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Localization of phospholipase C-γ1 signaling in caveolae: importance in EGF-induced phosphoinositide hydrolysis but not in tyrosine phosphorylation

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Abstract **Upon epidermal growth factor treatment, phospholipase C-γ1 (PLC-γ1) translocates from cytosol to membrane where it is phosphorylated at tyrosine residues. Caveolae are small plasma membrane invaginations whose structural protein is caveolin. In this study, we show that the translocation of PLC-γ1 and its tyrosine phosphorylation are localized in caveolae by caveolin-enriched low-density membrane (CM) preparation and immunostaining of cells. Pretreatment of cells with methyl-β-cyclodextrin (MβCD), a chemical disrupting caveolae structure, inhibits the translocation of PLC-γ1 to CM as well as phosphatidylinositol (PtdIns) turnover. However, MβCD shows no effect on tyrosine phosphorylation level of PLC-γ1. Our findings suggest that, for proper signaling, PLC-γ1 phosphorylation has to occur at $PtdInsP_2$-enriched sites.**

Key words: Phospholipase C-γ1; Caveola; Tyrosine phosphorylation

1. Introduction

Phospholipase C-γ (PLC-γ) hydrolyzes phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$), and one product of this hydrolysis, inositol 1,4,5-trisphosphate, mobilizes Ca^{2+} from the intracellular stores, while the other product of the hydrolysis, 1,2-diacylglycerol, is an activator of protein kinase C [1]. It has been suggested that in A431 cells, or other cultured cells that express high levels of the human epidermal growth factor (EGF) receptor, it is the tyrosine phosphorylation of PLC-γ by the EGF receptor that is responsible for the EGF-stimulated inositol phosphate production [2,3]. It has also been reported that stimulation of A431 cells with EGF leads to the translocation of PLC-γ1 from cytosol to membrane [4].

Caveolae are 50–100 nm sized plasma membrane invaginations where cholesterol and glycosphingolipids are concentrated, making these domains resistant to extraction by Triton X-100 [5,6]. Caveolin, a 21–24 kDa integral membrane protein, is a major structural component of the caveolae [7]. Caveolae can be isolated as a low-density membrane fraction where caveolin is enriched, denoted by CM (caveolin-enriched low-density membrane), by sucrose density step gradient centrifugation [8,9]. It has been suggested that caveolae may function as subcellular compartments in which to store inactive signaling molecules for regulated activation and to facilitate cross-talk between distinct signaling cascades [10,11]. Many proteins involved in signal transduction have been found in CM, such as heterotrimeric G proteins, Src family kinases, platelet-derived growth factor receptors, and EGF receptors [6,12–14]. It has been reported that in A431 cells approximately half of the total cellular $PtdInsP_2$ is localized in CM [15]. The cholesterol binding agent methyl-β-cyclodextrin (MβCD), which has been reported to cause the loss of compartmentalization of molecules in caveolae [16,17], delocalizes $PtdInsP_2$ from CM [12]. In this paper, we show that EGF-induced translocation and tyrosine phosphorylation of PLC-γ1 are both localized in CM. Immunostaining of cells also shows a co-localization of PLC-γ1 and caveolin-1 after EGF stimulation. MβCD pretreatment of the cells inhibits PLC-γ1 translocation to CM and phosphatidylinositol (PtdIns) turnover. But MβCD has no effect on the phosphorylation of PLC-γ1. These results suggest that the co-localization of the PLC-γ1 phosphorylation and its substrate at an enriched site is required for proper PLC-γ1 signaling.

