Enhancement of Calcium Uptake and Phosphatidylinositol Turnover by Epidermal Growth Factor in A-431 Cells†

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ABSTRACT: Epidermal growth factor (EGF) stimulates the incorporation of $^{32}P_i$ and [^{3}H]inositol into phosphatidylinositol (5–10-fold) in A-431 cells. EGF also stimulates the incorporation of $^{32}P_i$ into phosphatidic acid (up to 10-fold). These effects are attributed to an acceleration of the turnover of phosphatidylinositol as a consequence of the binding of EGF to its membrane receptor. The extent of the phosphatidylinositol response to EGF parallels the extent of hormone binding. The phosphatidylinositol response to EGF appears to be dependent on an influx of calcium since (1) external calcium is required for the enhancement of phosphatidylinositol turnover, (2) the accumulation of ^{45}Ca by A-431 cells is stimulated by EGF, (3) blockage of calcium influx with $LaCl_3$ inhibits stimulation of phosphatidylinositol turnover, and (4) calcium influx via ionophore A23187 is sufficient to stimulate phosphatidylinositol turnover. Since the binding, internalization, and degradation of ^{125}I-labeled EGF in A-431 cells are unaffected by the omission of calcium from the medium, external calcium and phosphatidylinositol turnover are not necessary for the internalization and degradation of the EGF–receptor complex.

Increased turnover of phosphatidylinositol occurs in many animal cells under the influence of a wide variety of stimuli. However, the exact nature of the mechanism by which the turnover of this minor membrane phospholipid is initiated and the role that the turnover plays in cell physiology are unclear [reviewed by Michell (1975)]. Current interest regarding the role of phosphatidylinositol turnover center on (1) the coupling of calcium gates to the breakdown of phosphatidylinositol (Jones et al., 1979; Michell, 1975), (2) the release of arachidonic acid used in prostaglandin synthesis from diacylglycerol, a transitory product of phosphatidylinositol turnover (Bell et al., 1979; Marshall et al., 1981), and (3) the control of protein kinase activity by diacylglycerol derived from phosphatidylinositol turnover (Kishimoto et al., 1979).

Epidermal growth factor initiates a wide variety of biological effects in responsive cells, usually including enhanced cell division [reviewed by Carpenter & Cohen (1979)]. The human epidermoid carcinoma cell line A-431 has been utilized in recent studies on the mechanism of action of EGF,[1] because these cells have an extraordinarily high number of EGF receptors [(2–3) × 10^6 receptors/cell]. These cells bind and internalize EGF (Haigler et al., 1979a) and undergo rapid morphological changes (Chinkers et al., 1979, 1981), which appear to be associated with increased fluid pinocytosis (Haigler et al., 1979b). Membranes prepared from these cells show enhanced protein phosphorylation in response to EGF (Carpenter et al., 1979).

The present study reports that phosphatidylinositol turnover and calcium influx in A-431 cells are markedly enhanced by EGF. The interrelation of these events and possible correlations of calcium influx and phosphatidylinositol turnover to hormone binding, internalization, and degradation were examined.

Materials and Methods

Materials. Mouse EGF was isolated by published procedures (Savage & Cohen, 1972). [^{32}P]Orthophosphate ($^{32}P_i$), [2-^{3}H]inositol (12.5 Ci/mmol), $^{45}CaCl_2$, and Aquasol were purchased from New England Nuclear. Lipid standards and thin-layer chromatographic plates were from Supelco. Ionophore A23187 was a gift of Eli Lilly Co.

Growth of A-431 Cells. A-431 cells are grown in 60-mm Falcon dishes containing Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (Flow Laboratories) and gentamycin (Schering Corp.).

Extraction and Analysis of Lipids. Total cellular lipid was extracted by the method of Bligh & Dyer (1959) modified by the addition of 2% acetic acid. Lipids were analyzed by two-dimensional thin-layer chromatography (TLC) on silica gel H as described previously (Sawyer & Greenawalt, 1979). One-dimensional TLC was carried out on silica gel H in chloroform–methanol–acetic acid–H_2O (25:15:4:2). Phosphatidic acid was analyzed by a TLC system described by Lapetina & Cuatrecasas (1979). For analysis of phosphorylated phosphatidylinositols, lipid was extracted in the presence of HCl as described by Dawson & Eichberg (1965) and analyzed by the TLC method of Gonzalez-Sastre & Folch-Pi (1968).

Labeling of A-431 Cellular Lipids. Short-term experiments were done in Hepes-buffered saline (HBS) [20 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 μM P_i, and 0.1% glucose)]. Confluent cultures of A-431 cells were washed 3 times in HBS and then incubated at 37 °C with either $^{32}P_i$ or [^{3}H]inositol and other additions in a total volume of 1.5 mL of HBS. The incorporation was terminated by washing the dishes 5 times with cold 10% trichloroacetic acid Cl_3CCOOH containing 0.01 M sodium pyrophosphate. Acid-soluble radioactivity was estimated from a Cl_3CCOOH extract after cultures were washed 5 times with HBS. The cells then were scraped from the dish in 1.0 mL of H_2O, and the lipid was extracted by the addition of $CHCl_3$ and CH_3OH. Lipid extracts labeled with $^{32}P_i$ were evaporated to a small volume and

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‡ S.T.S. holds a Postdoctoral Fellowship from the U.S. Public Health Service (EY 07007-04).

