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Hippocalcin Increases Phospholipase D2 Expression Through Extracellular Signal-Regulated Kinase Activation and Lysophosphatidic Acid Potentiates the Hippocalcin-Induced Phospholipase D2 Expression

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Abstract We have previously isolated a 22 kD protein from a rat brain which was found to be involved in activating phospholipsae D (PLD), and identified the protein as hippocalcin through sequence analysis. Nevertheless, the function of hippocalcin for PLD activation still remains to be resolved. Here, we proposed that hippocalcin was involved in extracellular signal-regulated kinase (ERK)-mediated PLD2 expression. To elucidate a role of hippocalcin, we made hippocalcin transfected NIH3T3 cells and showed that the expression of PLD2 and basal PLD activity were increased in hippocalcin transfected cells. We performed PLD assay with dominant negative PLD2 (DN-PLD2) and hippocalcin co-transfected cells. DN-PLD2 suppressed increase of basal PLD activity in hippocalcin transfected cells, suggesting that increased basal PLD activity is due to PLD2 over-expression. Hippocalcin is a Ca^{2+}-binding protein, which is expressed mainly in the hippocampus. Since it is known that lysophosphatidic acid (LPA) increases intracellular Ca^{2+}, we investigated the possible role of hippocalcin in the LPA-induced elevation of intracellular Ca^{2+}. When the intracellular Ca^{2+} level was increased by LPA, hippocalcin was translocated to the membrane after LPA treatment in hippocalcin transfected cells. In addition, treatment with LPA in hippocalcin transfected cells markedly potentiated PLD2 expression and showed morphological changes of cell shape suggesting that increased PLD2 expression acts as one of the major factors to cause change of cell shape by making altered membrane lipid composition. Hippocalcin-induced PLD2 expression potentiated by LPA in hippocalcin transfected cells was inhibited by a PI-PLC inhibitor, U73122 and a chelator of intracellular Ca^{2+}, BAPTA–AM suggesting that activation of hippocalcin caused by increased intracellular Ca^{2+} is important to induce over-expression of PLD2. However, downregulation of PKC and treatment of a chelator of extracellular Ca^{2+}, EGTA had little or no effect on the inhibition of hippocalcin-induced PLD2 expression potentiated by LPA in the hippocalcin transfected cells. Interestingly, when we over-express hippocalcin, ERK was activated, and treatment with LPA in hippocalcin transfected cells significantly potentiated ERK activation. Specific inhibition of ERK dramatically abolished hippocalcin-induced PLD2 expression. Taken together, these results suggest for the first time that hippocalcin can induce PLD2 expression and LPA potentiates hippocalcin-induced PLD2 expression, which is mediated by ERK activation. J. Cell. Biochem. 97: 1052–1065, 2006.

Key words: hippocalcin; phospholipase D2 (PLD2); lysophosphatidic acid (LPA); extracellular signal-regulated kinase (ERK)

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Hippocalcin is a protein which is expressed mainly in the pyramidal nerve cell of the hippocampus and belongs to the neuronal calcium sensor, neuronal calcium sensor (NCS) protein family, whose members show multiple functions in nerve cell physiology [Burgoyne and Weiss, 2001]. It comprises of three EF-hand Ca^{2+}-binding structural domains and a myristoylation site at the N-terminal [Kobayashi et al., 1993]. When the intracellular Ca^{2+} level is increased, hippocalcin is translocated to the membrane after binding with Ca^{2+} [O'Callaghan et al., 2003]. Myristoylation of the protein is proved to be essential for the translocation to the membrane [O'Callaghan et al., 2002]. Recent studies have shown that hippocalcin interacts with the neuronal apoptosis inhibitor protein (NAIP) that is an antiapoptotic protein expressed by neuron [Lindholm et al., 2002]. Hippocalcin also binds other proteins, such as mitogen-activated protein kinase, MLK2 [Nagata et al., 1998], and cytochrome b5 [Oikawa et al., 2004]. Little is known about the function of hippocalcin, nevertheless hippocalcin suggests that it may be involved in Ca^{2+}-dependent cellular signaling.

PLD is a ubiquitous enzyme, which catalyzes the hydrolysis of phosphatidylcholine to produce choline and the putative second messenger phosphatidic acid. It is widely distributed in mammalian cells and genes encoding mammalian PLD1 and PLD2 have already been cloned. Whereas PLD1 is highly regulated by protein kinase C or small G proteins such as ADP-ribosylation factor (ARF) and Rho families both in vitro and in vivo, PLD2 having high basal activity shows little or no response to those activators [Exton, 1999; Jang et al., 2004]. PLD has been hypothesized to play a role in Golgi vesicular transport and the regulation of vesicular trafficking and cytoskeletal reorganization [O'Luanaigh et al., 2002], as well as exocytosis [Dohke et al., 2002]. This conclusively suggests that, regardless of extensive studies performed on regulation of PLD, its cellular roles nonetheless remain ambiguous. In addition, PLD enzymes can be activated by numerous types of hormones, neurotransmitters, growth factors, and cytokines. As a result, activation of PLD has been applied in a wide variety of cellular processes, including stimulation of DNA synthesis, actin polymerization, coatomer assembly, vesicle transport, neutrophil activation, platelet aggregation, and more [Exton, 1999]. However, the functional role of PLD in brain is not fully understood.

Lysophosphatidic acid (LPA) is a bioactive molecule synthesized and released by platelets present in serum [Nietgen and Durieux, 1998]. The potential role of LPA as an autocrine or paracrine mediator has not yet been clearly established. LPA is known to be involved in numerous cellular activities such as stimulating several intracellular signaling pathways via distinct G-protein-receptors and accumulating Ras-GTP. LPA also activates platelets and inhibits adenylyl cyclase through the pertussis-toxin-sensitive G_i protein. It is also involved in activating phospholipase C (PLC) via G_q protein [Yu et al., 2004] as well as transmiting signals to small GTPase through $G_{12/13}$ proteins [Fang et al., 2002]. In particular, LPA is an effective activator of Rho, a small GTP-binding protein involved in signaling mediated by cell adhesion [Mukai et al., 2002]. The signal transduction pathways utilized by LPA have been studied mainly in fibroblasts, in which LPA can activate the cytosolic tyrosine kinase c-src via G-protein $\beta\gamma$ subunits and also increases phospholipase D (PLD) activity [Kam and Exton, 2001]. Although it is known that LPA activates its cognate G protein receptor to stimulate multiple signaling pathways including PLD activation, the relationship between LPA and hippocalcin-induced PLD signaling has not yet been discovered. We have however confirmed through our previous studies that PLD can be activated by hippocalcin in the presence of Ca^{2+} and hippocalcin is one of the major regulatory proteins in Ca^{2+}-mediated PLD signaling pathway [Hyun et al., 2000]. LPA also activates mitogen-activated protein kinases (MAPKs) via G-protein-coupled pathway requiring the activation of Ras [Howe and Marshall, 1993]. Although LPA stimulates ERK activation and PLD activation, the relationship between PLD and ERK have not been studied. In this experiment, we showed that PLD2 expression and ERK activation were increased by over-expression of hippocalcin and that LPA significantly potentiated PLD2 expression and ERK activation in hippocalcin transfected cells. Therefore, we hypothesized that ERK activation is involved in hippocalcin-induced PLD2 expression.