2. Materials and methods

2.1. Antibodies and chemicals

For Western blot analysis, anti-caveolin-1 (C13620) antibody, anti-flotillin antibody, and anti-EGF receptor antibody were from Transduction Laboratories. Anti-PLC-γ1 monoclonal antibody and anti-phosphotyrosine monoclonal antibody were prepared as described previously [18]. For immunostaining of cells, anti-caveolin-1 polyclonal antibody (cat # sc-894) was from Santa Cruz Biotech, Inc., and rhodamine-conjugated goat anti-rabbit antibody (PN 31670) was from Pierce. Anti-PLC-γ1 monoclonal antibody was conjugated with FITC by ABI (Chonju, South Korea). EGF was obtained from the Daewoong Pharmaceutical Company (Seoul, South Korea). Myo-[^{3}H]inositol was from Amersham Pharmacia Biotech. All other chemicals were from Sigma.

2.2. Cell culture

A431 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum. Cells were serum-starved for 24 h before use in the experiments. Cells were pre-

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Abbreviations: EGF, epidermal growth factor; PLC-γ1, phospholipase C-γ1; CM, caveolin-enriched low-density membrane; MβCD, methyl-β-cyclodextrin; PtdIns, phosphatidylinositol; $PtdInsP_2$, phosphatidylinositol 4,5-bisphosphate

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incubated with phosphatase inhibitor (sodium vanadate, activated, 0.1 mM, 37°C, 10 min), and EGF treatment (100 ng/ml) was performed for 2 min at 37°C. MβCD (5 mM) pretreatment was given for 30 min at 37°C before the preincubation with sodium vanadate. COS-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum.

2.3. Detergent-free isolation of caveolin-enriched membranes

Caveolin-enriched membrane fractions were prepared as described previously [9]. A431 cells (one or two 150-mm dishes) were suspended in 2 ml of 500 mM sodium carbonate buffer, pH 11. Sodium vanadate (1 mM) was present at all steps. The cell suspension was homogenized with 10 strokes of a Dounce homogenizer, three 10-s bursts of a Polytron tissue grinder, and three 20-s bursts of a sonicator. The homogenate was then adjusted to 45% sucrose by addition of 80% sucrose prepared in MES buffer (25 mM MES, pH 6.5, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4) and placed into ultracentrifugation tubes so that each tube would contain the same amount of protein. A 5–35% discontinuous sucrose gradient (both in MES buffer) was then formed on top. The samples were centrifuged at 39 000 rpm for 6 h in an SW41 rotor (Beckman Instruments). Fractions were collected from the top in 1 ml amounts except for fractions 4 and 13, which were collected as 0.5 ml fractions.

2.4. Membrane precipitation and Western blot analysis

100 μl of CM and 450 μl of non-caveolae containing membrane (non-CM) fraction, prepared by pooling fractions 9–13, were diluted five times with MES buffer and then centrifuged at 100 000×*g* for 1 h. Supernatants were discarded and pellets were analyzed by Western blotting after SDS–PAGE (6–16%). Horseradish peroxidase-conjugated secondary antibody (1:5000 dilution; KPL) and an enhanced chemiluminescence assay kit (Amersham Life Sciences) were used to visualize the bands.

2.5. Immunoprecipitation of PLC-γl

CM and non-CM preparations were resuspended in 1 ml of extraction buffer (10 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.5% NP-40, 60 mM β-octylglucoside, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4) and sonicated. Insoluble material was removed by centrifuging at 15 000×*g* for 15 min and the supernatant incubated for 2 h at 4°C with anti-PLC-γl IgG [18] prebound to pansorbin. The immune complexes were collected and washed three times with extraction buffer. The precipitates were then resuspended in 30 μl of SDS–PAGE sample buffer and subjected to electrophoresis and Western blot analysis. To determine the effect of MβCD on PLC-γl tyrosine phosphorylation, 1 mg of A431 cell lysate in extraction buffer was incubated with anti-PLC-γl IgG [18] prebound to Protein A-Sepharose beads. The following procedures were as described above.

