Phosphatidylinositol kinase is activated in membranes derived from cells treated with epidermal growth factor

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ABSTRACT   The ability of epidermal growth factor (EGF) to stimulate phosphatidylinositol (PtdIns) kinase activity in A431 cells was examined. The incorporation of ^{32}P from [γ-^{32}P]ATP into PtdIns by A431 membranes was increased in membranes prepared from cells that had been pretreated with EGF. Demonstration of a stimulation of the PtdIns kinase activity by EGF required the use of subconfluent cultures and was dependent on the inclusion of protease inhibitors in the buffers used to prepare the membranes. Stimulation of the PtdIns kinase activity was rapid. The activation peaked 2 min after the addition of EGF and declined slowly thereafter. Half-maximal stimulation of the PtdIns kinase occurred at 7 nM EGF. Kinetic analyses of the reaction indicated that treatment of the cells with EGF resulted in a decrease in the K_m for PtdIns with no change in the V_{max}. The kinetic parameters for the utilization of ATP were unchanged in the EGF-treated membranes compared to the control membranes. Pretreatment of the cells with the phorbol ester phorbol 12-myristate 13-acetate blocked the ability of EGF to stimulate PtdIns kinase activity. These findings demonstrate that a PtdIns kinase activity in A431 cells is regulated by EGF and provide a good system for examining the mechanism by which EGF stimulates the activity of this intracellular enzyme.

Epidermal growth factor (EGF) is a small polypeptide that stimulates cell growth in a large number of cell types. The effects of EGF are mediated by way of a specific cell surface receptor for the growth factor (for a review see ref. 1). The EGF receptor possesses an intrinsic protein tyrosine kinase activity that is stimulated by EGF (2–4). Although the kinase is capable of phosphorylating itself as well as other cellular proteins, the function of this kinase activity in the transduction of the mitogenic signal remains unknown.

For many hormones, one of the early steps in signal transduction is the generation of two intracellular second messengers, inositol 1,4,5-trisphosphate and diacylglycerol (acyl$_2$Gro). The production of these messengers appears to involve the stimulation of a phospholipase C that cleaves phosphatidylinositol 4,5-bisphosphate to acyl$_2$Gro and inositol 1,4,5-trisphosphate. Acyl$_2$Gro stimulates the activity of protein kinase C leading to the phosphorylation of several cellular proteins (5). Inositol trisphosphate induces the release of Ca^{2+} from intracellular stores (6–8), which results in the activation of calcium-dependent enzymes.

We (9) and others (10–12) have demonstrated that EGF is able to stimulate phosphatidylinositol (PtdIns) metabolism in A431 cells. Regulation by the growth factor occurs at two separate points in the pathway. First, EGF stimulates the breakdown of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and acyl$_2$Gro, presumably by the activation of a phospholipase C (9, 11, 12). In addition, EGF causes an increase in the level of phosphatidylinositol 4-

phosphate (PtdIns4P), most likely as a result of the stimulation of a PtdIns kinase (9).

To characterize the mechanism by which EGF regulates the activity of a PtdIns kinase, we have developed an *in vitro* system in which to examine the properties of this enzyme. We report here that elevated PtdIns kinase activity is retained in the particulate fraction prepared from A431 cells treated with EGF. The activation is rapid and appears to involve a decrease in the K_m for PtdIns.

EXPERIMENTAL PROCEDURES

Materials. EGF was purified from mouse submaxillary glands by the method of Savage and Cohen (13). The material ran as a single band on NaDodSO$_4$ gels and was identified as authentic EGF by amino acid composition analysis and competition binding against ^{125}I-labeled EGF. [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Silica 60 thin layer plates were from Merck and were impregnated with oxalate as described (9). PtdIns was obtained from Sigma.

Cells. A431 cells were grown in Dulbecco's modified Eagle's medium supplemented with 7% (vol/vol) newborn calf serum and 3% (vol/vol) fetal calf serum.