Recent studies have demonstrated that PLD2 translocates itself to the ruffling membranes formed upon epidermal growth factor (EGF) stimulation [Honda et al., 1999], and that the

over-expression of PLD2 in numerous cell types resulted in actin cytoskeleton rearrangements [Colley et al., 1997]. PLD activation and the increase of intracellular Ca^{2+} to a certain extent imply rearrangements of actin cytoskeleton [Yeo et al., 2002]. These findings indicate a putative role of both PLD2 and intracellular Ca^{2+} in regulation of membrane dynamics. In spite of our limited knowledge concerning the role of hippocalcin in the cerebral nerve cells, we also showed that Ca^{2+}-mediated PLD2 expression by hippocalcin was responsible for variations in lipid bilayer properties. Overall, our results denote that LPA potentiates the hippocalcin-induced PLD2 expression, which is mediated by ERK activation. Also, we can deduce that morphological changes of cells are the ultimate results of amplified PLD2 expression, which eventually lead to alterations in lipid bilayer properties and actin cytoskeleton rearrangements.

MATERIALS AND METHODS

Materials

Oleoyl-*sn*-glycero-3-phosphate (LPA), EGTA, and phorbol myristate acetate (PMA) were purchased from Sigma-Aldrich (St. Louis, MO), and phosphatidylethanol (PEt) standard from Avanti Polar Lipids (Avanti, Alabaster, AL), and [9,10(n)-^{3}H] Palmitic acid from Amersham Pharmacia Biotech (Amersham Place, Little Chalfont, Buckinghamshire, England). Fetal bovine serum (FBS), penicillin/streptomycin solution, and Dulbecco's modified Eagle's medium (DMEM) with L-glutamine and low glucose was purchased from Gibco-BRL (Gaithersburg, MD). U73122 (91-[6-((17β-3-methoxy-estra-1,3,5(10)-trien-17-yl) amino) hexyl]-1 H-pyrrole-2,5-dione) and BAPTA-AM (1,2-bis (o-amino-phenoxy) ethane-N,N,N′,N′-tetraacetic acid tetra (acetoxymethyl) ester), and PD98059 (2′amino-3′methoxyflavone) were from Calbiochem (San Diego, CA). p-ERK1/2, ERK antibodies were from Cell Signaling. Anti-GFP antibody was from Roche (Indianapolis, IN). The Silica gel 60A plates for thin layer chromatography (TLC) were obtained through Whatman (Clifton, NJ). All other chemical agents were of analytical trace amount.

Cell Culture

NIH3T3 cells were obtained from American Type Culture Collection (ATCC CRL-1658) and were cultured for 2 days at 37°C in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Fibroblasts [$(1-2)\times10^5$/dish] were firstly seeded in 100 mm-diameter tissue culture dishes and were grown at 37°C in humidified CO_2-controlled (5%) incubator. These were then washed with DMEM containing 0.1% (w/v) bovine serum albumin, 100 U/ml penicillin, and 100 μg/ml streptomycin (serum-free medium), and subsequently incubated in a serum-free medium at 37°C for 1 day prior to stimulation with LPA.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

cDNAs was made from total mRNA extracted from vector transfected cells and hippocalcin transfected cells. Total RNA was isolated with Tri Reagent according to the manufacture's instruction, and 2 μg of RNA was reverse transcribed by using random hexamer mixed primers. cDNAs was amplified by PCR using PLD primers (PLD1 forward primer: 5′-TCACGGCAGCATCAGTAGAGTCTATG-3′, PLD1 reverse primer: 5′-AGTTCCAGCGCTGGATGAAGTGTC-3′, PLD2 forward primer: 5′-ATCTGAACTCCAGCCAGTTG-3′, PLD2 reverse primer: 5′-CATGGCGTGGTAATTGCGATAG-3′). The condition for PLD were as follows; denaturation 94°C, 45 s; annealing 60°C, 40 s; extension 72°C, 40 s. The number of cycles determined for PLD 1a, PLD1b, and PLD2 were 30. Amplified DNA fragments were identified on 1.5% agarose gel and visualized afterwards through ethidium bromide staining.

Transient Transfection With Plasmid DNA in NIH3T3 Cells

NIH3T3 cells were transiently transfected by 4 μg of each pcDNA3.1 (Vector), pcDNA3.1-hippocalcin, pcDNA3.1-dominant negative PLD1, pcDNA3.1-dominant negative PLD2, enhanced green fluorescent protein EGFP-C1 (Vector), and EGFP-hippocalcin plasmid using Nucleofector™ Kit (Amaxa).

Determination of PLD Activity

PLD activity was determined by the formation of phosphatidylethanol (PEt), as described elsewhere [Han and Shin, 2000]. Briefly, NIH3T3 cells cultured on six-well plates were labeled radioactively with 1 μCi/ml of [^{3}H]-palmitic acid in serum-free medium for 24 h.

The cells were then pretreated with 1% (v/v) ethanol for 15 min before stimulation with LPA. Following stimulation, the cells were quickly washed with ice-cold phosphate buffered saline (PBS) and suspended in ice-cold methanol. Lipids were extracted according to the method of Bligh and Dyer, and PEt was separated by TLC using a solvent system of acetate/isooctane/acetic acid/water (110:50:20:100, by vol.). The regions corresponding to the authentic PEt bands were identified with 0.002% (w/v) primulin in 80 vol.% acetone, scraped, and counted using a scintillation counter.

Digital Calcium Imaging

Cells from the stock culture were plated onto glass cover-slip (25-mm round) at a density of 5×10^5/coverslip. The cells were loaded with 2 μM fura-2 acetoxymethyl ester (Molecular Probes) in HEPES-buffered Hanks' salt solution, containing 0.5% bovine serum albumin, for 45 min at 37°C. The HEPES buffer was composed of the following (in mM): HEPES, 20; NaCl, 137; $CaCl_2$, 1.26; $MgSO_4$, 0.4; $MgCl_2$ 0.5; KCl, 5.0; KH_2PO_4, 0.4; Na_2HPO_4, 0.6; $NaHCO_3$, 3.0; glucose, 5.0. The loading procedure was terminated by washing with HEPES-buffered Hanks' solution for 15 min prior to start of an experiment. The coverglass was then mounted in a flow-through chamber, superfused at a rate of 2 ml/min. The chamber containing the fura-2-labeled cells was then mounted on the stage of an inverted microscope (Nikon Diaphot) and alternately excited at 340 or 380 nm in wavelength by rapidly switching optical filters (10-nm band) mounted in a computer-controlled wheel (Sutter Instrument) placed between 1100-W Xe arc lamp and the epiflourescence port of the microscope. Excitation light was reflected from a dichroic mirror (400 nm) through a 20× objective (Nikon) and digital fluorescence images were collected with a cooled CCD camera (Photometric; $1{,}280 \times 1{,}035$ binned to 256×207 pixels). $[Ca^{2+}]_i$ was calculated from the ratio of the two background-subtracted digital images. Ratio values were converted to free $[Ca^{2+}]_i$ by applying to the following equation, $[Ca^{2+}]_i = K\ \beta\ (R - R_{min})/(R_{max} - R)$, in which R is the 340/380-nm fluorescence emission ratio and $K = 224$ nM, the dissociation constant for fura-2 (Grynkiewicz's method, 1985). The maximum ratio (R_{max}), the minimum ratio (R_{min}), and the constant β (the ratio of the fluorescence measured at 380 nm in Ca^{2+}-free solution) were previously determined by treating cells with 10 μM ionomycin in Ca^{2+}-free and saturating (5 mM Ca^{2+}) solution.

