Activation of Phospholipase D in Rat Thymocytes by Sphingosine

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Sphingosine is known to regulate a wide range of cell physiology including growth, differentiation, and apoptosis. In this study, we examined the effect of sphingosine on the phospholipase D (PLD) activity in rat thymocytes. Sphingosine potently stimulated PLD in the absence of extracellular calcium, while depletion of intracellular calcium by BAPTA/AM treatment completely blocked activation of PLD by sphingosine. Sphingosine-induced increase of the intracellular calcium concentration was confirmed using a fluorescent calcium indicator Fluo-3/AM. A phosphoinositide-specific phospholipase C inhibitor U73122 partially inhibited the stimulation of PLD by sphingosine. When mouse PLD2 gene was transfected into mouse thymoma EL4 cells, which lack intrinsic PLD activity, sphingosine could stimulate PLD2 significantly while overexpression of human PLD1 had no effect. Taken together, the sphingosine-stimulated PLD activity in rat thymocytes is dependent on the mobilization of intracellular calcium and appears to be due to the PLD2 isoform.

Key Words : Sphingosine, Phospholipase D, Calcium, Thymocytes

Introduction

Sphingosine is a highly bioactive lipid involved in regulation of cell growth, differentiation, and apoptosis.[1-3] However, in spite of their pivotal role in cell fate, little is known about signal transduction pathways involving sphingosine. Initial interest in sphingosine was focused on its inhibitory action on protein kinase C (PKC).[4] Since then, sphingosine has been widely used as an inhibitor of PKC *in vitro* and in intact cells for investigating the roles of PKC in cellular physiology. Sphingosine is also involved in generation of cellular phosphatidic acid (PA), another putative second messenger.[5] Furthermore, when exogenously added to cells, sphingosine mobilizes Ca^{2+} from intracellular stores.[6,7] Recently, several laboratories demonstrated that sphingosine stimulates phospholipase D (PLD) in mammalian cells such as neural cells, fibroblasts, and smooth muscle cells.[8-10] However, neither signaling mechanism involved nor PLD isoform stimulated by sphingosine has been known.

Phospholipase D (PLD), which hydrolyzes phosphatidylcholine (PC) to produce phosphatidic acid (PA) and choline, is believed to play a role in growth control, meiosis, differentiation, cytoskeletal reorganization, membrane trafficking, and apoptosis.[11,12] To date, two isoforms, namely PLD1 and PLD2 have been cloned in human, mouse, and rat.[12] PLD1 has low basal activity and is highly activated by small G proteins (such as ARF and Rho A) and PKC, whereas PLD2 has high basal activity and shows no dependency on G proteins. Both isoforms require PIP_2 as a cofactor. It has been suggested that factors such as PKC, protein tyrosine kinases, and Ca^{2+} ion regulate the activity of PLD *in vivo*.[11]

In the present study, we investigated activation of PLD by sphingosine in rat thymocytes. Our results indicate that the activation of PLD by sphingosine is highly correlated with intracellular mobilization of Ca^{2+}. Our experiments further demonstrate that the PLD2 isoform is activated by sphingosine.

Experimental Section

Materials. [9,10-^{3}H(N)]Palmitic acid (5 mCi/mL) was purchased from Dupont NEN (USA). 1,2-bis-(O-aminophenoxyl)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (BAPTA/AM) and {1-[2-amino-5-(2,7,-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2-(2'-amino-5'-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid pentaacetoxymethyl ester (Fluo 3/AM) was from Calbiochem (Nottingham, UK). Precoated silica gel 60 plate was obtained from Merck (Damstadt, Germany). Phosphatidylethanol (PEt) was prepared from egg PC using cabbage PLD according to the procedures described previously.[13] RPMI 1640 medium and fetal bovine serum (FBS) were from GIBCO (Gaithersburg, USA). D-erythro-sphingosine, U73122, thapsigargin, and DEAE dextran were purchased from Sigma (St. Louis, USA).

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Abbreviations: ARF, ADP ribosylation factor; BAPTA/AM, 1,2-bis-(O-aminophenoxyl)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester; DTPA, diethyltriaminepentaacetic acid; FLUO 3/AM, {1-[2-Amino-5-(2,7,-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2-(2'-amino-5'-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid pentaacetoxymethyl ester; G3PDH, Glyceraldehyde 3-phosphate dehydrogenase; HBSS, Hanks balanced salt solution; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); PA, Phosphatidic acid; PC, Phosphatidylcholine; PIP_2, Phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD, Phospholipase D; RT-PCR, Reverse Transcription-Polymerase Chain Reaction.; U73122, {1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione}.