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[1] Abbreviations used: EGF, epidermal growth factor; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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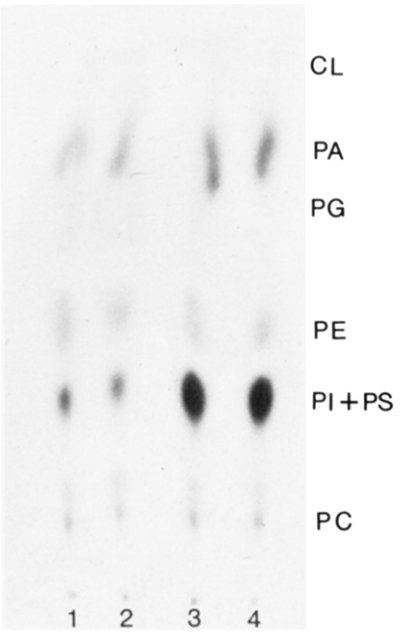

FIGURE 1: Effect of EGF on $^{32}P_i$ incorporation into A-431 phospholipids. Confluent cultures were incubated with 1.0 μCi of $^{32}P_i$/mL in the presence or absence of EGF for 60 min at 37 °C. The experiment was terminated, and the lipid was extracted and analyzed by TLC as described under Materials and Methods. Lipid standards were visualized with I_2 vapor. Autoradiography was performed with Kodak RP Royal X-O Mat film for 2 days. Lanes 1 and 2 are replicates from control cells; lanes 3 and 4 are from cells treated with 200 ng of EGF/mL.

analyzed by TLC. Lipid extracts labeled with [^{3}H]inositol were evaporated to dryness in a scintillation vial and counted by liquid scintillation spectrophotometry in Aquasol.

Binding of ^{125}I-Labeled EGF to A-431 Cells. EGF was iodinated and binding determined by the method of Carpenter & Cohen (1976). HBS was supplemented with 0.1% bovine serum albumin (BSA) for binding experiments. Nonspecific binding was determined by the binding of ^{125}I-labeled EGF in the presence of 5 μg of unlabeled EGF. The percentage of cell bound counts which were internalized was estimated by the acetic acid extraction method of Haigler et al. (1980), and the proportion of ^{125}I-labeled EGF which was degraded was estimated by the column chromatographic method of Carpenter & Cohen (1976).

Results

Effect of EGF on the Incorporation of $^{32}P_i$ into Phosphatidylinositol and Phosphatidic Acid. The incorporation of $^{32}P_i$ into A-431 cells in the presence and absence of EGF was examined. These experiments were carried out in Hepes-buffered saline containing 50 μM phosphate. The radioactivity incorporated into the total cellular lipids during a 1.0-h incubation at 37 °C was 1.5–3-fold higher in the presence of EGF than in its absence. These lipid extracts were fractionated by thin-layer chromatography, and the distribution of radioactivity was examined by autoradiography (Figure 1) and by measuring the radioactivity of individual spots (Table I). The phosphatidylinositol and phosphatidic acid from cells exposed to EGF were 5.4- and 2.7-fold, respectively, more radioactive than those from control cells.

The increased labeling of phosphatidylinositol and phosphatidic acid in the presence of EGF did not appear to be the result of increased transport of $^{32}P_i$ or of an increased specific activity of ATP since EGF did not significantly affect the incorporation of $^{32}P_i$ into other phospholipids (Table I). In addition, EGF had no detectable effect on the labeling of Cl_3CCOOH-insoluble, lipid-depleted cellular material, and EGF had only a slight effect on the Cl_3CCOOH-soluble pool of radioactivity during these experiments (20% increase; data not shown). We believe that the effect of EGF on the labeling of phosphatidylinositol and phosphatidic acid is the result of phosphatidylinositol turnover since we were unable to detect any significant change in the phospholipid composition when EGF was added for either 1 or 24 h to cells prelabeled to equilibrium (72 h) with $^{32}P_i$.

Time Course of the Incorporation of $^{32}P_i$ into Phosphatidic Acid and Phosphatidylinositol. The time course of $^{32}P_i$ incorporation into the phospholipids of A-431 cells in the presence and absence of EGF is shown in Figure 2. Enhancement of the incorporation of $^{32}P_i$ into both phosphatidic acid and phosphatidylinositol in the presence of EGF was clearly seen. However, the effect on phosphatidic acid was

Table I: Distribution of ^{32}P in Phospholipids of A-431 Cells[a]

incubation conditions	expt	radioactivity in isolated lipid fractions (cpm) PA	PI	PC	PE	PS	others
control	1	900	2120	1510	2200	780	500
	2	900	2640	1790	1470	1030	
EGF	1	2040	13800	1830	1700	1240	500
	2	2520	11680	1650	1810	830	

[a] Confluent cultures were incubated with 1.0 μCi of $^{32}P_i$ for 60 min at 37 °C as described under Materials and Methods. EGF (200 ng/mL) was added just prior to the addition of $^{32}P_i$. The reaction was terminated, and lipids were extracted and analyzed by two-dimensional TLC as described under Materials and Methods. Phospholipids identified by using authentic standards were scraped from the plate and counted in a gas-flow counter.

