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Phospholipase D Is Not Involved in Rho A-Mediated Activation of Stress Fiber Formation

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Abstract : In order to investigate the role of a small GTP-binding protein RhoA in lysophosphatidic acid (LPA)-induced stress fiber formation, C3 ADP-ribosyltransferase was prepared by expressing in E. coli and then applied to Rat-2 fibroblasts. C3 transferase isolated from *E. coli* was as effective as the toxin from *Clostridium botulinum* in ADP-ribosylation of RhoA. Incubation of the cells with C3 transferase for 2 days induced ADP-ribosylation of RhoA by a dose-dependent manner, with a sub-maximal induction at 25 μg/ml. As expected, LPA-induced stress fiber formation was completely blocked by pre-incubation with C3 transferase for 2 days. However, exogenously added C3 transferase had no significant effect on the formation of phosphatidylethanol by LPA. These results suggested that phospholipase D was not activated by RhoA in the LPA-induced stress fiber formation.
Key words : C3 transferase, lysophosphatidic acid, phospholipase D, RhoA, stress fibers

Regulation of phospholipase D (PLD) has been intensively studied since hydrolysis of phosphatidylcholine by PLD produces second messengers such as phosphatidic acid (PA) and diacylglycerol, which are important in transmembrane cell signaling (Billah *et al.*, 1990; Exton, 1994). PA is involved in various signaling pathways such as activation of protein phosphorylation (Bocckino *et al.*, 1991), stimulation of DNA synthesis (Kanuss *et al.*, 1990), increase of intracellular Ca^{2+} (Salmon and Honeyman, 1980; Putney *et al.*, 1984) and stress fiber formation (Ha and Exton, 1993a). Diacylglycerol derived from PC is reported to activate a novel protein kinase C (PKC) ε in IIC9 fibroblasts (Ha and Exton, 1993b).

It has been known that PC-hydrolyzing PLD is regulated by PKC, tyrosine kinase and small GTP-binding proteins (Exton, 1994; Yeo and Exton, 1995). PKCα activated PLD isolated from porcine brain (Singer *et al.*, 1996). In HL60 cells, PKCα was more effective than PKCβ in the activation of plasma membrane PLD (Ohguchi *et al.*, 1996). The possible involvement of tyrosine kinase in the regulation of PLD was shown by the inhibition of PLD activation by herbimycin A in E6CH cultures (Mangoura and Dawson, 1993). In cerebellar astrocytes, phorbol ester activation of PLD was blocked by genistein (Mangoura *et al.*, 1995). PC-hydrolyzing PLD is also regulated by Arf in HL60 cells and porcine brain (Cockcroft *et al.*, 1994; Martin *et al.*, 1996; Singer *et al.*, 1996). Furthermore, it has been reported that Arf is synergistic with PKC in the activation of PLD partially isolated from porcine brain (Singer *et al.*, 1996). In addition to Arf, RhoA is also known to activate PLD in cell free systems (Brown *et al.*, 1995; Kuribara *et al.*, 1995; Siddiqi *et al.*, 1995). RhoA was involved in the cell-free activation of PLD by GTPγS in human neutrophils (Bowman *et al.*, 1993; Kwak *et al.*, 1995). In HL 60 cells, RhoA activated PC-hydrolying PLD synergistically with PKC (Ohguchi *et al.*, 1996). Recently, there have been two reports suggesting a role of RhoA in the regulation of PLD *in vivo* (Malcolm *et al.*, 1996; Schmidt *et al.*, 1996).

LPA is known to activate stress fiber formation by regulating RhoA (Moolenaar, 1995). It has been reported that PLD was involved in LPA-induced stress fiber formation (Ha *et al.*, 1994; Cross *et al.*, 1996). In IIC9 fibroblasts, LPA activated PLD and stress fiber formation, which were inhibited by pertussis toxin (Ha *et al.*, 1994). Butanol, a primary alcohol, inhibited PLD and actin polymerization induced by LPA in PAE cells (Cross *et al.*, 1996). However, it is not still known whether RhoA activates stress fiber formation by activating PLD or not.

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In this study, we present results suggesting that RhoA was involved in LPA-induced stress fiber formation, but not in the activation of PLD. C3 transferase of *Clostridium botulinum* inhibited LPA-induced formation of intracelllar stress fibers, but had no significant effect on LPA-stimulated PLD activity in Rat-2 fibroblasts.

