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# Stimulation by epidermal growth factor of inositol phosphate production in plasma membranes from A431 cells

Bernard Payrastre, Monique Plantavid and Hugues Chap

*INSERM Unité 326, Phospholipides membranaires, Signalisation cellulaire et Lipoprotéines, Hôpital Purpan, Toulouse (France)*

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**Plasma membranes were isolated from A431 cells previously labelled with *myo*-[$^3$H]inositol during exponential growth, using a rapid procedure on Percoll gradients. They displayed a significant phospholipase (PLC) activity against phosphoinositides, which was stimulated by guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS), epidermal growth factor (EGF) and fetal calf serum (FCS) (24%, 11% and 97% over controls, respectively). The effect of EGF was not significantly increased by GTPγS. Upon addition of cytosol, EGF promoted an almost 100% stimulation of inositol 1,4,5-trisphosphate and inositol bisphosphate generation, which displayed an absolute requirement for GTPγS. This dose-dependent effect of cytosol was linear until 60 μg/ml of cytosolic protein and decreased afterwards; it was abolished by heat treatment and trypsin hydrolysis, and it was not reproduced by an identical amount of bovine serum albumin. The same biphasic stimulation was observed with phosphotyrosyl proteins immunopurified from cytosol of A431 cells previously stimulated by EGF. Since phosphotyrosyl proteins displayed PLC activity, our data suggest that soluble protein substrates of EGF receptor tyrosine kinase, including PLC, could be involved in the regulation of phosphoinositide hydrolysis in response to EGF. Using phosphatidyl[$^3$H]inositol 4,5-bisphosphate ($PIP_2$) dispersed with unlabelled phosphatidylethanolamine and phosphatidylserine as an exogenous substrate, no stimulation of PLC activity by EGF could be detected, either with membranes or with membranes plus cytosol. It is concluded that EGF might stimulate hydrolysis of phosphoinositides by PLC through complex interactions between plasma membrane and cytosolic factors which still remain to be identified.**

## Introduction

It is now well established that the tyrosine kinase activity intrinsic to epidermal growth factor (EGF) receptor is essential to the cellular effects of this growth factor [1–3]. EGF binding leads, probably through several mechanisms including dimerization of the receptor [4–7], to activation of tyrosine kinase, resulting in phosphorylation of some specific target proteins, all of which are not yet identified [6].

On the other hand, the involvement of phospholipase (PLC) in the transduction of signals evoked by EGF is much more controversial and appears to depend on the cell type. For instance, EGF fails to induce phosphoinositide breakdown in Balb/c 3T3 fibroblasts [8], in Swiss 3T3 fibroblasts [9] and in CHL fibroblasts [10], whereas the formation of inositol 1,4,5-trisphosphate ($IP_3$) is stimulated in hepatocytes [11,12]. In human A431 carcinoma cells, studies dealing with either phosphoinositide metabolism [13–17] or measurements of cytoplasmic free $Ca^{2+}$ concentration [14,18] are now in agreement with the view that EGF mobilizes $Ca^{2+}$ from an intracellular source through $IP_3$ generation [14,18], in addition to a large influx of extracellular $Ca^{2+}$ [13,19,20]. Since A431 cells overexpress EGF receptors, this suggests that the number of EGF receptors on the cell surface could be the crucial point for this biochemical effect to occur [15,21].

The most intriguing question is to understand the

Abbreviations: EGF, epidermal growth factor; FCS, fetal calf serum; G-protein, guanine nucleotide-binding protein; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); $IP_3$, inositol 1,4,5-trisphosphate; $IP_2$, inositol bisphosphate; $IP_1$, inositol monophosphate; PDGF, platelet-derived growth factor; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; DMEM, Dulbecco's modified Eagle's medium.

Correspondence: H. Chap, INSERM Unité 326, Phospholipides membranaires, Signalisation cellulaire et Lipoprotéines, Hôpital Purpan, 31059 Toulouse Cedex, France.

