Stimulation of Phospholipase C–γ1 Membrane Association by Epidermal Growth Factor

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Epidermal growth factor (EGF) treatment of A-431 epidermoid carcinoma cells elicited a redistribution of phospholipase C–γ1 (PLC-γ1) from a predominantly cytosolic localization to membrane fractions. The temporal coincidence of this redistribution with EGF stimulation of inositol phosphate formation and EGF increased phosphorylation of PLC-γ1 suggests that the membrane association of PLC-γ1 is a significant event in second messenger transduction.

SIGNAL TRANSDUCTION STIMULATED by EGF occurs through the activation of the tyrosine kinase activity of the EGF receptor (*1*). Kinase-deficient receptors, produced by site-directed mutagenesis, are unable to elicit any biologic responses to EGF, although ligand binding is not perturbed (*2*). Distal to the receptor, however, an incompletely defined signaling pathway transmits a message resulting in nuclear activation and cell division (*3*). EGF rapidly induces the phospholipase C (PLC)–mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce inositol 1,4,5-trisphosphate [$I(1,4,5)P_3$] and 1,2-diacylglycerol, two intracellular second messenger molecules that regulate Ca^{2+} levels and protein kinase C activity, respectively (*4*). Although several PLC isozymes have been described (*5*), only PLC-γ1 is a phosphorylation substrate for the EGF receptor kinase in intact cells and in vitro (*6, 7*). EGF treatment of A-431 cells causes a rapid increase in the phosphorylation of tyrosine and serine residues in PLC-γ1 (*5, 6*); almost 70% of the total PLC-γ1 is phosphorylated within 2 min of EGF addition to cells (*7, 8*), and four modified tyrosine residues have been identified by sequencing (*8, 9*). Sites of serine phosphorylation have not been identified.

It has not been shown whether these phosphorylations are involved in enhancement of PLC-γ1 catalytic activity or in other aspects of the enzyme's physiology. Since PLC-γ1 is a cytosolic enzyme and its substrate PIP_2 represents a very minor component (<0.05%) of the total membrane phospholipid, we investigated whether the cytosolic enzyme becomes membrane-associated after the addition of EGF to intact cells.

We determined the location of PLC-γ1 in particulate and soluble fractions after disruption of control and EGF-treated A-431 cells by quantitative immunoblotting with PLC-γ1–specific monoclonal antibodies (MAbs) or enzyme activity assays. We prepared three subcellular fractions by differential centrifugation using the method of Cohen and Fava (*10*). The low-speed fraction (LSF) comprises most plasma membrane EGF receptors. The high-speed fraction (HSF) consists of membranes that in cells treated with EGF at 37°C, but not at 4°C, contain activated EGF-receptor complexes whose tyrosine kinase activity is elevated, and from which EGF-receptor complexes are excluded in the presence of energy poisons (*10*). The HSF, therefore, is enriched in endosomal membranes. The cytosol fraction consists of the supernatant remaining after the high-speed centrifugation, and contains no EGF receptors.

Within 1 min, EGF-treated A-431 cells increase the phosphorylation of PLC-γ1 and the formation of inositol phosphates (*4, 6, 7*). The redistribution of immunoreactive PLC-γ1 from cytosol to membrane-associated fractions was equally rapid (Fig. 1A). In unstimulated cells approximately 88% and 12% of the immunoreactive PLC-γ1 was present in the cytosol fraction and membrane fractions, respectively. One minute after EGF addition, about 68% of the immunoreactive PLC-γ1 was membrane-associated. The amount of PLC-γ1 associated with the membrane fractions coincides with the stoichiometry of growth factor–induced tyrosine phosphorylation of PLC-γ1 (*7, 8*). After 1 hour of EGF treatment, nearly 55% of the total immunoreactive PLC-γ1 was still membrane-associated; EGF stimulation of inositol polyphosphate formation is sustained for this amount of time (*4*).

