Differential Phospholipase D Activation by Bradykinin and Sphingosine 1-Phosphate in NIH 3T3 Fibroblasts Overexpressing Gelsolin*

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Gelsolin, an actin-binding protein, shows a strong ability to bind to phosphatidylidylinositol 4,5-bisphosphate (PIP2). Here we showed in vitro experiments that gelsolin inhibited recombinant phospholipase D1 (PLD1) and PLD2 activities but not the oleate-dependent PLD and that this inhibition was not reversed by increasing PIP2 concentration. To investigate the role of gelsolin in agonist-mediated PLD-activated NIH 3T3 fibroblasts stably transfect with the cDNA for human cytosolic gelsolin, Gelsolin overexpression suppressed bradykinin-induced activation of phospholipase C (PLC) and PLD. On the other hand, sphingosine 1-phosphate (SIP)-induced PLD activation could not be modified by gelsolin overexpression, whereas PLC activation was suppressed. PLD activation by phorbol myristate acetate or Ca2+ ionophore A23187 was not affected by gelsolin overexpression. Stimulation of control cells with either bradykinin or SIP caused translocation of protein kinase C (PKC) to the membranes. Translocation of PKC-α and PKC-β1 but not PKC-ε was reduced in gelsolin-overexpressed cells, whereas phosphorylation of mitogen-activated protein kinase was not changed. SIP-induced PLC activation and mitogen-activated protein kinase phosphorylation were sensitive to pertussis toxin, but PLD response was insensitive to such treatment, suggesting that SIP-induced PLD activation via certain G protein distinct from Gi for PLC and mitogen-activated protein kinase pathway. Our results suggest that gelsolin modulates bradykinin-mediated PLD activation via suppression of PLC and PKC activities but did not affect SIP-mediated PLD activation.

Hydrolysis of phosphatidylcholine (PC)1 by phospholipase D

(Received for publication, May 7, 1999, and in revised form, June 29, 1999)

* This work was supported in part by Grants-in-aid for Scientific Research on Priority Areas 09273104 and 10212204 and Grants-in-aid for Scientific Research (B) 09480162 and (C) 09670150 from the Ministry of Education, Science, Sports, and Culture of Japan. A special Coordination Fund for Promoting Science and Technology was from the Science and Technology Agency of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PC, phosphatidylcholine; ARF, ADP-ribosylation factor; DMEM, Dulbecco’s modified Eagle’s medium; G2 and G6, gelsolin-overexpressing clones 2 and 6, respectively; GTP-γ-S, guanosine 5'-(γ-thiotriphosphate); InsP, inositol phosphate; MAP kinase, mitogen-activated protein kinase; PA, phosphatidic acid; PBut, phosphatidylbutanol; PE, phosphatidyethanolamine; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC and PLD, phospholipase C and D, respectively; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin; S1P, sphingosine 1-phosphate; Vect, vector-transfected; Ab, antibody; MAP, mitogen-activated protein; BSA, bovine serum albumin.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidylcholanelamine (PE), PC, PIP2, sodium oleate, genetin (G418), A23187, and phorbol myristate acetate (PMA) were purchased from Sigma. Sphingosine 1-phosphate (SIP) was from Matreya, Inc. (Pleasant Gap, PA). [9,10-3H]Palmitic acid (54.0 Ci/mmol), myo-[3H]inositol (90 Ci/mmol), and [choline-methyl-3H]dipalmi-
toyl-PC (26.5 Ci/mmol) were from NEN Life Science Products. LipombFAMINE was from Life Technologies, Inc. Rabbit polyclonal antibodies were prepared to the carboxyl-terminal 15 residues of human PLD1a (Ab-224) and carboxyl-terminal 16 residues of rat PLD2.
Polyclonal antibodies to PKC isoforms were from Santa Cruz Biotechnology (Santa Cruz, CA), and the antibody to phosphorylated mitogen-activated protein (MAP) kinase was from New England Biolabs (Boston, MA). Anti-rabbit antibody conjugated with horseradish peroxidase and chemiluminescence kit (ECL system) were from Amer sham Pharmacia Biotech.

Cell Culture and Transaction of Gelsolin cDNA—NIH 3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 4% fetal bovine serum, 100 units of penicillin/ml, and 100 μg of streptomycin/ml at 37 °C in a humidified, CO2-controlled (5%) incubator.