Thymocytes and isotope labeling. Thymocytes were prepared by the method described by Errasfa *et al.*.[14] Rat thymus glands were washed in Hanks balanced salt solution (HBSS) and gently homogenized in a Tenbroeck glass homogenizer with a loose fitting pestle. After removing tissue remnants by filtering through nylon mesh, thymocytes were collected by centrifugation at 600 × g for 3 min and washed once with HBSS. Murine thymoma EL4 cells were grown in RPMI 1640 medium supplemented with 0.12 mg/mL penicillin G, 0.2 mg/mL streptomycin, 2 mg/mL sodium bicarbonate, 20 mM HEPES, pH 7.3, and 10% heat-inactivated FBS at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For isotope labeling of cellular phosphatidylcholine, thymocytes or EL4 cells (10^6 cells/mL) were incubated in serum-free RPMI 1640 medium containing 2 μCi/mL [^{3}H]palmitic acid for 3 h at 37 °C as previously described.[15]

PLD assay. PLD activity in intact cells was determined by the formation of phosphatidylethanol (PEt) in the presence of ethanol.[15] Isotope labeled cells were washed twice with HBSS supplemented with 2.5 mg/mL bovine serum albumin (BSA) to eliminate free [^{3}H]palmitic acid. Cells were then resuspended in physiological saline containing 140 mM NaCl, 5 mM KCl, 1.1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM Na_2HPO_4, 5.5 mM glucose, 0.1 mM diethylenetriaminepentaacetic acid (DTPA), and 20 mM HEPES, pH 7.4. Cells were adjusted to a concentration of 1.2×10^6 cells/400 μL and preincubated with 1.5% (v/v) ethanol at 37 °C for 30 min. After further 30 min incubation with added chemical stimuli, reactions were terminated by addition of 1.2 mL chloroform/methanol/12 N HCl (100 : 50 : 0.25, v/v/v). After vortexing, organic layer was separated by centrifugation and washed with 0.1 M KCl. Lipid extracts were separated on Silica Gel 60 TLC plates with PEt standard using the upper phase of ethyl acetate/iso-octane/acetic acid/water (75 : 10 : 15 : 50, v/v/v/v) as solvent. PEt band was scraped off the plate, eluted with 300 μL of ethanol/1 N HCl (100 : 1, v/v) and the radioactivity was measured in a liquid scintillation counter.

Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$). Changes in $[Ca^{2+}]_i$ was measured using a fluorescent indicator, Fluo 3/AM. Thymocytes were loaded with 5 μM Fluo 3/AM at a density of 3×10^6 cells/mL in HBSS for 1 h at 37 °C. Cells were washed twice with HBSS supplemented with 2.5 mg/mL BSA. Cells were resuspended at a density of 10^6 cells/mL in HBSS containing 1 mM probenecid. Fluorescence was measured at excitation and emission wavelengths 506 and 526 nm, respectively, in a SFM 25 spectrofluorometer (Kontron Instruments, Italy) with continuous stirring at room temperature.

Extraction of RNA and RT-PCR. Total RNA was isolated from thymocytes with RNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's protocols. PCR primers for PLD and glyceraldehydes 3-phosphate dehydrogenase (G3PDH) were prepared according to the reports of Yoshimura *et al.*[16] and Li *et al.*,[17] respectively. RNA (250 ng) was reverse transcribed and amplified with ProSTAR HF single-tube RT-PCR system (Stratagene, U.S.A.) according to the manufacturer's instructions. RNA was reverse transcribed at 42 °C for 15 min. After inactivation of reverse transcriptase at 95 °C for 1 min, PCR was performed for 40 cycles with denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec, and extension at 68 °C for 2 min. The RT-PCR products were analyzed by 1% agarose gel electrophoresis.