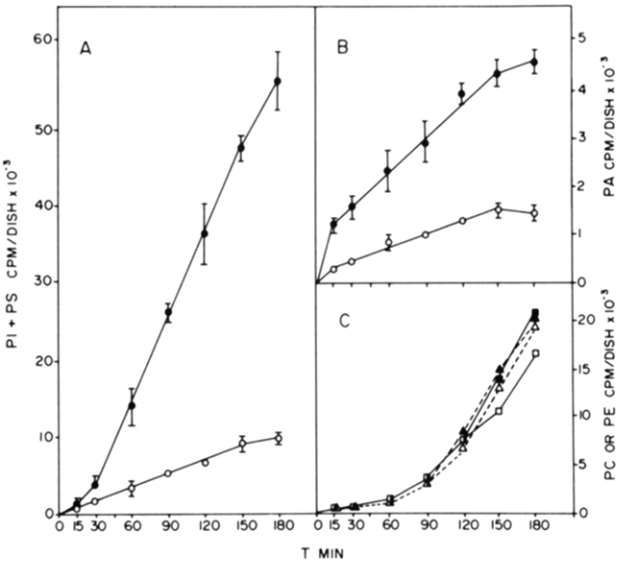

FIGURE 2: Time course of $^{32}P_i$ incorporation into A-431 cellular phospholipids. Confluent cultures of A-431 cells were incubated for the indicated time at 37 °C with 1.0 μCi of $^{32}P_i$/mL in the presence or absence of 200 ng of EGF/mL. EGF and $^{32}P_i$ were added simultaneously at 0 time. The incorporation was terminated, and the lipids were extracted and analyzed by one-dimensional TLC as described under Materials and Methods. Spots identified with authentic standards were scraped and counted in a gas-flow counter. $^{32}P_i$ incorporation into phosphatidylinositol plus phosphatidylserine is shown in panel A. Control (○); plus EGF (●). $^{32}P_i$ incorporation into phosphatidic acid is shown in panel B. Control (○); plus EGF (●). Incorporation of $^{32}P_i$ into phosphatidylcholine (△, ▲) and phosphatidylethanolamine (□, ■) are shown in panel C. Control, open symbols; plus EGF, closed symbols. Error bar represents the variation between duplicate experiments.

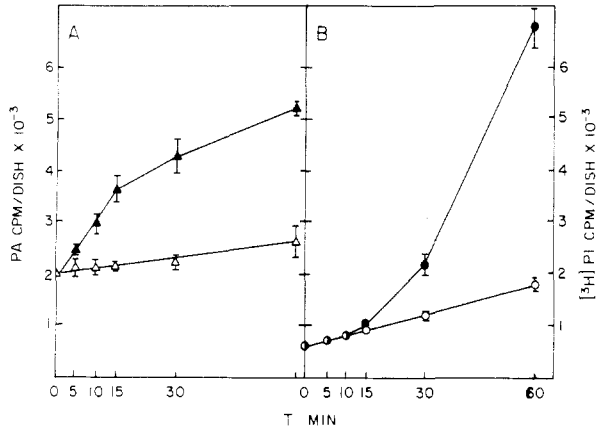

FIGURE 3: Time course of labeling of phosphatidic acid and phosphatidylinositol in A-431 cells in the presence and absence of EGF. Confluent cultures of A-431 cells were prelabeled with either 10.0 μCi of $^{32}P_i$/mL or 1.0 μCi of [^{3}H]inositol/mL for 30 min at 37 °C. EGF (200 ng/mL) was added to replicate dishes, and the incubation was continued at 37 °C for the indicated time. The cultures were processed as described under Materials and Methods. (Panel A) Phosphatidic acid, separated by TLC, was scraped and counted [control (△); plus EGF (▲)]. (Panel B) The total lipid extracts from cells labeled with [^{3}H]inositol were evaporated and counted by liquid scintillation spectrophotometry [control (○); plus EGF (●)]. Error bars indicate the variation between duplicate experiments.

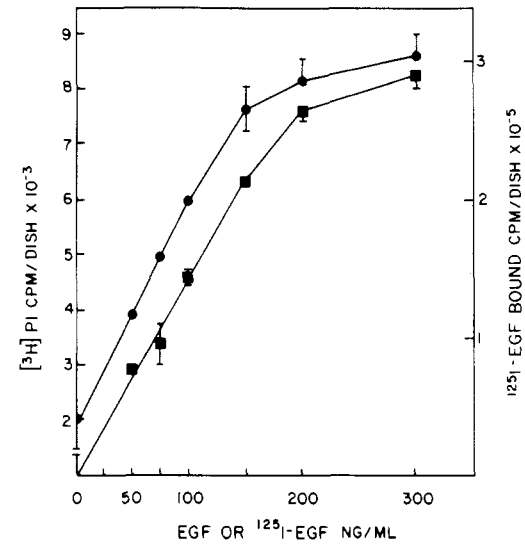

FIGURE 4: Relationship of the extent of binding of ^{125}I-labeled EGF to A-431 cells to the stimulation of phosphatidylinositol synthesis. Confluent cultures of A-431 cells were incubated with [^{3}H]inositol (5.0 μCi/mL) and varying concentrations of EGF for 60 min at 37 °C. Cultures were processed and counted as described under Materials and Methods. Replicate cultures were incubated with varying concentrations of ^{125}I-labeled EGF (5000 cpm/ng) under the same conditions. Binding was determined as described under Materials and Methods. Error bar represents the variation between duplicate experiments. [^{3}H]Inositol accumulation (●); ^{125}I-labeled EGF bound (■).

apparent at the earliest time point examined, 15 min (Figure 2B), whereas the effect of EGF on $^{32}P_i$ accumulation into phosphatidylinositol was apparent only after a lag of approximately 30 min (Figure 2A). After an incubation of 180 min, the radioactivity in phosphatidylinositol (plus phosphatidylserine) from cells treated with EGF was 5.6-fold higher than that from control cells; the radioactivity in phosphatidic acid was 3-fold higher in cells treated with EGF. At this time point (180 min), only minor increases (20%) were noted in the incorporation of label into phosphatidylcholine and phosphatidylethanolamine from cells treated with EGF compared to control cells.