Epidermal growth factor, bradykinin, and adenosine triphosphate (ATP) each elicit increased formation of $I(1,4,5)P_3$ in A-431 cells (*4*), but only EGF increases the phosphorylation of PLC-γ1 (*6*). As shown in Fig. 1A, neither bradykinin nor ATP elicited redistribution of PLC-γ1 protein.

If increased amounts of PLC-γ1 protein become associated with the cell surface after EGF treatment, there also should be a redistribution of PLC activity to the membrane fractions. In the same fractions represented in Fig. 1A, PLC activity was measured (Fig. 1B). EGF treatment did not increase the total PLC activity at any time analyzed; however, there was a rapid decrease in the amount of PLC activity in the cytosol fraction and a corresponding increase in PLC activity in both membrane fractions. The PLC agonist bradykinin resulted in no redistribution of PLC activity in subcellular frac-

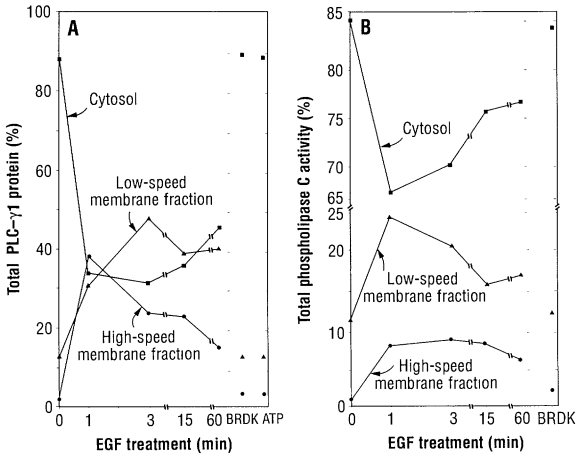

Fig. 1. (**A**) Time course of EGF-induced PLC-γ1 membrane association in A-431 cells. The amount of immunoreactive PLC-γ1 was determined in the cytosol fraction, LSF, or HSF. Subconfluent A-431 cells were treated with EGF (200 ng/ml) for the time indicated and fractionated as described (*10*). Portions of the resuspended subcellular fractions were subjected to SDS-PAGE and immunoblot analysis (*15*). Where indicated, bradykinin (BRDK, 10 μM) or ATP (20 μM) were added to the cells for 1 hour at 37°C. The percentage of the total HSF, LSF, and cytosol fractions analyzed were 27%, 8.9%, and 4%, respectively. (**B**) Time course of EGF-induced redistribution of PLC activity among subcellular fractions. The total PIP_2-specific PLC activity was quantitated in each fraction shown in (A) after EGF or bradykinin treatment. PLC activity was determined as described previously (*4*), with final concentrations of Triton X-100 (0.025%), octylglucoside (0.14%), and 1 μM free Ca^{2+}. The reaction proceeded for 15 min at 35°C, with [^{3}H]PIP_2 (specific activity 1.37 cpm/pmol). The total enzyme activity in the plates at each point averaged 250 nmol of IP_3 formed per 15 min. The percentage of the total HSF, LSF, and cytosol fractions analyzed were 2.5%, 0.8%, and 0.25%, respectively. The data shown were calculated for the total amount of activity present in each fraction. These data are representative of at least 15 independent experiments with variations of less than 15%.

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tions, but did produce a small increase (~20%) in total PLC activity. In these measurements of PLC activity it is not possible to isolate activity caused only by the PLC-γ1 isozyme. Although PLC-γ1 accounts for a significant fraction of the total PLC activity, considerable activity remains after quantitative immunoprecipitation of PLC-γ1. Therefore, it is not possible to compare the increases or decreases in A and B of Fig. 1. In our initial report (*6*), in which a more vigorous homogenization procedure and different buffer were used, the EGF-sensitive PLC activity remained cytosolic. The more gentle methodology we now use allows the detection of membrane-associated PLC in EGF-treated cells.