Immunocytochemistry

In order to observe cellular morphology and translocation of hippocalcin using fluorescence microscope (Nikon), EGFP-hippocalcin transfected NIH3T3 cells were seeded on a bottom 24-well plate dish in serum-free medium for 24 h before treating with LPA. Cells were fixed with 4% buffered paraformaldehyde for 20 min, washed with 0.1% bovine serum albumin in PBS three times for 5 min, permeabilized with 0.3% Triton X-100 for 30 min, and blocked with 10% goat serum in PBS for 30 min. The cells were then subsequently immunostained using primary antibody (1:400 dilution of GFP antibody) for 1 h and washed with 0.1% bovine serum albumin in PBS three times for 5 min. This procedure was followed by consequent incubation for 1 h at room temperature with secondary antibodies (1:400 dilution of Fluorescence-tagged (Jackson ImmunoResearch, West Grove, PA) and 1:200 dilution of biotinylated (Vector Laboratories, Burlingame, CA). Following incubation, the cells were extensively washed with distilled water, and lastly the coverslips were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA). The cells were analyzed with a fluorescence microscope (Nikon), and hence produced images were further processed using Photoshop 6.0 software. In order to eliminate any anomalies, all experiments were repeated for at least three times.

Western Blot Analysis

Cells were firstly lysed in 20 mM Tris, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1% Triton X-100, 1 mM PMSF, and 1 mM Na_3VO_4. Amounts ranging from 10 to 20 μg of proteins were subsequently loaded onto sodium dodecylsulfate (SDS)–polyacrylamide gels (12%), electrophoresed, and were transferred to nitrocellulose membrane (Amersham Pharmacia Biotech). After blocking with 5% dried skim milk for 2 h, the membrane was incubated with primary antibodies. The blots were further incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2,000, New England Biolabs, Inc., Beverly, MA) and specific

bands were detected through ECL (Amersham Pharmacia Biotech).

Statistical Analysis

All experiments were performed in groups of triplicate. Results were expressed as means ± SEM of the number of experiments indicated.

RESULTS

Hippocalcin Increases PLD2 Expression

To investigate whether hippocalcin is involved in PLD2 expression, we generated NIH3T3 cells over-expressing hippocalcin. Over-expression of hippocalcin led to higher expression of PLD2 and basal PLD activity (Fig. 1A,B). Next, we investigated which PLD isozyme is involved in hippocalcin-mediated PLD signaling. When both of hippocalcin transfected cells and vector transfected cells were transfected with either pcDNA3.1-dominant negative PLD1 (DN-PLD1) or pcDNA3.1-dominant negative PLD2 (DN-PLD2) genes, only the DN-PLD2 inhibited basal PLD activity (Fig. 1C). This result suggests that hippocalcin is involved in PLD2 expression.

LPA-Induced Intracellular Ca^{2+} Increase is Involved in Hippocalcin-Mediated Cellular Morphological Changes and Translocation of Hippocalcin to the Membrane

Hippocalcin is a Ca^{2+}-binding protein of the neuronal Ca^{2+} sensor protein (NCS) family. When the intracellular Ca^{2+} level was increased, hippocalcin was translocated to the

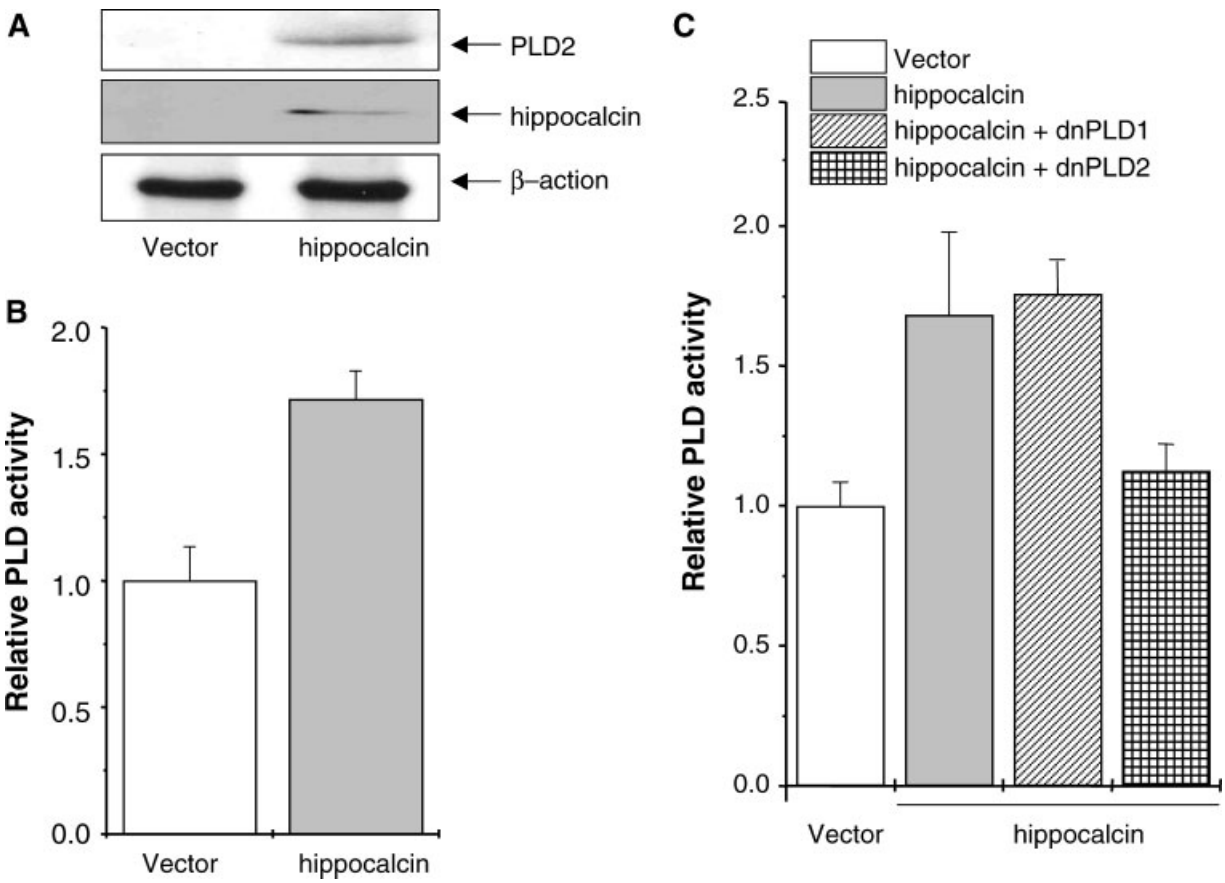

Fig. 1. Characterization of NIH3T3 cells over-expressing hippocalcin. **A**: NIH3T3 cells over-expressing hippocalcin were generated as described under ''Experimental Procedures.'' The cell lysates were immunoblotted with anti-PLD, hippocalcin, and β-actin antibodies. **B**: The level of basal PLD activity was measured as described under ''Materials and Methods.'' The data were obtained from all of pcDNA3.1 transfected NIH3T3 cells and hippocalcin expressing NIH3T3 cells simultaneously. **C**: The level of basal PLD activity was measured as described under ''Materials and Methods.'' The data were obtained from all of pcDNA3.1 transfected NIH3T3 cells and DN-PLD1 transfected and DN-PLD2 transfected hippocalcin expressing NIH3T3 cells simultaneously. These experiments have been repeated for three times and the data presented are means ± SE from triplicated experiments.