A HindIII/Stol fragment of human cytoplasmic gelsolin cDNA (21) was cloned into HindIII-HpaI site of plNLC retroviral vector (a generous gift from Dr. A. D. Miller, Fred Hutchinson Cancer Research Center) (22). The resulting construct, plNChGsn or plNLCX alone was transfected into Bosc 23, a highly efficient ecotrophic virus-packaging cell line (23), using LipofectAMINE. Two days after lipofection, the virus-containing supernatant was collected and centrifuged to remove cells and debris. NIH 3T3 cells were infected with the virus-containing supernatant. The vector and gelsolin-transfected cells were maintained in the presence of 0.5 mg/ml G418. The expression of gelsolin or PLD proteins was examined by Western blotting with specific antibodies using the ECL detection system.

Baculovirus Expression and Assay of PLD Activity—PLD1 and PLD2 were expressed in Sf9 cells that had been infected with recombinant baculoviruses harboring human PLD1 or PLD2 cDNAs (kindly supplied by Dr. M. A. Frohman, State University of New York) as described previously (11). Cells were suspended in ice-cold lysis buffer (20 mM HEPES, pH 7.4, 1 mM EGTA, 1 mM MgCl2, 1 mM dithiothreitol, 1 mM phenylmethylsulfonfluoride, 20 μg/ml (β-3-trans-carboxyoxiran-2-carbonyl)-l-leucyl-agmatine, E-64) and lysed by sonication. The lysates were centrifuged at 1,000 × g for 5 min, and the resulting supernatant was centrifuged at 100,000 × g for 60 min to obtain the membrane fraction.

PLD1 activity was assayed essentially as described previously (20) by measuring the generation of 3H-labeled choline from [choline-methyl-3H]dipalmitoyl-phosphatidylcholine (PC) as substrate. Briefly, 20 μl of lipid vesicles containing PE, PIP2, and PC in a molar ratio of 16:1:4:1 with [choline-methyl-3H]dipalmitoyl-PC (total 4 × 107 cpm/assay) were added to 100 μl of a mixture containing PLD source, 10 mM ARF, 5 μg GTP/S, 50 mM HEPES-NaOH, pH 7.5, 3 mM MgCl2, 80 mM KCl, 2.5 mM MgCl2, and 2 mM CaCl2. PLD2 activity was assayed using the procedure used for PLD1 except for omitting PLD1, ARF, and GTP/S. Oleate-dependent PLD activity was assayed by measuring the generation of 3H-labeled choline from [choline-methyl-3H]dipalmitoyl-phosphatidylcholine in the presence of 0.5 mM oleate (20).

Measurement of Total Inositol Phosphates—NIH 3T3 cells in 6-well plates were preloaded with 1 μCi/ml myo[3H]inositol for 24 h in inositol-free DMEM medium containing 0.3% bovine serum albumin (BSA). Cells were then washed twice with the HEPES/Tyrode buffer (10 mM HEPES/NaOH, pH 7.4, 134 mM NaCl, 12 mM NaHCO3, 2.9 mM KCl, 0.36 mM NaH2PO4, 1.0 mM MgCl2, 1.8 mM CaCl2, 1 mg/ml BSA, and 1 mM phenylmethylsulfonyl fluoride, 20 mM NaCl, 25 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml E-64, 20 mM β-glycerophosphate, 1 mM sodium fluoride, and 1 mM sodium orthovanadate, pH 7.4), and then homogenized by sonication. Protein concentrations were assayed with Bradford protein assay reagent using BSA as the standard. Total cell lysates (20 μg) were subjected to electrophoresis on 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% BSA. Phosphorylation of MAP kinase 1/2 was determined by immunoblotting with a polyclonal antibody (anti-phospho- MAP kinase) that recognizes only activated MAP kinase 1/2. Total MAP kinase 1/2 was detected by blotting with an antibody against MAP kinases. After washing in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM NaCl and 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-linked secondary antibody. After repeated washings, the bound antibody was detected by using the ECL Western blotting detection system.

PKC Translocation—For measurement of PKC translocation, NIH 3T3 cells were subjected to serum-free DMEM containing 0.3% BSA for 10 min before stimulation. The washed cells were incubated and harvested in ice-cold buffer A (25 mM HEPES, pH 7.4, containing 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) and then lysed by sonication. Cell lysates were centrifuged at 1,500 × g for 5 min, and the supernatants were further centrifuged at 100,000 × g for 30 min. The pellets were resuspended in buffer A. For Western blot analysis, these membranes were separated on 8% gels by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking in 5% (w/v) BSA, the membranes were incubated with specific antibodies for PKC isozymes. The membranes were incubated with horseradish peroxidase-linked secondary antibody. After repeated washings, the bound antibody was detected by using the ECL Western blotting detection system.

