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# Epidermal Growth Factor Stimulates Phosphatidylinositol Turnover in Human Foreskin Fibroblasts Without Activation of Protein Kinase C

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Epidermal growth factor stimulates phosphatidylinositol turnover in human foreskin fibroblasts. This is a primary cell culture with normal numbers of epidermal growth factor receptors that is stimulated to divide by epidermal growth factor. Increases are seen in the inositol phospholipids and inositol phosphates. Despite this activation of phosphatidylinositol turnover, there is no detectable activation of protein kinase C.

**Key words: EGF, cell proliferation, tyrosine kinase, second messenger**

The role of phosphatidylinositol (PI) turnover in mediating the action of epidermal growth factor (EGF) remains uncertain. A number of groups have shown that EGF stimulates PI turnover in A431 cells [1–6]. These cells are unusual, however, in that they contain a large number of EGF receptors and are stimulated to divide only by very low concentrations of EGF [7]. Results are variable in other cell types. Wahl et al. [5] found that EGF stimulated PI turnover in other cell lines containing high numbers of EGF receptors, but Bjorge and Kudlow [8] could not demonstrate this in one of the same cell lines. Raben and Cunningham showed PI activation by EGF in II-C9 and chick embryo fibroblasts [9]. They also showed stimulation in Swiss 3T3 cells, whereas Vara and Rozengurt did not [10]. Muldoon et al. found an increase in inositol phosphates in Rat-1 cells but did not measure lipids [11]. Negative results have been reported in Balbc/3T3 [12] and CHL cells [13].

We studied the effect of EGF on PI turnover and protein kinase C activation in human foreskin fibroblasts that are stimulated to divide by EGF.

## MATERIALS AND METHODS

Human foreskin fibroblasts were obtained from Dr. U. Steinbrecher and Dulbecco's modified Eagle's medium (DME) from Gibco. EGF, fetal calf serum (FCS), PI,

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phosphatidylinositol-4-phosphate (PIP), phosphatidylinositol-4,5-bisphophate ($PIP_2$), phosphatidic acid (PA), phosphatidylserine (PS), phorbol myristic acid (PMA), and histone were from Sigma. [$^{32}$Pi] and [$\gamma^{32}$P]ATP were from ICN and AG1-X8, 100–200 mesh, formate form from Biorad. All other chemicals were from BDH, and TLC plates were from Merck.

Cells were grown in 24-well plates in DME with 2% FCS for growth and PI turnover experiments. For protein kinase C assays, they were grown in 60 mm plates and placed in serum-free medium for 24 h before the assay.

For PI turnover experiments, the cells were labelled with 100 $\mu$Ci/plate of $^{32}$P in phosphate-free DME supplemented with 100 $\mu$M $PO_4$ and 2% serum for 72 h to achieve isotopic equilibrium. Ten millimeters of $LiCO_3$ was added for 1 h, and then EGF was added for the indicated time at a final concentration of 100 nM.

The experiments were stopped by washing the cells three times with cold incubation buffer. One milliliter of cold 2.4 N HCl was added to each plate, and the cells were scraped off and transferred to glass tubes. The extraction was a modification of the protocol described by Schacht [14]. Three milliliters of $CHCl_3$:MeOH (1:2) and then 1 ml of $CHCl_3$ were added and mixed. After separation, the lower organic phase was removed and the upper phase re-extracted with 2 ml of $CHCl_3$. The combined lower phases were washed with 5 ml of MeOH:1 N HCl (1:1) and then dried under nitrogen. The lipids were redissolved in 40 $\mu$l $CHCl_3$:MeOH (2:1) and separated on TLC. PIP and $PIP_2$ were resolved using a basic buffer system of $CHCl_3$:MeOH:30% $NH_4OH$:$H_2O$ (43:38:10:5) [14]. PI and PA were separated in an acidic system of $CHCl_3$:Pet Ether:MeOH:HAc (5:3:1.6:1) [15]. TLC plates were exposed to autoradiography and the radioactive spots identified by co-migration with iodine-stained standards. The spots were scraped off and quantitated by liquid scintillation counting.

The inositol phosphates were separated as described by Berridge et al. [16] using ion-exchange resins. One milliliter of the upper phase of the extraction was applied to a 1 ml column of AG1-X8. Inositol-1-phosphate ($IP_1$) was eluted with 13 ml of 0.1 M formic acid, 0.2 M ammonium formate, inositol-1,4-bisphophate ($IP_2$) with 11 ml of 0.1 M formic acid, 0.4 M ammonium formate, and inositol-1,4,5-trisphosphate ($IP_3$) with 7 ml of 0.1 M formic acid, 1.0 M ammonium formate. Although this method does not separate the inositol phosphates as completely as do the newer HPLC techniques [17], it was adequate for the purposes of this study.