2.6. Immunostaining of COS-7 cells

2×10^5 COS-7 cells were transfected with 0.5 μg DNA of PLC-γl [27] using LipofectAMINE following the manufacturer's instructions. Cells were serum-starved for 24 h before use in the experiments. EGF treatment (100 ng/ml) was performed for 5 min at 37°C. Cells were washed with phosphate-buffered saline (PBS) and fixed for 10 min at room temperature with 3.7% paraformaldehyde in PBS. Fixed cells were rinsed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were rinsed with PBS and incubated with blocking solution (2% bovine serum albumin and 100 μl/ml equine serum in PBS) for 1 h at room temperature. Cells were then incubated overnight at 4°C with anti-caveolin-1 polyclonal antibody diluted 1/200 with blocking solution. After washing six times with PBS (10 min each), cells were incubated with FITC-conjugated anti-PLC-γl monoclonal antibody (1.5 μg/ml in blocking solution) and rhodamine-conjugated goat anti-rabbit antibody (diluted 1/1000 with blocking solution after reconstitution). Then cells were washed six times with PBS (10 min each). Slides were mounted and examined by a fluorescence microscope (Nikon, Inc., Melville, NY, USA).

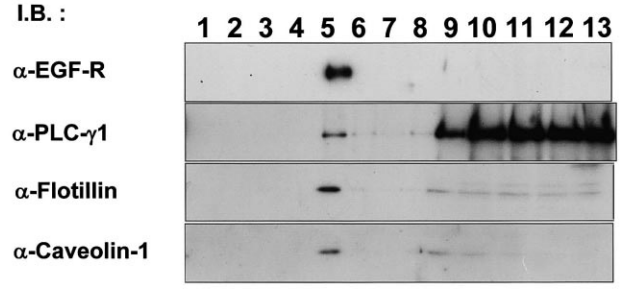

Fig. 1. PLC-γl is present in the CM fraction. A431 cells in 150-mm plates were harvested after 12 h of serum starvation into 500 mM Na_2CO_3, pH 11, homogenized, and fractionated by sucrose density gradient centrifugation as described in Section 2. Fractions were collected from the top, and 20 μl aliquots from each fraction were subjected to SDS–PAGE followed by Western blot analysis with the indicated antibodies. Caveolae are enriched in fraction 5. Fractions 9–13 were pooled for the non-caveolae membrane (non-CM) fraction.