RESULTS

Effects of Gelsolin on Activities of Phospholipase D Isoforms in Vitro—Since gelsolin is a potential actin-binding protein that has high affinity for PIP2 (15, 16), we examined whether gelsolin inhibits PLD activity by binding the cofactor PIP2 using Sf9 cells overexpressing PLD1 and PLD2. Gelsolin (5 μM) inhibited ARF-dependent PLD1 and PLD2 activities of membrane fractions from transfected Sf9 cells using PE-PIP2-PC micelles as substrate (Fig. 1). On the other hand, gelsolin had no effect on oleate-dependent PLD activity abundant in PC12 cells. Further examination showed that PLD2 activity was
 inhibited by gelsolin in a concentration-dependent manner with maximum inhibition at 5 μM (Fig. 2A). On the other hand, PLD2 activity was stimulated by adding PIP2 to PE/PC vesicles in a concentration-dependent manner with a maximum at 40 μM in the absence of gelsolin (Fig. 2B). The inhibitory effect of gelsolin (1 μM) was not abrogated by increasing PIP2 concentration, suggesting that gelsolin did not inhibit PLD2 activity by binding PIP2.

Overexpression of Gelsolin in NIH 3T3 Cells—To examine the effect of gelsolin on PLD activation in vivo, we studied various clones stably overexpressing gelsolin in NIH 3T3 fibroblasts. Cells were transfected with a construct containing gelsolin cDNA, and two clones (G2 and G6) were obtained. The expression level of gelsolin was higher in G2 clone than G6 clone, as inferred by Western blot analysis (Fig. 3A). A clone of vector-transfected NIH 3T3 cells (Vect) was used as control. No significant differences were observed in the growth rate and morphology between Vect, G2, and G6 cells (data not shown).

To examine the expression levels of PLD isoforms in the transfected NIH3T3 cells, the lysates were subjected to Western blot analysis using two PLD antibodies (Ab-224 and Ab-226). Both antibodies could react with recombinant human PLD1a, PLD1b, and mouse PLD2 used as standards. Ab-224 can detect PLD1a, PLD1b, and PLD2, and Ab-226 detected PLD1b and PLD2 but not PLD1a in HaCaT cell lysates (Fig. 3B, upper panels, 1 and 2). Western blot analysis using these two antibodies revealed the presence of a significant amount of PLD2 and much less PLD1a (23% of PLD2) but not PLD1b in vector-transfected control NIH 3T3 cells (Vect) (Fig. 3B, lower panel). However, there were no significant differences in the amount of these PLD isoforms among Vect, G2, and G6 clones. Expression levels of other signaling enzymes, such as PKC isozymes (α, β1, δ1, and ε) and PLC isozymes (PLCβ1, β3, and γ1) were also not different between Vect- and gelsolin-overexpressing clones (data not shown).

Effects of Gelsolin Overexpression on Bradykinin-induced PLD Activation—To examine the effects of gelsolin overexpression on PLD activation, formation of PBut was examined in Vect, G2, and G6 cells stimulated by bradykinin. The time course of PBut formation by bradykinin (2 μM)-stimulated Vect, G2, and G6 cells is shown in Fig. 4. Gelsolin overexpression reduced PBut formation induced by bradykinin stimulation. G2 clone with the highest level of gelsolin expression showed the lowest PBut formation compared with control (Vect) cells. PLD activation was reduced to a lesser extent in G6 clone, which had a lower gelsolin expression. These results suggest that inhibition of bradykinin-stimulated PBut formation appear to correlate with the expression level of gelsolin.

Involvement of PLC Activation in Bradykinin-induced PLD Activation in Gelsolin-overexpressing Cells—PLD activation is known to be dependent on phosphoinositide hydrolysis by PLC, since PKC activation by diacylglycerol derived from PIP2 breakdown is involved in the activation of PLD by agonist stimulation (27, 28). We have previously demonstrated that gelsolin inhibited PLC activity in vitro (17), and Sun et al. (18) also demonstrate that gelsolin overexpression suppresses bradykinin-stimulated PLCβ activity. To examine the effect of gelsolin overexpression on PLC activation in NIH 3T3 cells, we
measured the formation of inositol phosphates (InsPs) in response to bradykinin. As shown in Fig. 5, bradykinin stimulation increased InsP formation in a concentration-dependent manner in control cells (Vect), whereas gelsolin overexpression reduced PLC response to bradykinin by approximately 50% in G2 cells (Fig. 5A). Similar repression of PBut formation was observed in G2 cells stimulated with bradykinin at all concentrations tested (Fig. 5B).