molecular events linking EGF receptor to the stimulation of inositol phosphate production. A guanine nucleotide regulatory protein (G-protein) appears to be involved in the activation of PLC by many hormones and neurotransmitters, including 5-hydroxy tryptamine, $\alpha_1$-adrenergic and $M_1$-muscarinic agonists, as well as angiotensin II [22–26]. However, the knowledge on the structure of a number of receptors coupled to G-proteins has recently greatly improved [27,28] and it is obvious that EGF receptor belongs to a different class of receptors, containing only one transmembrane region, together with a large cytoplasmic domain displaying tyrosine kinase activity. This is in contrast with the seven membrane spanning segments present in receptors coupled to G-proteins [27,28]. Although this does not exclude EGF receptor be coupled to G-proteins, as suggested by several recent studies [11,29], the interaction mechanism is probably different. Several recent reports demonstrating that the intrinsic tyrosine kinase of EGF receptor is essential for the activation of $PIP_2$ degradation [30] and that an isozyme of PLC, PLC-$\gamma$, is both a substrate, in intact cells and in vitro, of EGF receptor [31–35] as well as PDGF receptor [33,36], suggest that tyrosine phosphorylation of soluble PLC-$\gamma$ may represent the mechanism linking EGF receptor and stimulation of $PIP_2$ hydrolysis. However, if in intact cells, it exists a correlation between tyrosine phosphorylation of PLC-$\gamma$ with the increased hydrolysis of phosphoinositides [31,36], the activity in vitro of the phosphorylated PLC-$\gamma$ is not affected [34,36,37].

We recently developed a rapid method to isolate plasma membranes from A431 cells, where EGF receptor still displays its most essential characteristics [38]. The present studies were undertaken in order to examine the effect of EGF on PLC in a cell-free system. EGF was compared to fetal calf serum (FCS), which was previously shown to stimulate $Ca^{2+}$ mobilization from internal stores of intact A431 cells more efficiently than EGF [19]. This procedure was chosen to investigate the role of G-proteins in PLC activation and to examine in a more physiological approach than in previous studies [34] the effects of cytosoluble proteins and cofactors on the hydrolysis of plasma membrane phosphoinositides.

## Materials and Methods

*Materials*

Percoll (poly(vinyl pyrollidone)-coated silica particles) was from Pharmacia, Uppsala, Sweden. Dulbecco's modified Eagle's medium (DMEM) and FCS were from Gibco, Cergy-Pontoise, France. Calf serum was from I.B.F, Villeneuve-la-Garenne, France. Guanosine 5′-*O*-(3-thiotriphosphate) (GTP$\gamma$S) was from Boehringer, Mannheim (F.R.G.). EGF and all other chemicals were obtained from Sigma, Saint-Louis, MO (U.S.A.). Sepharose-linked monoclonal antiphosphotyrosine antibody, *myo*-[2-$^3$H]inositol, aqueous solution (18.7 Ci/mmol), phosphatidyl[2-$^3$H]inositol 4,5-bisphosphate (1.0 Ci/mmol), [$\gamma$-$^{32}$P]ATP (3000 Ci/mmol) were purchased from Amersham International (U.K.).

*Cell culture*

A431 cells were routinely cultured in DMEM containing 5% (v/v) FCS, 100 U/ml penicillin and 100 $\mu$g/ml streptomycin in 75 cm$^2$ flasks gassed with humidified 5% $CO_2$, 95% air at 37°C.

*[$^3$H]Inositol labelling of intact A431 cells*

Cells were incubated in culture dishes (140 mm × 20 mm), using inositol-free DMEM (Institut J. Boy, Reims, France) supplemented with 5% (v/v) calf serum, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 2 $\mu$Ci/ml of *myo*-[2-$^3$H]inositol, at 37°C for approximately 48 h. Cells were then about 2/3 confluent. They were washed twice with 15 ml NaCl (0.9%, w/v), scraped with a rubber policeman into NaCl and harvested by centrifugation at 200 × *g* for 10 min.

*Plasma membrane isolation*

Cells were disrupted at 4°C by the nitrogen cavitation technique in a Kontes pressure homogenizer (Vineland, NJ, U.S.A.) as previously described [38,39], except that leupeptin (50 $\mu$g/ml), phenylmethylsulfonyl fluoride (2 mM) and EGTA (2 mM) were added to the lysis buffer as proteinase inhibitors. Plasma membranes were isolated on Percoll density gradient at pH 9.6 as described [38]. After fractionation, the plasma membrane fraction was immediately diluted (3-fold) with 50 mM Tris-buffer (pH 7.4) containing 1 mM ATP. Percoll beads were removed by centrifugation at 130 000 × *g* for 40 min. Plasma membranes stuck at the surface of Percoll were collected in buffer A containing 100 mM KCl, 20 mM NaCl, 0.96 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 1 mM EGTA and 5 mM $MgSO_4$ (pH 7.2) and they were homogenized by sonication on ice for 3 × 10 s, using an MSE sonicator at maximal output.