Membrane Preparation. A431 cells for the assay were grown to a density of 10–15×10^4 cells per cm^2 in 150-mm plates. Unless otherwise indicated, the cells were incubated for 2 min either with or without 30 nM EGF. The medium was removed, and the cells were scraped into 15 ml of ice-cold 5 mM Hepes (pH 7.0), 2 mM MgCl$_2$, containing 10 mM benzamidine, leupeptin at 1 μg/ml, α_2-macrogobulin at 5 μg/ml, 500 μM phenylmethylsulfonyl fluoride, and 2 mM EGTA as protease inhibitors. The cells were homogenized with 15 strokes in a 40-ml Dounce homogenizer. The homogenates were centrifuged at $30,000 \times g$ for 30 min in a Sorvall RC5B centrifuge to pellet the membrane fraction. This fraction was resuspended in 1 ml of 50 mM glycerol 2-phosphate (pH 7.4) containing 1 mM EGTA and protease inhibitors (at the same concentrations as in the homogenization buffer) to give a protein concentration of 300–400 μg/ml.

PtdIns Kinase Assay. The activity of the PtdIns kinase was measured by quantitating the transfer of phosphate from [γ-^{32}P]ATP to PtdIns. A 65-μl aliquot of membranes was used in a final assay volume of 100 μl that also contained the following components (in final concentrations): 500 μM [γ-^{32}P]ATP (4–6 $\times 10^6$ cpm per assay), 20 mM MgCl$_2$, and 2 μM PtdIns resuspended in water containing Triton X-100 to a final concentration of 0.2%. Assays were begun by the addition of the [γ-^{32}P]ATP and MgCl$_2$ to the reaction mixture and were incubated for 5 min at 30°C. A 2.25-ml aliquot of methanol/concentrated HCl, 10:1 (vol/vol), was added to terminate the assays. The lipids were then extracted with

Abbreviations: EGF, epidermal growth factor; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; acyl$_2$Gro, diacylglycerol; PMA, phorbol 12-myristate 13-acetate.

chloroform and separated by thin layer chromatography as described (9). The labeled PtdIns4P was identified by comigration with authentic PtdIns4P standard. The PtdIns4 [^{32}P]P bands on the silica plates were located by autoradiography and scraped into vials containing scintillation fluid, and ^{32}P was measured. PtdIns kinase activity was linear with protein up to at least 100 μg per assay and was linear with time for at least 5 min at all concentrations of ATP and PtdIns examined. The PtdIns4[^{32}P]P formed did not appear to be further metabolized under the assay conditions. Following addition of a 100-fold excess of unlabeled ATP to the assay, the cpm in PtdIns4[^{32}P]P did not decline during a further 30-min incubation, and no new labeled phospholipid bands appeared on the thin layer plates.

Protein Assay. Protein was determined by a modification (14) of the Lowry method (15).

Quantitation of PtdIns in A431 Membranes. The PtdIns present in the particulate fraction of A431 cells was extracted in chloroform/methanol as described above. The PtdIns was then separated from other phospholipids by thin layer chromatography using the method of Bell *et al.* (16). The PtdIns was scraped from the silica plate and quantitated as described by Vaskovsky *et al.* (17).

RESULTS

Identification of an EGF-Stimulated PtdIns Kinase Activity. Preliminary experiments on the subcellular distribution of PtdIns kinase activity in A431 cells demonstrated that the majority of the activity was present in the particulate fraction (data not shown). All experiments were, therefore, performed using the particulate fraction as the source of the PtdIns kinase.

To demonstrate a stimulation of the PtdIns kinase by EGF, whole cells were incubated in the absence or presence of 30 nM EGF. Particulate fractions were prepared and assayed for PtdIns kinase activity. Two factors were critical for detecting an increase in PtdIns kinase activity in particulate fractions prepared from EGF-pretreated cells. The first was the addition of protease inhibitors to the buffers used in preparing the membranes. As shown in Fig. 1A, when particulate fractions were prepared in the absence of protease inhibitors, the basal

levels of PtdIns kinase activity were high but were unaffected by pretreatment of the cells with EGF. By contrast, when protease inhibitors were included during preparation of the particulate fractions the level of PtdIns kinase activity in control cells was much lower, but EGF was able to induce an increase in the activity of the enzyme.

The second critical factor was that the stimulation was strongly dependent on the density of the cell cultures. If subconfluent cultures of A431 cells were used, EGF induced a marked stimulation in PtdIns kinase activity (Fig. 1B). As the density of the cells increased, the ability of EGF to stimulate the PtdIns kinase declined. In fully confluent cultures, EGF failed to stimulate the activity of the enzyme. For all further experiments, cultures at a cell density of 10–15 × 10^4 cells per cm^2 (≈30% of the density of a fully confluent plate) were used to optimize the stimulation by EGF. This will be referred to as a subconfluent culture. Even with subconfluent cultures, the stimulation by EGF varied from experiment to experiment, ranging from a 1.4- to 2.5-fold increase with an average of ≈2-fold.