Materials and Methods

Materials

Cell culture medium components were obtained from GIBCO-BRL (Gaithersburg, USA). Glutathione-S-transferase fusion vector pGEX- 4T-2 was obtained from Invitrogen (Carlsbad, USA). C3 ADP-ribosyltransferase from *C. botulinum* was from Biomol Laboratory (Plymouth Meeting, USA). Phosphatidylethanol was obtained from Avanti Polar Lipids (Alabaster, USA). [^{32}P]-NAD was from Du Pont-New England Nuclear (Wilmington, USA). All other chemicals were of analytical grade.

Cell culture

Rat-2 fibroblasts were cultured in DMEM supplemented with 25 mM HEPES, 10% (w/v) fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified, CO_2-controlled (5%) incubator. For experiments, cells were grown on multi-well plates for 2 days and serum-starved for 2 days in serum-free DMEM supplemented with 5 μg/ml apo-transferrin, 1 mg/ml bovine serum albumin, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 25 mM HEPES, pH 7.4.

Expression and purification of C3 transferase

EFC3 plasmid containing C3 transferase gene was cut with *BamH1* and *EcoRI* and then ligated with pGEX-4T-2 vector. *E. coli* (XL1 blue) transformed with the vector were grown at 30°C in LB medium supplemented with 100 μg/ml ampicillin. GST-C3 transferase fusion protein was induced by incubation with 0.1 mM isopropylthiogalactoside for 2 h. After harvesting at 8,000 rpm for 10 min, the cells were frozen to -80°C in a deep freezer, thawed, and then disrupted with a French Pressure Cell in a phosphate buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.3) containing 1% Triton X-100. The cell extract was loaded onto a prepacked glutathione sepharose 4B column (Pharmacia Biotech) and then C3 transferase was eluted from the column with PBS after incubating overnight with 5 units of thrombin.

ADP-ribosylation

Rat-2 fibroblasts grown on a 100 mm culture dish were washed with PBS and trypsinized. The cell pellets were then suspended in 0.5 ml of suspension buffer (1 mM EDTA, 1.5 mM $MgCl_2$, 0.1 mM PMSF, 10 mM Tris-HCL, pH, 7.5). The cells were then disrupted by sonication for 5 s and centrifuged at 13,000 rpm for 10 min at 4°C. Twenty μg of the supernatant was mixed with 0.1 μg of C3 transferase. The ADP-ribosylation reaction was started by addition of 1 μCi of [^{32}P]NAD to the mixture of cell homogenate and C3 transferase in 60 μl of reaction buffer (1 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 10 mM thymidine, 0.2 μM NAD, 20 mM Tris-HCl, pH 7.5). After incubation at 37°C for 30 min, the reaction was terminated by adding the same volume of 2X SDS sample buffer containing 2% SDS, 10% glycerol, 100 mM dithiothreitol, 0.001% bromophenol blue (w/v) and 60 mM Tris-HCl, pH 6.8. The samples were then subjected to SDS-PAGE analysis and subsequent autoradiography.

Observation of F-actin

Rat-2 cells were cultured on coverslips in 12 well plates and incubated with serum-free medium containing 25 ng/ml C3 transferase for 2 days. The cells were then washed with fresh serum-free DMEM and stimulated with 1 μg/ml of LPA for 30 min. The cells were stained with NBD-phallacidin by the procedures of Ha and Exton (1993b). Briefly, cells were quickly washed with PBS and fixed with 3.7% formaldehyde in PBS for 30 min. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 30 min on ice and then stained with 0.165 μM NBD-phallacidin in PBS for 30 min. The stained cells were washed three times with PBS for 15 min and mounted with gelvatol on slide glasses. The cells were then observed with a confocal laser scanning microscope (Carl Zeiss LSM 410). The sample was excited by a 488 nm argon laser and the images were filtered by a longpass 515 nm emission filter. Three dimensional images were constructed from 5-10 serial images made by automatical optical sectioning.

Determination of PLD activity

Cells were cultured on 6-well plates and incubated with serum-free medium containing 2 μCi/well [^{3}H]myristic acid for 2 days. The labelled cells were incubated with 1% ethanol for 10 min and then treated with 1 μg/ml LPA for 15 min. Sometimes, cells were pretreated with 25 μg/ml of C3 transferase for 2 days before stimulating with LPA. Subsequently, the cells were quickly washed with ice-cold PBS and scraped into 2 ml of ice-cold methanol. Lipids were extracted according to the procedures of Bligh and Dyer (1959) and then phosphatidylethanol (PEt) was separated by a thin layer chromatography using a solvent system of ethylacetate/iso-octane/acetic acid/water (110:50:20:100, v/v).