Transient transfection. Transient transfection of EL4 cells was performed by the method of Kullmann *et al.*.[18] Briefly, 1 μg of DNA (pCGN vectors containing wild type human PLD1b and mouse PLD2)/2×10^6 cells was resuspended in 200 μL of Tris-buffered saline (25 mM Tris-HCl, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.6 mM Na_2HPO_4, pH 7.4) containing 500 μg/mL DEAE-dextran for 20 min at room temperature. The cells were then treated with 1% Me_2SO for 3 min and thereafter washed twice with Tris-buffered saline and resuspended in culture medium. After 24 h, cells were used for experiments.

Results and Discussion

Effect of sphingosine on PLD activity in thymocytes. First we determined the PLD activity in thymocytes in the presence of varying concentrations of sphingosine. Figure 1A shows that sphingosine increased the production of PEt, the unique PLD product in the presence of ethanol. Stimulation of PLD by sphingosine was maximal at the concentration of 30 μM and the stimulated PLD activity decreased to the basal level at higher concentrations of sphingosine. Next we examined time-course of the PLD stimulation by sphingosine at its optimum concentration (30 μM). PLD activity increased time-dependently over 60-min incubation period, as shown in Figure 1B.

Effect of calcium on the sphingosine-stimulated PLD activity. Calcium has been thought as one of major regulatory factors for PLD activities in many cell types.[11] Figure 2A shows that sphingosine stimulated the PLD in thymocytes to the same extent in the presence or absence of extracellular Ca^{2+} (1.1 mM), showing that existence of extracellular Ca^{2+} is not essential for the activation of PLD by sphingosine. On the other hand, when intracellular Ca^{2+} was depleted by 30 min preincubation of the cells with an intracellular calcium chelator BAPTA/AM, sphingosine failed to stimulate PLD (Figure 2B). Upon this observation, we next measured the increase of $[Ca^{2+}]_i$ by sphingosine using a cell permeable fluorescent Ca^{2+} indicator Fluo 3/AM (Figure 3). When Fluo3-loaded thymocytes were treated with sphingosine, Fluo3 fluorescence increased about 2-fold indicating an increase of $[Ca^{2+}]_i$. Together with Figure 2, these data suggest that sphingosine stimulates increase of $[Ca^{2+}]_i$, and the Ca^{2+} in turn activates PLD in thymocytes. To confirm the role of intracellular Ca^{2+} in stimulating PLD in thymocytes, we treated the cells with thapsigargin (1 μM), a Ca^{2+}-ATPase inhibitor, to disrupt internal Ca^{2+} stores. As expected, thapsigargin significantly stimulated PLD activity (approximately 2-fold over control) in thymocytes (data not shown).