Characterization of an EGF-Stimulated PtdIns Kinase Activity in the Particulate Fraction of A431 Cells. The divalent cation requirements of the PtdIns kinase were examined. Either Mg^{2+} or Mn^{2+} alone were able to support PtdIns kinase activity. PtdIns kinase activity increased with increasing concentrations of Mg^{2+}, plateauing at a concentration of 20 mM. With Mn^{2+} as the divalent cation, optimal PtdIns kinase activity was observed with 2 mM Mn^{2+} with activity dropping by 50% when assays were performed in 20 mM Mn^{2+}. Maximal activity observed in 20 mM Mg^{2+} was about 3-fold greater than that seen when the optimal concentration of Mn^{2+} was used as the divalent cation. At every concentration of metal ion tested, the EGF-stimulated increase in PtdIns kinase activity was greater when assays were performed in Mg^{2+} as opposed to Mn^{2+}. Since equivalent concentrations of Mg^{2+} and Mn^{2+} support vastly different levels of PtdIns kinase activity, it is unlikely that the observed metal ion effects are due merely to differences in ionic strength. Ca^{2+} alone was unable to support activity and concentrations of 100 μM or greater inhibited the enzyme activity by 50%–75% when included in assays containing 20 mM Mg^{2+}.
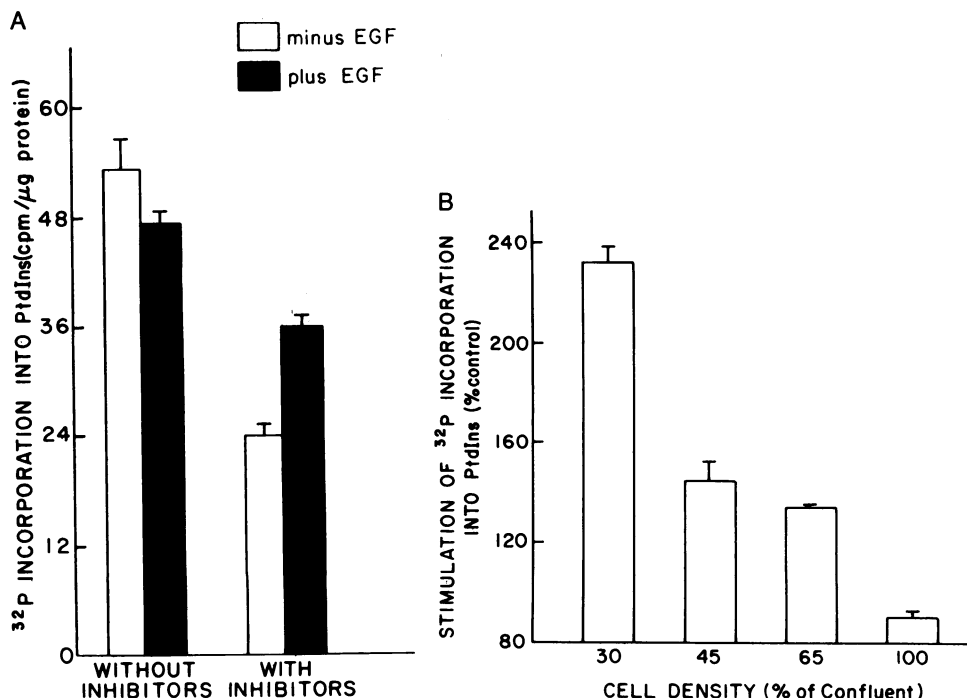