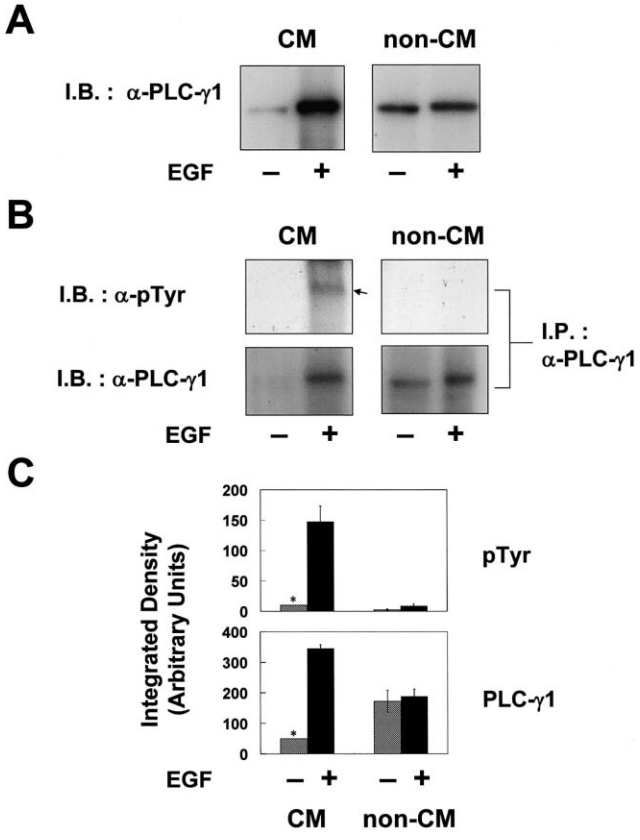

Fig. 2. PLC-γl translocation and tyrosine phosphorylation are localized in the CM fraction. A: The 100 ng/ml EGF stimulation was performed at 37°C for 2 min. To exclude cytosolic proteins in the non-caveolae membrane (non-CM) fraction, 100 μl of CM and 450 μl of the non-CM fraction were diluted five times with MES buffer and centrifuged at 100 000×*g* for 1 h. Supernatants were discarded, and pellets were resuspended in 30 μl SDS–PAGE sample buffer and subjected to Western blot analysis. B: Pellets prepared as in A were extracted with extraction buffer and subjected to immunoprecipitation with anti-PLC-γl monoclonal antibody. The immunoprecipitated complexes were analyzed by Western blot analysis. ← indicates the region corresponding to the molecular weight of PLC-γl. C: Densitometric analysis of translocated PLC-γl in CM and non-CM. The blots in the lower panels of B underwent densitometry using the Molecular Analyst Software (Bio-Rad). Means ± S.E.M. from two independent assays are shown. Columns marked by asterisks represent the controls.

2.7. Preparation of cholesterol–MβCD complexes

Cholesterol–MβCD complexes were prepared as described by Pike et al. [12]. Briefly, 6 mg of cholesterol was dissolved in 80 μl of isopropyl alcohol:chloroform (2:1). MβCD (200 mg) was dissolved in 2.2 ml of water and heated to 80°C with stirring in a water bath. The cholesterol was then added in small aliquots and the solution stirred until clear. This solution contained 6.8 mM cholesterol.

2.8. Measurement of PtdIns turnover

A431 cells were plated in six-well dishes. For labeling, confluent cultures were incubated in inositol-free DMEM containing 1 μCi/ml myo-[^{3}H]inositol but no serum for 24 h. The cells were then treated with MβCD as described above followed by 20 mM LiCl pretreatment for 10 min. Then the cells were stimulated with EGF (100 ng/ml) for 30 min. The reactions were terminated by aspiration of the medium and addition of 400 μl of 5% perchloric acid. After incubation on ice for 30 min and centrifugation at 15 000×*g* for 5 min, 10 μl of the supernatant was sampled and used to determine total [^{3}H]inositol. Inositol phosphates were isolated on an AG 1-X8 column as described previously [19].

3. Results

3.1. Small amount of PLC-γl is found in CM of unstimulated A431 cells

The homogenate of A431 cells after 12 h of serum starvation was fractionated by sucrose density centrifugation in the absence of detergent, a widely used method to isolate caveolae-enriched membrane subdomains. As shown in Fig. 1, caveolin, EGF receptor, and flotillin, which is caveolae-associated integral membrane protein [14,20], were enriched in fraction 5. Fraction 5 was therefore selected as the caveolae fraction, denoted by CM, and fractions from 9 to 13 were pooled as the non-caveolae membrane (non-CM) fraction. Only a minor amount of PLC-γl was detected in the CM fraction (Fig. 1).

3.2. Translocation and tyrosine phosphorylation of PLC-γl occurs only in CM

The CM fraction and non-CM fraction were pelleted at 100 000×*g* to exclude cytosolic proteins. Upon treatment of the cells with EGF, the translocation of PLC-γl to the membrane was observed mainly in CM (Fig. 2A). CM and non-

