrosine *in vitro* by the EGF receptor purified from A431 cells (6). Moreover, tyrphostins, selective inhibitors of the EGF receptor tyrosine kinase, block EGF-induced PLC-γ phosphorylation on tyrosine and EGF-induced Ca^{2+} release in intact A431 and HER14 cells (5, 23). Finally, it has been demonstrated that PLC-γ activity can be increased by *in vitro* tyrosine phosphorylation and decreased by *in vitro* treatment with a phosphotyrosine-specific phosphatase (7).

Four tyrosine phosphorylation sites have been identified in the PLC-γ protein (3). In HSC-1 cells, a human squamous cell carcinoma-derived cell line that expresses high levels of the EGF receptor, EGF rapidly induces tyrosine phosphorylation of PLC-γ *in vivo* on two sites, tyrosine residues 771 and 1254. Two additional tyrosine phosphorylation sites, tyrosine residues 472 and 783, were identified through *in vitro* tyrosine phosphorylation of purified bovine brain PLC-γ with the EGF receptor. The role of tyrosine phosphorylation in activation of PLC-γ has been investigated by substituting phenylalanine for tyrosine at PLC-γ phosphorylation sites 771, 783, and 1254 and expressing the mutant PLC-γ in NIH 3T3 cells (31). Phenylalanine substitution at Tyr-783 completely blocked the ability of PDGF to activate PLC. However, the mutation at Tyr-1254 inhibited and the mutation at Tyr-771 enhanced the activation of PLC-γ by PDGF. These results suggest that phosphorylation on Tyr-783 is essential for PLC-γ activation and strongly suggest that phosphorylation on Tyr-783 is essential for PLC-γ activation and strongly suggest that phosphorylation of PLC-γ on tyrosine plays an important role in EGF- and PDGF-stimulated PLC activity. A counterpoint to this hypothesis is provided by experiments performed with cells transfected with constructs expressing EGF receptors missing 126 amino acids in the carboxyl terminus of the molecule. Stimulation of these cells with EGF increases the phosphorylation of PLC-γ on tyrosine but does not activate inositol lipid hydrolysis (32). Taken together, all the results suggest that the phosphorylation of PLC-γ on tyrosine may be necessary, but not sufficient, for its activation.

Most of the data in support of the hypothesis that phosphorylation of PLC-γ on tyrosine is important for its activation have been obtained using A431 cells or other cultured cells expressing high levels of EGF receptors. Interestingly, data from normal cells suggest that other pathways may also be involved in EGF signaling. For example, in rat cardiac membranes, EGF stimulates adenylate cyclase activity, potentially via a G_s-like protein (13). In rat inner medullary collecting tubule cells, EGF increases prostaglandin E_2 production (12), and in rat hepatocytes (8) or renal epithelial cells (11) it stimulates production of inositol phosphates. In the latter three cell types, the effects of EGF can be blocked by pertussis toxin, suggesting that a G_i-like protein may be involved. In support of this hypothesis, there are kinetic differences between cultured cells and normal cells in their response to EGF. In cultured rat WB cells, EGF causes a much slower increase in inositol polyphosphates than do peptide hormones such as angiotensin II (24, 25), whereas in normal rat hepatocytes both EGF and angiotensin II cause rapid responses (8). For these reasons, it was important to investigate whether EGF induces any phosphorylation of PLC-γ on tyrosine in cells such as hepatocytes in which a G protein is apparently involved in EGF action. The data from this study demonstrate that, although similar amounts of PLC-γ are present in hepatocytes and A431 cells (with slightly smaller amounts in Rat1hER cells), the effect of EGF on tyrosine phosphorylation of PLC-γ is very different in the different cell types. Whereas EGF strongly stimulated tyrosine phosphorylation of PLC-γ in A431 and Rat1hER cells, its effect on PLC-γ phosphorylation was minor in hepatocytes (Figs. 2, 3, and 6).[2] Therefore, tyrosine phosphorylation of PLC-γ is not likely to account for PLC activation by EGF in hepatocytes. This suggestion is supported by the observation that pertussis toxin has no effect on the EGF-induced phosphorylation of PLC-γ on tyrosine (Fig. 5), whereas it completely abolishes the EGF enhancement of intracellular Ca^{2+} concentration in rat hepatocytes (Fig. 4).

A study with objectives similar to those of the present work appeared recently (10). Although the overall conclusions of the

[2] Based on the data in Figs. 2, 3, and 6, it appears that phosphotyrosine antibodies offer the most sensitive method for measurement of the small increases in the tyrosine phosphorylation of PLC-γ observed in hepatocytes.

study by Yang *et al.* agree with those presented in this report, one aspect of their work contrasts with the results presented in Fig. 5. Using phosphotyrosine antibodies to immunoprecipitate phosphotyrosine-containing proteins from ^{32}P-labeled hepatocytes, Yang *et al.* (10) found that pertussis toxin did attenuate the EGF-stimulated increase in the amount of ^{32}P incorporated into a 150-kDa protein. The labeled protein did contain PLC-γ, based on immunoblotting using monoclonal antibodies to PLC-γ. However, it was not determined whether the labeled band contained proteins other than PLC-γ,[3] and the amount of phosphate incorporated into PLC-γ on tyrosine was not measured directly. This latter consideration could be important because PLC-γ contains a significant amount of phosphoserine (see Fig. 3). Overall, these problems make an unequivocal interpretation of the data difficult.