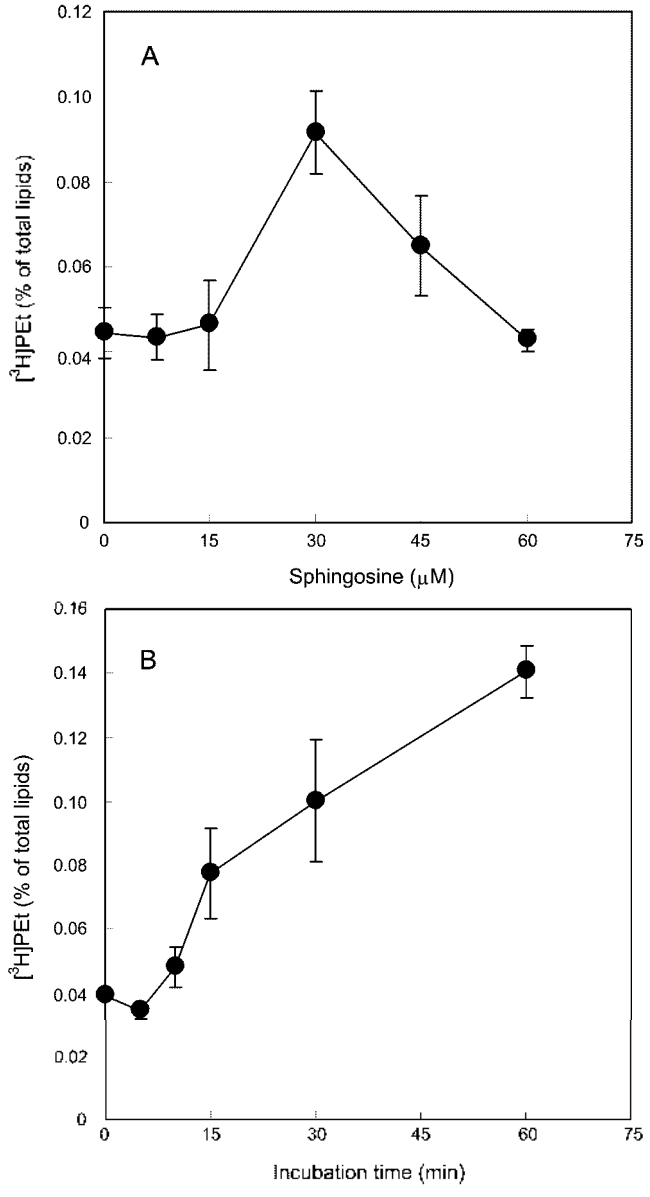

Figure 1. Effect of sphingosine on PLD activity in rat thymocytes. PLD activity was measured by determining the formation of [^{3}H]PEt from the cells labeled with [^{3}H]palmitic acid in the presence of ethanol. A, effect of sphingosine on the PLD activity during 30 min incubation. B, time course of PLD activation by sphingosine (30 μM). Data are means ± S.D. of triplicate determinations.

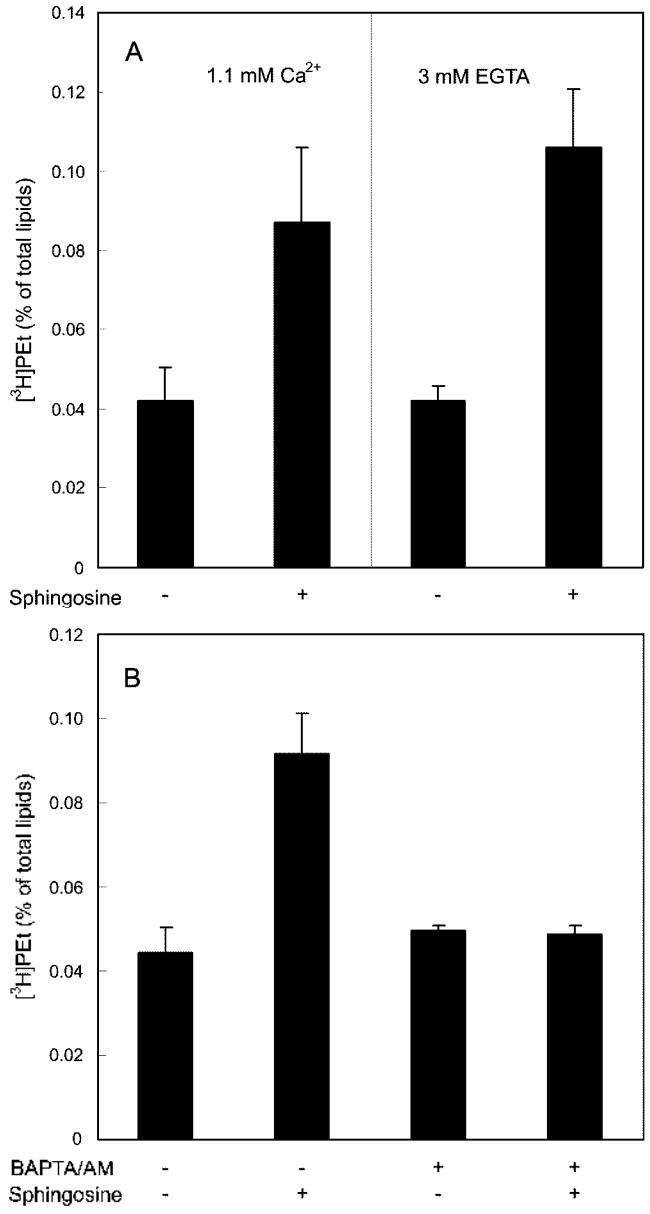

Figure 2. Effect of Ca^{2+} on the sphingosine-stimulated PLD activity in thymocytes. A, effect of extracellular Ca^{2+} on the PLD activation by sphingosine. [^{3}H]Palmitic acid-labeled thymocytes were incubated in physiological saline (1.1 mM Ca^{2+}) or 3 mM EGTA-added Ca^{2+}-free physiological saline with 30 μM sphingosine as described in experimental section. B, effect of intracellular Ca^{2+} chelator on the PLD activity stimulated by sphingosine. Cells were preincubated for 30 min with 80 μM BAPTA/AM and then incubated further for 30 min in the absence or presence of 30 μM sphingosine. Data are means ± S. D. of triplicate determinations.

