Thrombin-mediated Phosphoinositide Hydrolysis in Chinese Hamster Ovary Cells Overexpressing Phospholipase C-δ1*

(Received for publication, December 13, 1993, and in revised form, March 7, 1994)

Yoshiko Banno, Yukio Okano, and Yoshinori Nozawa
From the Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi-40, Gifu 500, Japan

The regulatory mechanism(s) of a phosphoinositide-specific phospholipase C, PLC-δ1, was investigated using a cell line of stably overexpressed PLC-δ1 (PLCδ30 cells) in Chinese hamster ovary cells. Thrombin stimulation of PLCδ30 cells exhibited 6.5-fold increase in total inositol phosphates (InsP), which was significantly higher than that in the vector-transfected (V1) cells (2.0-fold). AIF; increased InsP accumulation in both V1 and PLCδ30 cells, and pertussis toxin partially blocked InsP accumulation in thrombin-stimulated PLCδ30 cells. Guanosine triphosphatase (GTPyS) markedly potentiated thrombin-stimulated InsP generation in permeabilized PLCδ30 cells compared with V1 cells, suggesting possible involvement of a G-protein (s) in the activation of PLC-δ1. In PLCδ30 cells, ionomycin-induced significant InsP generation and thrombin-stimulated InsP generation were completely inhibited by addition of EGTA. Furthermore, the stimulatory effects of thrombin plus GTPyS in PLCδ30 cells were more sensitive to change in free calcium concentration than in V1 cells. Suppression by 12-O-tetradecanoylphorbol 13-acetate of thrombin-stimulated InsP accumulation was not affected by increasing Ca2+ concentration. These results indicate that thrombin overexpressed PLC-δ1 activation is regulated via both G-protein(s) and calcium.

Phosphoinositide-specific phospholipase C (PI-PLC) is a key signal transducing enzyme to generate two second messengers, inositol trisphosphate and diacylglycerol (1–3). There are three classes of PI-PLC isozymes (β, γ, and δ), each containing 2 to 4 subtypes, and nine distinct isozymes in total have been cloned (β1, β2, β3, β4, γ1, γ2, δ1, δ2, and δ3) (4–6). These classes of PI-PLC isozymes are activated by differential mechanisms upon receptor stimulation. Tyrosine phosphorylation has been established as an important intracellular signaling mechanism. PLC-γ is a unique subclass containing src homology region, called SH2 and SH3, indicating the involvement of tyrosine phosphorylation in the activation mechanism. When receptor-type tyrosine kinases, such as epidermal growth factor or platelet-derived growth factor receptors are stimulated, tyrosine phosphorylation of PLC-γ occurs and induces phosphoinositide breakdown (7–9). Activation of receptors having no intrinsic kinase also induces phosphorylation of PLC-γ via non-receptor-type tyrosine kinases (2).

Receptors for muscarinic acetylcholine, bradykinin, and thrombin belong to the family of receptors containing seven membrane-spanning domains characteristic of those coupled to effector enzymes through guanine nucleotide-binding protein (G-protein) (10). Recent investigations provided evidences that receptor-mediated activation of PLC-β isozymes is caused by two distinct mechanisms; one through α subunits of Gq family which is insensitive to pertussis toxin (PT) and the other through the βγ subunits of G-proteins (11–17). In vitro reconstitution studies show that activation of the PLC-β isozymes mediated by Gq0 is in the order of PLC-β1 > PLC-β3 > PLC-β2, whereas βγ subunits-mediated stimulation of PLC-β isozymes is in the order of PLC-β3 > PLC-β2 > PLC-β1 (18, 19).

In contrast, despite the wide distribution of PLC-δ isozymes from mammalian cells (20) to yeast (21, 22), their receptor-linked activation mechanism(s) has not fully been clarified. The PLC-δ isozymes with an approximate molecular mass of 85 kDa are considerably smaller than either PLC-β (130–155 kDa) or PLC-γ (145 kDa). PLC-δ isozymes also contain two conservative domains, X and Y, but they lack src homology regions and are therefore unlikely to be a substrate for tyrosine kinases (2). It is also to be noted that PLC-δ isozymes only contain calcium-binding EF-hand motif (23). Therefore, it is conceivable that PLC-δ isozymes are sensitive to calcium and therefore that the intracellular calcium elevation alone may provoke PLC-δ to hydrolyze polyphosphoinositides in vivo. Or the calcium binding to the EF-hand motif may modulate activation process of the PLC-δ such as the translocation to membrane or interaction with putative G-protein(s). Little is known about the type(s) of receptor coupled to PLC-δ except that the PLC-δ is associated with thrombin-triggered mitogenic response (24, 25). Two mutants of CCL39 cells lacking PLC-δ1 showed significant different behaviors in thrombin-induced PI-turnover: decreased and enhanced responses (26). In the present study, in order to get more insight into the regulatory mechanisms of PLC-δ1, we have investigated the thrombin receptor-mediated activation of phosphoinositide hydrolysis in CHO cells overexpressing PLC-δ1.