*Cytosol preparation*

A431 cells were suspended in a 50 mM Tris-maleate buffer (pH 7.35) and lysed by the nitrogen cavitation procedure as described above. The homogenate was rid of cellular debris by centrifugation (1000 × *g* for 10 min, 4°C) and the supernatant was subjected to a second centrifugation at 105 000 × *g* for 60 min at 4°C. The final pellet corresponded to the crude membrane fraction and supernatant to the soluble fraction (cytosol).

*Immunopurification of phosphotyrosyl proteins from cytosol*

About 2/3 confluent cells were treated during 5 min at 37°C with 250 ng/ml EGF. After washing twice with

isotonic NaCl, cells were scraped into NaCl and harvested by centrifugation at 200 × *g* for 10 min. A431 cells were then suspended in a buffer containing 20 mM Hepes (pH 7.2), 50 mM NaCl, 30 mM sodium pyrophosphate, 5 mM β-glycerophosphate, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate and 10 μg/ml leupeptin, and lysed by the nitrogen cavitation procedure. The cytosol was then obtained as described above and treated during 2 h 30 min at 4°C with 50 μl of Sepharose-linked antiphosphotyrosine antibody. The bead matrix was then batch-washed with the following buffers: (1) 138 mM NaCl, 8 mM $Na_2HPO_4$ and $KH_2PO_4$ (pH 7.4); (2) 500 mM LiCl and 100 mM Tris-HCl (pH 7.6); (3) distilled water; and (4) 20 mM Tris-HCl, 100 mM NaCl and 1 mM EDTA (pH 7.6). Phosphotyrosyl proteins were eluted by addition of 100 μl of a buffer containing 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EGTA and 15 mM phenylphosphate for 10 min at 4°C.

*Measurement of endogenous phosphoinositide breakdown*

[$^3$H]Inositol-labelled plasma membranes (100 μg proteins) were incubated immediately in 0.5 ml of buffer A containing 1 mM ATP and 10 mM LiCl. The free $Ca^{2+}$ concentration was adjusted to $10^{-6}$ M by addition of appropriate amounts of $CaCl_2$ as described [40]. Growth factors and other effectors were added as indicated. Incubations were carried out at 37°C for 10 min and they were stopped by adding 0.05 ml of 200 mM EDTA (pH 7.4). Lipids were immediately extracted with an acidic procedure [41,42]. The upper phase contained free [$^3$H]inositol plus [$^3$H]inositol phosphates, whereas [$^3$H]inositol phosphoinositides were in the lower phase.

*Assay of PLC against exogenous substrate*

Membrane-associated or soluble PLC activities were also determined using [$^3$H]$PIP_2$ as exogenous substrate. Vesicles were prepared essentially as described [43,44] by mixing [$^3$H]$PIP_2$ (30000 dpm, 1 nmol/assay) with phosphatidylethanolamine and phosphatidylserine in a molar ratio 1 : 8 : 2. Membranes (10 μg protein) or membranes plus soluble fraction (10 μg protein) were incubated in 20 mM Tris-maleate buffer (pH 7.4), containing 1.3 μM free $Ca^{2+}$ (adjusted with $Ca^{2+}$/EGTA), in the presence or absence of 100 μM GTPγS, 250 ng/ml EGF or 10% (v/v) FCS as indicated, under a final volume of 0.1 ml. Incubation proceeded at 37°C for 10 min and was terminated as described above.

*Lipid analysis*

Inositol lipids were separated by monodimensional thin-layer chromatography on silica gel-coated plates (Merck, Darmstadt, F.R.G.) using chloroform/methanol/4.3 M $NH_4OH$ (90 : 70 : 20, v/v) as a solvent [42]. The radioactive spots were located and quantified with a thin-layer chromatography radioactivity monitor (Berthold LB 2842) or scraped off and counted in Instagel (Packard, Groningen, The Netherlands).

*Separation of inositol phosphates*

Analysis of individual inositol phosphates was carried out from upper phases of lipid extraction. They were neutralized with NaOH, diluted with water to 5 ml and applied to 1 ml columns of AG1-X8 anion exchange resin (Bio-Rad, Richmond, CA, U.S.A.). Sequential elutions were performed according to Berridge et al. [45]. Anion-exchange HPLC separation of inositol phosphates was performed according to Ref. 46.

*Other analytical methods*

Protein was determined by the procedure of Lowry et al. [47] using bovine serum albumin as a standard. Separation of proteins by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed using 10–15% polyacrylamide gradient from Phastsystem (Pharmacia-LKB, Uppsala, Sweden) and silver staining with a kit provided by the furnisher. Phosphorus measurements were performed according to Böttcher et al. [48].