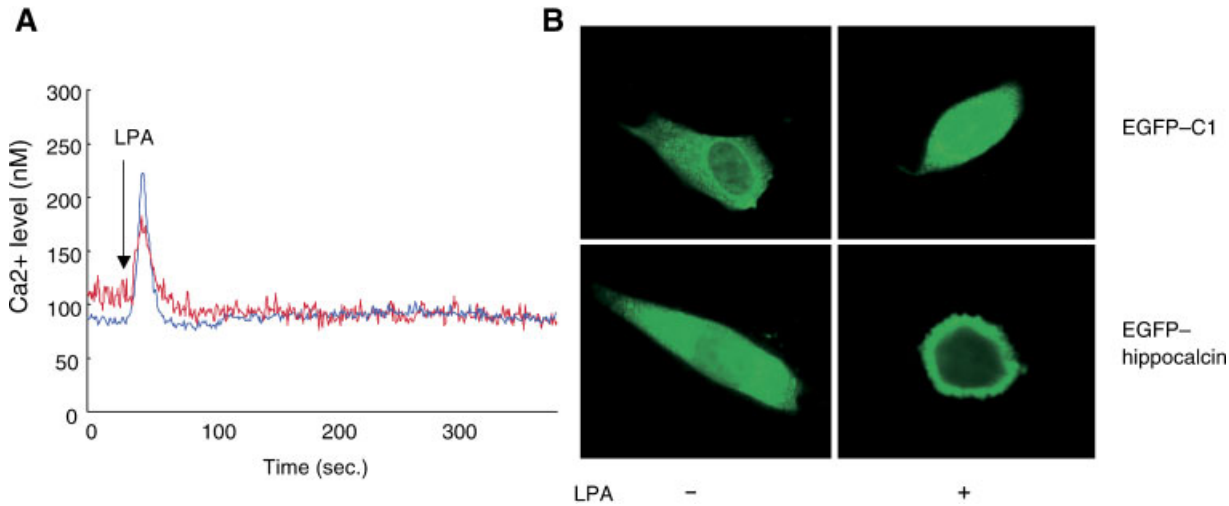

Fig. 2. LPA-induced intracellular Ca^{2+} increase is involved in hippocalcin-mediated cellular morphological changes and translocation of hippocalcin to the membrane in EGFP-hippocalcin transfected cells. **A**: LPA induced an increase in $[Ca^{2+}]_i$ in EGFP-hippocalcin transfected cells. Measurement of $[Ca^{2+}]_i$ derived from fura-2-based digital images as described Materials and Methods, where image pairs were collected within time intervals ranging from 6 s to 1 min consequently addition of LPA for 15 min. The result showed two repeated experiments (red and blue). **B**: EGFP-C1 transfected and EGFP-hippocalcin transfected NIH3T3 cells were serum-starved for 18 h and were washed twice in PBS. Subsequently, these were treated with LPA (10 μM). Cells were fixed at specified time after plating and staining for GFP antibody. Thus immunostained cells were then observed through fluorescence microscopy (magnification, ×400). The results shown represent the average of three repeated experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

membrane after binding with Ca^{2+} in hippocalcin transfected cells. When we treated hippocalcin transfected cells with LPA, intracellular Ca^{2+} levels increased (Fig. 2A). Figure 2B showed that while LPA treatment did not induce any morphological changes in the EGFP-C1 transfected cells, cell shapes of EGFP-hippocalcin transfected cells changes after 15-min treatment of LPA. We also found that hippocalcin was translocated to the membrane after treatment of LPA (Fig. 2B). These data suggest that hippocalcin is responsible for the morphological changes induced by increase of intracellular Ca^{2+}.

LPA Potentiates Hippocalcin-Induced PLD2 Expression

We next investigated whether LPA is involved in the signal transduction pathways leading to hippocalcin-induced PLD2 expression in hippocalcin transfected cells. As shown in Figure 3A, transfection of hippocalcin induced increased expression of PLD2 and LPA treatment potentiated PLD2 expression in hippocalcin transfected cells. The other PLD isoform, PLD1, was not detected in this experiment (data not shown). Like western data, no significant changes were observed in the mRNA levels of PLD 1a and 1b after LPA treatment in both of the control cells and hippocalcin transfected cells. Contrarily, PLD2 mRNAs levels were markedly increased after transfection of hippocalcin and LPA treatment potentiated elevation of PLD2 mRNA level, compared those of the control cells (Fig. 3B). In summary, these results indicate that LPA potentiates hippocalcin-induced PLD2 expression.

PKC is not Involved in the Hippocalcin-Induced PLD2 Expression

There have been a few reports suggesting that some of the intracellular LPA actions require the PLD signaling via protein kinase C (PKC) [Gustavsson et al., 1994; Kim et al., 2004]. To determine whether PKC is involved in hippocalcin-induced PLD2 expression potentiated by LPA, we examined the effect of PKC downregulation on hippocalcin-induced PLD2 expression potentiated by LPA. LPA treatment increased PLD activity to about twofold in control cells. The effect of prolonged pretreatment of control cells with PMA showed complete inhibition of LPA-induced PLD activity. In case of hippocalcin transfected cells LPA treatment potentiated PLD activity approximately to three fold and the effect of prolonged pretreatment with PMA showed only partial inhibition of PLD activity activated by LPA (Fig. 4A).

