Examination of the role of serine phosphorylation in phospholipase C-γ and its related P47 in cAMP-mediated depression of epidermal growth factor receptor signal transduction

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Forskolin-pretreatment of A431 cells reduced both intrinsic and epidermal growth factor (EGF)-induced EGF receptor phosphorylation, however, phosphorylation of pospholipase C-γ (PLC-γ) was stimulated under the same conditions. No significant difference was detected in the amount of phosphotyrosine of PLC-γ between two cultures with or without forskolin treatment followed by EGF. On the other hand, phosphorylation of a 47 kDa protein (P47) which cross-reacted with an anti-PLC-γ monoclonal antibody, was stimulated by both forskolin and EGF. Phosphorylation was exclusively on serine residues in this case. These results indicate that both PLC-γ and P47 are posphorylated by a cAMP-dependent protein kinase and the EGF-stimulated serine kinase, and suggest that serine phosphorylation of PLC-γ has no effect on ligand-dependent coupling with the EGF receptor.

Epidermal growth factor; Tyrosine phosphorylation; Phospholipase C; Cyclic AMP-dependent kinase; Human carcinoma A431 cell

1. INTRODUCTION

EGF receptor tyrosine kinase is essential for EGF-induced biochemical reactions such as phosphatidylinositol turnover which plays an important role in the receptor-mediated signal transduction pathway for DNA synthesis. Several enzymes regulate the chain of reactions and PLC is the key enzyme involved in the production of two important second messengers, diacylglycerol and inositoltriphosphate. Recently PLC-γ has been shown to be coupled with receptors of EGF and platelet-derived growth factor in a ligand-dependent fashion and to be phosphorylated on tyrosine residues [1–5]. Furthermore PLC-γ was found to be a substrate for cAMP-dependent protein kinase (protein kinase A) [6].

On the other hand, we recently demonstrated that cAMP modulated not only EGF receptor phosphorylation but also EGF-mediated cell growth inhibition at

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Abbreviations: EGF, epidermal growth factor; PLC-γ, phospholipase C-γ type; cAMP, cyclic AMP; protein kinase A, cAMP-dependent protein kinase; P47, 47 kDa phosphoprotein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; F12, Ham's F12 medium

the physiological concentration of EGF in human epidermoid carcinoma A431 cells [7]. These results indicate cAMP-mediated biochemical reactions, perhaps through protein kinase A, regulate the EGF receptor signal transduction pathway. The system of cAMP-mediated depression of EGF receptor signal transduction in A431 cells therefore provides a good opportunity to examine the role of serine phosphorylation of PLC-γ on its ability to couple with EGF receptors.

In this report, we demonstrate that cAMP-mediated serine phosphorylation of PLC-γ has no effect on the degree of tyrosine phosphorylation induced by EGF, strongly suggesting that serine phosphorylation of PLC-γ does not regulate the interaction of PLC-γ with the EGF receptor.

2. MATERIALS AND METHODS

2.1. Cell culturing
A431 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Prior to each experiment, the cells were seeded in chemically defined medium DMEM plus Ham's F12 (DMEM/F12) supplemented with insulin, transferrin, ethanolamine and fibronectin as described previously [8], except that 15 mM Hepes buffer (pH 7.4) and 30 nM Na₂SeO₃ were added.

2.2. Labeling of cells with [³²P]orthophosphate and isolation of the radio-labeled EGF receptor, PLC-γ and P47 proteins
The methods for labeling of the cells with [³²P]orthophosphate in chemically defined medium and for immunoprecipitation of EGF receptor, PLC-γ and P47 were essentially the same as described previously [9].

2.3. *Phosphoamino acid analysis and tryptic phosphopeptide mapping*

Phosphoamino acid analysis was carried out as described previously [9] with minor modifications [10]. The proteins recovered from polyacrylamide gels were filtered through an Ultra Free tube (Millipore). Samples were transferred to screw-capped polypropylene tubes (Sarstedt) to minimize loss. The samples were hydrolyzed in 6 N HCl for 2 h at 105°C and HCl was removed by centrifugation under reduced pressure (Speed Vac, Savant). The hydrolysates were separated by two-dimensional electrophoresis using a Flat Bed Apparatus Model FBE 3000 (Pharmacia). With the same apparatus, tryptic phosphopeptide mapping was carried out essentially as described previously [9].

2.4. *Materials*

Mouse EGF was obtained from Collaborative Research Inc., forskolin from Sigma, [^{32}P]orthophosphate (carrier free) from ICN. All other materials were the same as described previously [9]. Monoclonal antibodies against PLC-γ were prepared and mixtures of these antibodies were used for immunoprecipitation and immunoblotting as reported by Meisenhelder et al. [3]. Monoclonal antibody against EGF receptor (clone 528) was obtained from Oncogene Science Inc. Molecular weight markers for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad.