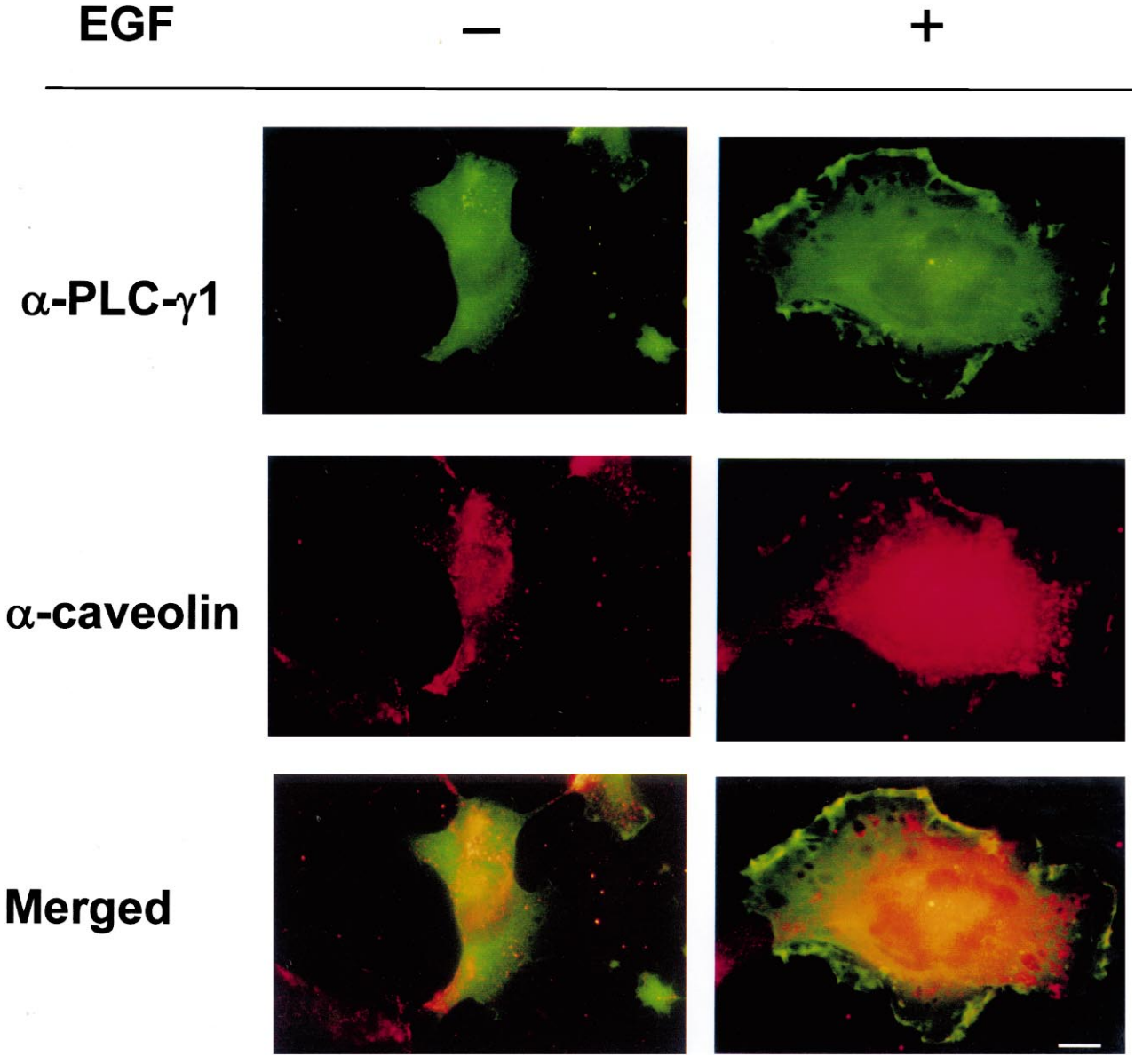

Fig. 3. Immunostaining of COS-7 cells shows the co-localization of PLC-γl and caveolin-1 after EGF treatment. COS-7 cells were transfected with PLC-γl. At 24 h after transfection, the cells were starved for 24 h prior to stimulation with EGF for 5 min. Then the cells were fixed and stained with anti-caveolin-1 antibody. Rhodamine-conjugated anti-rabbit antibody was used as a secondary antibody for staining of caveolin-1, and FITC-conjugated anti-PLC-γl antibody was used to visualize PLC-γl. The yellow spots of the merged image in the lower panel indicate the co-localization of PLC-γl and caveolin-1 after EGF treatment. Bar, 10 μm.