EXPERIMENTAL PROCEDURES

Materials—myo-[3H]inositol (90 Ci/mmol) was obtained from American (Buckinghamshire, United Kingdom) and [3H]phosphatidylinositol 4,5-bisphosphate (IPP; 8.8 Ci/mmol) from DuPont NEN. Thrombin was obtained from Mochida Pharmaceutical Company (Tokyo, Japan). Guanosine 5′-O-(γ-thiotriphosphate) (GTPγS) and guanosine 5′-O-(β-thiodiphosphate) (GDPβS) were from Boehringer Mannheim. Ionomycin and phosphoryl myristate acetate (TPA) were purchased from Sigma. PT and digitoxin were from Wako (Osaka, Japan).
Genistein and Geneticin (G418) were from Funakoshi (Tokyo, Japan) and Life Technologies, Inc., respectively.

Cell Culture and Transfection of PLC-61 cDNA—CHO cells were maintained in a growth medium of Ham's F-12 supplemented with 10% fetal calf serum, PLC-61 cDNA subcloned in a plasmid vector, pBlus2, was kindly supplied by Dr. S. G. Rhee (National Institutes of Health, Bethesda, MD). pIBIBO, a mammalian expression vector, pSRn, containing human immunodeficiency virus promoter and neo gene (27). The constructed plasmid DNA or the vector DNA was transfected into CHO cells seeded on a collagen-coated 60-mm culture dish by the modified CaPO4 method using a mammalian transfection kit (Stratagene, La Jolla, CA). Two days after the transfection, the cells were reseeded in a 100-mm culture dish in growth medium containing 0.8 mg/ml G418 and further cultured for 3 days. The G418-resistant cells were then subcultured in two 96-well microtiter plates to obtain single clones by limiting dilution. Three and 20 clones for vector- and PLC-61-transfected cells, respectively, were obtained and maintained in the presence of 0.2 mg/ml G418. The expression of PLC-61 protein was examined by Western blotting with monoclonal anti-PLC-61 antibody using the ECL detection system (Amersham).

Measurement of Inositol Phosphates—CHO cells were seeded at a density of 7 × 104 cells/well in six-well plates in complete growth medium. At near confluence, the growth medium was changed to 1.0 ml of inositol-free minimum Eagle's medium containing 0.3% bovine serum albumin and myo-[3H]inositol (1 µCi/ml). After 36-h labeling, the cells were washed twice with modified Krebs-Ringer buffer (KRB) consisting of 125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 5 mM Hepes (pH 7.4), and 20 mM LiCl and were further incubated for 15 min at 37 °C. Experiments were conducted by adding agonists to the labeled cells, and the reaction was terminated by the addition of 0.5 ml of 10% perchloric acid to each well at the indicated times. Inositol phosphates were separated using Dowex AG1-X8 anion exchange resin (200-400 mesh, formate form, Bio-Rad) as described elsewhere (28). [3H]Inositol and [3H]glycerophosphoinositol were run to exchange resin (200-400 mesh, Bio-Rad) as described elsewhere (28). [3H]Inositol and [3H]glycerophosphoinositol were run to exchange resin (200-400 mesh, Bio-Rad) as described elsewhere (28).

Cell Permeabilization—[3H]Inositol-labeled cells were permeabilized by incubating with 15 µg digitonin for 6 min at 25 °C in potassium glutamate (KG) buffer consisting of 139 mM potassium glutamate, 20 mM Pipes (pH 6.9), 1 mM Mg-ATP, 1 mM MgCl2, and 5 mM EGTA containing 20 mM LiCl as described previously (29). Under these conditions, more than 98% of the cells were permeabilized, as assessed by the trypan blue exclusion test. Then, the overlaying medium was aspirated, and the cells were incubated with the indicated concentration of free Ca2+ or various agents in potassium glutamate buffer at 37 °C.