Effect of U73122 on PLD activation by sphingosine. We next investigated sphingosine-induced signal pathway that is involved in the activation of PLD. To determine the role of phospholipase C (PLC) signaling in the PLD activation by sphingosine in thymocytes, effect of U73122, a phosphoinositide-specific PLC (PI-PLC) inhibitor, was examined. As shown in Figure 4, U73122 (20 μM) significantly inhibited the sphingosine-induced PLD activity. This observation implicates that the PLD activation could be a downstream event of PI-PLC upon stimulation with sphingosine.

Isoform specificity of PLD activation by sphingosine. Two isoforms of PLD, namely PLD1 and PLD2 have been identified and cloned so far.[12] To determine which PLD isoform is responsible for the PLD activation by sphingosine, we examined mRNA expressions of PLD1 and PLD2 in rat thymocytes (Figure 5A). RT-PCR analysis revealed similar levels of expression of PLD1 and PLD2 in rat thymocytes making identification of sphingosine-activated PLD isoform difficult. Thus we used murine thymoma EL4 cells, which lack detectable PLD expression[19] (Figure 5A), as control cells to examine the effect of sphingosine on

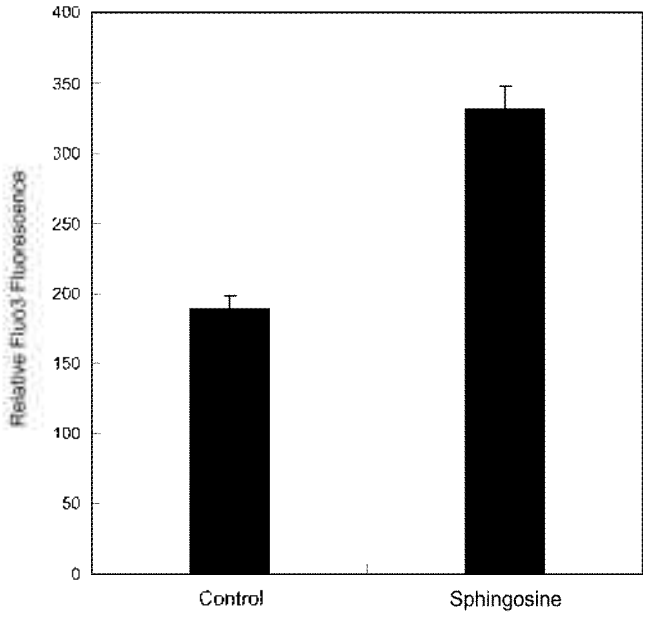

Figure 3. Effect of sphingosine on intracellular Ca^{2+} mobilization. Thymocytes were loaded with fluorescent calcium indicator FLUO 3/AM. Cells were washed, resuspended in HBSS, and treated with 30 μM sphingosine for 30 min. Changes in the fluorescence were measured at excitation and emission wavelengths 506 and 526 nm, respectively. Data are means ± S. D. from three experiments.

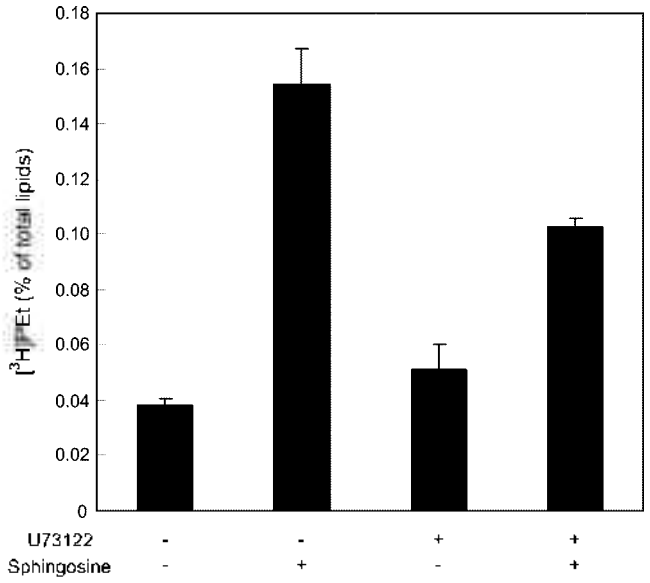

Figure 4. Effect of U73122 on PLD activity stimulated by sphingosine. [^{3}H]Palmitic acid-labeled thymocytes were pretreated with 20 μM U73122 during 30 min preincubation period and further incubated with 30 μM sphingosine for 30 min. Data are means ± S. D. of triplicate determinations.