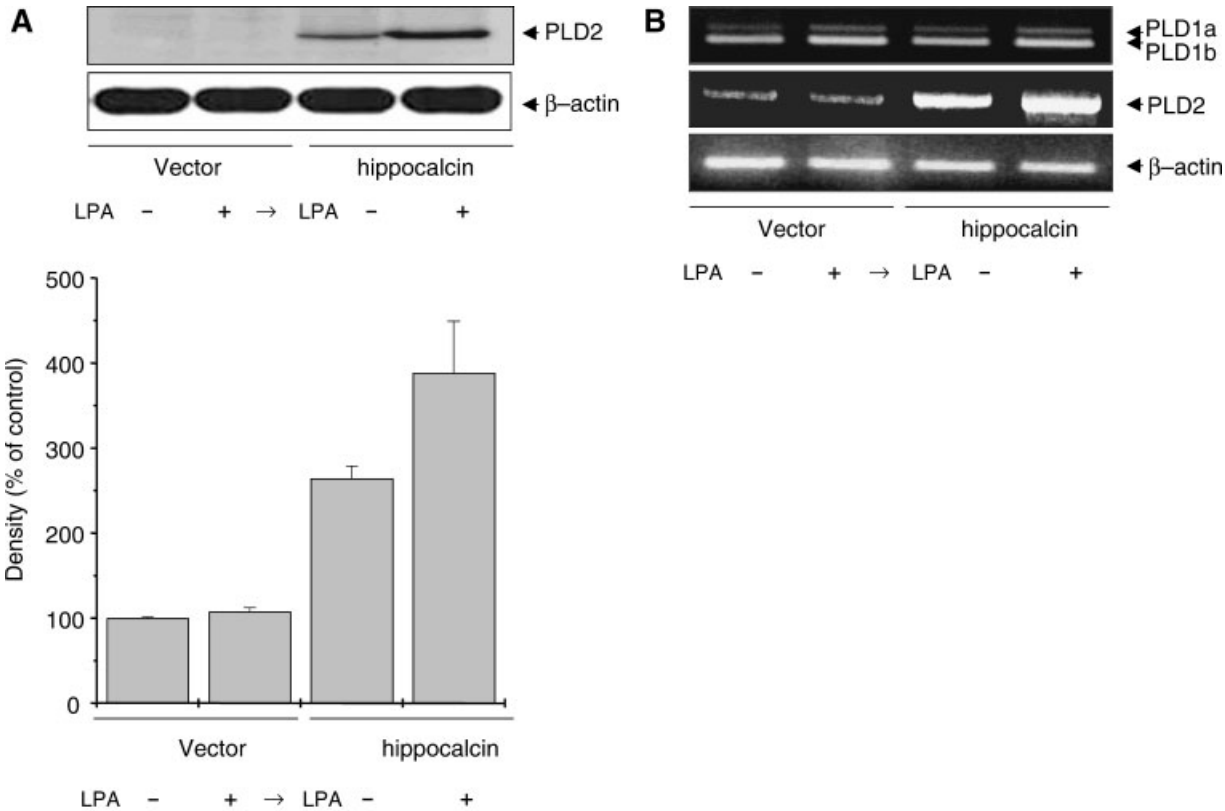

Fig. 3. LPA potentiates hippocalcin-induced PLD2 expression in hippocalcin transfected NIH3T3 cells. **A**: Western blot analysis of PLD expression in pcDNA3.1 transfected (Vector) and hippocalcin transfected NIH3T3 cells. The pcDNA3.1 transfected and hippocalcin transfected NIH3T3 cells were treated sequentially with LPA (10 μM) for 15 min. These cells were then lysed and immunobloted using anti-PLD antibody and β-actin antibody. The intensity of bands was quantified using QuantityOne software (Bio-Rad). **B**: Following 15 min of LPA treatment (10 μM) in both pcDNA3.1 transfected and hippocalcin transfected NIH3T3 cells, the mRNA levels of mPLD1a, mPlD1b and mPLD2 were determined using RT-PCR method.

Moreover, prolonged pretreatment of the hippocalcin transfected cells with PMA did not inhibit hippocalcin-induced PLD2 expression potentiated by LPA (Fig. 4B). These results suggest that while LPA activates PLD1 through PKC activation, PKC activation is not involved in the hippocalcin-induced PLD2 expression potentiated by LPA in hippocalcin transfected cells.

Inhibition of Hippocalcin-Induced PLD2 Expression Potentiated by LPA and Cellular Morphological Changes by Blocking Phosphatidyl-Inositol-4,5-Biphosphate-Phospholipase C (PI-PLC) and Intracellular Ca^{2+}

Since PI-PLC activation by LPA leads to production of two second messengers, DAG and IP_3, which is responsible for inducing release of Ca^{2+} from intracellular stores, LPA increased intracellular Ca^{2+} level in the hippocalcin transfected cells (Fig. 2A). Pretreatment with PI-PLC inhibitor, U73122 suppressed the hippocalcin-induced PLD2 expression potentiated by LPA. Intracellular Ca^{2+} chelator, BAPTA-AM also showed suppression of the hippocalcin-induced PLD2 expression, whereas extracellular Ca^{2+} chelator, EGTA did not suppress the hippocalcin-induced PLD2 expression (Fig. 5A). We could confirm that U73122 and BAPTA-AM dramatically decreased intracellular Ca^{2+} level to basal levels within 1 min. However, pretreatment with EGTA did not suppress intracellular Ca^{2+} levels (Fig. 5B). Since the addition of LPA to EGFP-hippocalcin transfected cells significantly induced morphological changes within the first 15-min, we additionally examined effects of U73122, BAPTA-AM, and EGTA on LPA-induced morphological changes and translocation of hippocalcin in EGFP-hippocalcin transfected cells. Pretreatment of the EGFP-hippocalcin transfected cells with U73122 and

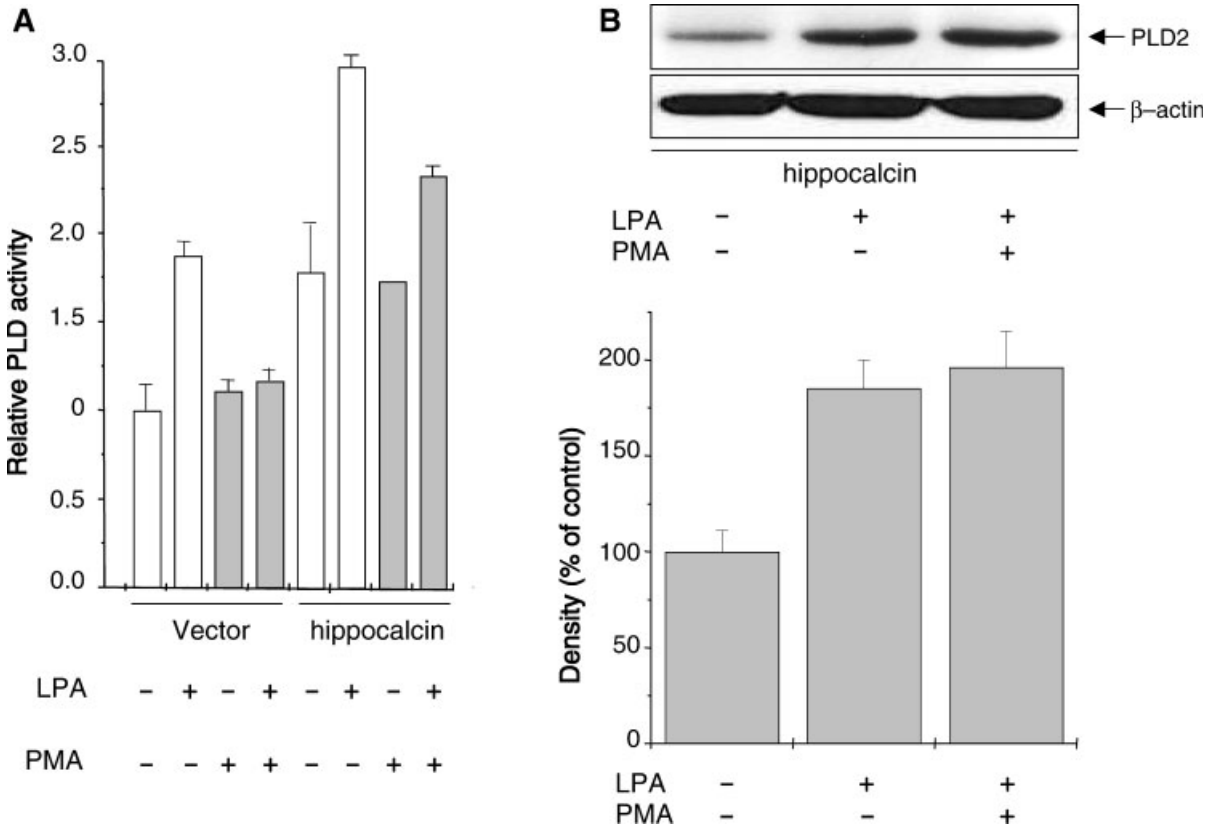

Fig. 4. PKC is not involved in hippocalcin-induced PLD2 expression in hippocalcin transfected NIH3T3 cells. **A**: PLD activity was measured as described under ''Materials and Methods'' in response to 15 min of LPA (10 μM) treatment in both pcDNA3.1 transfected and hippocalcin transfected NIH3T3 cells. Cells were pretreated with 200 nM of PMA for 24 h prior to LPA stimulation for effective downregulation of PKC activity. Data are means of ± SE from triplicates of experiments. **B**: Western blot analysis of PLD2 expression in hippocalcin transfected NIH3T3 cells. The hippocalcin transfected NIH3T3 cells were extracted and were treated sequentially with LPA (10 μM) for 15 min. Cells were pretreated with 200 nM of PMA for 24 h prior to LPA stimulation for effective downregulation of PKC activity. These cells were then lysed and immunobloted using anti-PLD antibody. The intensity of bands was quantified using QuantityOne software (Bio-Rad).

