Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 324 (2004) 458–465
BBRC
www.elsevier.com/locate/ybbrc

Lysophosphatidic acid is a mediator of Trp-Lys-Tyr-Met-Val-D-Met-induced calcium influx

Ha-Young Lee[a,b], Hyun-Kyu Kang[a], Hye-Ran Yoon[c], Jong-Young Kwak[a,b], Yoe-Sik Bae[a,b,*]

[a] Medical Research Center for Cancer Molecular Therapy, College of Medicine, Dong-A University, Busan 602-714, Republic of Korea
[b] Department of Biochemistry, College of Medicine, Dong-A University, Busan 602-714, Republic of Korea
[c] Department of Special Biochemistry, Seoul Medical Science Institute, Seoul Clinical Laboratories, Seoul 140-809, Republic of Korea

Received 13 September 2004
Available online 25 September 2004

Abstract

Intracellular calcium (Ca^{2+}) homeostasis is very strictly regulated, and the activation of G-protein-coupled receptor (GPCR) can cause two different calcium changes, intracellular calcium release, and calcium influx. In this study, we investigated the possible role of lysophosphatidic acid (LPA) on GPCR-induced Ca^{2+} signaling. The addition of exogenous LPA induced dramatic Ca^{2+} influx but not intracellular Ca^{2+} release in U937 cells. LPA-induced Ca^{2+} influx was not affected by pertussis toxin and phospholipase C inhibitor (U73122), ruling out the involvement of pertussis toxin-sensitive G-proteins, and phospholipase C. Stimulation of U937 cells with Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm), which binds to formyl peptide receptor like 1, enhanced phospholipase A_2 and phospholipase D activation, indicating LPA formation. The inhibition of LPA synthesis by phospholipase A_2-specific inhibitor (MAFP) or *n*-butanol significantly inhibited WKYMVm-induced Ca^{2+} influx, suggesting a crucial role for LPA in the process. Taken together, we suggest that LPA mediates WKYMVm-induced Ca^{2+} influx.

Keywords: Lysophosphatidic acid; Ca^{2+} influx; Phospholipase A_2; Phospholipase D

The maintenance of the cytosolic Ca^{2+} concentration is very tightly regulated by various mechanisms, including inositol-1,4,5-triphosphates (IP_3)-dependent intracellular Ca^{2+} release and Ca^{2+} influx via plasma membrane bound Ca^{2+} channels [1–3]. Many G-protein-coupled receptors (GPCR) are also involved in the regulation of the cytosolic Ca^{2+} concentration. The binding of agonist to its specific receptor causes certain morphological changes and activates heterotrimeric G-proteins, which cause the activation of phospholipase Cβ (PLCβ) [4]. PLCβ elicits the hydrolysis of phosphoinositol-4,5,-bis-phosphates (PIP_2), generating IP_3, and diacylglycerol, which are involved in the opening of endoplasmic reticulum (ER)-bound IP_3 receptor and in the activation of protein kinase C, respectively [4,5]. The mechanisms involved in intracellular Ca^{2+} release have been exhaustively investigated. However, the mechanisms of Ca^{2+} influx induced by the activation of GPCR remain controversial.

One of the suggested models for GPCR-induced Ca^{2+} influx is the existence of a soluble factor which mediates ER Ca^{2+} store depletion and plasma membrane Ca^{2+} channel activation [6–8]. According to this model, depletion of the ER Ca^{2+} store leads to the release of small diffusible factors that act on channels to stimulate Ca^{2+} entry [6–8]. Tsien and colleagues [6] termed referred to these molecules as a "calcium influx factor" (CIF), and suggested that CIF has hydroxyls on adjacent carbons, a phosphate, and a molecular weight of

* Corresponding author. Fax: +82 51 241 6940.
E-mail address: yoesik@donga.ac.kr (Y.-S. Bae).

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.09.072

under 500. Even though several reports have suggested a putative role for CIF on the modulation of Ca^{2+} influx downstream GPCR activation, the factors involved in GPCR-mediated Ca^{2+} influx are still unclear.

Lysophosphatidic acid (LPA) is a pluripotent lipid mediator and has been reported to regulate cell growth, differentiation, and cell motility [9–11]. Many previous reports demonstrated that LPA binds to cell surface receptors, which are G-protein-coupled [9–11]. Until now, three different LPA receptors have been reported, namely, LPA_1, LPA_2, and LPA_3 [12]. However, Lynch and colleagues [13] reported that LPA induces mitogenesis in an LPA cell surface receptor-independent manner, suggesting a new mode of LPA signaling. In this study, we examined the role of LPA on GPCR-mediated Ca^{2+} signaling, especially Ca^{2+} influx. Accordingly, we investigated the involvement of LPA on the Ca^{2+} signaling induced by activated formyl peptide receptor like 1 (FPRL1) by using Trp-Lys-Tyr-Met-Val-D-Met (WKYMVm) [14,15] in U937 cells. We also checked the direct effect of LPA on Ca^{2+} release and Ca^{2+} influx in U937 cells.