3. RESULTS

We have shown previously that when A431 cells were pretreated with reagents which increase cAMP levels, the degree of EGF receptor phosphorylation was decreased in both intrinsic and EGF-induced phosphorylation at a physiological concentration of EGF. On the other hand, protein kinase A phosphorylates PLC-γ but not the other forms of PLC [6].

To confirm the characteristics of the phosphorylation of EGF receptor and PLC-γ in response to increased intracellular cAMP, A431 cells labeled with ^{32}P$_i$ were pretreated with 10 μM forskolin for 60 min and then incubated with various concentrations of EGF for various times (2–10 min). Each cell lysate was separated into two groups and subjected to immunoprecipitation with antibodies against EGF receptor or PLC-γ, respectively. As shown in Fig. 1A, two major and two minor phosphoproteins were specifically immunoprecipitated with anti-PLC-γ monoclonal an-
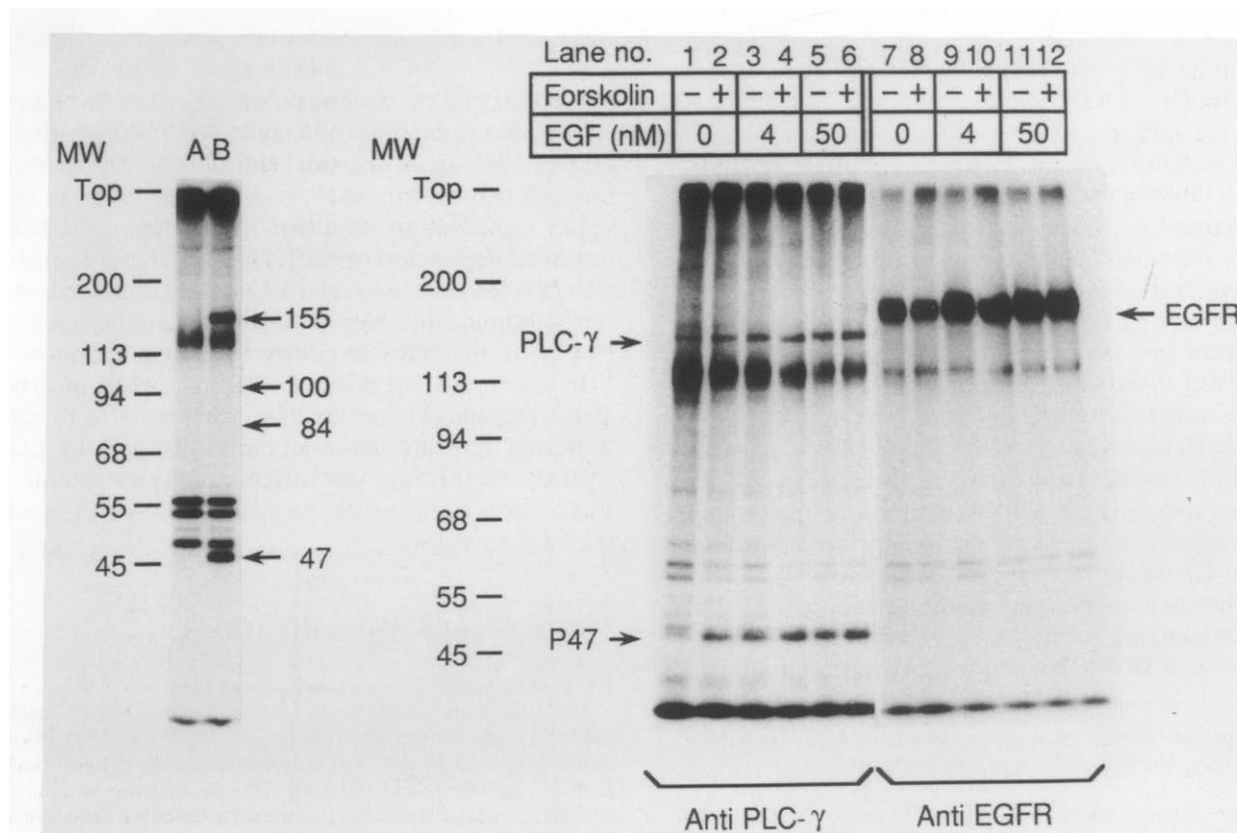