After incubation with EGF or PMA, protein kinase C was assayed by adding 0.5 ml of cold 20 mM Tris, pH 7.5, 5 mM EDTA, 10 mM mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 $\mu$g/ml Pepstatin A, and 10 $\mu$g/ml soybean trypsin inhibitor. The cells were scraped into 1.5 ml tubes and disrupted with sonication. The cells were spun in a microfuge at 4°C for 3 min. The supernatant was used as cytoplasmic enzyme. The pellet was solublized in 0.5 ml of the above buffer with 1% Triton X-100 for 20 min at room temperature and then recentrifuged. This supernatant was used as the solublized membrane enzyme. Both fractions were bound to 0.1 ml DEAE columns and eluted stepwise with 300 $\mu$l of the same buffer containing 50, 300, and 500 mM KCl. Protein kinase C activity eluted at 300 mM. The assay contained 50 $\mu$l of enzyme, 10 $\mu$l of 100 mM $MgCl_2$, 10 $\mu$l of histone III 1 mg/ml, and either 20 $\mu$l of 0.1 M EDTA or 10 $\mu$l of freshly sonicated PS (1 mg/ml) and 10 $\mu$l of 16 mM $CaCl_2$. After standing for 5 min, 10 $\mu$l of 1 mM ATP (1,000 cpm/pmol) was added and reacted for 5 min at 22°C. It was stopped by spotting 75 $\mu$l onto phosphocellulose paper and adding

this to 75 mM phosphoric acid. This was washed four times for 10 min, dried, and counted by liquid scintillation. The difference between the tubes with EDTA and those with PS and $CaCl_2$ was taken to represent protein kinase C activity.

Statistical comparisons were done using the Student's *t*-test.

## RESULTS

Table I shows that EGF stimulated the growth of human foreskin fibroblasts when added to DME in the presence of 2% FCS. We found that EGF stimulated PI turnover in human foreskin fibroblasts when tested at 1 and 5 min but not at 30 min. Table II shows significant increases in all inositol phosphates at 1 and 5 min. Table III shows an increase in $PIP_2$ at 1 min and PIP, $PIP_2$, and PA at 5 min. To eliminate the possibility that the increase in the $IP_3$ peak was due to an increase in ATP that co-migrates with $IP_3$ on these columns, we separated ATP from $IP_3$ by thin-layer chromatography and found that there was no difference in the amount of ATP between control and EGF columns (data not shown).

Since PI turnover is believed to activate protein kinase C [18] and some of the actions of EGF are proposed to be mediated by this enzyme (reviewed in [19]), we attempted to determine if EGF activated protein kinase C in human foreskin fibroblasts. Table IV shows no activation or translocation of protein kinase C, as measured by its ability to phosphorylate histone, at any time point. We also performed this experiment in the presence of 2% serum (the condition in which the growth data in Table I were obtained) and found identical results (data not shown). PMA was used as a positive control and showed the expected [18] translocation of activity from cytoplasm to

**TABLE I. EGF Stimulates Growth of Human Foreskin Fibroblasts**

| Day 0 | Day 6—Control | Day 6—EGF |
|---|---|---|
| 6,900 | 22,950 ± 9,000 | 67,050 ± 14,850* |

Cells were plated overnight in DME with 10% serum in 24-well plates. The cells were washed and placed in DME with 2% serum, either with or without 100 nM EGF. The media was changed every 2 days, and the cells were removed with trypsin and counted using a Neubauer chamber on day 6. Values, in cells/well, are the mean ± standard deviation of three experiments.
*$P < .05$.

**TABLE II. EGF Stimulates Inositol Phosphate Release in Human Foreskin Fibroblasts**

| | $IP_1$ | $IP_2$ | $IP_3$ |
|---|---|---|---|
| Control | 100 ± 39.4 | 100 ± 39.4 | 100 ± 27.8 |
| EGF—1 min | 188 ± 22.1* | 212 ± 34.4** | 158 ± 25.8* |
| EGF—5 min | 297 ± 19.8*** | 271 ± 15.0*** | 233 ± 16.8*** |
| EGF—30 min | 86 ± 2.0 | 104 ± 6.0 | 67 ± 8.0 |

Cells were grown in DME with 2% serum in 24-well plates and labelled for 72 h with $^{32}P$ as described in Materials and Methods. One hundred nM of EGF was applied for the indicated time and the inositol phosphates extracted as described in Materials and Methods. Values are % of control with no EGF being assigned 100%. They are expressed as mean ± standard deviation of four experiments.
*$P < .05$.
**$P < .02$.
***$P < .01$.