In view of the rapidity of the effect of EGF on the incorporation of $^{32}P_i$ into phosphatidic acid (15 min), the initiation of enhanced labeling of phosphatidic acid was examined during earlier time intervals. Figure 3A shows that the enhancement of phosphatidic acid labeling was apparent within 5 min after EGF was added. Under the conditions of this experiment, the rate of accumulation of $^{32}P_i$ into phosphatidic acid was linear in control cells. During the first 15 min of exposure to EGF, the linear rate of accumulation of $^{32}P_i$ into phosphatidic acid was stimulated approximately 10-fold. In this experiment, the cells were prelabeled with $^{32}P_i$ for 30 min.

Incorporation of [^{3}H]Inositol into Phosphatidylinositol. The accumulation of [^{3}H]inositol into A-431 cells was examined as an independent indicator of phosphatidylinositol synthesis. As shown in Figure 3B, treatment of the cells with EGF resulted in the stimulation of [^{3}H]inositol incorporation into the lipid fraction of these cells. Analysis of the lipid extract from cells labeled with [^{3}H]inositol showed that greater than 95% of the radioactivity was identified as phosphatidylinositol and virtually no radioactivity was found in TLC fractions corresponding to authentic standards of polyphosphoinositides (data not shown). The lag between the addition of EGF and the enhanced incorporation of [^{3}H]inositol into phosphatidylinositol by EGF was the same as in experiments using $^{32}P_i$ (30 min), and the magnitudes of enhancement by EGF were similar when either label was used. In this experiment, the cells were preincubated with [^{3}H]inositol for 30 min prior to the addition of EGF. Additional experiments (not shown) indicated that the length of preincubation time with [^{3}H]-inositol (0–90 min) had no effect on the lag time required for an EGF response.

Comparison of the Binding of ^{125}I-Labeled EGF to A-431 Cells and the Stimulation of the Labeling of Phosphatidylinositol. The stimulation of the incorporation of [^{3}H]inositol into phosphatidylinositol in A-431 cells at varying concentrations of EGF was compared, under identical conditions, to the extent of binding of ^{125}I-labeled EGF in replicate cultures. The results, shown in Figure 4, indicate that the effect of EGF on the labeling of phosphatidylinositol paralleled the extent of binding of ^{125}I-labeled EGF to the cells at all concentrations of EGF examined. Saturation of EGF binding sites was required to fully stimulate the incorporation of inositol into phosphatidylinositol. Half-maximal stimulation of inositol accumulation required 90 ng of EGF/mL, while half-maximal binding occurred at 100 ng of EGF/mL.

Effect of Extracellular Calcium and Magnesium on the Labeling of Phosphatidylinositol in A-431 Cells. It has been hypothesized that calcium gates might be controlled by phosphatidylinositol turnover (Michell, 1975). However, a few cell types have been shown to require extracellular calcium for the stimulation of phosphatidylinositol turnover (Cockcroft et al., 1980a,b). Therefore, the effect of the calcium concentration in the medium on the enhancement of the labeling of phosphatidylinositol by EGF in A-431 cells was examined. As shown in Figure 5, extracellular calcium was required for EGF to enhance the labeling of phosphatidylinositol, although the basal level of inositol incorporation into phosphatidylinositol was completely independent of the calcium concentration in the medium. Inclusion of EGTA (1.0 mM) had no effect on the labeling of phosphatidylinositol in calcium-free medium (data not shown). Half-maximal enhancement of the labeling of phosphatidylinositol by EGF occurred in medium containing 200 μM calcium, and maximal enhancement of the labeling of phosphatidylinositol required approximately 1.0 mM calcium in the medium.

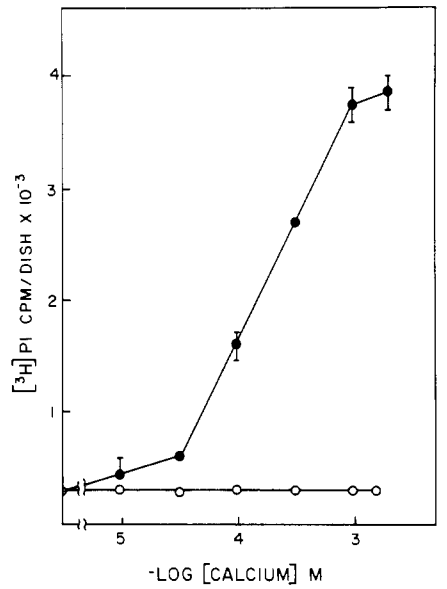

FIGURE 5: Effect of extracellular calcium on the stimulation of [^{3}H]inositol incorporation into phosphatidylinositol by EGF. Confluent cultures of A-431 cells were incubated with 1.0 μCi of [^{3}H]inositol/mL for 90 min at 37 °C. The media consisted of HBS free of calcium and magnesium or with calcium present at the indicated concentrations. Incorporation of [^{3}H]inositol into phosphatidylinositol was determined as a function of increasing concentrations of calcium in the presence and absence of EGF. Cultures were processed as described under Materials and Methods. Control (○); plus EGF (●). Error bars represent the variation in duplicate experiments.

Extracellular magnesium could not substitute for the requirement of calcium in the stimulation of the labeling of phosphatidylinositol by EGF. When the medium contained magnesium (1.0 mM) and no calcium, the basal incorporation of inositol into phosphatidylinositol was increased approximately 2-fold over that in medium devoid of both calcium and magnesium; however, no EGF effect was seen. In the presence of calcium (1.0 mM), the further addition of magnesium (1.0 mM) increased both the basal and EGF stimulated labeling of phosphatidylinositol by approximately 2-fold (data not shown).