Preparation of Cytosolic and Membrane Fractions—Confluent cultures of CHO cells in 100-mm dishes were washed three times with 4 ml of ice-cold phosphate-buffered saline and then 2 ml of cold hypotonic buffer (4 °C, 7.4), 100 mM NaCl, 1 mM EDTA, 5 mM EGTA, and 1 mM dithiothreitol containing protease inhibitors (10 µg/ml leupeptin, 0.5 µM phenylmethylsulfonyl fluoride) were added. The cells were gently scraped from the dish with a rubber policeman, homogenized for 30 strokes with a Dounce homogenizer. After unbroken cells were removed by centrifugation at 3,000 × g for 5 min, the supernatant fractions were centrifuged at 105,000 × g for 30 min to give a cytosolic and membrane fractions. The membrane pellet was then resuspended in the hypotonic buffer.

Assay for Phospholipase C Activity—The activity of phospholipase C in vitro was measured essentially as described previously (30). Reaction mixtures (50 µl) contained 20 µM Tris/maleate buffer (pH 6.8), 80 mM KCl, [3H]P(lipid phosphate (20,000 dpm/PiP), 100 µM phosphatidyl dilethanolamine (500 µM), 0.1% octyl glucoside, 1 mM Ca2+/EGTA, 2 mM MgCl2, and membrane protein (10 µg/ml). The reaction mixtures were incubated for 15 min at 37 °C, and the reaction was terminated by the addition of 0.25 ml of chloroform/methanol/concentrated HCl (100:100:6, v/v), followed by 0.1 ml of saturated HCl. After centrifugation at 3,000 × g for 10 min, the aliquots were assayed for [3H]InsP3 production. The formation of [3H]InsP3 was linear in 20-min incubation and linear with the protein concentrations up to 10 µg/assay.

Protein was determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard.

Electrophoresis and Immunoblotting—SDS-polyacrylamide gel electrophoresis was run through 8% acrylamide gel according to the method by Laemmli (31). Electrophoretic blotting onto nitrocellulose membrane (PVDF) was carried out as the procedure of Towbin (32). Blocking was performed in Tris-buffered saline containing 5% casein and 0.2% Tween 20 for 2 h. The Western blots were incubated with monoclonal antibodies against PLC-61, PLC-1, or PLC-61 for overnight. Monoclonal antibodies against PLC-61, PLC-g1, and PLC-61 were kindly supplied by Dr. S. G. Rhee (National Institutes of Health, Bethesda, MD).

RESULTS

Overexpression of PLC-61 in CHO Cells—CHO cells were transfected with a construct containing rat brain PLC-61 cDNA, and three clones (PLC62, -27, -30) were obtained. A clone PLC630 showing the highest level of PLC-61, as inferred by Western blot analysis, was selected and employed through the following experiments. A clone of vector-transfected CHO cells (V1) was used as a control. No significant differences were observed in growth rate and morphology between the PLC630 and V1 cells (data not shown).

Western blot analyses using antisera against bovine PLC-61, -p1, and -61 revealed marked overexpression of PLC-61 in the PLC630 cells (Fig. 1C, lane 3). Parental and vector-transfected (V1) cells have similar amount of PLC-61 (Fig. 1C, lane 3). Western blot analysis, was selected and employed through the following experiments. A clone of vector-transfected CHO cells (V1) was used as a control. No significant differences were observed in growth rate and morphology between the PLC630 and V1 cells (data not shown).

Western blot analyses using antisera against bovine PLC-61, -p1, and -61 revealed marked overexpression of PLC-61 in the PLC630 cells (Fig. 1C, lane 3). Parental and vector-transfected (V1) cells have similar amount of PLC-61 (Fig. 1B, lanes 1 and 2), but PLC-61 (Fig. 1A, lane 1 and 2) and PLC-61 (Fig. 1C, lane 1 and 2) are barely detectable with protein content up to 400 µg in both cell types. It could be possible that CHO cells also contain other PLC isozyme(s), probably PLC-63, which is widely expressed in a variety of tissues and cell lines, because PLC-p2 is known to be present only in HL-60 (18, 19). Distributions of PLC-61 and PLC-61 in cytosolic and membrane fractions were examined in CHO cells (Fig. 2). In contrast to PLC-61 which was mainly present in cytosol, a high level of PLC-61 was observed in the membrane fraction of PLC630 cells. When PLC activity was measured with PI, as substrate, the specific activities of homogenates of V1 and PLC630 cells were 37.9 ± 4.6 and 121.2 ± 1.4 nmol/min/mg of protein, respectively, and the activities of the membrane fractions were 2.5 ± 0.8 and 8.8 ± 1.2 nmol/min/mg of protein, respectively.