Since low temperature does not interfere with ^{125}I-labeled EGF binding to surface receptors but does prevent subsequent internalization of ligand-receptor complexes (*11*), we determined the influence of this parameter on PLC-γ1 distribution in the presence of EGF. Our previous studies showed that treatment of the cells with EGF at low temperature (4°C) resulted in rapid phosphorylation of PLC-γ1, but prevented increased formation of inositol phosphates (*4, 6, 7*). EGF treatment of cells at 37°C but not at 4°C led to redistribution of EGF receptor from the LSF to the HSF (Fig. 2), consistent with enhanced endocytosis of EGF-receptor complexes (*10*). In contrast, PLC-γ1 was associated with both membrane fractions after EGF treatment at 37°C and 4°C. In fact, at 4°C there was a greater EGF effect on PLC-γ1 membrane association than at 37°C. The small EGF-induced increase in PLC-γ1 associated with the HSF is difficult to interpret, as a small (6% to 7%) contamination of plasma membrane found in the LSF would be sufficient to produce the observed increase in the endosome-enriched HSF fraction.

The association of PLC-γ1 with the membrane is reversible and dependent on occupancy of the EGF receptor (Table 1). A pH 4 wash, which removes bound EGF from its receptor, results in the rapid dissociation of PLC-γ1 from both membrane fractions and reappearance in the cytosol fraction. PLC-γ1 in these acid-washed cells did not associate with membranes unless the cells were treated again with EGF.

We have also examined the phosphorylation state of PLC-γ1 recovered from the membrane and cytosol fractions of EGF-treated cells (Fig. 3). The analysis of these phosphotryptic peptides shows that the phosphorylation state of the two pools of PLC-γ1 is qualitatively similar.

These data show that EGF treatment of A-431 cells results in rapid subcellular redistribution of PLC-γ1. In untreated cells, approximately 10% of the total PLC-γ1 protein is membrane-associated, but after 1 min of EGF treatment nearly 70% of this enzyme becomes membrane-associated. We estimate, from radioimmunoassay data, that A-431 cells contain approximately 200,000 molecules of PLC-γ1. The EGF, therefore, induces the relocation of about 160,000 PLC-γ1 molecules. This association of PLC-γ1 with the membrane was as rapid as the EGF-stimulated formation of inositol phosphates and tyrosine phosphorylation of PLC-γ1. Since PLC-γ1 is a substrate for the EGF receptor kinase (*6, 7*), an association between the two is to be expected. However, the interaction between PLC-γ1 and

Table 1. Reversibility of EGF-dependent association of PLC-γ1 with membrane fractions. A-431 cells were treated without or with EGF (200 ng/ml) for 20 min at 4°C. After EGF treatment, indicated dishes were washed with pH 7 buffer or pH 4 buffer, 50 mM glycine, and 100 mM NaCl (*14*). The acid-washed dishes were fractionated immediately or treated for an additional 15 min at 4°C with or without EGF (200 ng/ml) before fractionation. The percentage of total PLC-γ1 associated with each fraction was measured by quantitative immunoblotting (*15–19*). The numbers expressed are the average of three experiments, with variation between experiments of less than 15%. CYT, cytosol fraction.

Cell treatment	Percent of total PLC-γ1 in		
	LSF	HSF	CYT
No EGF	6.7	1.5	91.8
EGF (30 min)	26.4	21.3	52.2
EGF (30 min), pH 7 wash	53.6	5.1	41.3
EGF (30 min), pH 4 wash, then			
Fractionate	9.7	2.3	77.8
EGF readded (15 min)	40.5	10.0	49.5
No EGF	7.6	1.1	91.3