Stimulation of the Labeling of Phosphatidylinositol by Ionophore A23187. In view of the requirement of calcium for the stimulation of the labeling of phosphatidylinositol by EGF, the calcium ionophore, A23187, was examined for its ability to stimulate the incorporation of inositol into phosphatidylinositol. Figure 6 shows that increasing the concentration of A23187 in the medium above 10^{-7} M resulted in enhanced accumulation of [^{3}H]inositol. Micromolar concentrations of A23187 enhanced the labeling of phosphatidylinositol to approximately the same extent as EGF. As shown in Figure 6, addition of EGF to cultures containing A23187 resulted in the additional enhancement of the labeling of phosphatidylinositol, although the responses were not quantitatively additive. In parallel to the results obtained with EGF (Figure 5), calcium was required for the ionophore to exert its effect on the labeling of phosphatidylinositol (Figure 6); in neither instance could calcium be replaced by magnesium.

Effect of EGF and $LaCl_3$ on Calcium Transport in A-431 Cells. For further substantiation of the role of calcium in the stimulation of the labeling of phosphatidylinositol by EGF, the effect of EGF on calcium transport in A-431 cells was examined. Figure 7 shows the effect of EGF on the cellular accumulation of ^{45}Ca. EGF and ^{45}Ca were added simultaneously to cells at 37 °C. An approximate 5-fold enhancement of ^{45}Ca accumulation by EGF occurred within 1.0 min. In

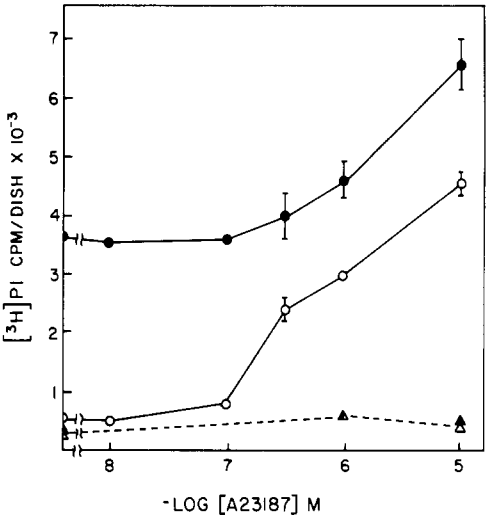

FIGURE 6: Effect of A23187 on the labeling of phosphatidylinositol by [^{3}H]inositol in A-431 cells. Confluent cultures of A-431 cells were incubated with 1.0 μCi of [^{3}H]inositol/mL for 90 min at 37 °C in the presence and absence of EGF (200 ng/mL), and cells were processed as described under Materials and Methods. The medium contained the indicated concentrations of A23187 and 1.0% Me_2SO. This concentration of Me_2SO had no effect on the labeling of phosphatidylinositol. The experiment was carried out in HBS [control (○); plus EGF (●)] and in HBS free of calcium and magnesium [control (△); plus EGF (▲)]. Error bars represent the variation between duplicate experiments.

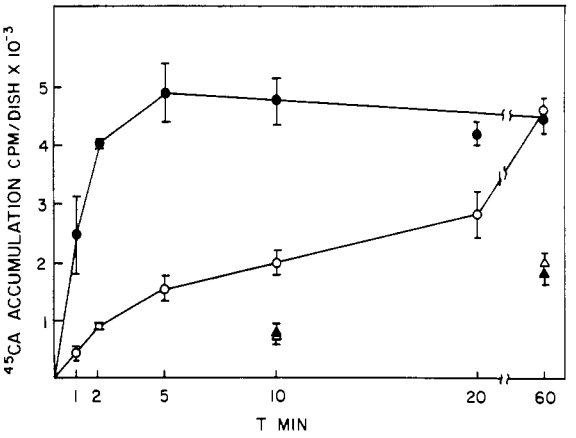

FIGURE 7: Effect of EGF on ^{45}Ca accumulation in A-431 cells. ^{45}Ca (2.0 μCi/mL) and EGF (500 ng/mL) were added simultaneously to confluent cultures of A-431 cells in HBS. In replicate cultures, ^{45}Ca accumulation was determined in the presence of 200 μM $LaCl_3$. After the indicated interval, the cells were washed rapidly 10 times with cold HBS containing 10.0 mM $CaCl_2$ and 1.0 mM $LaCl_3$. Cells were solubilized with 1.0 mL of 0.5 N NaOH and counted by liquid scintillation spectrometry in Aquasol. Control (○); plus EGF (●); $LaCl_3$ control (△); $LaCl_3$ plus EGF (▲). Data is the mean of three experiments; error bars indicate standard deviation.

the presence of EGF, a higher steady-state level of cellular ^{45}Ca was reached in 5 min, whereas in the absence of EGF, a slow continuous accumulation of ^{45}Ca was noted; at the end of 60 min, the cellular levels of ^{45}Ca in control and EGF-treated cells were similar. Addition of $LaCl_3$ (200 μM), a calcium antagonist and inhibitor of calcium flux (Weiss, 1974), greatly reduced the uptake of ^{45}Ca in control cells and completely abolished the effect of EGF (Figure 7).