activities of PLD isoforms. Sphingosine failed to stimulate PLD activity in these control EL4 cells (Figure 5B). Then we transiently transfected EL4 cells with PLD1 and PLD2. Expression of active PLD1 and PLD2 was confirmed by *in vitro* assay of PLD activity (data not shown). Overexpression of PLD1 did not affect PLD activity upon sphingosine treatment. However, sphingosine significantly stimulated

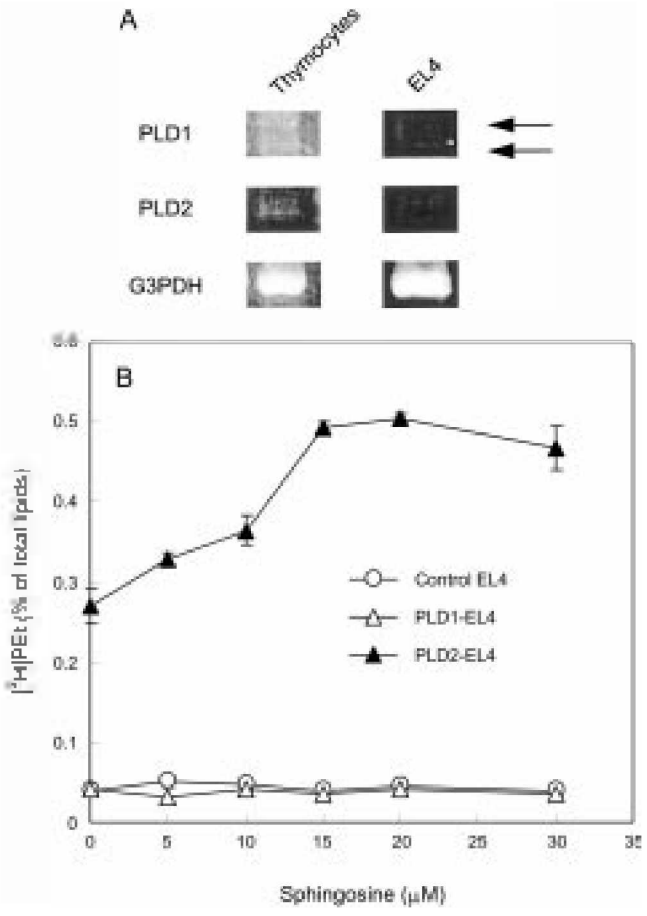

Figure 5. Effect of sphingosine on PLD isoforms transiently transfected in EL4 cells. A, mRNA expressions of PLD isoforms in rat thymocytes and EL4 cells. RNAs were extracted, reverse transcribed, and amplified as described in experimental section. Arrows indicate PLD1a (upper) and PLD1b (lower), respectively. B, effect of sphingosine on PLD activity in PLD1- and PLD2-transfected EL4 cells. Transfection of EL4 cells was done by DEAE-dextran method as described in experimental section. Cells were labeled with [^{3}H]palmitic acid and stimulated with sphingosine. Data are mean ± S. D. of triplicate determinations.

PLD activity in PLD2-expressing cells, suggesting that the PLD2 isoform is responsible for the PLD activity stimulated by sphingosine.

In summary, we have demonstrated that sphingosine stimulates thymocyte PLD in intracellular calcium-dependent manner. Furthermore, PLD2 isoform was responsible for the sphingosine-stimulated PLD activity. Activation of PLD by sphingosine in thymocytes could be physiologically significant since PA, the product of PLD, acts as a lipid second messenger during cell growth, differentiation, and apoptosis.[11,12] Sphingosine has been known to affect development, proliferation, and activation of lymphocytes upon antigenic stimuli.[20] Recent reports indicate that the PLD activity in thymocytes is coupled to the immune receptor signaling.[21,22] Considering the close correlation between sphingosine signaling and PLD activation, it is possible that some immunomodulatory role of sphingosine in thymocytes might be mediated through the activation of PLD.

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