FIG. 1. Effects of protease inhibitors and cell density on stimulation of the PtdIns kinase by EGF. (A) Cultures of A431 cells (≈50% confluent) were incubated with or without EGF and particulate fractions were prepared in the presence or absence of protease inhibitors. The membranes were then assayed in triplicate for PtdIns kinase activity. (B) Cultures of A431 cells were grown to the densities indicated. Cell density was determined by comparing the total protein recovered from each plate with the total from confluent plates. Plates were incubated with or without EGF, and particulate fractions were prepared and assayed in triplicate for PtdIns kinase activity. Values represent the mean ± SEM.
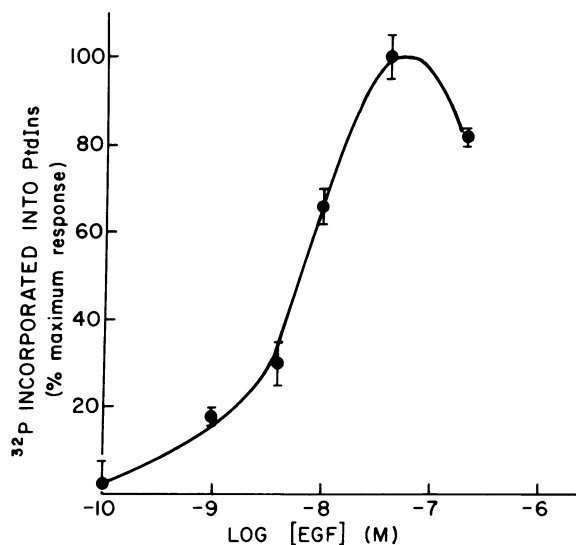
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FIG. 2. Dose–response curve for the EGF stimulation of PtdIns kinase activity. Individual subconfluent cultures of A431 cells were incubated with the indicated concentrations of EGF. Particulate fractions were prepared and assayed in triplicate for PtdIns kinase activity. Values represent the mean ± SEM from three separate experiments in which the maximum stimulation by EGF ranged from 2.1- to 2.3-fold.

Fig. 2 shows a dose–response curve for the stimulation of PtdIns kinase activity by EGF. Half-maximal activation of the PtdIns kinase occurred at 7 nM EGF, and maximal stimulation was observed at 30 nM EGF. At EGF concentrations >30 nM, EGF stimulation of the PtdIns kinase activity declined.

The time course of the stimulation of the PtdIns kinase by EGF is shown in Fig. 3. Stimulation of the PtdIns kinase by EGF was rapid, with maximal activation occurring within 2 min of the addition of EGF. The stimulation remained high for ≈5 min and then declined to basal levels by 60 min.

Kinetic Properties of the EGF-Stimulated PtdIns Kinase. Particulate fractions from cells treated with or without EGF were prepared and assayed for PtdIns kinase activity at increasing concentrations of added PtdIns. The concentration of endogenous PtdIns present in the particulate fraction

was determined to be 6.5 nmol of PtdIns per mg of protein. This represents a concentration of 1–2 μM under our standard assay conditions. To determine whether there was a difference in the concentration of PtdIns in control and EGF-treated cells, membranes were prepared from cells that had been labeled for 24 hr with *myo*-[^{3}H]inositol and then incubated with or without EGF. No difference in the amount of PtdIns extracted from the membranes derived from control vs. EGF treated cells could be detected. This is consistent with our findings of no change in PtdIns levels in whole cells treated with EGF (9). The total concentration of PtdIns used for calculating the data shown in Fig. 4 represents the sum of the exogenous and endogenous PtdIns. As can be seen from the Lineweaver–Burk plots in Fig. 4, treatment of the cells with EGF resulted in a decrease in the apparent K_m of the PtdIns kinase for PtdIns with little change in the V_{max}. Ultracentrifugation of A431 membranes incubated for 5 min with 0.2% Triton X-100 demonstrated that the PtdIns kinase was soluble under the conditions of our assay, thus analysis of the kinetic data using Michaelis–Menten equations seems justified.

The change in the utilization of PtdIns was confirmed in experiments to determine the kinetic parameters for ATP as a substrate for PtdIns kinase. Fig. 5 shows Lineweaver–Burk plots for the utilization of ATP in subsaturating (Fig. 5A) and saturating (Fig. 5B) concentrations of PtdIns. When the concentration of PtdIns was subsaturating, EGF did not induce a significant change in the apparent K_m for ATP but caused a 2-fold increase in the apparent V_{max}. This change in the apparent V_{max} was not observed when the PtdIns kinase was assayed using saturating concentrations of PtdIns. Again the apparent K_m values were essentially unchanged following treatment of the cells with EGF. These data are consistent with the interpretation that EGF stimulates the activity of the PtdIns kinase by inducing a decrease in the K_m for PtdIns and not by altering the V_{max} for the reaction.