Previous experiments in this laboratory have shown that EGF stimulates the release of cellular calcium from A-431 cells prelabeled with ^{45}Ca (Chinkers et al., 1981). We have repeated and extended these experiments. During a 5-min incubation at 37 °C, treatment of prelabeled cells with EGF enhanced the release of ^{45}Ca. Cells treated with 500 ng of

Table II: Effect of $LaCl_3$ on the Phosphatidylinositol Response to EGF and Ionophore A23187[a]

incubation conditions	expt	additions during [^{3}H]inositol accumulation (cpm/dish) none	200 μM $LaCl_3$
control	1	690	640
	2	780	680
EGF (200 ng/mL)	1	5240	680
	2	5120	690
A23187 (1.0 μM)	1	4510	1860
	2	4750	1850

[a] Confluent cultures were incubated 90 min at 37 °C with 1.0 μCi/mL [^{3}H]inositol as described under Materials and Methods. EGF, ionophore A23187, and $LaCl_3$ were added simultaneously with the [^{3}H]inositol. Ionophore A23187 was added in Me_2SO such that the final concentration of Me_2SO was 0.1%. This concentration of Me_2SO had no effect on inositol accumulation.

EGF/mL released 60% more radioactivity into the medium than control cells. $LaCl_3$ (200 μM) completely blocked the EGF effect on calcium release from prelabeled cells (data not shown). Thus EGF appears to stimulate both the influx and efflux of calcium; however, other alterations in the calcium pools of the cell, resulting in the same quantitative data, cannot be excluded.

Effect of $LaCl_3$ on the Phosphatidylinositol Response. Since the effect of EGF on calcium accumulation and release from A-431 cells was abolished by $LaCl_3$, the effect of this compound on the incorporation of [^{3}H]inositol into phosphatidylinositol was examined. As shown in Table II, $LaCl_3$ (200 μM) had no effect on the basal accumulation of inositol but completely blocked the enhancement of the labeling of phosphatidylinositol by EGF. Ionophore, A23187, enhanced the labeling of phosphatidylinositol in both the presence and absence of $LaCl_3$. $LaCl_3$ (200 μM) had no effect on the binding or internalization of ^{125}I-labeled EGF in A-431 cells (data not shown).

Phosphatidylinositol Response and the Binding, Internalization, and Degradation of EGF. The dependence of the phosphatidylinositol response on extracellular calcium affords an opportunity to determine if the presence of calcium and the subsequent labeling of phosphatidylinositol are necessary for the internalization and degradation of ^{125}I-labeled EGF. The binding, internalization, and degradation of EGF by A-431 cells was examined in the presence and absence of extracellular calcium and/or magnesium. Internalization was determined by the method of Haigler et al. (1980) which utilizes a buffer at pH 2.5 to remove surface-bound EGF. From Table III (last column), it can be seen that the extent of ^{125}I-labeled EGF binding to the cell is not affected by the presence or absence of calcium and/or magnesium in the medium. Further, the extent of internalization (nonextractable radioactivity) was almost identical in the presence or absence of extracellular calcium and/or magnesium. The inclusion of EGTA (1 mM) in calcium-free medium did not reduce the extent of binding or internalization of ^{125}I-labeled EGF (data not shown). In control experiments, when ^{125}I-labeled EGF was bound to cells at 4 °C, to prevent internalization, over 95% of the radioactivity was extracted by buffer at pH 2.5.

Degradation of cell-bound ^{125}I-labeled EGF in the presence and absence of extracellular calcium was also compared (Table IV). After 120 min of incubation at 37 °C, a total of 43% of cell-bound radioactivity was released into the medium in the control experiment and 46% of the cell-bound radioactivity was released in calcium- and magnesium-free medium. Of the radioactivity in the medium, 11.0% of the total was identified as iodotyrosine in the control experiment and 12.8% in the calcium- and magnesium-free medium. The remainder of the radioactivity was associated with higher molecular weight material. The omission of calcium and magnesium from the medium thus did not impair the ability of the cell to degrade EGF. As seen in Table IV (last column), as the time of incubation lengthened, an increasing proportion (almost 50%) of the released radioactivity could be accounted for as iodotyrosine in both control and experimental cultures. Thus the phosphatidylinositol response of the A-431 cell to EGF does not appear to be required for either the internalization or the degradation of the hormone.

Discussion

The widespread nature of the phenomenon of stimulated phosphatidylinositol turnover suggests it has a fundamental role (or roles) in cellular physiology (Irvine & Dawson, 1980). The turnover has been interpreted as a closed cycle of phosphatidylinositol breakdown and resynthesis via diacylglycerol and phosphatidic acid. The data presented herein adds EGF to the long list of stimuli (Michell, 1975) which evoke a phosphatidylinositol response.

The enhancement by EGF of the labeling of phosphatidylinositol was demonstrated both with $^{32}P_i$ and [^{3}H]inositol (Figures 1–3). Experiments with $^{32}P_i$ further suggest that phosphatidic acid synthesis is an intermediate in the phosphatidylinositol response. Addition of EGF appeared to have little effect on the incorporation of $^{32}P_i$ into phospholipids in general.