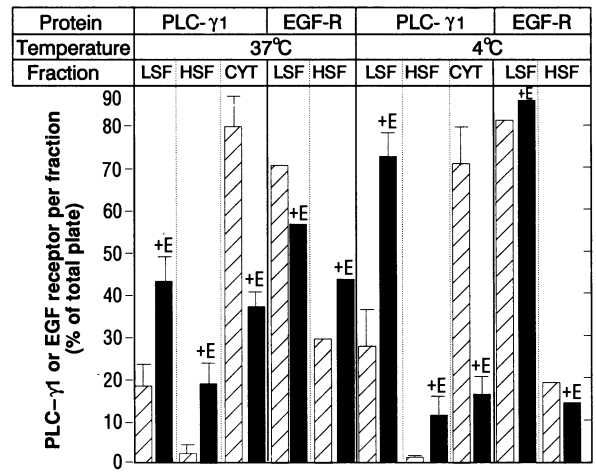

Fig. 2. Influence of temperature on EGF-stimulated PLC-γ1 and EGF receptor redistribution among A-431 subcellular fractions. Cells were treated with EGF (200 ng/ml) for 1 hour at 37°C or 4°C, fractionated (*10*), and the amount of PLC-γ1 or EGF receptor was determined (*15*). Hatched and solid boxes represent the amount of immunoreactive protein present in the absence or presence of EGF (E), respectively. The data represent the average of three separate determinations ± SEM for PLC-γ1, and two separate determinations for the EGF receptors. CYT, cytosol fraction.

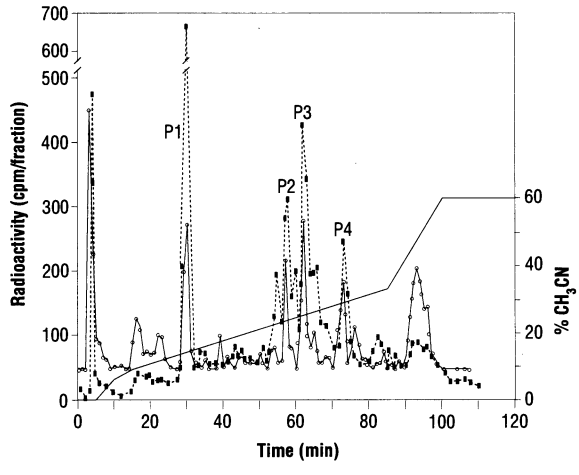

Fig. 3. High-performance liquid chromatography (HPLC) elution profile of membrane and cytosolic PLC-γ1 tryptic phosphopeptides. PLC-γ1 from cytosol (■) and pooled membrane fractions (○) of ^{32}P-labeled EGF-treated A-431 cells was immunoprecipitated in the presence of 1% Triton X-100 and isolated by SDS-PAGE. After autoradiography, the protein was trypsin digested, and phosphopeptides were analyzed by reversed-phase HPLC as described (*8*), with the indicated gradient of acetonitrile and collecting 1-ml fractions. Peptide P1 contains phosphoserine and P2, P3, and P4 contain phosphotyrosine (*8, 9*). P2 corresponds to phosphorylation of Tyr^{771} and P3 to phosphorylation of Tyr^{1254} (*8, 9*). P4 probably corresponds to phosphorylation of Tyr^{783} (*8, 9*). The early peak (4 to 5 min) contains inorganic phosphate. The elution profile was the same in two separate experiments.

membranes is probably more complex than a tyrosine kinase: PLC-γ1 interaction as enzyme-substrate, which would be expected to result in only a transient association. In fact, it has been reported that primarily nontyrosine phosphorylated PLC-γ1 molecules are associated with activated EGF receptors (*12*). When PLC-γ1 is immunoprecipitated from EGF or platelet-derived growth factor (PDGF) treatment of different cells, a similar spectrum of coprecipitating proteins has been noted (*6, 7, 13*). It is possible that these proteins may be involved in mediating the membrane association of PLC-γ1.

These results could suggest a role for the cellular redistribution of PLC-γ1 in the EGF stimulation of cellular PLC activity. EGF-induced membrane association of PLC-γ1 may stimulate the kinetics of phosphatidylinositide breakdown by increasing association of the enzyme with its substrate. This would suggest major differences in the mechanism by which G protein–dependent agonists such as bradykinin (which produces no relocalization or tyrosine phosphorylation of PLC) and growth factors (for which there is no evidence of G protein dependence) communicate with PLC to increase PIP_2 hydrolysis.