BAPTA-AM suppressed morphological changes and translocation of hippocalcin by LPA. In contrast, EGTA did not suppress morphological changes and translocation of hippocalcin by LPA at all (Fig. 5C). These results indicate that LPA potentiates hippocalcin-induced PLD2 expression and induces cellular morphological changes by increasing intracellular Ca^{2+} level, which is responsible for activation of hippocalcin through PI-PLC activation.

Regulation of Hippocalcin-Induced PLD2 Expression by ERK

To investigate how hippocalcin increased PLD2 expression, we checked MAPkinase signaling because there are reports which suggest that LPA induces ERK phosphorylation and PLD activation [Hong et al., 2001]. Interestingly, when we transfected hippocalcin into NIH3T3 cells, we found that immediate activation of ERK1/2 through western blot assay with anti-phospho-ERK antibody. Moreover, LPA potentiated hippocalcin-induced ERK activation (Fig. 6A). However, LPA did not affect the level of ERK. As shown in Figure 6B, pretreatment with MEK inhibitor, PD98059 completely suppressed the hippocalcin-induced PLD2 expression showing complete inactivation of ERK. Also LPA-induced cellular morphological changes in hippocalcin transfected cells were disappeared after treatment of PD98059 (Fig. 6C). These results convincingly indicate that ERK acts as an essential factor for PLD2 expression induced by hippocalcin.

DISCUSSION

Hippocalcin is a member of neuron-specific protein family characterized by four hand-like subunits, of which three of them are able to bind

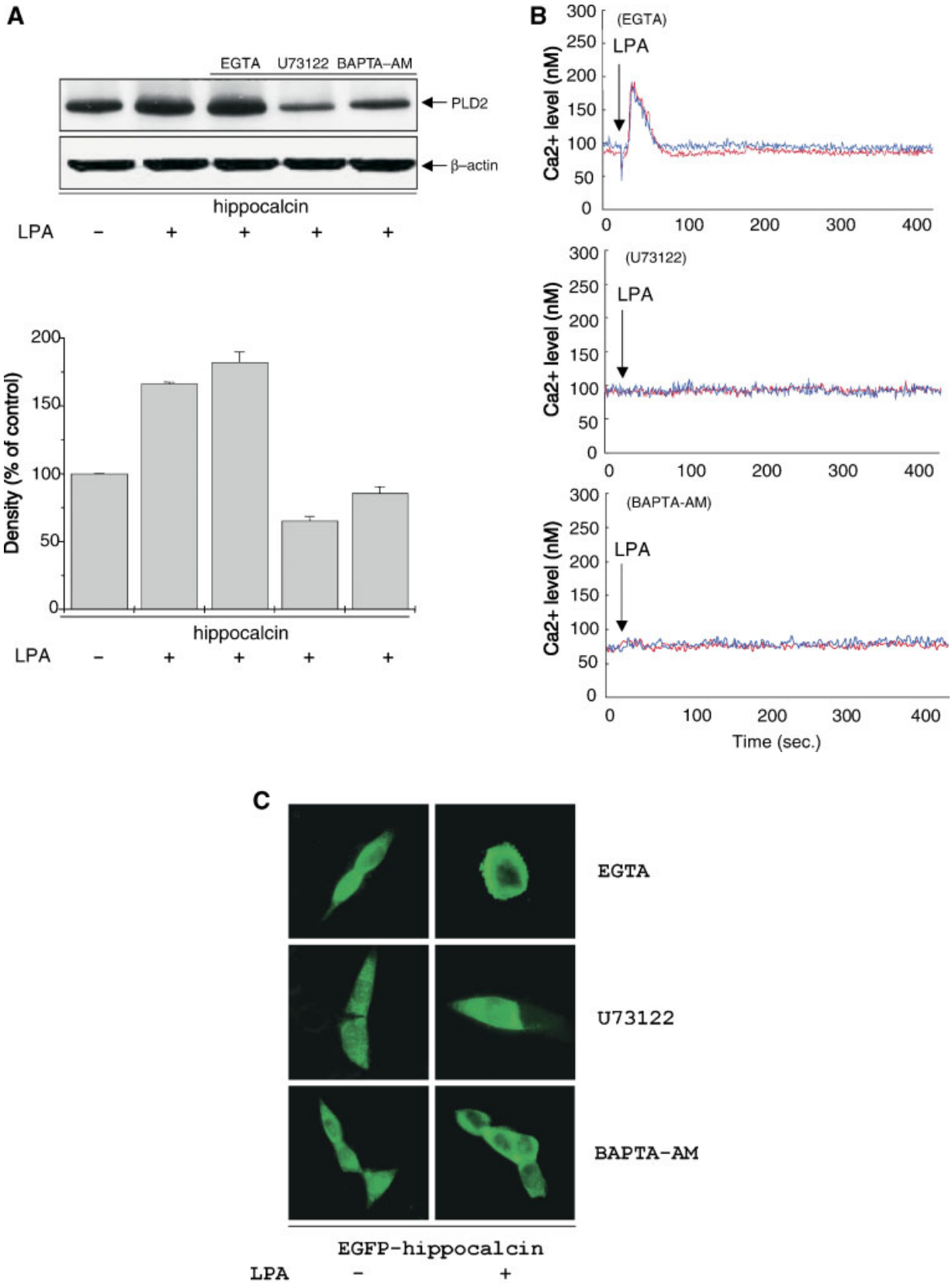
A
EGTA U73122 BAPTA-AM
PLD2
β-actin
hippocalcin
LPA – + + + +
Density (% of control)
200
150
100
50
0
hippocalcin
LPA – + + + +
B
(EGTA)
LPA
Ca2+ level (nM)
300
250
200
150
100
50
0
0 100 200 300 400
(U73122)
LPA
Ca2+ level (nM)
(BAPTA-AM)
LPA
Ca2+ level (nM)
Time (sec.)
C
EGTA
U73122
BAPTA-AM
EGFP-hippocalcin
LPA – +

Fig. 5.