In the present study, the effect of EGF on the tyrosine phosphorylation of PLC-γ was measured directly using two methods, phosphotyrosine antibodies and phosphoamino acid analysis. The results obtained with either method demonstrate that EGF does not cause a significant degree of tyrosine phosphorylation of PLC-γ in hepatocytes, suggesting that phosphorylation is not the method for activating PLC in these cells. Even though the mechanism for PLC activation by EGF receptors in rat hepatocytes is not understood, the data strongly suggest that a G_i-like protein may be involved. For example, pertussis toxin completely abolished EGF-stimulated PLC activation in rat hepatocytes (8). In hepatic membranes, EGF enhanced the initial rate of GTP/GDP exchange, suggesting a direct interaction of the EGF receptor with a G protein (9). Moreover, a G_i-like protein has been demonstrated to coimmunoprecipitate with the hepatic EGF receptor (10). Interestingly, there appear to be other receptors in the rat hepatocyte with signaling mechanisms similar to those used by the EGF receptor. Based on its cDNA sequence, the activin A receptor has a single membrane-spanning domain (33), yet activin A causes an increase in inositol phosphates and intracellular Ca^{2+} in hepatocytes that is blocked by pretreatment of the cells with pertussis toxin (34). In addition to the examples provided by the EGF and activin A receptors in hepatocytes, there is increasing evidence that receptors with a single transmembrane domain can interact with G proteins in other cell types. Such interactions have been observed with receptors for PDGF (35), colony-stimulating factor 1 (36), transforming growth factor β1 (37, 38), insulin (39), and insulin-like growth factor II (40).

These results raise the interesting question of how a receptor with a single membrane-spanning region can interact with G proteins, because most models of a typical G protein-coupled hormone receptor are based on a structure with seven membrane-spanning domains. There are at least two possible mechanisms by which the EGF receptor might interact with G proteins. First, the EGF receptor may directly interact with a G protein via mechanisms similar to those used by typical G protein-coupled receptors. The study of receptors with seven transmembrane domains suggests that certain intracellular domains play important roles in the G protein-receptor coupling (41), a hypothesis supported by the observation that peptides such as mastoparan can activate G proteins (42). These results suggest that a short intracellular sequence in a receptor may be sufficient to interact with G proteins. This hypothesis is supported by the observation that a peptide based on an intracellular domain of the human insulin-like growth factor II receptor activates G_{i2} in a manner similar to that of G protein-coupled receptors (40). The interaction of this receptor and G_{i2} can be blocked by an antibody against the peptide (40). Second, the EGF receptor may activate G proteins via tyrosine phosphorylation. The phosphorylation of G proteins on tyrosine has been observed using purified insulin receptors and purified G_o and G_i reconstituted into a phospholipid vesicle system (43). The EGF receptor has also been observed to stimulate the phosphorylation of a purified 22-kDa G protein on tyrosine (44), although no correlation between tyrosine phosphorylation of a G protein and its function has been reported.

Regardless of the uncertainty in the mechanism by which EGF receptors stimulate PLC activity in hepatocytes, it seems clear that different mechanisms are used by this receptor to activate PLC in hepatocytes and in cultured cells expressing high levels of EGF receptors. The use of different signaling mechanisms by the EGF receptor in different cell types could be explained by the existence of subtypes of EGF receptors that couple to different signaling mechanisms. Alternatively, the EGF receptor may couple to different PLC isozymes in different cells. There are at least four PLC isozymes (α, β, γ, and δ) as identified by their cloned cDNAs (3) and it is possible that the EGF receptor (or a novel subtype of the receptor) may activate PLC-α, PLC-β, or PLC-δ via a pertussis toxin-sensitive G protein in hepatocytes. PLC-β has been purified from hepatic membranes (45) and must be considered as one candidate for regulation by a toxin-sensitive G protein. The reconstitution of EGF-stimulated PLC activity using purified EGF receptors, different G proteins, and different PLC isozymes *in vitro* will ultimately be required for a clear understanding of the mechanism by which EGF stimulates PLC activity in hepatocytes.

Acknowledgments

We thank Dr. E. L. Hewlett for a generous supply of pertussis toxin, Dr. J. T. Parsons for phosphotyrosine antibodies, Dr. T. Pawson for the gift of the PLC-γ-TrpE pATH II vector to the Cancer Center, Dr. H. S. Earp for antiserum against EGF receptor, and Dr. W. J. Wasilenko and Dr. M. J. Weber for the Rat1hER cells. We are also very grateful to Ms. G. S. Harris for her excellent technical assistance.

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[3] For example, hepatocytes prepared by collagenase digestion contain a 150-kDa fragment of the EGF receptor that migrates with the PLC-γ band on a SDS gel. The tyrosine phosphorylation state of this protein is increased by treatment of the cells with EGF (unpublished observations).

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