**TABLE III. EGF Stimulates Inositol Phospholipids in Human Foreskin Fibroblasts**

| | PI | PIP | $PIP_2$ | PA |
|---|---|---|---|---|
| Control | 100 ± 19.8 | 100 ± 49.2 | 100 ± 32.5 | 100 ± 51.6 |
| EGF—1 min | 138 ± 59.8 | 97 ± 40.1 | 376 ± 61.1* | 162 ± 12.2 |
| EGF—5 min | 139 ± 20.3 | 269 ± 21.8* | 332 ± 17.2** | 403 ± 21** |
| EGF—30 min | 128 ± 57 | 77 ± 26 | 181 ± 74 | 77 ± 4 |

Cells were grown in DME with 2% serum in 24-well plates and labelled for 72 h with $^{32}P$ as described in Materials and Methods. One hundred nM of EGF was applied for the indicated time and the inositol phospholipids extractd as described in Materials and Methods. Values are % of control with no EGF being assigned 100%. They are expressed as mean ± standard deviation of four experiments.
*$P < .05$.
**$P < .02$.

**TABLE IV. EGF Does Not Activate Protein Kinase C in Human Foreskin Fibroblasts**

| | Cytoplasm | Membrane | Membrane/cytoplasm |
|---|---|---|---|
| Control | 8 ± 4.6 | 0.41 ± .24 | 42% ± 25% |
| EGF—2 min | 15.2 ± 9.6 | 0.19 ± .05 | 12% ± 1% |
| EGF—10 min | 18.2 ± 9.8 | 0.14 ± .04 | 10% ± 1% |
| EGF—30 min | 11.6 ± 7 | 0.12 ± .03 | 12% ± 5% |
| PMA | 0.6 ± 0.4 | 0.85 ± .06* | 550% ± 7%** |

Cells were grown in 60 mm plates in DME with 2% serum but kept serum free for 24 h before the experiment. One hundred nM of EGF was added for the indicated time in DME with 1% BSA. One μM PMA was added for 10 min as a positive control. Values are expressed as pmol/min $^{32}P$ incorporated except for the membrane/cytoplasm ratio, which is expressed as %. They represent mean ± standard deviation of three experiments.
*$P < .05$.
**$P < .001$.

membrane fractions. We also assessed the phosphorylation of an 80K protein that has been used as a marker of protein kinase C activation [20,21] and did not find any stimulation by EGF (data not shown).

## DISCUSSION

Most reports of EGF stimulating PI turnover have been in cells with increased numbers of EGF receptors [1–6]. These cell types often respond atypically to EGF, perhaps because of the increased receptor number. For example, A431 cells are stimulated to divide only at very low concentrations of EGF and higher levels cause growth inhibition [7]. Such cells, therefore, may not be good models for investigating possible second messengers for the mitogenic actions of EGF.

We have found that EGF stimulates PI turnover in a primary cell culture, human foreskin fibroblasts, that responds mitogenically to EGF. Others [9,11] have also shown PI turnover by EGF in normal but non-primary cell lines. Although this in no way proves that PI turnover is essential for the mitogenic action of EGF, it may provide a useful system for further tests of this hypothesis. It also shows that activation of PI turnover is not simply due to an increased number of EGF receptors.

We were surprised to find no apparent activation of protein kinase C as this enzyme is usually activated by diacylglycerol, one of the second messengers released by

the PI cycle [18]. Table IV shows no increase in enzyme activity or translocation from cytoplasm to membrane by EGF, as compared with the phorbol ester PMA. In fact, EGF apparently decreases the amount of protein kinase C activity found in the membrane, although this did not achieve statistical significance. We performed the assays after serum was absent for 24 h to prevent serum from masking any EGF stimulation. However, we cannot exclude the possibility that C kinase is activated at a level that is not detected by our assay. Although EGF is generally assumed to activate protein kinase C (reviewed in [19]), close examination of the literature finds that only one paper [22] shows a direct increase in protein kinase C activity in response to EGF and this was in A431 cells with a large number of EGF receptors. Thus, protein kinase C activation may not be stimulated by EGF in more typical cell lines with normal numbers of EGF receptors.

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