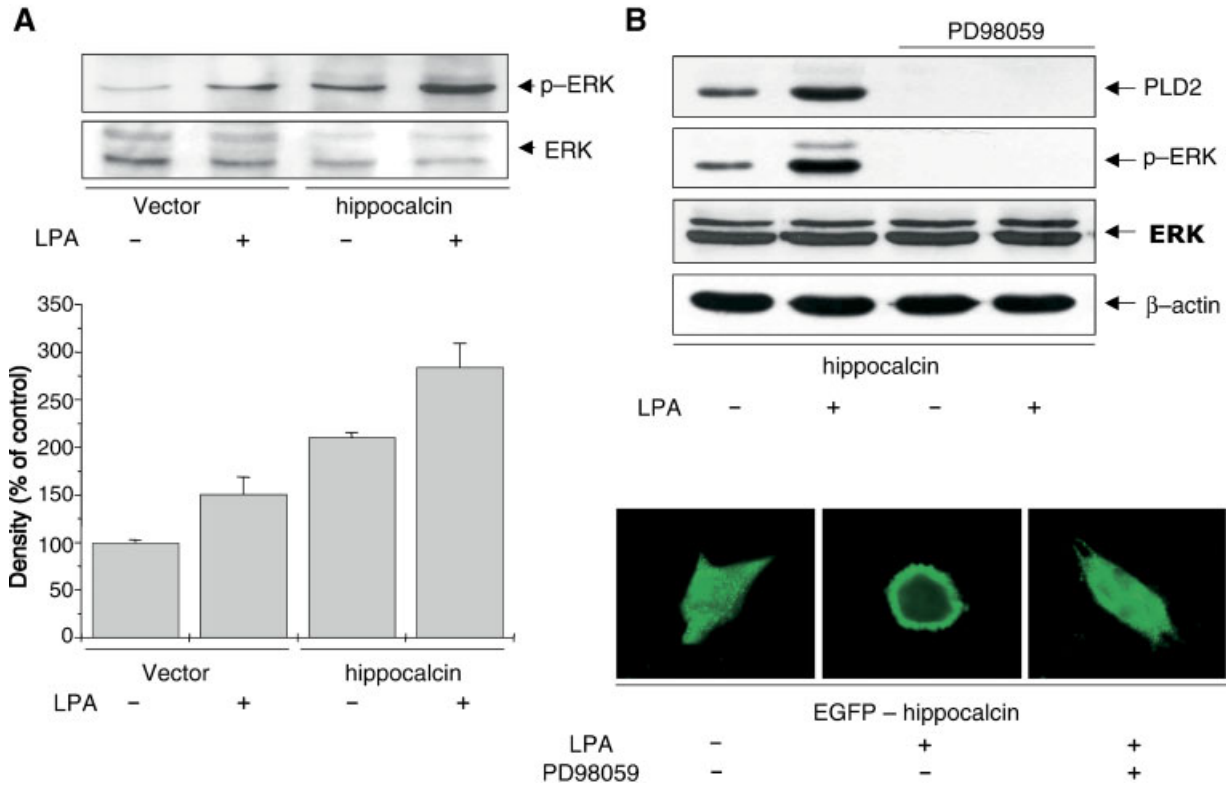

Fig. 6. Hippocalcin induces PLD2 expression via ERK activation in hippocalcin transfected NIH3T3 cells. **A**: Western blot analysis of ERK activation in pcDNA3.1 transfected (Vector) and hippocalcin transfected NIH3T3 cells. The pcDNA3.1 transfected and hippocalcin transfected NIH3T3 cells were extracted and were treated sequentially with LPA (10 μM) for 15 min. These cells were then lysed and immunobloted using p-ERK antibody and ERK antibody. The intensity of bands was quantified using QuantityOne software (Bio-Rad). **B**: Hippocalcin transfected NIH3T3 cells were treated LPA (10 μM) for 15 min. Cells were pretreated with 40 μM of PD98059 for 1 h prior to LPA stimulation. The cells were then lysed and immunobloted with anti-PLD antibody and p-ERK antibody. **C**: EGFP-hippocalcin transfected cells were serum-starved for 18 h and washed twice with PBS and subsequently LPA was added. Cells were pretreated with 40 μM of PD98059 for 1 h prior to LPA stimulation. These cells were then fixed at specified time after plating and staining with GFP antibody. Immunostained cells were observed by fluorescence microscopy (magnification, ×400). The results shown derive from three repeated experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to Ca^{2+} and have a myristoylation site at the N-terminal. It is likely that these subunits may be required for their Ca^{2+}-dependent membrane association deducing from their structures. Most of the myristoylated proteins tend to be involved in cellular signal transduction, since myristoylation is vital for interaction between proteins and cell membranes. Hippocalcin is

Fig. 5. PI-PLC inhibitor and intracellular Ca^{2+} chelator inhibit hippocalcin-induced PLD2 expression potentiated by LPA and cellular morphological changes in hippocalcin transfected NIH3T3 cells. **A**: Hippocalcin transfected NIH3T3 cells were treated LPA (10 μM) for 15 min. Cells were pretreated with 10 μM of U73122 for 30 min, 2 mM of EGTA for 30 min, and 100 μM of BAPTA-AM for 15 min prior to LPA stimulation. The cells were then lysed and immunobloted with anti-PLD antibody. The intensity of bands was quantified using QuantityOne software (Bio-Rad). **B**: LPA induced an increase in $[Ca^{2+}]_i$ in EGFP-hippocalcin transfected cells. Pretreatments with both 10 μM of U73122 for 30 min and 100 μM of BAPTA-AM for 15 min did not elicit $[Ca^{2+}]_i$ in EGFP-hippocalcin transfected cells. However, pretreatment with 2 mM EGTA for 30 min did not inhibit $[Ca^{2+}]_i$ in the identical cells. Measurement of $[Ca^{2+}]_i$ derived from fura-2-based digital images as described Materials and Methods, where image pairs were collected within time intervals ranging from 6 s to 1 min consequently addition of LPA for 15 min. The result showed two repeated experiments (red and blue). **C**: EGFP-hippocalcin transfected cells were serum-starved for 18 h and washed twice with PBS and subsequently LPA was added. Cells were pretreated with 10 μM of U73122 for 30 min, 100 μM of BAPTA-AM for 15 min, and 2 mM of EGTA for 30 min prior to LPA stimulation. These cells were then fixed at specified time after plating and staining with GFP antibody. Immunostained cells were observed by fluorescence microscopy (magnification, ×400). The results shown derive from three repeated experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

myristoylated at N-terminal glycine residue, binds with Ca^{2+} in a submicromolar concentration, and assist the translocation of hippocalcin to a membrane with bound Ca^{2+}. At low Ca^{2+} concentration, hippocalcin remains itself as Ca^{2+}-free form and is predominantly cytosolic, whereas at higher concentration of Ca^{2+}, hippocalcin binds to Ca^{2+}, inducing a conformational change, which promotes membrane association [Kobayashi et al., 1993]. Immunohistochemical analysis of the adult rat brain has shown that hippocalcin is readily expressed in the pyramidal cells of the hippocampus and weakly expressed in the pyramidal cells of the cerebral cortex and the Purkinje cells of the cerebellum [Saitoh et al., 1994]. Hippocalcin is located in cytoplasm and plasma membrane of the cell body and in dendrites, and hippocalcin mRNA seems to be present in the proximal dendrites of hippocampal CA pyramidal cells [Saitoh et al., 1993]. However, there are no reported data on the role of the molecule to suggest its involvement in Ca^{2+}-dependent cellular signaling.