The PEt bands were identified with 0.002% primulin in 80% acetone, scraped and then counted using a scintillation counter.

Results and Discussion

Since C3 transferase is known to inhibit RhoA activity by ADP-ribosylation (Malcolm *et al.*, 1996), we have applied C3 transferase expressed in *E. coli* to Rat-2 fibroblasts to determine the roles of RhoA in positive activation of PLD and stress fiber formation in response to LPA. C3 transferase expressed in *E. coli* was successfully purified and its activity was tested as shown in Fig. 1. As a positive control, commercially available C3 transferase was also used to compare its ADP-ribosylating activity toward RhoA with our purified enzyme. Two C3 transferases revealed similar ADP-ribosylating activities on approximately 25 kDa protein, consistent with previous reports (Quillam *et al.*, 1989; Just *et al.*, 1992; Ménard *et al.*, 1992).

In order to determine whether exogenously added C3 transferase is able to inhibit RhoA by ADP-ribosylation, Rat-2 cells were incubated with various concentrations of C3 transferase for 2 days and then cell lysates were incubated with [^{32}P]NAD and C3 transferase. As shown in Fig. 2, C3 transferase inhibited the incorporation of [^{32}P]NAD into RhoA by a concentration-dependent manner, with about 50% inhibition by 25 μg/ml. Similar results have been reported in HL 60, HEK and various tumor cells (Koch *et al.*, 1994; Aepfelbacher *et al.*, 1995; Schmidt *et al.*, 1996; Udagawa, and McIntyre, 1996). Considering that RhoA is inhibited by ADP-ribosylation, these results suggested that exogenous C3 transferase was successfully moved into Rat-2 cells and able to inhibit RhoA in Rat-2 cells.

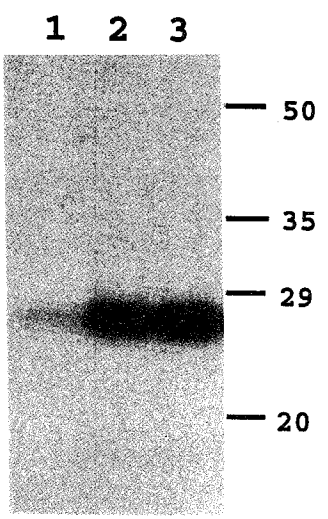

Fig. 1. *In vitro* ADP-ribosylation of Rho A by C3-transferase. Twenty μg of cell lysate was mixed with control or 0.1 μg of C3 transferase and ADP-ribosylation reaction was carried out at 37°C for 30 min using [^{32}P]NAD as a ribose donor. The samples were then subjected to SDS-PAGE and subsequent autoradiography as described in Materials and Methods. 1, control; 2, commercial C3 transferase; 3, C3 transferase expressed in *E. coli*.

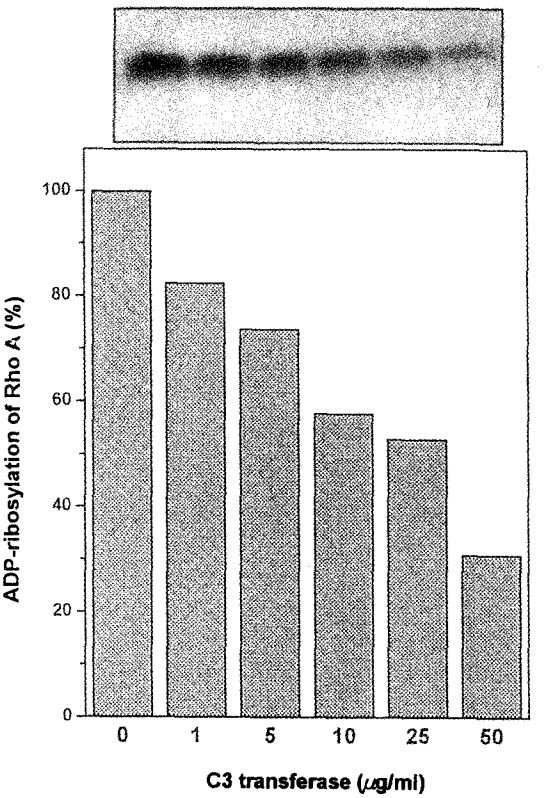

Fig. 2. *In vivo* ADP-ribosylation of RhoA in Rat-2 cells. Rat-2 cells were incubated with various concentrations of C3 transferase for 2 d in serum-free medium and were disrupted by sonication. Cell lysates were ADP-ribosylated with 0.1 μg C3 transferase *in vitro* as described in Materials and Methods. The labeled proteins were then analyzed by SDS-PAGE followed by phosphoimager (lower panel) and autoradiography (upper panel).