CM were treated with anti-PLC-γ1 monoclonal antibody and the immunoprecipitates were probed with anti-phosphotyrosine antibody. Phosphorylated PLC-γ1 was exclusively detected in the CM fraction (Fig. 2B). Since tyrosine phosphorylation of PLC-γ1 is critically required for this enzyme's activation in vivo [21], our results suggest that the enzymatic activation of PLC-γ1 is localized in CM.

3.3. Immunostaining of COS-7 cells shows the co-localization of PLC-γ1 and caveolin-1 after EGF stimulation

We showed that PLC-γ1 translocates to CM (Fig. 2), but it was not sure that the CM fraction contains caveolae only. Therefore, to investigate the PLC-γ1 translocation further, we stained COS-7 cells after transfection of PLC-γ1. As shown in Fig. 3, PLC-γ1 (green) translocates from cytosol to plasma membrane after EGF stimulation. Caveolin-1 (red) is a structural protein of caveolae. Merged images show the co-localization (yellow) of PLC-γ1 and caveolin-1 in the plasma membrane after EGF treatment, which tells that PLC-γ1 translocates to caveolae upon EGF stimulation.

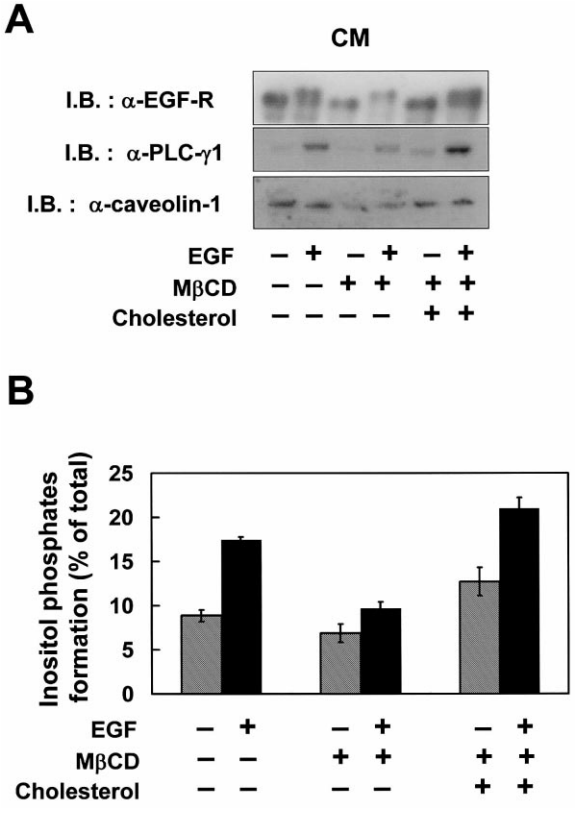

Fig. 4. MβCD pretreatment inhibits EGF-stimulated PLC-γ1 translocation to CM and PtdIns turnover. A: After serum starvation, A431 cells were preincubated with or without 5 mM MβCD for 30 min at 37°C. The medium of one MβCD-treated culture was removed and replaced with 0.2 mM cholesterol complex with MβCD for 30 min at 37°C. EGF stimulation was performed and the cells were subjected to a detergent-free method of isolating the caveolin-enriched membranes. 100 μl of the CM fraction was diluted five times with MES buffer and centrifuged at 100 000×*g* for 1 h. Supernatants were discarded, and pellets were resuspended in 30 μl of SDS–PAGE sample buffer, electrophoresed, and analyzed by Western blotting. B: A431 cells were labeled with [^{3}H]inositol and then switched into inositol-free DMEM. MβCD treatment and cholesterol add-back were performed as described above. Then the cells were treated with 20 mM LiCl for 10 min at 37°C. Subsequently the cultures were stimulated with 100 ng/ml EGF for 30 min at 37°C. [^{3}H]Inositol phosphates were isolated as described in Section 2. Data shown represent the means ± S.E.M. of duplicate determinations.