To examine whether inhibition of PLD activation by gelsolin overexpression is a downstream event in the PLC-PKC pathway, we examined the effect of Ca²⁺ ionophore, A23187, and PMA on PLD activation. PBut formation was increased by stimulation with 1 μM A23187 (Fig. 6A) or 100 nM PMA (Fig. 6B) in a time-dependent manner. PBut formation induced by either stimulator was not affected by gelsolin overexpression. These observations suggested that bradykinin-mediated PLD activation was dependent upon PLC activation in NIH 3T3 fibroblasts.

Effects of Gelsolin Overexpression on PLD Activation Induced by Sphingosine 1-Phosphate—S1P is known to stimulate PLD activity in various cells (29–31). To examine the effect of gelsolin on S1P signaling in NIH 3T3 cells, we investigated PLC and PLD activation by S1P in G2 cells. InsPs formation was increased by S1P stimulation in a concentration-dependent manner in control cells (Vect) but was reduced in G2 cells at the concentrations examined (Fig. 7A). PBut formation by S1P stimulation increased in a concentration-dependent manner in control cells, reaching a peak level with 5 μM S1P at 2 min. In sharp contrast to InsPs formation, S1P-stimulated PBut formation was not inhibited in G2 cells (Fig. 7B). These results suggested that gelsolin overexpression repressed PLC activation but not PLD activation in S1P-stimulated cells.

Effects of Pertussis Toxin on Agonist-induced PLC and PLD

![Fig. 4. Effects of gelsolin overexpression on bradykinin-induced PLD activation.](image)

![Fig. 5. Effects of gelsolin overexpression on bradykinin-induced PLC and PLD activation.](image)

![Fig. 6. Effects of gelsolin overexpression on PLD activation induced by ionophore A23187 or PMA.](image)
Activation—The bradykinin receptor has been known to couple to Gq (32), whereas the S1P receptor associates with a heterotrimeric G protein (EDG-1) (33, 34). A number of EDG receptor subfamilies are coupled to G proteins such as pertussis toxin (PTX)-sensitive G_{i1}/G_{i2} or -insensitive G_{i3}/G_{12/13} proteins (35). As shown in Fig. 8, PTX pretreatment (200 ng/ml) markedly reduced InsP formation induced by S1P but not by bradykinin, suggesting that PLC activation by S1P may be mediated via PTX-sensitive G protein. On the other hand, PBut formation induced by S1P was not affected by pretreatment with PTX, even at a high concentration of PTX (2 μg/ml). These results suggest that the G protein involved in S1P-evoked PLD activation is distinct from that in PLC activation.

Effects of Gelsolin Overexpression on Translocation of Protein Kinase C Isozymes Induced by Agonists—PKC is one of the most potent stimulators of agonist-induced PLD activation (1). To study the involvement of PKC in the agonist-induced PLD activation, membrane fractions from bradykinin or S1P-stimulated Vect and G2 cells were examined by using antibodies to PKC isozymes. Treatment of Vect cells with bradykinin or S1P induced translocation of PKC-α, PKC-β1, and PKC-ε (Fig. 9). The addition of EGTA inhibited the translocation of PKC-α and PKC-β1 but not PKC-ε. Furthermore, S1P- but not bradykinin-induced translocation of PKC isozymes was suppressed by PTX treatment. PKC-α and PKC-β1 levels did not increase in membranes from G2 cells incubated with bradykinin or S1P. On the other hand, translocation of PKC-ε induced by both agonists was not affected in gelsolin overexpressing cells.

Agonist-induced Mitogen-activated Protein Kinase Activation—Treatment of various cells with bradykinin or S1P resulted in activation of the MAP kinase pathway. Recently, Rizzo et al. (6) reported that PA formation via activation of PLD by insulin induced stimulation of the MAP kinase pathway via Raf-1 kinase activation. We examined the effect of gelsolin overexpression on the agonist-induced MAP kinase activation using an antibody that recognized the phosphorylated form of MAP kinase. Stimulation of Vect cells with S1P caused a marked phosphorylation of MAP kinase (Fig. 10). PKC down-regulation by long term treatment with PMA (300 nM for 24 h) and PKC inhibitor Ro31–8220, but not tyrosine kinase inhibitor ST638, reduced S1P-induced MAP kinase phosphorylation by 80–90%. Pretreatment of Vect cells with PTX abolished S1P-induced MAP kinase phosphorylation, but EOTA had no effect. These results suggest that S1P-induced MAP kinase activation was PKC-dependent and PTX-sensitive. As shown in Fig. 10C, MAP kinase phosphorylation induced by PMA, bradykinin, and S1P was similar in Vect and G2 cells, suggesting that gelsolin overexpression had no effect on agonist-stimulated MAP kinase activation.