Thrombin-stimulated Inositol Phosphate Production—To examine the effects of PLC-61 overexpression on phosphoinositide hydrolysis, production of InsP3 was examined in vector-transfected control cells and PLC-61 cDNA transfected clones (827, 830) stimulated by thrombin. As shown in Fig. 3A, Western blotting analysis with anti-PLC-61 antibody revealed that PLC-61 was expressed in different levels among clones: undetectable in V1 cell, very low in PLC827 clone, and high in PLC62 and PLC830 clones. The thrombin-stimulated InsP3 formation appears to positively correlate with the levels of PLC-61.
brane fractions. Soluble fractions were separated by centrifugation as described under "Experimental Procedures." Soluble fractions (100 μg of protein) and membrane fractions (200 μg of protein) solubilized in lysis buffer containing 1% cholate and 1% Triton X-100 were subjected to SDS-PAGE, then transferred to the PVDF membrane and probed with antibodies against PLC-γ1 (A) and PLC-δ1 (B).

Fig. 2. Distribution of PLC-γ1 and -δ1 in soluble and membrane fractions. The vector-transfected cells (lane 1) and PLC δ30 cells (lane 2) were homogenized and the soluble (S) and membrane (M) fractions were separated by centrifugation as described under "Experimental Procedures." Soluble fractions were transferred to PVDF membrane and probed with antibodies against PLC-γ1 (A) and PLC-δ1 (B).

Fig. 3. Western blot with PLC-δ1 and InsP formation by thrombin stimulation in various cell lines. A, vector-transfected cells (V1) and PLC-δ1-overexpressed cell lines (PLCδ27, PLCδ2, and PLCδ30) were lysed, and 200 μg of protein was subjected to SDS-PAGE, then transferred to PVDF membrane and probed with antibodies against PLC-δ1.

2-fold increase. Higher concentrations of thrombin were more effective in PLCδ30 cells than in the V1 cells for 30-min incubation in [3H]InsP formation, with the maximum response at approximately 2 units/ml (Fig. 4B).

In contrast to the marked responses to thrombin, bradykinin known to cause receptor-mediated phosphoinositide hydrolysis in a variety of cells did not show significant increase in InsP level both in the V1 cells and PLCδ36 cells (Fig. 5). AIF decreased [3H]InsP production in V1 and PLCδ30 cells 2.8-fold and 3.6-fold, respectively. Pretreatment with PT (100 nM for 10 h) of PLCδ30 cells partially prevented thrombin-stimulated [3H]InsP formation (34.5 ± 11% inhibition). Prolonged treatment with PT (24 h) did not enhance the inhibitory effects. On the other hand, under the same condition, only small inhibition was observed in thrombin-stimulated [3H]InsP formation of the V1 cells (16.3 ± 4.7% inhibition).

Ca2+ dependence of PLC activation by Ca2+-mobilizing hormones has been a matter of controversy. For example, hormone-stimulated polyphosphoinositide breakdown in intact hepatocytes is independent of the intracellular calcium rise (33, 34), whereas Ca2+ ionophore A23187 stimulated PLC-mediated breakdown of polyphosphoinositides in rabbit neutrophils (35). This discrepancy may be due to differences of PLC isozymes. Thrombin receptors are known to elevate intracellular Ca2+ levels by stimulating the extracellular Ca2+ influx (36). Thus, the effect of Ca2+ on thrombin-stimulated [3H]InsP formation was examined. The marked increase of [3H]InsP levels in
Thrombin Stimulation of Overexpressed PI-PLC-81

PLC-630 cells induced by thrombin was completely blocked by addition of 3 mM EGTA in the incubation medium (60 ± 19.9% for thrombin; 130.2 ± 60.4% for thrombin plus EGTA compared with the unstimulated level) (Fig. 5). Artificial rise of intracellular Ca²⁺ by ionomycin (1 μM, 30 min) in the presence of extracellular Ca²⁺ (1.5 mM) promoted InsP₃ generation (281.9 ± 24.6%) in PLC-630 cells, but it caused only a slight increase of the InsP levels in the control cells (120 ± 11.4%). When assayed in the presence of EGTA with no added Ca²⁺, PLC-61 partially purified from yeast cannot hydrolyze PIP₂ (21). These results suggest that intracellular Ca²⁺ is essential for thrombin-stimulated PLC-61 activation in the PLC-630 cells.