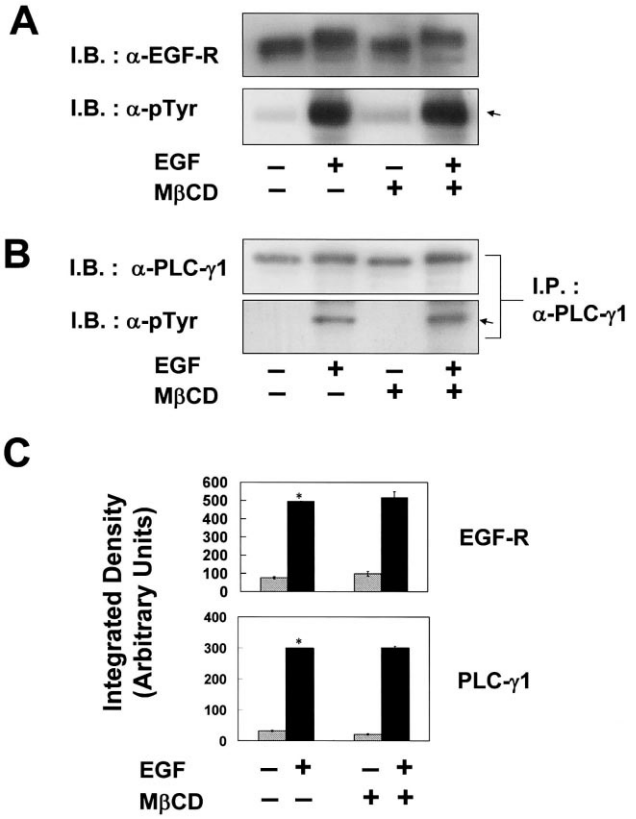

Fig. 5. MβCD has no effect on the phosphorylation of EGF receptor and PLC-γ1. A: A431 cells were incubated with 5 mM MβCD for 30 min at 37°C before 0.1 mM sodium vanadate was added for 10 min. Subsequently the cultures were stimulated with 100 ng/ml EGF for 2 min at 37°C. 600 μl extraction buffer was added to 1 mg of cells, and 20 μl aliquots were subjected to SDS–PAGE followed by Western blot analysis with anti-EGF receptor antibody and anti-phosphotyrosine antibody. ← indicates the region corresponding to the molecular weight of the EGF receptor. B: The cell lysates prepared in A were incubated with anti-PLC-γ1 monoclonal antibody. The immunoprecipitated complexes were analyzed by Western blot analysis with anti-PLC-γ1 antibody and anti-phosphotyrosine antibody. ← indicates the region corresponding to the molecular weight of PLC-γ1. C: The blots in A and B underwent densitometry using the Molecular Analyst Software (Bio-Rad). In determining the MβCD effect on EGF receptor phosphorylation, the integrated densities of blots in the lower panel of A were divided by the integrated densities of blots in the upper panel of A. In determining MβCD effect on PLC-γ1 phosphorylation, the integrated densities of blots in the lower panel of B were divided by the integrated densities of blots in the upper panel of B. Means ± S.E.M. from two independent assays are shown. Columns marked by asterisks represent the controls.

3.4. MβCD pretreatment inhibits PLC-γ1 translocation to CM and PtdIns turnover

Depletion of cellular cholesterol by MβCD was reported to cause the loss of compartmentalization of signaling molecules in caveolae [12]. To determine what effect the disruption of the caveolar structure would have on PLC-γ1 signaling, we treated the cells with MβCD before EGF stimulation. As seen in Fig. 4A, MβCD pretreatment delocalized EGF recep-

tor and caveolin-1 from CM. And MβCD pretreatment inhibited EGF-induced PLC-γ1 translocation to CM (Fig. 4A) and PtdIns turnover (Fig. 4B). The inhibition was recovered when cholesterol was added back, which tells that MβCD effects were not simply the artifacts caused by its cellular toxicity. These results suggest that the integrity of caveolae is important for PLC-γ1 signaling.