*Expression of data and statistical analysis*

Owing to a large variability in the [$^3$H]inositol-labelling pattern between membranes isolated during different experiments, a direct comparison of radioactivity counts could not be performed. Thus in all experiments, the control values were normalized to a conventional value of 100%, and comparison with various assays (upon addition of EGF, GTPγS, or cytosol, for instance) were compared to this 100% value using paired Student's *t*-test.

## Results

*Distribution of [$^3$H]inositol in phosphoinositides of plasma membranes isolated from A431 cells*

As previously described [38], purified plasma membranes were obtained within 2 h and displayed an enrichment ratio of 4.5 for [$^3$H]concanavalin A bound to intact cells prior to disruption, and of 4.4 for 5'-nucleotidase activity (not shown). As illustrated in Table I, this was accompanied by a relative enrichment in phosphatidylinositol 4-phosphate (PIP) and $PIP_2$. Indeed, the sum of [$^3$H]PIP and [$^3$H]$PIP_2$ accounted for 27.1% of the plasma membrane radioactivity, against 3.1% in whole cells. This suggests a preferential localization of polyphosphoinositides in the plasma membrane, as previously shown in human platelets [49]. The [$^3$H]$PIP_2$/[$^3$H]PIP ratio was 0.72 in intact cells against 0.46 in plasma membrane, indicating some specific de-

gradation of the former phospholipid during the isolation procedure, despite the presence of 1 mM ATP in the washing buffer. These membranes were then used to study PLC activation.

*PLC activation by EGF and FCS in isolated plasma membranes from A431 cells*

Plasma membranes isolated from A431 cells previously labelled with [$^3$H]inositol were incubated in a medium mimicking the ionic composition of cytosol, also containing 1 mM ATP and $10^{-6}$ M free $Ca^{2+}$. Under these conditions, EGF induced a slight increase of inositol phosphate production (46–47% over controls for $IP_3$ and inositol bisphosphate or $IP_2$), significant only for $IP_2$ (Table II). In contrast, FCS promoted a higher increase of $IP_2$ and $IP_3$ (168% and 258% over controls, respectively, see Table II). GTPγS alone was also able to stimulate PLC activity (25–35% over controls), suggesting a possible involvement of G-proteins similar to that previously described in other cells [22–26]. However, no synergism could be observed upon simultaneous addition of EGF and GTPγS (Table II).

The effect of FCS was not due to the increase in free $Ca^{2+}$ concentration (0.25 mM) brought about by endogenous $Ca^{2+}$ present in serum, since it was verified that increasing free $Ca^{2+}$ concentration up to 1 mM did not change inositol phosphate production under our assay conditions. Moreover, calf serum, which does not contain the same growth factors as those present in FCS, did not exert any significant effect on inositol phosphate production (106 ± 12% of controls, mean ± S.E., three experiments, in the presence of GTPγS).

In some experiments giving the highest increase in $IP_3$ radioactivity (mainly with FCS), $IP_3$ was identified as the 1,4,5-isomer by anion-exchange HPLC analysis. When GTPγS was added together with growth factors, $IP_3$ displayed a further significant increase only with FCS ($P < 0.02$), indicating that at least part of PLC activation by FCS is mediated by G-proteins, which is not the case with EGF (Table II).

TABLE I

*Distribution of radioactivity in phosphoinositides of whole cells and plasma membranes after incorporation of [$^3$H]inositol*

Data correspond to percentages of total radioactivity present in lipid extracts from intact cells or plasma membranes (5800 ± 1000 dpm/μg protein) isolated by Percoll gradient and are means ± S.E. of five and four different preparations for whole cells and for plasma membranes, respectively).

| | Whole cells (%) | Plasma membranes (%) |
|---|---|---|
| Phosphatidylinositol | 92.9 ± 1.0 | 71.9 ± 6.0 |
| Lysophosphatidylinositol | 4.0 ± 1.0 | 0.9 ± 0.2 |
| Phosphatidylinositol 4-phosphate | 1.8 ± 0.2 | 18.5 ± 2.4 |
| Phosphatidylinositol 4,5-bisphosphate | 1.3 ± 0.3 | 8.6 ± 0.3 |

TABLE II

*Effects of growth factors and guanine nucleotides on inositol phosphate production in A431 plasma membranes*