Fig. 1. Stimulation of phosphorylation of PLC-γ and P47 by forskolin and EGF. A431 cells (6 × 10^5 cells) were seeded in a 60-mm dish containing 5 ml of DMEM/F12 + 4F and cultured for 2 days. After replacement of the medium with phosphate-free F12 + 4F, the cells were cultured in the presence of ^{32}P$_i$ (0.5 mCi/ml) for 6 h. The cells were treated with 10 μM forskolin for the last 1 h and incubated for 5 min with various concentrations of EGF as indicated at the top of the panel. After lysing the cells, each cell extract was incubated with the complex of *Staphylococcus aureus* (SA)-preimmune IgG for 1 min at 0°C and centrifuged at 10000 × g for 10 min. The cleared lysates were separated into two groups, each extract was subjected to immunoprecipitation with SA-preimmune IgG (A) or SA-anti-PLC-γ (B). (Right panel) Each equal amount of cleared lysate, obtained by the incubation with SA-preimmune IgG twice, was immunoprecipitated with anti-PLC-γ antibody or anti-EGF receptor antibody. Proteins in the immunoprecipitated material were separated by electrophoresis through 10% SDS-PAGE.

tibodies as reported previously [3]; 155 kDa (PLC-γ), 100, 84 and 47 kDa (P47). At least two proteins (PLC-γ and P47) were also detected in immunoblotting with anti-PLC-γ (data not shown) indicating that these two phosphoproteins have similar antigenicity, but are likely distinct proteins. As expected, while forskolin pretreatment decreased both intrinsic and EGF-induced (4 nM EGF) phosphorylation of the EGF receptor, it stimulated the phosphorylation of both PLC-γ and P47 (Fig. 1B). The approximately 1.5-fold stimulation was reproducible. While a cAMP-mediated depression of EGF receptor phosphorylation disappeared in response to high concentration of EGF (50 nM), no such a dose effect was detected in phosphorylation of PLC-γ and P47.

We have carried out phosphoamino acid analysis of both PLC-γ and P47. As shown in Fig. 2A and B, while EGF-dependent tyrosine phosphorylation was observed in PLC-γ, P47 contained entirely phosphoserine in the presence or absence of EGF. The amounts of phosphotyrosine and phosphoserine in PLC-γ were estimated both in the cells pretreated with and without forskolin followed by EGF. Although the degree of serine phosphorylation of PLC-γ increased in the forskolin-treated culture compared to that in the untreated culture, the content of phosphotyrosine was almost the same in both cultures (Table I). The relative amount of phosphorylation on serine and tyrosine residues of PLC-γ in both cultures was not affected
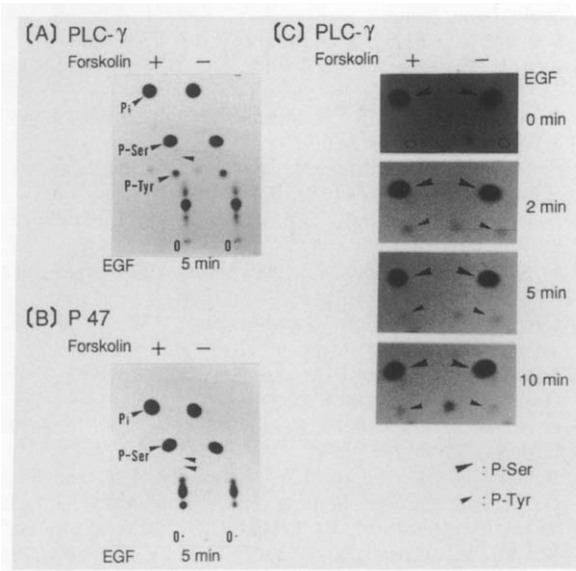
Table I

Quantitative analysis of phosphotyrosine in PLC-γ

Experiment no.	EGF (nM)	Ratio of degree of PLC-γ phosphorylation[a] (forskolin +/−)	Ratio of phosphotyrosine content[b] (forskolin +/−)
1	0	1.13 ± 0.08*	ND
2	4	1.50 ± 0.27**	1.03 ± 0.40**
3	10	1.16 ± 0.04**	1.30 ± 0.08**

[a] The degree of PLC-γ phoshorylation was determined by measuring the radioactivity in 155 kDa bands from the SDS-PAGE gel
[b] Radioactivity in phosphotyrosine on thin-layer plate was measured by scraping the spots identified by ninhydrin-stained standard marker of each phosphoamino acid and autoradiography. Each sample of scraped powder was counted in 1 ml of ACS II (Amersham) liquid scintillation solution. The background count was subtracted from each value in both cases

^{32}P-labeled PLC-γ obtained from the cells with or without forskolin treatment followed by exposure to 0, 4 or 10 nM EGF for 5 min were subjected to 2-dimensional phosphoamino acid analysis. ND, not determined. * Range of 2 independent experiments. ** SD of 3 independent experiments

during the incubation with EGF (2–10 min) (Fig. 2C). Comparable tyrosine phosphorylation of PLC-γ in both cultures was confirmed using another method; the combination of immunoprecipitation with anti-PLC-γ and immunoblotting with anti-phosphotyrosine antibody (data not shown).