Effect of Phorbol 12-Myristate 13-Acetate (PMA) on EGF Stimulation of the PtdIns Kinase. We have demonstrated (9) that treatment of whole cells with the tumor promoting phorbol ester PMA inhibited the ability of EGF to stimulate the accumulation of PtdIns4P and production of inositol phosphates. To determine whether PMA also inhibited the stimulation of the PtdIns kinase in the current system, whole cells were treated with 0.1 μM PMA for 1 hr prior to challenge with EGF and preparation of the particulate fraction. In untreated cells, basal and EGF-stimulated PtdIns kinase activities were 334 ± 14 and 595 ± 8 cpm per μg of protein,
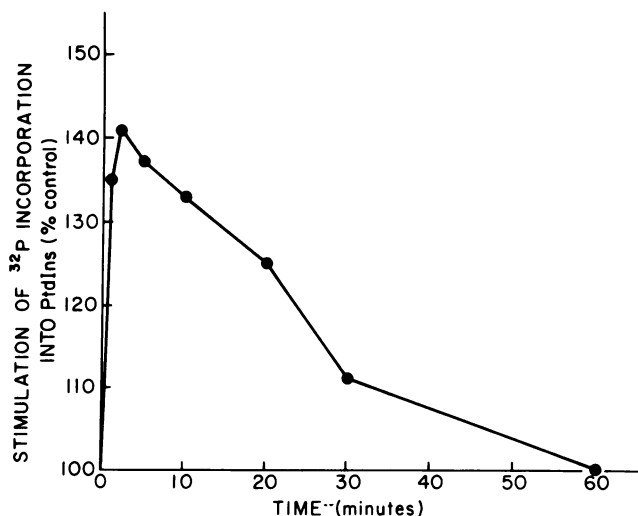

FIG. 3. Time course of EGF stimulation of PtdIns kinase activity. Individual subconfluent cultures of A431 cells were incubated with 30 nM EGF for the indicated times, then were homogenized, and assayed in triplicate for PtdIns kinase activity.
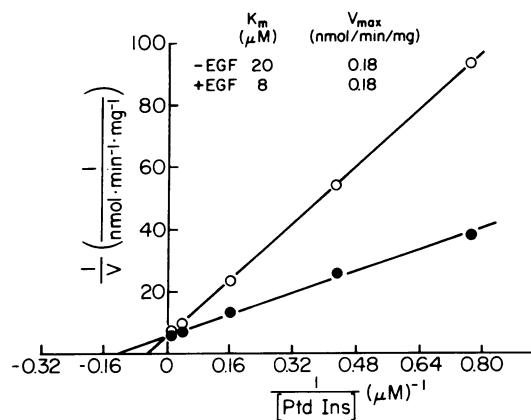