Plasma membranes (100 μg proteins) from A431 cells prelabelled with *myo*-[2-$^3$H]inositol were incubated at 37°C for 10 min, alone or in the presence of 100 μM GTPγS, 250 ng/ml EGF, 250 ng/ml EGF plus 100 μM GTPγS, 10% (v/v) FCS or 10% (v/v) FCS plus 100 μM GTPγS. Incubation was performed as described under Materials and Methods. Mean distribution of radioactivity in control samples (40000 ± 10000 dpm) was as follows (free inositol, 34%; $IP_1$, 26%; $IP_2$, 31%; $IP_3$, 9%). Data are expressed as percentages of control values (plasma membranes alone) and are means ± S.E. of eight or five separate experiments performed in duplicate in the first and second series, respectively.

| Assays | $IP_1$ (%) | $IP_2$ (%) | $IP_3$ (%) |
|---|---|---|---|
| First series | | | |
| Plasma membranes | 100 | 100 | 100 |
| Plasma membranes plus GTPγS | 148 ± 15 | 136 ± 6 [a] | 126 ± 3 [c] |
| Plasma membranes plus EGF | 199 ± 30 | 147 ± 7 [a] | 146 ± 9 |
| Plasma membranes EGF plus GTPγS | 186 ± 27 | 161 ± 11 | 153 ± 7 [b] |
| Plasma membranes plus FCS | 239 ± 37 | 268 ± 33 [a] | 358 ± 49 [a] |
| Plasma membranes plus FCS plus GTPγS | 248 ± 33 | 295 ± 25 [a] | 462 ± 55 [b] |
| Second series | | | |
| Plasma membranes plus EGF plus GTPγS | 125 ± 5 | 135 ± 9 | 148 ± 12 |
| Plasma membranes plus EGF plus GTPγS plus soluble fraction | 149 ± 11 [d] | 190 ± 25 [d] | 196 ± 18 [d] |

[a] $P < 0.05$; [b] $P < 0.02$; [c] $P < 0.001$: comparison to plasma membranes; [d] $P < 0.05$: comparison to plasma membranes plus EGF plus GTPγS plus soluble fraction versus plasma membranes plus EGF plus GTPγS without soluble fraction.

*Effect of cytosolic proteins on the hydrolysis of plasma membrane phosphoinositides*

Baldassare and Fisher [50] previously reported that addition of cytosol to platelet plasma membranes results in an increased degradation of $PIP_2$ when GTPγS is present in the assay. Data of Table II (second series of experiments) indicate that addition of cytosol to plasma membranes incubated with EGF plus GTPγS resulted in a further significant increase of inositol phosphate production. In order to study in more detail the mechanism of this increase promoted by cytosolic fraction, the release of hydrosoluble radioactivity was

TABLE III

*Effects of cytosolic fraction on inositol phosphate production induced by EGF and FCS*

Plasma membranes from [$^3$H]inositol-labelled A431 cells (100 μg protein) were incubated alone or with soluble fraction (30 μg protein) in the presence or absence of growth factors as indicated, for 10 min, at 37°C. Assays were performed as described under Materials and Methods. Data correspond to total water soluble radioactivity and are expressed as percentages of control values (incubation of plasma membranes alone). They are means ± S.E. of three different experiments performed in duplicate.

| Effectors added | Plasma membranes (%) | Plasma membranes plus soluble fraction (%) |
|---|---|---|
| None | 100 | 105.9 ± 6.3 |
| GTPγS (100 μM) | 123.9 ± 4.2 | 134.9 ± 2.2 [a] |
| EGF (250 ng/ml) | 110.8 ± 4.8 | 106.7 ± 6.8 |
| EGF (250 ng/ml) plus GTPγS (100 μM) | 122.5 ± 3.3 | 188.8 ± 7.7 [a,b,c] |
| FCS (10%, v/v) | 197.5 ± 17.7 | 284.7 ± 22.2 [a] |
| FCS (10%, v/v) plus GTPγS (100 μM) | 214.7 ± 22.0 | 306.5 ± 26.0 [a] |

[a] Probability of significance according to Student's *t*-test (plasma membranes plus soluble fraction versus plasma membranes without soluble fraction): $P < 0.05$.

[b] $P < 0.02$, comparison to the same assay with GTPγS alone.

[c] $P < 0.05$, comparison to the same assay with EGF alone.

determined under various conditions. As shown in Table III, the 105 000 × *g* supernatant from A431 cells did not increase the basal hydrolysis of phosphoinositides, even in the presence of EGF alone. Surprisingly, when both EGF and GTPγS were present in the incubation medium, then the addition of soluble fraction significantly increased the formation of inositol phosphates (from 23% over controls in the assay without cytosol to 89% over controls in the presence of cytosol). The same effect was observed with either FCS alone or FCS plus GTPγS.