Furthermore to evaluate the contribution of phosphorylation on specific sites of PLC-γ, the phosphopeptide maps of PLC-γ were compared between the two cultures. No distinctive decrease of any phosphopeptides was detected in the tryptic phosphopeptide maps of PLC-γ between them (Fig. 3a,b), while forskolin induced one new phosphopeptide in P47 (Fig. 3c,d). The peptides produced from P47 are quite different from those of PLC-
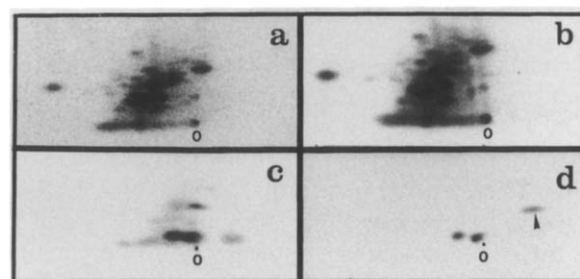

Fig. 2. Phosphoamino acid analysis of PLC-γ and P47 from A431 cells treated with forskolin followed by EGF. ^{32}P-labeled A431 cells were treated with or without 10 μM forskolin for 60 min followed by 4 nM EGF for the times indicated. The cell lysates were immunoprecipitated with anti-PLC-γ monoclonal antibodies and PLC-γ and P47 were separated by electrophoresis through SDS-PAGE and subjected to phosphoamino acid analysis as described in section 2. Autoradiograms of PLC-γ (A, C) and P47 (B) are presented. 'O' indicates where the samples were applied.

Fig. 3. Tryptic phosphopeptide maps of PLC-γ and P47 from A431 cells treated with or without forskolin followed by EGF. Radiolabeled PLC-γ and P47 were isolated as described in the legend of Fig. 1 and subjected to tryptic peptide mapping as described in section 2. Results for PLC-γ from EGF-treated cells (a), from forskolin-pretreated and EGF-treated cells (b), for P47 from untreated (c) and forskolin-treated cells (d) are shown. The arrowhead in (d) indicates the new phosphopeptide induced by forskolin. 'O' indicates where the samples were applied.

γ indicating that P47 is not a degraded form of PLC-γ. These results indicate that serine phosphorylation of PLC-γ stimulated by forskolin has no effect on the tyrosine phosphorylation of PLC-γ by the EGF receptor tyrosine kinase.

Assuming that the degree of tyrosine phosphorylation of PLC-γ reflects the strength of the interaction between the EGF receptor and PLC-γ, our data suggest that serine phosphorylation of PLC-γ by protein kinase A does not regulate the coupling ability of PLC-γ with EGF receptors in a ligand-dependent fashion.

4. DISCUSSION

Since the cAMP-mediated modulation of the EGF receptor signal transduction system in A431 cells is related to the inhibition of DAG production [7], we supposed that serine phosphorylation of PLC-γ by protein kinase A might be involved in this regulatory process. We found no effect of serine phosphorylation of PLC-γ on inhibition of PLC-γ tyrosine phosphorylation by the EGF receptor. We also focused on phosphorylation of P47 to examine its possible contribution to the system. The results demonstrated that treatment of A431 cells with forskolin, EGF or forskolin followed by EGF increased the degree of phosphorylation of PLC-γ and P47. EGF-dependent tyrosine phosphorylation was found in PLC-γ but not in P47. We compared the degree of tyrosine phosphorylation of PLC-γ from cells with or without forskolin treatment followed by exposure to EGF at a physiological concentration for various periods (2–10 min). However, no distinctive difference in the amount of phosphotyrosine in the proteins was detected between the two cultures, while EGF-induced EGF receptor phosphorylation was depressed by forskolin-pretreatment.

Since no inhibition of EGF-induced tyrosine phosphorylation of PLC-γ was detected, we concluded that serine phosphorylation of PLC-γ stimulated by protein kinase A does not regulate the ligand-dependent coupling ability of PLC-γ with EGF receptors.

On the other hand, it is noteworthy that phosphorylation patterns of PLC-γ and P47 were quite similar to each other in response to forskolin, EGF and forskolin followed by EGF. Although PLC-γ and P47 are different molecules, both are phosphorylated by protein kinase A and by EGF-stimulated serine kinase probably through a common pathway. In platelets, a 47 kDa protein is known to be phosphorylated by protein kinase C in a ligand-dependent fashion [11,12], although its function remains unclear.

Several groups have recently shown that small G proteins also might be involved in thrombin-mediated signal transduction in platelets [13,14] where cAMP enhancing agents also induce inhibitory effects [11,15]. One of the small G proteins was phosphorylated by protein kinase A both in vitro [16,17] and in vivo [16]. Although we cannot as yet correlate the relationship between P47 in A431 cells described here and the 47 kDa protein in platelets, nor the involvement of G proteins in the EGF receptor system, these kinds of molecules might be involved in the regulation of the ligand-dependent activation of PLC, not only in platelets but also in EGF receptor pathways [18].

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