Effects of GTPyS and Thrombin in Permeabilized Cells—
Thrombin receptor has been known to couple to two G-proteins, Gq and G₁ in CCL39 fibroblasts cells (37). In order to obtain further evidence for involvement of G-protein in PLC-61 activation, the effect of GTPyS on InsP₃ formation was examined in digitonin-permeabilized cells of both V1 and PLC-630. The digitonin-permeabilized cells were incubated in the presence or absence of thrombin with increasing concentrations of GTPyS (10–100 μM). As shown in Fig. 6, GTPyS over a range of 10–100 μM markedly enhanced InsP₃ levels in the presence of thrombin in PLC-630 cells, although GTPyS alone produced only a small increase. In contrast, V1 cells exposed to thrombin in the presence of GTPyS showed merely a very small increase in [³H]InsP₃ level. The effects of GDP½S on the GTPyS-stimulated InsP₃ production were further examined in the both types of cells (Fig. 7). GDP½S (1 μM) inhibited the GTPyS (50 μM)-stimulated [³H]InsP₃ productions in the V1 and PLC-630 cells (99.8 ± 10.0% and 70.4 ± 9.8% inhibition, respectively), and its partial inhibitions in the V1 and PLC-630 cells (65.2 ± 10.5% and 51.3 ± 12.5% inhibition, respectively) was observed in the thrombin plus GTPyS-stimulated [³H]InsP₃ production.

In order to see whether the PLC-61 takes a major part in stimulation of phosphoinositide breakdown in PLC-630 cells by thrombin, we examined the effect of anti-PLC-61 antibody on PIP₂ breakdown in the membrane fractions prepared from the V1 and PLC-630 cells. The membrane fractions were incubated at 30 °C for 15 min in the presence or absence of thrombin (1 unit/ml) plus 100 μM GTPyS. The PIP₂ hydrolyzing activities of V1 and PLC-630 cell membranes were stimulated by thrombin plus GTPyS 1.7 ± 0.4-fold and 2.6 ± 0.5-fold, respectively. The elevated PIP₂ hydrolyzing activity in the PLC-630 membranes was reduced to 1.6 ± 0.6-fold by preincubation of the membranes with anti-PLC-61 antibody, whereas the stimulation of PLC activity by thrombin plus GTPyS in the V1 membranes was not affected by the pretreatment with the antibody. These results provide additional support to the view that the PLC-61 activity is regulated by G-protein(s) linked to thrombin receptor in the PLC-61-overexpressed PLC-630 cells. However, unlike intact or permeabilized PLC-630 cells, its membrane fraction exposed to thrombin plus GTPyS did not show a marked increase in InsP₃ level.

Effect of Ca²⁺ on Inositol Phosphate Production in Permeabilized Cells—Experiments with EGTA and ionomycin in intact PLC-630 cells indicated Ca²⁺ dependence in PLC-61 activation. The requirement of Ca²⁺ for thrombin-stimulated phosphoinositide breakdown was examined in permeabilized cells (Fig. 8). In the absence of Ca²⁺ (3 mM EGTA with no added Ca²⁺), neither thrombin nor GTPyS caused any InsP₃ generation in both cells. Thrombin or GTPyS alone caused marginal InsP₃ production at low Ca²⁺ concentration (10⁻⁶ M), and the InsP₃ level was, to a small but significant extent, elevated at 1 μM Ca²⁺ in both cells. Ca²⁺ (1 μM) alone stimulated InsP₃ generation by 2.5-fold in PLC-630 cells. In the V1 cells, Ca²⁺ alone did not affect the InsP level. On the other hand, [³H]InsP₃ generation induced with thrombin plus GTPyS was much greater than the sum of individual activation at all Ca²⁺ (10⁻⁴ to 10⁻⁶ M) concentrations in PLC-630 cells. These results suggest that Ca²⁺ serves as an essential factor in the elevation of thrombin receptor-coupled G-protein-mediated phosphoinositide hydrolysis by PLC-61 in PLC-630 cells.
**DISCUSSION**