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15. Samples of each fraction were subjected to SDS–7.5% polyacrylamide gel electrophoresis (PAGE) (*16*) and electroblotted to nitrocellulose (Schleicher & Schuell, 0.45 μM) overnight at 25 V and blocked with BLOTTO (*17*). The filters were probed with a 1:500 mixture of PLC-γ1 MAbs (*18*) and then detected with rabbit antibodies to mouse immunoglobulin G (1:500, Sigma) and ^{125}I-labeled protein A (2 μCi/10 ml BLOTTO). Where indicated, filters were probed to detect EGF receptors with 1:500 rabbit antiserum 986 (*19*) and ^{125}I-labeled protein A. The radioactive bands were quantitated by cutting the filters and determining the amount of ^{125}I present. The amount of PLC-γ1 in each band was normalized according to the entire amount of PLC-γ1 analyzed, to give the relative percent of the total PLC-γ1 present in each fraction.
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Angiotensin II–Induced Calcium Mobilization in Oocytes by Signal Transfer Through Gap Junctions

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Angiotensin II (AII) stimulates rapid increases in the concentration of cytosolic calcium in follicular oocytes from *Xenopus laevis*. This calcium response was not present in denuded oocytes, indicating that it is mediated by AII receptors on the adherent follicular cells. The endogenous AII receptors differed in their binding properties from mammalian AII receptors expressed on the oocyte surface after injection of rat adrenal messenger RNA. Also, the calcium responses to activation of the amphibian AII receptor, but not the expressed mammalian AII receptor, were blocked reversibly by octanol and intracellular acidification, treatments that inhibit cell coupling through gap junctions. In addition, AII increased the rate of progesterone-induced maturation. Thus, an AII-induced calcium-mobilizing signal is transferred from follicle cells to the oocyte through gap junctions and may play a physiological role in oocyte maturation.

ANGIOTENSIN II EXERTS NUMEROUS physiological actions in addition to those related to the control of blood pressure and extracellular volume (*1*). The actions of AII are mediated by specific plasma membrane receptors that are coupled to phosphoinositide hydrolysis and Ca^{2+} mobilization in target tissues including smooth muscle, adrenal, liver, kidney, and brain. AII receptors from mammalian tissues have been expressed in *Xenopus laevis* oocytes (*2, 3*). Using albino frog oocytes injected with adrenal glomerulosa mRNA and the Ca^{2+} indicator aequorin, we observed that activation of AII receptors expressed from mammalian mRNA elevated intracellular Ca^{2+} concentrations (*3*). During these studies, we also detected endogenous amphibian AII receptors that could trigger Ca^{2+} mobilization in *Xenopus* oocytes. We have investigated the properties and location of this AII receptor.

Amphibian AII receptor–mediated Ca^{2+} responses were comparable to those reported (*3*) for adrenal mRNA-injected oocytes and were characterized by a rapid and transient rise in luminescence of the Ca^{2+} indicator aequorin (Fig. 1A). There was a large degree of variability in the incidence and magnitude of endogenous receptor activity that may reflect cyclical and seasonal variations in the endocrine control of receptor expression. However, in individual animals the peak light responses were uniform and dose-dependent, with a rank potency order of AII > AIII >> AI (Fig. 1A, inset). Endogenous AII responses were maximal in freshly isolated oocytes and decreased progressively during the next 4 days of incubation (Fig. 1B); most of the oocytes (81%) did not exhibit detectable light responses by day 3. This decrease was not due to differences in the content of aequorin, since oocytes injected on day 4 yielded similar light emission after lysing with 1% SDS as those injected with aequorin on day 1 (*4*). In contrast to the declining response mediated by the endogenous AII receptor, AII-induced light responses in oocytes injected with both aequorin and rat adrenal mRNA increased progressively with time and were maximal on day 3 (Fig. 1B). We therefore determined amphibian receptor-mediated responses to AII in freshly isolated oocytes 1 to 6 hours after injection of aequorin and mammalian AII receptor–mediated responses 3 days after coinjection with aequorin and rat adrenal mRNA.

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