Since we were unable to detect any significant change in the relative proportion of phosphatidylinositol to other phospholipids during exposure of the cells to EGF, we believe that the phosphatidylinositol response to EGF is the result of phosphatidylinositol turnover. However, when the disappearance of label from phosphatidylinositol was examined, EGF had no apparent effect on the loss of label (data not shown). The enhanced turnover of less than 5% of the total phosphatidylinositol would not have been detected in this

Table III: Effect of External Calcium and Magnesium on the Binding and Internalization of EGF in A-431 Cells[a]

incubation conditions	time (min)	temp (°C)	^{125}I-labeled EGF bound (cpm/dish) nonextractable	extractable	total
HBS	30	4	1 200 ± 1 200	55 000 ± 5 600	56 000 ± 7 100
HBS	15	37	46 500 ± 500	66 100 ± 2 300	112 500 ± 1 900
Ca free	15	37	44 400 ± 1 700	78 200 ± 5 200	122 600 ± 3 500
Mg free	15	37	45 200 ± 5 200	59 600 ± 3 200	104 800 ± 8 300
Ca, Mg free	15	37	44 300 ± 2 000	66 900 ± 3 100	111 100 ± 4 800

[a] Confluent cultures of A-431 cells were incubated under the indicated conditions with 100 ng of ^{125}I-labeled EGF/mL (5000 cpm/ng) in HBS or calcium- and/or magnesium-free HBS. Unbound EGF was removed with eight washes of cold incubation medium. Surface-bound EGF was extracted with cold 0.2 M sodium acetate, pH 2.5, containing 0.5 M NaCl for 6 min. After the cultures were washed again with the acidic buffer, unextracted counts were removed with 1.0 mL of 0.5 M NaOH. Results shown are the mean of three experiments ± standard deviation.

Table IV: Effect of External Calcium and Magnesium on the Degradation of EGF in A-431 Cells[a]

incubation conditions	time interval (min)	^{125}I released into medium (% of initial cell bound)	[^{125}I]iodotyrosine (% of medium ^{125}I)
HBS	0–15	16.2	5.9
	15–45	11.9	23.3
	45–120	15.0	49.0
Ca, Mg free HBS	0–15	18.2	6.1
	15–45	12.7	33.1
	45–120	15.3	49.1

[a] Confluent cultures of A-431 cells were incubated at 4 °C for 1 h with 100 ng of ^{125}I-labeled EGF/mL (5000 cpm/ng) in either HBS or HBS in which $CaCl_2$ and $MgCl_2$ were omitted. BSA (0.1%) was present in both media. After unbound EGF was removed by eight washes with cold incubation medium, the cells were warmed to 37 °C. At the indicated intervals, the medium was exchanged with fresh medium. The medium was analyzed by gel filtration on Bio-Gel P-10 (as described under Materials and Methods) to determine the proportion of ^{125}I-labeled EGF which had been degraded to iodotyrosine. Data shown are the means of duplicate experiments.

experiment. Therefore we suspect that a small fraction of the total phosphatidylinositol is turned over in response to EGF.

It is of interest that a lag exists between the addition of EGF and the enhancement of phosphatidylinositol labeling whereas no lag was detected in the enhancement of phosphatidic acid labeling. This response is characteristic of phosphatidylinositol turnover in many systems. Phosphatidic acid labeling is typically enhanced within seconds after the addition of a stimulus, and enhanced phosphatidylinositol labeling usually follows in from 3 to 10 min after the addition of a stimulus (Michell, 1975).

The extent of the stimulation of the labeling of phosphatidylinositol by EGF appeared to be proportional to the extent of hormone occupancy of the EGF receptor (Figure 5). In view of the rapidity of the effects of EGF on the labeling of phosphatidic acid (Figure 2), hormone degradation probably is not a required step in this reaction. However, since internalization of EGF has been detected within minutes (Haigler et al., 1979a), it is not possible to exclude internalization as a required step.

The interrelationship between phosphatidylinositol turnover and calcium uptake is not clear. Phosphatidylinositol turnover has been proposed as a factor in the opening of calcium gates in the cell (Michell, 1975). On the other hand, others (Lapetina & Cuatrecasas, 1979; Bell & Majerus, 1980; Cockcroft et al., 1980a,b) have reported that certain cell types respond to a rise in intracellular calcium by accelerating the rate of phosphatidylinositol turnover.

Experiments reported here suggest that an EGF-stimulated calcium influx may trigger the stimulation of phosphatidylinositol turnover in A-431 cells. This interpretation is supported by the following observations: (1) stimulation of phosphatidylinositol turnover by EGF required extracellular calcium, (2) EGF stimulated the influx of calcium, (3) blocking calcium influx with $LaCl_3$ inhibited phosphatidylinositol turnover induced by EGF, and (4) calcium influx via A23187 was sufficient to stimulate phosphatidylinositol turnover. Enhancement of phosphatidylinositol turnover is independent of extracellular calcium in most cell types which have been examined. However, enhancement of phosphatidylinositol turnover in neutrophils has been reported to be dependent on extracellular calcium (Cockroft et al., 1980a,b).

EGF has been reported to enhance the active accumulation of nutrients in several cell types (reviewed by Carpenter & Cohen, 1979). Rozengurt & Heppel (1975) have shown that ^{86}Rb influx is enhanced by EGF within 2 min. Experiments reported here (Figure 7) show that EGF stimulates calcium influx by 5-fold within 1.0 min in A-431 cells. The accumulation of ^{45}Ca is 100-fold higher than the accumulation expected from bulk pinocytosis. Previous work in this laboratory Chinkers et al., 1981) reported that EGF increased calcium efflux from A-431 cells. The mechanism by which EGF apparently effects both calcium influx and efflux is not known. It is possible that the observed effects on the translocation of ^{45}Ca may be due not to direct changes in the transport of calcium across the plasma membrane but to alterations in the calcium pools of the cell, giving rise to the same quantitative data. It is of interest that EGF reduced the requirement of extracellular calcium for the growth of human fibroblast cells by 50-fold (McKeehan & McKeehan, 1979).