Although the regulatory mechanisms of PLC-α and PLC-γ activation have well been studied and characterized, much less is known about the activation mechanism of PLC-δ. In order to gain more insight into the mechanism of PLC-δ activation, in the present study, we have investigated its regulatory mechanism using a line of CHO cells transfected with PLC-δ1 cDNA. It was shown that the production of InsP stimulated with thrombin was markedly enhanced in PLC-δ1-overexpressed PLC630 cells compared with vector-transfected control cells, whereas bradykinin (BK) which can stimulate phosphoinositide hydrolysis in a variety of cell types did not produce InsP in these cells. Furthermore, investigations using both intact and permeabilized cells demonstrated that thrombin-induced InsP formation was modulated by the receptor-coupled G-protein(s), the intracellular Ca2+ concentrations, and the activation of PKC, indicating that the activation mechanisms of PLC-δ1 were similar but not identical to those of PLC-β type. Although it is generally known that all PLC isoforms can hydrolyzePIP2 dependently on Ca2+ in *vitro*, most of the previous studies have shown that hormone-induced phosphoinositide hydrolysis by PLC in intact cells was observed at low intracellular Ca2+ levels of unstimulated cells (10-7 M) (39). Downes and Michell (40) suggested that agonist-induced hydrolysis of phosphoinositides is relatively insensitive to extracellular Ca2+ removal and that artificial elevation of Ca2+ does not promote the hydrolysis. Our previous data using a cell line of osteoblasts MC3T3-E1 were consistent with this notion (41). The MC3T3-E1 cells contain much higher amounts of PLC-β1 relative to PLC230 cells. It was shown that in MC3T3-E1 cells BK-stimulated InsP generation was neither affected by extracellular Ca2+ chelation with EGTA.
nor intracellular Ca\(^{2+}\) elevation by ionomycin. BK receptors have recently been considered to be coupled to PLC-\(\beta1\) through a family of G-proteins, Gq (13). Thus, BK-induced InsP formation is probably mediated by the activation of PLC-\(\beta\), and an increase in [Ca\(^{2+}\)](i) is not required for its activation. However, the present investigation indicated the absolute requirement of extracellular Ca\(^{2+}\), because its depletion caused a nearly complete inhibition of thrombin-induced InsP production in PLC630 cells. Furthermore, Ca\(^{2+}\) ionophore ionomycin alone induced greater increases in InsP levels in PLC630 cells than in vector-transfected (V1) cells which contain PLC-\(\beta\) type and much less PLC-\(\beta1\). In permeabilized PLC630 cells, the [Ca\(^{2+}\)](i) level up to 1 \(\mu\)M was sufficient to cause a small but significant InsP production, whereas no significant InsP production was observed at the same Ca\(^{2+}\) concentration in V1 cells. These results suggest a preferential association of Ca\(^{2+}\) with PLC-\(\delta\) compared with PLC-\(\beta\) in activation in vitro. Eberhard and Holz (42) have proposed a hypothetical view that the initial transient cytosolic Ca\(^{2+}\) rise induced by InsP\(_2\) resulting from receptor-G-protein-mediated PLC(A) activation may in turn contribute to activation of PLC(B). Activation of PLC by 450 in PLC-61 is followed by 450 in vitro.


carboxyl-terminal residues in PLC-p1 but by only 10 residues hand,

Recent investigations have shown that PLC-p isozymes are

inhibited by increasing the Ca\(^{2+}\) concentration. Therefore, we as-

sessed that G\(\beta\) subunits could bind to the amino-terminal region of

PLC-\(\delta\). CHO cells contain several receptor-coupled G-proteins

(Gi2, Gi3, Gs, and Gq) (46, 47). Pretreatment with PT of

PLC630 cells partially inhibited InsP production induced by thrombin, indicating partial involvement of PT-sensitive G-protein(s). Thrombin receptor is known to couple to two G-proteins, Gq and Gi2 (37). Inhibition by PT of thrombin-stimulated InsP formation was much higher in PLC630 cells relative to that of V1 cells which may couple to Gq, suggesting that a different type of G-protein may be involved in PLC-\(\delta1\) activation by thrombin. Stimulation of InsP formation by thrombin plus GTP\(_S\) was also observed in the membrane from PLC630 cells, but the stimulation level was much less compared with those obtained in whole cell or permeabilized cells.

The PLC-\(\gamma\) isozyme has SH domains which are required for their activation (7–9), but the PLC-\(\delta\) isozyme has no such domains, indicating that tyrosine phosphorylation may not be implicated in activation of PLC-\(\delta\) isozymes. Actually, our study has shown that genistein, a potent tyrosine kinase inhibitor, did not affect InsP levels. Activation of PLC-\(\delta\) isozymes by thrombin stimulation in PLC630 cells (data not shown).

Several lines of evidence are accumulating to support the view that activation of PKC attenuates receptor-mediated PLC activation in many types of cells by its negative feedback action (48, 49). The target of PKC can be receptor, G-protein, or PLC itself. Phosphorylations by PKC of epidermal growth factor receptor and PLC-\(\gamma1\) were proposed to explain the attenuation mechanism of phosphoinositide hydrolysis (50, 51). PLC-\(\beta1\) also appears to be a target for the PKC-mediated attenuation in certain cells (2, 38). Pretreatment with TPA of cells containing PLC-\(\beta1\), PLC-\(\gamma1\), and PLC-\(\delta1\) elicited large increases in phospho-

phorylation of serine residues in PLC-\(\beta1\), but only small in-

creases in PLC-\(\gamma1\) and no effect in PLC-\(\delta1\), but does not affect its catalytic activity (38). It was also suggested that phospho-

phorylation of PLC-\(\beta1\) by PKC may interfere with its binding to Gq (38). We demonstrated in PLC630 cells that PKC activa-