As shown in Fig. 1A, cytosol increased the generation of water soluble [$^3$H]inositol compounds promoted by EGF plus GTPγS in a dose dependent manner up to a concentration of 60 μg/ml protein, and then levelled off. In contrast to Cockcroft and Stutchfield [51], who showed a selective increase of inositol monophosphate ($IP_1$) upon addition of cytosol to membranes from HL 60 cells, $IP_3$ as well as $IP_2$ and $IP_1$ were all increased upon addition of homologous cytosol to plasma membranes from A431 cells incubated with EGF plus GTPγS (Table II).

In order to better characterize the cytosolic factor(s) responsible for the stimulation of inositol phosphate production in plasma membranes, cytosol was submitted to heat treatment (5 min at 100°C, three experiments) or incubated with trypsin linked to agarose beads (2 U per 60 μg cytosolic proteins, for 5 min at 37°C, two experiments). This resulted in 96% and 69% loss of stimulatory activity, respectively. It was also verified that bovine serum albumin (60 μg/ml, i.e., the concentration of cytosolic proteins giving the highest stimulation) did not modify the activity of PLC in plasma membranes. Finally, phosphotyrosyl proteins were isolated from cytosol obtained from A431 cells stimulated by EGF, using an antiphosphotyrosine antibody linked to Sepharose.

Analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of proteins immunoprecipitated with antiphosphotyrosine antibody revealed an enrichment in three main bands running with an apparent molecular mass of 67, 55 and 27 kDa, together with several other minor polypeptides (Fig. 2).

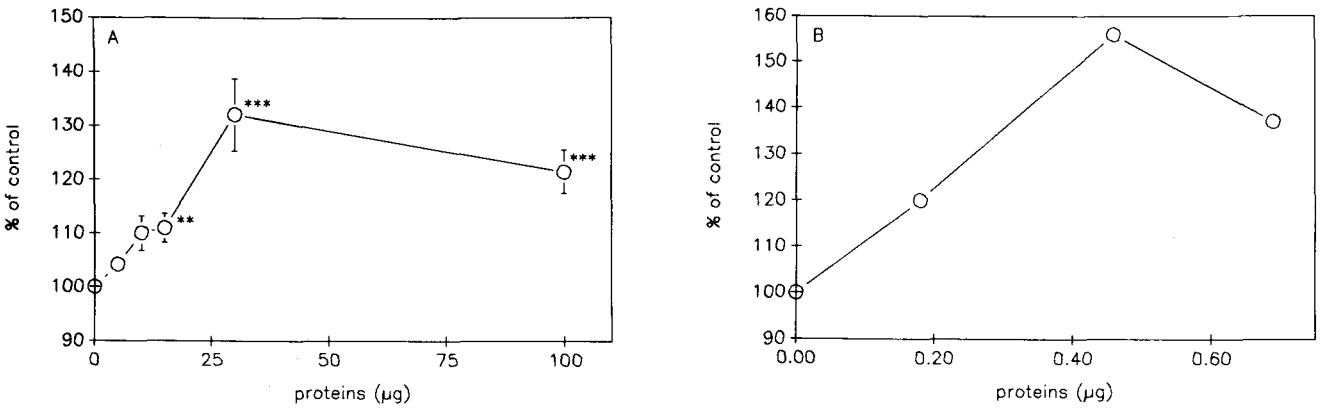


Fig. 1. Effect of increasing concentrations of soluble proteins and phosphotyrosyl proteins on inositol phosphate production induced by EGF in the presence of GTPγS. Plasma membranes (100 μg proteins) from [$^3$H]inositol-labelled A431 cells were incubated in the presence of increasing amounts of cytosolic proteins (A) or of soluble proteins immunoprecipitated with antiphosphotyrosine antibody (B). Assays were performed as described under Materials and Methods in the presence of EGF (250 ng/ml) and GTPγS (100 μM). Controls contained identical volumes of 50 mM Tris-maleate buffer (pH 7.35) (A) or of the buffer used for elution of phosphotyrosyl proteins (B). Data correspond to total water soluble radioactivity and are expressed as percentages of control values (plasma membranes incubated with EGF and GTPγS). They are means ± S.E. of five experiments (A) or from two experiments (B). Probability of significance according to paired Student's *t*-test: **, $P < 0.02$; ***, $P < 0.01$.