FIG. 4. Lineweaver–Burk plots for the utilization of PtdIns by the PtdIns kinase. Subconfluent cultures of A431 cells were incubated with (solid circles) or without (open circles) 30 nM EGF prior to preparation of the particular fractions. Membranes were assayed in triplicate for PtdIns kinase activity as described except that various concentrations of PtdIns were added from 0 to 300 μM.
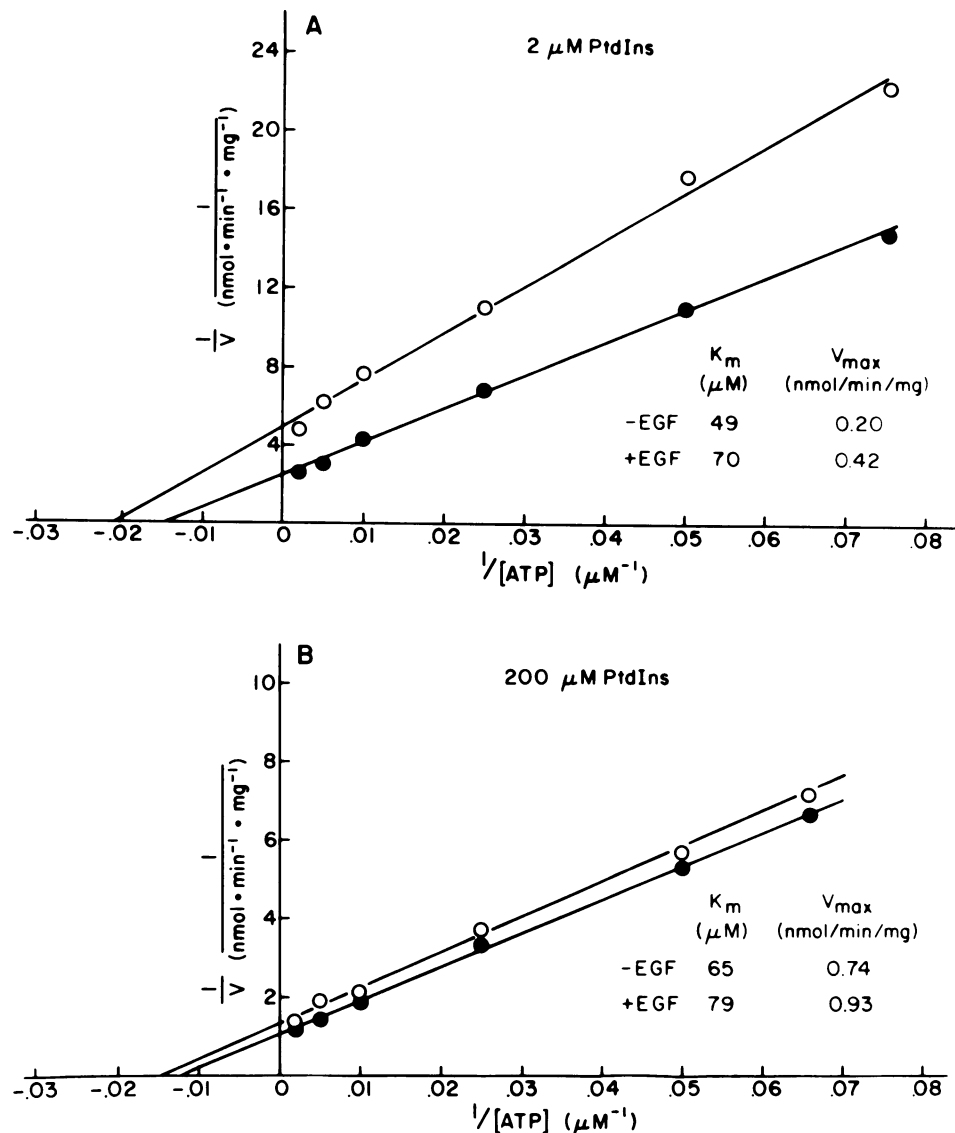
FIG. 5.   Lineweaver–Burk plots for the utilization of ATP by the PtdIns kinase. Subconfluent cultures of A431 cells were incubated with (solid circles) or without (open circles) 30 nM EGF prior to the preparation of the particulate fractions. Membranes were assayed in duplicate for PtdIns kinase activity for 3 min with various concentrations of ATP. (*A*) Lineweaver–Burk plot of activity using 2 μM PtdIns. (*B*) Lineweaver–Burk plot of activity using 200 μM PtdIns.

respectively. By contrast, in PMA-treated cells, basal and EGF-stimulated PtdIns kinase activities were 407 ± 17 and 474 ± 17 cpm per μg of protein, respectively. Thus, pretreatment of cells with PMA inhibited the ability of EGF to stimulate PtdIns kinase activity.

DISCUSSION

We have shown (9) that EGF stimulates an increase in the levels of PtdIns4*P* in A431 cells. The data presented here show that a PtdIns kinase activity present in the particulate fraction of A431 cells is increased when the whole cells are incubated with EGF prior to disruption. Initial attempts to elicit a stimulation of PtdIns kinase activity by the addition of EGF directly to the particulate fraction were unsuccessful and frequently resulted in an inhibition of the activity. When EGF was added to whole cells, however, a rapid and stable stimulation of the PtdIns kinase activity present in preparations of cell membranes was observed. This is similar to the situation with the S6 kinase where the addition of insulin, EGF, or other growth factors to whole cells stimulates the

phosphorylation of ribosomal protein S6 on a serine residue (18–22). Stimulation of the PtdIns kinase by EGF in A431 cells is nonetheless different from activation of the S6 kinase since the PtdIns kinase is a membrane-bound rather than a soluble enzyme and its activation is more rapid than that of the S6 kinase.