DISCUSSION
We showed in the present study that gelsolin inhibited recombiant PLD1 and PLD2 activities but not oleate-dependent PLD, which requires PIP_2 as a cofactor in the exogenous substrate in vitro assay (1, 2). There are a number of inhibitory proteins for PLD activity such as synaptotanin and fodrin. These are not direct inhibitors of PLD activity but are related to reduced PIP_2 in agonist-stimulated cells (36, 37). Ceramide also modulates agonist-mediated PLD activation by inhibiting PKC-α, ARF, or RhoA translocation (28, 38, 39). Our previous study, however, indicated that C2-ceramide directly inhibited both GTPyS-dependent and -independent PLD activities in membrane fractions from HaCaT cells when assayed using the...
and bradykinin and sphingosine 1-phosphate and effects of EGTA
phoresis and immunostained with the indicated antibodies.
A, thermore, recent studies have demonstrated that PKC-
induced PLD activation in a variety of cell types (1, 2). Fur-
more, evidence indicates that PKC plays a major role in agonist-
activation by PMA, ionophore A23187, and S1P. Several lines
of evidence indicate that PKC plays a major role in agonist-
activation of PLD1 or PLD2 from overexpressed NIH 3T3 cells.
However, we could not find any association with gelsolin based on experiments using partially
purified PLD from rabbit brain. However, we could not find any
association with gelsolin when PLD1 or PLD2 from overex-
pressed NIH 3T3 cells was immunoprecipitated using specific antibodies.

Our study demonstrated that overexpression of gelsolin in
NIH 3T3 cells modified agonist-stimulated PLC and PLD activ-
ation. Thus, gelsolin overexpression repressed bradykinin-
mediated PLC and PLD activation but did not affect PLD
activation by PMA, ionophore A23187, and S1P. Several lines
of evidence indicate that PKC plays a major role in agonist-
induced PLD activation in a variety of cell types (1, 2). Fur-
thermore, recent studies have demonstrated that PKC-α and
PKC-β are the principal regulator of PLD activity (2, 41, 42)
and that the regulatory domain of PKC-α itself is an effective
activator of PLD (43). PKC activation is thought to be due to
activation of PLC isoforms, which hydrolyze PIP2 to generate
inositol 1,4,5-trisphosphate and diacylglycerol. The resultant
increase in diacylglycerol and Ca²⁺ leads to activation and
translocation of conventional PKC isoforms. Thus, the inter-
action of PKC with PLD in membranes may be sufficient to
induce PLD activation. PLD activation was abolished in PKC-
down-regulated cells, suggesting that PLC acts upstream of
PLD. The involvement of PLC/PKC in PLD regulation by
growth factors has been shown by studies using mouse embry-
onic fibroblasts with disrupted PLCγ1 gene (44). Our studies
demonstrated here that gelsolin overexpression inhibited
bradykinin-stimulated PLCβ activity, thereby leading to repres-
sion of activation of PKC-α and PKC-β but not PKC-ε. There-
fore, the reduced PLD response to bradykinin in gelsolin-
overexpressed cells may be secondary to repressed PKC-α
activation. This notion was further supported by the finding
that PMA- or A23187-induced PLD activation was unaffected
by gelsolin overexpression. Considered together, these results

Fig. 9. Membrane translocation of PKC isozymes induced by
bradykinin and sphingosine 1-phosphate and effects of EGTA
and PTX treatment. A, vector (Vect)- or gelsolin (G2)-overexpressing NIH 3T3 cells with or without pretreatment of PTX (200 ng/ml) (lanes 4 and 7) for 24 h were stimulated with bradykinin (2 μM) (lanes 2–4) for 1 min or S1P (5 μM) (lanes 5–7) for 2 min and lysed by sonication. In some cases, EGTA (3 mM) (lanes 3 and 6) was added in the buffer. Lane 1 is a control membrane. Membrane fractions were prepared as described under “Experimental Procedures.” Membrane fractions (5–20 μg of proteins) were subjected to 8% SDS-polyacrylamide gel electrophoresis and immunostained with the indicated antibodies. A, blot representative of three independent experiments. B, relative amounts of PKC isozymes in vector (Vect)- or gelsolin-overexpressing (G2) cells shown in A were quantified by scanning densitometry, and the mass of all membranes in each PKC isozyme was designated as 100%. Results are shown as mean of three independent experiment. □ control membranes; □, bradykinin-membranes; □, S1P membranes.