tion by TPA inhibited the thrombin-stimulated InsP produc-

tion, but the level of the inhibition was almost similar to the

vector-transfected (V1) cells. Furthermore, the TPA treat-

ment was without effect on InsP formation induced by GTP\(_S\)

stimulation in both cells. In HEL cells, PKC activation was

reported to cause loss of responsiveness to thrombin, and its

inhibition was thought to be attributed to blockage of the Ca\(^{2+}\)

entry by phosphorylation of thrombin receptor (52). However, it is not the case with PLC630 cells, because the suppression of thrombin-stimulated InsP generation by TPA was not abol-

ished by increasing the Ca\(^{2+}\) concentration. Therefore, we

assume that phosphorylation of thrombin receptor by PKC acti-

vation in the V1 and PLC630 cells suppresses PLC activation by interfering with binding of the receptor with G-protein(s).

Leonis and Silbert (26) have recently shown that two CCL

fibroblast mutant cell lines, which lack PLC-\(\delta\) isozyme, showed significant differences in thrombin-stimulated PI turn-

over, decreased and increased responses, and also that transfection of the deleted mutant with rat brain PLC-\(\delta1\) cDNA did not correct the agonist-induced PI turnover. We have observed in the present study that the increase of thrombin-stimulated InsP formation in the PLC630 cells was entirely dependent on extracellular Ca\(^{2+}\). Removal of extracellular Ca\(^{2+}\) in PLC630 diminished the marked response of thrombin-stimulated InsP formation. Therefore, it is conceivable that the CCL39 mutants have an additional mutation(s), except for lack of PLC-\(\delta1\), for example, defective Ca\(^{2+}\) influx-related component. However, for elucidation of the precise mechanism of PLC-\(\delta1\) activation, fur-

ther work, including a reconstitution experiment, should be

required and is currently under progress in our laboratory.
Acknowledgments—We thank Dr. S. G. Rhee (National Institutes of Health, Bethesda, MD) for the kind supply of PLC-81 cDNA and antibodies for PLCs (g1, y1, 81). We also thank to Dr. Y. Sugimoto (Shirakawa Institute of Animal Genetics of Japan, Fukushima, Japan) for providing a transfection vector SRα and discussion on cDNA construction.