The control mechanisms of phosphatidylinositol turnover are uncertain. Two types of phosphatidylinositol specific phospholipase C activities have been identified, a cytosolic, calcium dependent enzyme (EC 3.1.4.10) and a lysosomal, calcium independent enzyme. A speculative interpretation of the data presented here is that EGF stimulates an influx of calcium, activating cytosolic phospholipase C and resulting in phosphatidylinositol breakdown. However, there is some evidence that the state of the membrane substrate controls the turnover of phosphatidylinositol rather than the activation of phospholipase C (Irvine & Dawson, 1980; Irvine et al., 1979).

We have examined the possibility that the internalization and degradation of ^{125}I-labeled EGF may be dependent on phosphatidylinositol turnover and extracellular calcium. Calcium has been reported to be necessary for the clustering and internalization of EGF in 3T3 cells (Maxfield et al., 1978). However, our data in Tables III and IV show that EGF was internalized and degraded equally well in the presence and absence of extracellular calcium. These results imply that neither extracellular calcium nor enhanced phosphatidylinositol turnover is involved in the adsorptive pinocytosis of the hormone–receptor complex or in the fusion of the endocytosed vesicles with the lysosomes. Additional experiments (not shown) revealed that the internalization of EGF in human fibroblast cells also was not dependent on extracellular calcium.

Rapid phosphorylation of A-431 membrane proteins as a consequence of the binding of EGF to its receptor (Carpenter et al., 1979) may be involved in opening calcium gates or in potentiation of phosphatidylinositol turnover after a calcium influx triggers the event. In turn, phosphatidylinositol turnover may influence protein kinase activities in the A-431 cell other than the protein kinase closely associated with the EGF receptor. A calcium-dependent protein kinase from brain which is activated by diacylglycerol has been reported (Kishimoto et al., 1980).

In view of the similarities between the protein kinase activities associated with the EGF receptor and the Rous sarcoma transforming gene product (src) (Ushiro & Cohen, 1980; Chinkers & Cohen, 1981), it is interesting that transformation of cells with the Rous sarcoma virus also results in enhanced phosphatidylinositol turnover (Diringer & Friis, 1977; Michell, 1975).

Acknowledgments

We acknowledge the technical assistance of Marty Reich and Katherine Chen.

References

Bell, R. L., & Majerus, P. W. (1980) *J. Biol. Chem. 255*, 1790–1792.

Bell, R. L., Kennerly, D. A., Stanford, N., & Majerus, P. W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3238–3241.
Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.
Carpenter, G., & Cohen, S. (1976) *J. Cell Biol. 71*, 159–171.
Carpenter, G., & Cohen, S. (1979) *Annu. Rev. Biochem. 48*, 193–216.
Carpenter, G., King, L., Jr., & Cohen, S. (1979) *J. Biol. Chem. 254*, 4884–4891.
Chinkers, M., & Cohen, S. (1981) *Nature (London) 290*, 516–519.
Chinkers, M., McKanna, J. A., & Cohen, S. (1979) *J. Cell Biol. 83*, 260–265.
Chinkers, M., McKanna, J. A., & Cohen, S. (1981) *J. Cell Biol. 88*, 422–429.
Cockcroft, S., Bennett, J. P., & Gomperts, B. D. (1980a) *FEBS Lett. 110*, 115–118.
Cockcroft, S., Bennett, J. P., & Gomperts, B. D. (1980b) *Nature (London) 288*, 275–277.
Dawson, R. M. C., & Eichberg, J. (1965) *Biochem. J. 96*, 634–643.
Diringer, H., & Friis, R. R. (1977) *Cancer Res. 37*, 2979–2984.
Gonzalez-Sastre, F., & Folch-Pi, J. (1968) *J. Lipid Res. 9*, 532–533.
Haigler, H. T., McKanna, J. A., & Cohen, S. (1979a) *J. Cell Biol. 81*, 382–395.
Haigler, H. T., McKanna, J. A., & Cohen, S. (1979b) *J. Cell Biol. 83*, 82–90.
Haigler, H. T., Maxfield, F. R., Willingham, M. C., & Pastan, I. (1980) *J. Biol. Chem. 255*, 1239–1241.
Irvine, R. F., & Dawson, R. M. C. (1980) *Biochem. Soc. Trans. 8*, 376–377.
Irvine, R. F., Hemington, N., & Dawson, R. M. C. (1979) *Eur. J. Biochem. 99*, 525–530.
Jones, L. M., Cockcroft, S., & Michell, R. H. (1979) *Biochem. J. 182*, 669–676.
Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U., & Nishizuka, Y. (1980) *J. Biol. Chem. 255*, 2273–2276.
Lapetina, E. G., & Cuatrecasas, P. (1979) *Biochim. Biophys. Acta 573*, 394–402.
Marshall, P. J., Boatman, D. E., & Hokin, L. E. (1981) *J. Biol. Chem. 256*, 844–847.
Maxfield, F. R., Willingham, M. C., Davies, P. J. A., & Pastan, I. (1978) *Nature (London) 277*, 661–663.
McKeehan, W. L., & McKeehan, K. A. (1979) *Exp. Cell Res. 123*, 397–400.
Michell, R. H. (1975) *Biochim. Biophys. Acta 415*, 81–147.
Rozengurt, E., & Heppel, L. A. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 4492–4495.
Savage, C. R., Jr., & Cohen, S. (1972) *J. Biol. Chem. 247*, 7609–7611.
Sawyer, S. T., & Greenawalt, J. W. (1979) *Biochem. Pharmacol. 28*, 1735–1744.
Ushiro, H., & Cohen, S. (1980) *J. Biol. Chem. 255*, 8363–8365.
Weiss, G. B. (1974) *Annu. Rev. Pharmol. 14*, 343–354.