Since C3 transferase expressed in *E. coli* was able to inhibit RhoA *in vivo*, we investigated the possible roles of RhoA in the activation of PLD and stress fiber formation. In these experiments, 25 μg/ml of C3 transferase, a concentration of half-maximal inhibition, was used to inhibit RhoA rather than higher concentrations because cells might be killed by complete inhibition of RhoA. First, cells were incubated with 25 μg/ml C3 transferase for 2 days, treated with 1 μg/ml LPA for 30 min and then changes of filamentous actin (F-actin) were observed by the use of a laser confocal scanning microscope (Fig. 3). As expected from the previous reports (Ha, and Exton, 1993b; Ha *et al.*, 1994; Cross *et al.*, 1996), incubation of serum-starved Rat-2 cells with 1 μg/ml of LPA resulted in an enhanced level of actin po-

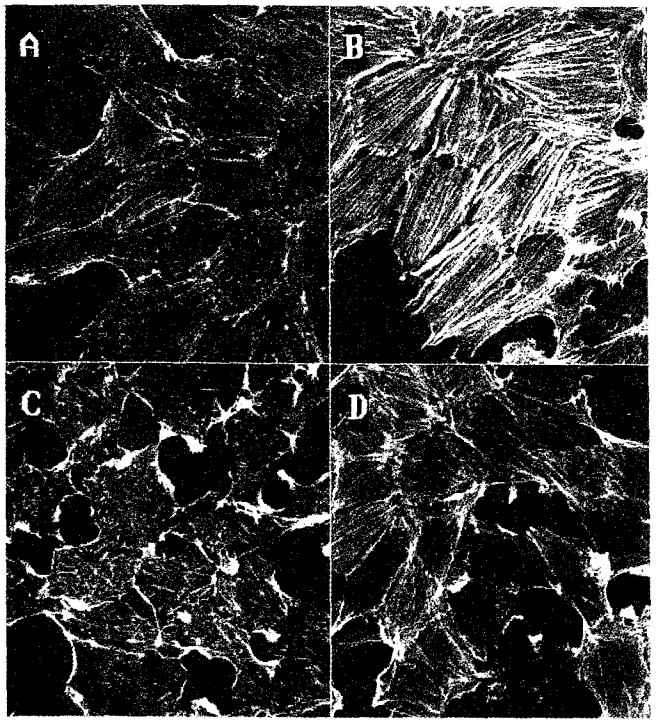

Fig. 3. Effect of C3 transferase on LPA-induced stress fiber formation. Rat-2 fibroblasts were grown on coverslips in 12-well plates and incubated with 25 μg/ml of C3 transferase (C and D) for 2 days. Then, cells were stimulated with control (A and C) or 1 μg/ml of LPA (B and D) for 30 min. F-actin was stained with NBD-phallacidin as described in Materials and Methods. The stained cells were observed by a laser scanning confocal microscope.