REFERENCES
1. Berridge, M. J. (1993) Nature 361, 315–324
2. Rhee, S. G., and Choi, K. D. (1992) J. Biol. Chem. 267, 12939–12996
3. Meldrum, K., Parker, J. P., and Carozzi, A. (1991) Biochim. Biophys. Acta 1069, 49–71
4. Krz, H., Lin, L. L., Sultman, L., Ellis, C., Heldin, C.-H., Pawson, T., and Knopf, J. (1990) Ciba Found. Symp. 150, 112–177
5. Kim, M. J., Bahk, Y. Y., Min, D. S., Lee, S.-J., Ryu, S. H., and Suh, P.-G. (1993) Biochem. Biophys. Res. Commun. 194, 706–712
6. Lee, C. W., Park, D. J., Lee, K. H., Kim, C. G., and Rhee, S. G. (1993) J. Biol. Chem. 268, 21318–21327
7. Meissnerfelder, J., Suh, G. P., Rhee, S. G., and Hunter, T. (1989) Cell 57, 1109–1122
8. Wahl, M. I., Nishida, S., Kim, J. W., Kim, H., Rhee, S. G., and Carpenter, G. (1990) J. Biol. Chem. 265, 3944–3948
9. Martin, T. F. J., Lewis, J. E., and Kowalsky, J. A. (1991) Biochem. J. 280, 753–760
10. Birbrairauwer, L., Abramowitz, J., and Brown, A. M. (1990) Biochim. Biophys. Acta 1041, 185–224
11. Smrcka, A. V., Hepler, J. R., Brown, K. O., and Sternweiss, P. O. (1991) Science 250, 804–807
12. Taylor, S. J., Chase, H. Z., Rhee, S. G., and Exton, J. H. (1991) Nature 350, 516–518
13. Waldo, G., Boyer, J., Morris, A., and Harden, T. K. (1991) J. Biol. Chem. 266, 14217–14225
14. Wu, D., Lee, C. H., Rhee, S. G., and Simoni, M. I. (1992) J. Biol. Chem. 267, 1811–1817
15. Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P. J., and Gierschik, P. (1992) Nature 360, 684–686
16. Katz, A., Wu, D., and Simon, M. I. (1992) Nature 360, 686–689
17. Hestrin, J. R., Kozasa, T., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweiss, P. C., and Gilman, A. G. (1993) J. Biol. Chem. 268, 14367–14375
18. Park, D., Tho, D.-Y., Lee, C.-W., Lee, K.-H., and Rhee, S. G. (1993) J. Biol. Chem. 268, 4557–4563
19. Smrcka, A. V., and Sterneis, P. C. (1993) J. Biol. Chem. 268, 9667–9675
20. Rhee, S. G., Kim, H., Suh, P. G., and Choi, W. C. (1990) Biochem. Soc. Trans. 18, 237–241
21. Flick, J. S., and Thurman, J. (1993) Mol. Cell. Biol. 13, 8661–8676
22. Yoko-o, T., Mataus, Y., Yagisawa, H., Nojima, H., Uno, I., and Toh-e, A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1804–1808
23. Bairoch, A., and Cox, J. A. (1990) FEBS Lett. 269, 454–456
24. Rath, H. M., Fee, J. A., Rhee, S. G., and Silbert, D. F. (1990) J. Biol. Chem. 265, 3080–3087
25. Cho, Y. S., Han, M. K., Chae, S. W., Park, C. V., and Kim, U. H. (1993) FEBS Lett. 334, 257–260
26. Leonis, M. A., and Silbert, D. F. (1993) J. Biol. Chem. 268, 9416–9424
27. To, T., Sugimoto, Y., Okano, Y., Kanoh, H., and Nozawa, Y. (1993) FEBS Lett. 307, 201–204
28. Berridge, M. J., Dawson, R. M. C., Downes, P., Heslop, J. P., and Irvine, R. F. (1983) Biochem. J. 212, 473–482
29. Kanes, H., Kanahe, Y., and Notsaw, Y. (1992) J. Neurochem. 59, 176–1794
30. Banno, Y., Nakashima, T., Kumada, T., Ebisawa, K., Nonomura, Y., and Nozawa, Y. (1992) J. Biol. Chem. 267, 6486–6494
31. Laemmli, U. K. (1970) Nature 227, 680–685
32. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
33. Palmer, S., Hawkins, P. T., Michell, R. H., and Kirk, C. J. (1966) Biochem. J. 258, 421–495
34. Takashima, A., and Kenimer, J. G. (1989) J. Biol. Chem. 264, 10654–10659
35. Meade, C., Turner, G. A., and Bateman, P. E. (1986) Biochem. J. 238, 425–436
36. Noyon, C. R., Nickahai, A., Little, P. J., Tkachuk, V. A., and Bokih, A. (1992) J. Biol. Chem. 267, 7295–7302
37. Hung, D. T., Wong, Y. H., Vu, T. K. H., and Coughlin, S. R. (1992) J. Biol. Chem. 267, 20631–20634
38. Ryu, S. H., Kim, U. H., Wahl, M. I., Brown, A. B., Carpenter, G., Huang, K. P., and Rhee, S. G. (1990) J. Biol. Chem. 265, 17941–17945
39. Cockcroft, S. (1992) Biochim. Biophys. Acta 1118, 135–160
40. Downes, P., and Michell, R. H. (1992) Cell Calcium 13, 467–502
41. Banno, Y., Sakai, T., Kumada, T., and Nozawa, Y. (1993) Biochem. J. 292, 401–408
42. Eberhard, D. A., and Hola, W. R. (1988) Trends Neurosci. 11, 517–520
43. Wu, D., Jiang, H., Katz, A., and Simon, M. I. (1993) J. Biol. Chem. 268, 3794–3799
44. Smrcka, A. V., and Sterneis, P. C. (1993) J. Biol. Chem. 268, 9667–9674
45. Clapham, D. E., and Neer, E. J. (1993) Nature 365, 403–406
46. Dell’Acqua, M. J., Carroll, C. R., and Perulata, E. G. (1993) J. Biol. Chem. 268, 5678–5685
47. Mitchell, P. M., Buckley, N. J., and Milligan, G. (1993) Biochem. J. 293, 455–499
48. Ozawa, K., Yamada, K., Kazanietz, M. G., Blumberg, P. M., and Beaven, M. A. (1993) J. Biol. Chem. 268, 2280–2283
49. Pachter, J., Pai, J. K., Mayer-Essel, R., Petrin, J. M., Dobek, E., and Bishop, W. R. (1992) J. Biol. Chem. 267, 9626–9630
50. Deckers, S. J., Ellis, C., Pawson, T., and Veilu, T. (1990) J. Biol. Chem. 265, 7099–7105
51. Park, D. J., Min, H. K., and Rhee, S. G. (1992) J. Biol. Chem. 267, 1496–1501
52. Brass, L. F. (1992) J. Biol. Chem. 267, 6044–6050