Stimulation of the PtdIns kinase by EGF was dependent on protease inhibitors in the buffers used to prepare the particulate fractions and on the use of subconfluent cells. In the absence of protease inhibitors, basal activity increased, and the stimulation by EGF decreased concomitantly. This suggests that the PtdIns kinase or a protein that regulates its activity is susceptible to proteolysis. Proteolysis may result in activation of the enzyme and preclude further stimulation by EGF. This is reminiscent of the situation with protein kinase C that can be cleaved to a form that is persistently active and unresponsive to acyl$_2$Gro and phosphatidylserine (23). Stimulation of the PtdIns kinase by EGF was significantly greater when subconfluent cultures of cells were treated with the growth factor than when confluent cultures were used. Similar findings have been reported for the

induction of c-fos and c-myc mRNA levels by EGF in BALB/c 3T3 cells (24). The molecular basis for this observation is unknown.

The activity and the stimulation by EGF of the PtdIns kinase were greater when assays were performed in Mg^{2+} as compared to Mn^{2+}. It has been suggested that protein tyrosine kinases may function as PtdIns kinases (25–28), although later reports have indicated that the tyrosine kinase and the PtdIns kinase are two distinct activities (29–31). The data presented here also suggest that the two kinase activities are separate since the divalent cation requirements for the EGF-stimulated PtdIns kinase are different than those reported for the EGF receptor/kinase (32) using protein substrates. In particular, the strong preference of the PtdIns kinase for Mg^{2+} as opposed to Mn^{2+} contrasts with the preference of the EGF receptor/kinase for Mn^{2+}.

Stimulation of the PtdIns kinase by EGF was rapid and biphasic. This paralleled the time course for PtdIns kinase activation by EGF in whole cells (9). MacDonald *et al.* (33) have described an increase in PtdIns kinase activity in Swiss 3T3 cells in response to platelet-derived growth factor. In that report, no acute stimulation of the PtdIns kinase was observed, but a cycloheximide-sensitive increase in activity was noted at time points beginning ≈2 hr after the addition of platelet-derived growth factor. Since our time course did not cover these later time points, it is possible that a slow induction of the PtdIns kinase may also occur in A431 cells exposed to EGF.

Kinetic analyses of the reaction catalyzed by the PtdIns kinase suggested that EGF stimulated the enzyme by inducing a decrease in the apparent K_m of the kinase for PtdIns. The calculated K_m of 20 μM for the unstimulated PtdIns kinase was well above the experimentally determined value of 1–2 μM for the contribution of endogenous PtdIns. Thus, under physiological conditions, an EGF-stimulated decrease in the K_m would result in an increase in PtdIns kinase activity. The PtdIns kinase was found to be soluble under the conditions of our assay. It should be noted, however, that both the enzyme and the substrate are likely to be present in mixed micelles that may alter the local concentration of PtdIns. Therefore, the true K_m value may be different quantitatively from that calculated in these experiments, but the qualitative nature of the difference between treated and untreated cells should be retained.

The mechanism by which EGF activates the PtdIns kinase is unknown. PtdIns kinase activities have been reported to be associated with several tyrosine kinase activities including the EGF receptor/kinase (28), the insulin receptor/kinase (25, 28), pp60$^{v\text{-}src}$ (26), pp68$^{v\text{-}ros}$ (27), and the Abelson gene product (34). In addition, Kaplan *et al.* (35) have demonstrated that in polyoma virus-infected cells, the middle-sized tumor antigen is found in a complex with pp60$^{c\text{-}src}$ and a PtdIns kinase activity. Thus, there may be a physical association between protein tyrosine kinases and a PtdIns kinase, possibly as a result of an enzyme–substrate relationship. An alternative mechanism for the activation of the PtdIns kinase would be interaction of the enzyme with a regulatory protein, such as a GTP binding regulatory protein. Our observation that the EGF-stimulated rise in PtdIns4P levels in whole cells is inhibited by cholera toxin is consistent with this possibility. Further work will be required to distinguish between the various possibilities.

One metabolic role for the PtdIns kinase is likely to be the generation of PtdIns4P as a precursor for phosphatidylinositol 4,5-bisphosphate. PtdIns4P can also serve as a substrate for a phospholipase C, to generate acyl$_2$Gro in the absence of inositol 1,4,5-trisphosphate production. This may be important in maintaining the activation of some enzymes while terminating the activation of others. Whether PtdIns4P or any of its direct metabolites act as regulators of intracellular enzyme activities is unknown at this time but regulation of the synthesis of PtdIns4P by EGF suggests that the PtdIns kinase plays an important role in the metabolism of PtdIns.

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