Fig. 10. Effects of gelsolin overexpression and various inhibi-
tors on phosphorylation of MAP kinase A, vector-transfected NIH 3T3 cells were pretreated with PMA (300 nM) or PTX (200 ng/ml) for 24 h and with ST638, EGTA (3 mM) or Ro31–8220 (RO, 50 μM) for 15 min and stimulated with S1P (5 μM) for 2 min. The cell lysates (20 μg protein) were analyzed by Western blotting using anti-MAP kinase (MAPK 1/2) or -phospho-specific MAP kinase (P-MAPK 1/2) antibodies. A, blot representative of three independent experiments. CONT, control. B, amounts of phosphorylated MAP kinase 2 shown in B were quantified by scanning densitometry and expressed as fold increase relative to unstimulated control. Results represent the mean ± S.E. of three separate experiments. C, vector (lanes 1, 3, 5, and 7) or gelsolin-overexpressed NIH 3T3 cells (lanes 2, 4, 6, and 8) were stimulated with PMA (100 nM) for 2 min, bradykinin (BK) (2 μM) for 1 min, and S1P (5 μM) for 2 min. Cell lysates were analyzed by Western blotting using anti-phospho-specific MAP kinase (P-MAPK 1/2). A blot representative of three independent experiments is shown.
suggest that bradykinin-mediated PLD activation may be attributed to PKC-dependent PLD1. PLD1 activation is mediated by several factors such as PKC (α, -β) and small G proteins (ARF, Rho family, and Rap) (1, 2, 42, 45). We also noted that bradykinin stimulation increased RhoA level in membranes of control cells, whereas its level was decreased in gelsolin-overexpressed cells (data not shown). Therefore, reduced RhoA levels might be also responsible for the reduced PLD response to bradykinin in gelsolin overexpressed cells.

On the other hand, PLD2 is independent of PLC activation, since it is not activated by PKC and small G proteins (1, 2). S1P levels might be also responsible for the reduced PLD response expressed cells (data not shown). Therefore, reduced RhoA levels are also increased by bradykinin stimulation increased RhoA level in membranes of ARF, Rho family, and Ral (1, 2, 42, 45). We also noted that three subfamilies of S1P receptor (EDG1, EDG3, and EDG5) signaling pathway in Swiss 3T3 fibroblasts (30). There are evidences indicate that PLD activation is independent of these signaling components. This notion is consistent with the S1P signaling pathway in Swiss 3T3 fibroblasts (30). There are three subfamilies of S1P receptor (EDG1, EDG3, and EDG5) (35). EDG1 is coupled to G12 protein, which mediates the S1P signaling pathway involving PLC, adenylate cyclase, and MAP kinase (47). In contrast, EDG3 and EDG5 mediate the pathways via the G12, G13, and G12/13 (35, 46). Furthermore, it has been reported that in Chinese hamster ovary cells stably expressing EDG1 receptor, S1P induces MAP kinase activation in a PTX- and genistein-sensitive but PKC-independent manner (30). In comparison, our study demonstrated that S1P-stimulated MAP kinase activation was PTX-sensitive and PKC-dependent, but tyrosine kinase-insensitive. These results indicate that in NIH3T3 fibroblasts, S1P-induced MAP kinase activation was mediated via G12, but not EDG1. PLC activation induced by bradykinin and S1P stimulation were suppressed by gelsolin overexpression, whereas MAP kinase activation by both agonists was not affected. These results suggest that S1P-induced PLC activation via certain G proteins distinct from G12 for PLC and the MAP kinase pathway in NIH 3T3 fibroblasts. Recently, PLD2 was demonstrated to be involved in insulin-dependent MAP kinase pathway (8). Our results, however, suggest that neither PLC nor PLD is associated with MAP kinase activation in S1P-stimulated NIH 3T3 fibroblasts.

Acknowledgment—We thank Dr. M. A. Frohman (State University of New York) for providing cDNA of human PLD1 and PLD2.

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