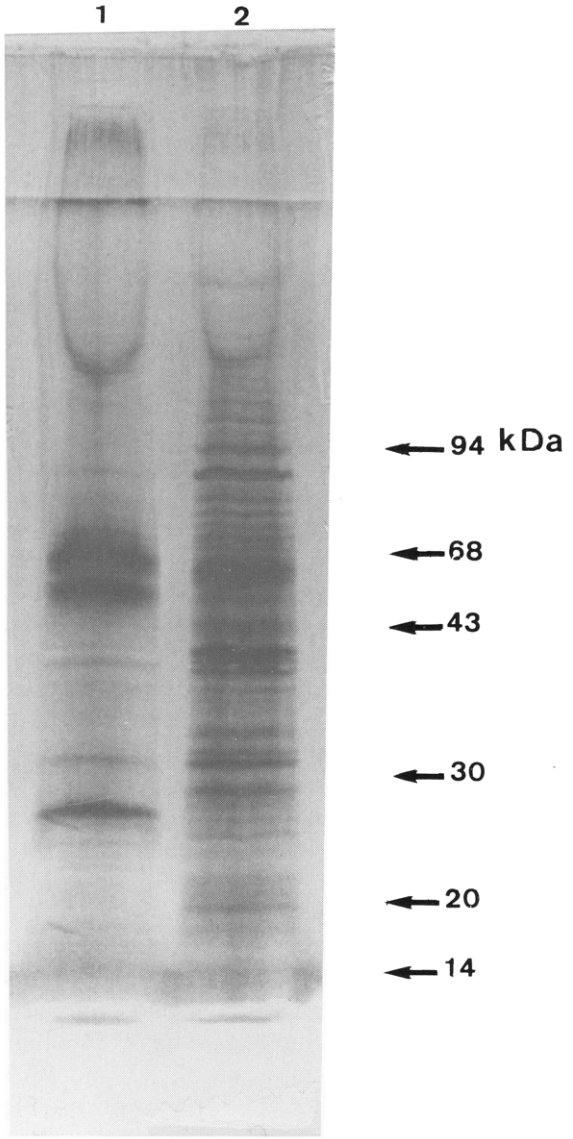


Fig. 2. SDS-PAGE of proteins from antiphosphotyrosine immunoprecipitate and from cytosol. Proteins from antiphosphotyrosine immunoprecipitate (lane 1, 0.03 μg) or from crude cytosol (lane 2, 0.2 μg) were electrophoresed as described under Materials and Methods and detected by silver staining, Arrows indicate the positions of molecular mass standards: phosphorylase *b* (94 kDa); bovine serum albumin (68 kDa); ovalbumin (43 kDa); carbonic anhydrase (30 kDa); trypsin-inhibitor (20.1 kDa); α-lactalbumin (14.1 kDa).

Immunoprecipitated proteins displayed PLC activity against exogenous [$^3$H]$PIP_2$ (2.8 and 6.4 pmol · $min^{-1}$ · $10^{-8}$ cells in two separate experiments), and this activity was sensitive to trypsin treatment (not shown). In contrast, no detectable PLC activity was measured in phosphotyrosine proteins isolated from a control cytosol, in agreement with Wahl et al. [37]. Furthermore, previous experiments revealed that no phosphotyrosine proteins could be immunoprecipitated from A431 cells non treated with EGF [52].

As shown in Fig. 1B, much lower amounts of proteins were required to increase inositol phosphate production in plasma membranes. However in this case, a more pronounced biphasic response could be observed, maximal stimulation occurring at 0.92 μg/ml phosphotyrosyl proteins, followed by inhibition of PLC activity at higher protein concentrations.

*Hydrolysis of exogenous [$^3$H]inositol-$PIP_2$ by membrane associated and soluble PLC from A431 cells*

An about twice higher PLC activity against exogenous [$^3$H]$PIP_2$ could be detected in cytosol from A431 cells, as compared to plasma membranes (607 ± 79 versus 335 ± 51 pmol · $min^{-1}$ · $mg^{-1}$, means ± S.E., three experiments). This contrasts with the failure of cytosol to promote hydrolysis of $PIP_2$, PIP or phosphatidylinositol (PI) present in plasma membranes, unless both EGF and GTPγS were present in the medium (Table III). However, PLC activities detected in cytosol and plasma membranes were not additive (684 ± 13 pmol · $min^{-1}$ · $mg^{-1}$), despite the fact that both soluble and membrane-bound PLC activities were determined in the linear range of protein concentration. Moreover, at a variance with data previously obtained with endogenous substrates present in the plasma membranes, no significant effect of EGF plus GTPγS on PLC could be detected with [$^3$H]$PIP_2$ as an exogenous substrate (652 ± 15 pmol · $min^{-1}$ · $mg^{-1}$).