For the first time, the data we have produced strongly suggest that hippocalcin increases PLD2 expression through ERK activation and LPA potentiates the hippocalcin-induced PLD2 expression. Concomitant with the increased PLD2 expression, morphological changes of hippocalcin transfected cells were found with the treatment of LPA, which increased intracellular Ca^{2+} level through PI-PLC activation. Unlike PLD2, PLD1 was not detected in both control cells and hippocalcin tranfected cells suggesting that hippocalcin can only induce expression of PLD2, not PLD1.

The changes in mRNA levels of the mouse PLD isoforms such as mPLD1a, mPLD1b, and mPLD2 were examined by RT-PCR in both control cells and hippocalcin transfected cells respectively. The result showed that mRNA level of mPLD2 dramatically increased in hippocalcin transfected cells, but no changes were observed in control cells. These findings were consistent with results obtained for the hippocalcin-induced PLD2 expression in hippocalcin transfected cells.

There has been a report showing that the increased Ca^{2+} level can activate PLD activity even in the condition of PKC downregulation [Gustavsson et al., 1994]. However, our experimental results surprisingly showed that downregulation of PKC could not abolish hippocalcin-induced PLD2 expression potentiated by LPA in hippocalcin transfected cells. These observations suggest that PKC is not involved hippocalcin-induced in PLD2 expression pathway.

Currently, there have been no reports on the effect of LPA in the hippocalcin-induced PLD2 expression, although there has been a few reports suggesting that LPA activate PLD1 [Hong et al., 2001]. In this study, we showed that PLD2 expression was dependent on hippocalcin, and consequently that activated hippocalcin after binding with Ca^{2+} plays an important role in ERK-mediated PLD2 expression and morphological changes of the cells. It has been known that LPA activates PLD through PI-PLC activation [Exton, 1996]. Thus, we tried to figure out whether PLC signaling pathways is involved in hippocalcin-induced PLD2 expression. Pretreatment of the hippocalcin transfected cells with U73122, a specific PI-PLC inhibitor completely inhibited hippocalcin-induced PLD2 expression potentiated by LPA and intracellular Ca^{2+} level increased by LPA. These results indicate that PI-PLC is a major enzyme to activate hippocalcin by providing intracellular Ca^{2+} and activation of hippocalcin through binding with Ca^{2+} is critical to over-express PLD2 in hippocalcin transfected cells.

It has been found that PI-PLC activation results in the generation of diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate which activates protein kinase C (PKC) and mobilizes Ca^{2+}, respectively [Kim et al., 2004]. We have already demonstrated that PKC is not involved in hippocalcin-induced PLD2 expression pathway. Treatment of LPA evokes a rapid but transient rise in intracellular Ca^{2+}, indicative of PI-PLC activation. There are two known major pathways of intracellular Ca^{2+} mobilization. One is the influx from extracellular space and another is the release from intracellular Ca^{2+} store. We have examined effects of chelating extracellular Ca^{2+} with EGTA and intracellular Ca^{2+} with BAPTA-AM on the hippocalcin-induced PLD2 expression potentiated by LPA. We showed that BAPTA-AM did inhibit hippocalcin-induced PLD2 expression potentiated by LPA in hippocalcin transfected cells. As expected, the pretreatment of the hippocalcin-transfected cells with BAPTA-AM completely inhibits the increase of intracellular Ca^{2+} level. These results imply conclusively that intracellular Ca^{2+} mobilization is important for

hippocalcin-induced PLD2 expression potentiated by LPA. On the other hand, EGTA showed little effects on hippocalcin-induced PLD2 expression potentiated by LPA in hippocalcin transfected cells and pretreatment of the hippocalcin transfected cells with EGTA did not show any inhibition on the increase of intracellular Ca^{2+} level. Hence, the effects of Ca^{2+} influx from extracellular space may be nullified in hippocalcin-induced PLD2 expression potentiated by LPA.

We investigated morphological changes induced by LPA treatments in EGFP-hippocalcin transfected cells. The treatment of LPA resulted in noticeable morphological changes at 15 min after treatment and translocation of hippocalcin to the membrane after binding with Ca^{2+}. The addition of LPA, on the contrary, did not induce any morphological changes in the EGFP-C1 transfected cells. U73122, BAPTA-AM abolished morphological changes induced by LPA, whereas EGTA did not. These results suggest that maintenance of intracellular Ca^{2+} level is important to activate hippocalcin and activated hippocalcin-induced PLD2 expression has a main role in morphological changes in the cells.

PLD is required for RhoA-dependent stress fiber formation in fibroblast. Kam and Exton [2001] came up with theory based on their researches that PLD2 was not directly involved in the formation of stress fibers. However, Colley et al. [1997] reported that the cytoskeletal rearrangements induced by over-expression of PLD2 in fibroblasts were restricted only to the cortical region and filopodia. In fibroblasts and neuroblastoma cells, LPA is known to initiate rapid RhoA-mediated rearrangements of the actin cytoskeleton [Seasholtz et al., 2004]. Consequently, it is possible that LPA-induced RhoA-mediated PLD2 expression is involved in cytoskeletal rearrangements. Phosphatidylinositol 4,5-bisphosphate (PIP_2), phosphatidic acid (PA), Ca^{2+}, and G proteins, including heterotrimeric G_{13}, and the small G protein Rho family Rac, Rho have been assumed to play a role in actin cytoskeleton rearrangement [Ritter et al., 1997; Gohla et al., 1998; Carton et al., 2003]. Moreover, there have been numerous in vitro assays using over-expressed PLD1 which led to conclusions such that PLD1 was the major isoform regulated by PKC, ARF, Rho [Hammond et al., 1997; Du et al., 2000; Kim et al., 2000; Siddiqi et al., 2000]. On the contrary, PLD2 was not at all stimulated by Rho GTPases [Sung et al., 1999]. Thus, these reports suggest that actin cytoskeleton rearrangement by LPA requires RhoA-independent PLD2 signaling. Here we showed that LPA potentiates the PKC-independent hippocalcin-mediated PLD2 expression pathway by increasing intracellular Ca^{2+} through PI-PLC activation. Furthermore, PLD2, which localizes itself primarily to the membrane, unlike PLD1 localized mainly in organelles and vesicles of the endosomal/lysosomal compartment, has been suggested to play a critical role in cytoskeletal rearrangement [Colley et al., 1997]. Therefore, it can be assumed that the PLD2 expressed by hippocalcin might play a role in cytoskeletal rearrangement, although no direct evidence can be provided at present. The possibility of the role of hippocalcin-induced PLD2 expression in cytoskeletal rearrangement is a possible candidate for further experimental studies.

Some reports have described an effect of LPA on ERK activation [Hong et al., 2001]. Thus, we investigated that whether hippocalcin-induced PLD2 expression is regulated by ERK. Surprisingly, we found that expression of hippocalcin resulted in immediate activation of ERK. Moreover, LPA potentiated ERK activation in hippocalcin transfected cells two fold higher than in control cells. To confirm ERK is an essential mediator in hippocalcin-induced PLD2 expression, we blocked MEK using PD98059. Blockade of MEK showed complete inhibition of ERK activation as well as PLD2 expression in hippocalcin transfected cells stimulated by LPA. Also morphological changes of cells in hippocalcin transfected cells were completely abolished with treatment of this MEK inhibitor. Taken together, we can propose that hippocalcin induce PLD2 expression through ERK activation.

Also our data suggest that activation of hippocalcin by increasing intracellular Ca^{2+} is important in PLD2 expression and ERK activation.

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