3.5. MβCD pretreatment has no effect on the phosphorylation of the EGF receptor and PLC-γ1

It has previously been reported that MβCD treatment delocalizes the EGF receptor and $PtdInsP_2$ from CM [12,22]. Our data show that the MβCD pretreatment of cells prevents the PLC-γ1 translocation to CM (Fig. 4A). Thus we expected that the inhibition of the PtdIns turnover could be the consequence of the loss of co-localization of the molecules involved. Still, it was not clear which step of the PLC-γ1 signaling would be affected. The inhibition of PtdIns turnover could be due to a decrease in EGF receptor tyrosine phosphorylation, a decrease in PLC-γ1 tyrosine phosphorylation, the delocalization of the substrate $PtdInsP_2$, or a combination of all of those events. In order to test these possibilities, we checked the effects of MβCD pretreatment on the phosphorylation level of the EGF receptor and PLC-γ1 using Western blot analysis. As seen in Fig. 5, there was no decrease in the tyrosine phosphorylation level of the EGF receptor or PLC-γ1. These findings suggest that the inhibition of the PtdIns turnover by the disruption of the caveolar structure is not a result of a failure in EGF receptor phosphorylation or PLC-γ1 phosphorylation. It is more likely a result of delocalization of $PtdInsP_2$.

4. Discussion

Caveolae are sites where many signaling molecules come together. Caveolae can be isolated as CM fraction. In this study, we show that the EGF receptor is found highly enriched in CM (Fig. 1) and the translocation and tyrosine phosphorylation of PLC-γ1 by EGF occur mainly in CM (Fig. 2). We also show that translocated PLC-γ1 and caveolin-1 are co-localized in the plasma membrane after EGF stimulation by immunostaining of cells (Fig. 3). PLC-γ1 is a substrate of the EGF receptor kinase [23], and many reports have suggested that the EGF receptor is localized in the caveolae [11,12,22,24,25]. Tyrosine phosphorylation of PLC-γ1 by the EGF receptor is crucial for the enzymatic activation of PLC-γ1 [21,26]. We therefore propose that the EGF-induced PLC-γ1 translocation and enzymatic activation are localized in the caveolae.

While the MβCD pretreatment of cells affected the integrity of the caveolae and inhibited the EGF-stimulated PtdIns turnover (Fig. 4), it was still not clear which step of the PLC-γ1 signaling was abrogated. Since the translocation of PLC-γ1 to CM was inhibited upon MβCD pretreatment (Fig. 4), a decrease in PLC-γ1 tyrosine phosphorylation was expected. However, we did not observe a decrease in the PLC-γ1 phosphorylation after MβCD pretreatment in EGF-stimulated A431 cells (Fig. 5). This suggests that the EGF receptor can phosphorylate PLC-γ1 properly even when the EGF receptor is delocalized from CM. Then, why was PtdIns turnover inhibited? There have been reports that about 50% of the total cellular $PtdInsP_2$ is concentrated in CM [15] and that MβCD pretreatment delocalizes $PtdInsP_2$ from CM [12]. These reports and our findings together suggest that the inhibition of the PtdIns turnover by the disruption of the caveolar structure is due to the delocalization of $PtdInsP_2$ and not a result of a failure in PLC-γ1 phosphorylation. We propose that the PLC-γ1 phosphorylation occurs at the $PtdInsP_2$-enriched site of the caveolae resulting in more efficient PtdIns turnover.

In summary, the data reported here demonstrate that the translocation and tyrosine phosphorylation of PLC-γ1 are localized in the CM domain. And we suggest that the localization of the PLC-γ1 phosphorylation at a site enriched in its substrate is a requisite for proper signaling.

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