## Discussion

The increase in inositol phosphate production induced by EGF is now well established in some cell types such as hepatocytes [11,12] or cells overexpressing EGF receptor such as A431 cells [13–17,21]. Based on the use of a cell-free system, the present study reports the first demonstration that EGF is able to stimulate membrane-bound PLC. However, this stimulation was always very weak, much less important compared to that evoked by FCS. In agreement with the present observation, the latter reagent, which is known to contain a mixture of various cofactors, was revealed previously more potent than EGF in inducing $Ca^{2+}$ mobilization from intracellular stores of A431 cells [19].

Furthermore, this study gives evidence that plasma membranes of A431 cells display a PLC activity stimulated by guanine nucleotides. However, our results also indicate that activation by EGF of membrane-bound PLC does not probably occur through a G-protein, since GTPγS did not enhance EGF effect, at variance with FCS. Similar findings have been recently described with PDGF in permeabilized WFB cells [53]. Like EGF, PDGF receptor contains one single membrane-spanning segment and a cytosolic tyrosine-specific protein kinase domain [54].

The main finding of the present study concerns the effect of cytosol, which greatly increased phosphodiesteratic cleavage of membrane-bound phosphoinositides.

This stimulation was observed for both EGF and FCS, but it required the presence of GTPγS only in the former case. This suggests a complex interaction between cytosol and plasma membranes, not necessarily involving G-proteins since GTPγS may produce some effects only due to its polyanionic structure (our results).

Heat and trypsin treatment helped us to establish the protein nature of the cytosolic factor(s) involved in the stimulation of inositol phosphate production by plasma membranes from A431 cells. In addition, the effect of cytosolic proteins appeared rather specific, since it could not be reproduced by equivalent amounts of bovine serum albumin. Several proteins could be proposed as putative soluble activators of inositol phosphate production. These include cytosolic PLC, a soluble G-protein identified in platelet cytosol [55], or even diacylglycerol kinase, which is translocated from cytosol to membrane in stimulated cells [56] and is able to generate phosphatidic acid, a powerful activator of PLC [57].

Based on the observation that EGF was required for the effects of cytosol to occur, and on the recent finding that cytosolic PLC-γ is a substrate of tyrosine kinase [31–35], immunopurified soluble proteins phosphorylated on tyrosine residues were examined for their ability to reproduce the stimulatory effect of cytosol. Our data strongly suggest that some soluble proteins phosphorylated by EGF receptor could regulate phosphodiesteratic cleavage of phosphoinositides. Moreover, the presence in antiphosphotyrosine immunoprecipitates of a PLC activity sensitive to trypsin might support the view that upon tyrosine phosphorylation, cytosolic PLC could be involved in an increased hydrolysis of plasma membrane phosphoinositides. However, recent data showed that tyrosine phosphorylation does not modify the in vitro activity of PLC [34].

In addition, a PLC inhibitor is probably also present among phosphotyrosyl proteins, as revealed upon increasing their concentrations. Although lipocortin I has previously been identified both as a substrate of EGF receptor tyrosine kinase [58,59] and as a nonspecific inhibitor of PLC [60], it does not seem to be responsible for the inhibition of PLC observed at higher protein concentrations, since no visible band was observed in the 35–38 kDa region of polyacrylamide gels. This further indicates a complex interaction between EGF receptor, plasma membrane and cytosol, and eliminates the possibility of a simple, direct stimulation of membrane-bound and/or soluble PLC by phosphorylation.

In conclusion, we have demonstrated that EGF alone is able to slightly stimulate inositol phosphate formation in isolated membranes by a GTP-independent mechanism. Furthermore, the soluble fraction greatly enhances phosphoinositide breakdown when EGF is present but GTPγS then becomes necessary. Although soluble PLC-γ phosphorylated by EGF receptor kinase appears as a good candidate responsible for this stimulation, our data leave open the possibility that complex interactions between EGF receptor tyrosine kinase, phospholipase(s) C, some G-proteins as well as other modulatory soluble factors are involved in the mechanism of EGF stimulation of inositol phosphate production. The experimental model used in the present study should prove useful to identify these putative factors.

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