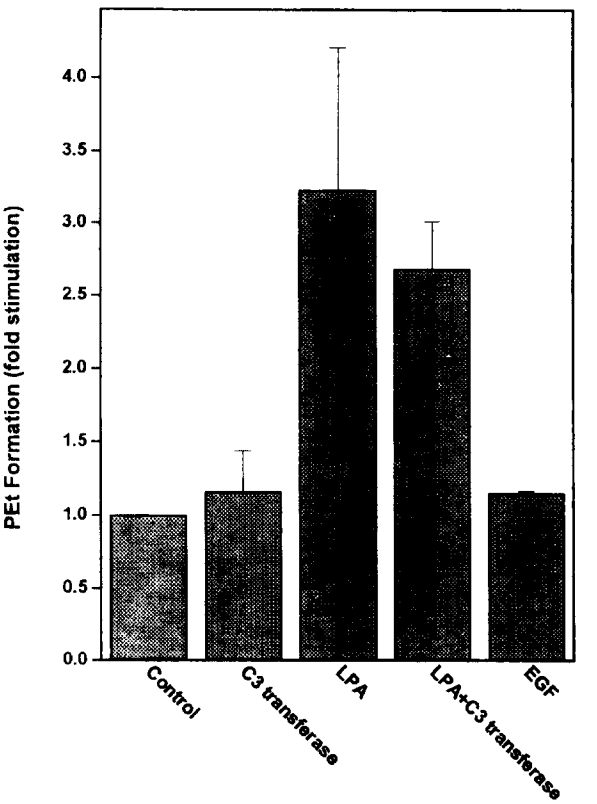

Fig. 4. No inhibitory effect of C3 transferase on LPA-induced PLD activation. Cells were labeled with [^{3}H]myristic acid for 2 days in serum-free medium, and then treated with 1 μg/ml LPA or 50 ng/ml EGF for 30 min. For inhibition of Rho A, 25 μg/ml of C3 transferase was added for 2 days during serum-starvation. Then, PEt formation was determined as described in Materials and Methods.

lymerization by the apparent enhancement in the number of stress fibers and extension of these fibers, whereas untreated control cells showed only a basal level of stress fibers (Fig. 3 A and B). But upon pretreatement of the cells with C3 transferase for 2 days during the course of serum-starvation, the LPA-induced formation of stress fibers was efficiently inhibited (Fig. 3 D). Interestingly, cells treated with C3 transferase alone exhibited some alterations in cell morphology, showing shrinked shape and protrusion-like redistribution of F-actin along plasma membrane (Fig. 3 C), which might result from the perturbed elongation of stress fibers by C3 transferase treatment. Taken together, the results suggest that Rho A is an absolute requirement for LPA-induced formation of stress fibers in Rat-2 cells.

Our results showed that C3 transferase inhibited LPA-induced stress fiber formation by ADP-ribosylation of RhoA (Fig. 3). LPA has been reported to induce stress fiber formation by activating PLD in IIC9 and PAE cells (Ha, and Exton, 1993b; Ha *et al.*, 1994; Cross *et al.*, 1996). So, it was hypothesized that Rho might produce stress fibers by activating PLD. To test this hypothesis, PLD activity was measured after incubating Rat-2 cells with 25 μg/ml of C3 transferase for 2 days. As shown in Fig. 4, LPA increased PLD activity by about 3 fold over the control level as determined by the formation of PEt in the presence of 1% ethanol. However, the pretreatment of C3 transferase had no significant inhibitory effect on LPA-stimulated PLD activity. Furthermore, EGF did not significantly activate PLD (Fig. 4), consistent with a previous report (Hess *et al.*, 1997), even though EGF produced stress fibers as did LPA (data not shown). Thus, it can be suggested that RhoA play a role as a downstream component of PLD rather than activating PLD in LPA-induced stress fiber formation.

Recent studies have been focused on the elucidation of intracellular signaling pathways leading to PLD activation since PA, the primary product of PC-hydrolyzing PLD, is known to have various functions such as stress fiber formation, stimulation of DNA synthesis and increase of intracellular Ca^{2+} (Salmon, and Honeyman, 1980; Putney *et al.*, 1984; Kanuss *et al.*, 1990; Ha, and Exton, 1993b). It is now accepted that PLD is regulated by PKC and a small G-protein Arf (Exton, 1994). Recently, there have been several reports suggesting a role

of RhoA in the activation of PLD (Bowman *et al.*, 1993; Kwak *et al.*, 1995; Siddiqi *et al.*, 1995; Malcolm *et al.*, 1996; Ohguchi *et al.*, 1996; Schmidt *et al.*, 1996). However, the possible involvement of RhoA in the activation of PLD has been a matter of debate since most of the studies were mainly conducted *in vitro* (Malcolm *et al.*, 1996). In the pesent report, we propose a mechanism that Rho A-induced stress fiber formation is mediated by a PLD-independent pathway. Considering PA activation of stress fiber formation in IIC9 cells and PAE cells (Ha, and Exton, 1993b; Ha *et al.*, 1994; Cross *et al.*, 1996), it is assumed that PA, a primary product of PLD, could somehow bring about the activation of Rho A and the subsequent activation of stress fiber formation.

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