Materials and methods

Materials. Lysophosphatidic acid (1-myristyl-2-hydroxy-*sn*-glycero-3-phosphate, sodium salt), lysophosphatidylcholine (1-myristyl-2-hydroxy-*sn*-glycero-phosphocholine), lysophosphatidylethanolamine (1-myristyl-2-hydroxy-*sn*-glycero-phosphoethanolamine), and lysophosphatidylserine (1-myristyl-2-hydroxy-*sn*-glycero-phosphoserine) were from Avanti polar lipids (Alabaster, AL). Sphingosine-1-phosphate, phytosphingosine-1-phosphate, MAFP (methyl arachidonylfluorophosphonate), $AACOCF_3$ (arachidonyltrifluoromethyl ketone), and BEL (bromoenol lactone) were from Biomol (Plymouth Meeting, PA). WKYMVm was synthesized, purified, and prepared in the Peptide Library Support Facility at Pohang University of Science and Technology as described previously [16]. Precoated silica gel TLC plates (F-254) were obtained from Merck (Darmstadt, FRG). RPMI 1640 was purchased from Invitrogen (Carlsbad, CA). Dialyzed fetal bovine serum and supplemented bovine calf serum were purchased from Hyclone Laboratory (Logan, UT). Fura-2 pentaacetoxymethyl ester (fura-2/AM) was purchased from Molecular Probes (Eugene, OR).

Measurement of intracellular calcium concentration. The level of calcium concentration ($[Ca^{2+}]_i$) was determined by Grynkiewicz's method using fura-2/AM [17]. Fluorescence changes at the dual excitation wavelength of 340 and 380 nm were measured, and the calibrated fluorescence ratio was translated into $[Ca^{2+}]_i$.

Measurement of the formation of inositol phosphates in cells. Agonist-induced formation of inositol phosphates was determined as described previously [18]. *myo*[^{3}H]inositol-labeled U937 cells were incubated with 20 mM Hepes at pH 7.2, 20 mM LiCl for 15 min, and PIP_2 hydrolysis was initiated by adding stimuli or solvents for the indicated times. Total inositol phosphate formation was quantified using Bio-Rad Dowex AG 1-X 8 anion exchange columns as described before [18].

Measurement of phospholipase A_2 (PLA_2) activity in cells. Cultured U937 cells (10^7 cells/ml) were prelabeled with 0.5 μCi/ml [^{3}H]arachidonic acid (AA) in RPMI 1640 medium for 24 h at 37 °C in a humidified incubator supplied with 95% air and 5% CO_2, as described before [19]. The labeled cells were then washed twice with serum-free RPMI 1640 and incubated in RPMI 1640 medium containing 0.1% fatty acid-free BSA for 15 min at 37 °C. After discarding the medium, the cells were stimulated with various concentrations of the peptide for indicated periods of time. The radioactivity in the medium and the collected cells was determined with a liquid scintillation counter.

Measurement of phosphatidylbutanol formation in U937 cells. The production of phosphatidylbutanol (PBtOH) was determined as described in a previous report [19] with a slight modification. U937 cells were resuspended to 1×10^6 cells/ml in RPMI 1640 medium containing 2.5% FBS and loaded with [^{3}H]myristic acid (5 μCi/ml) for 90 min at 37 °C. The loaded cells were then washed twice with serum-free RPMI 1640 medium and stimulated with the peptide in the presence of 0.5% *n*-butanol. Lipids were extracted and separated on silica gel 60 TLC plates using a solvent containing chloroform:methanol:acetic acid (90:10:10) as described previously [19]. To determine the amounts of PBtOH and total lipids, a Fuji BAS-2000 image analyzer (Fuji Film) was used.

Results

LPA stimulates Ca^{2+} influx without Ca^{2+} release in U937 cells

In this study, we examined the effect of LPA on calcium influx in U937 cells. When U937 cells were stimulated with various concentrations of LPA in the absence of extracellular Ca^{2+}, no significant change in the cytosolic Ca^{2+} concentration was observed (Fig. 1A). However, the addition of extracellular Ca^{2+} to LPA-stimulated U937 cells caused a dramatic Ca^{2+} influx (Fig. 1A). This LPA-induced Ca^{2+} influx increased according to the added Ca^{2+} concentration and showed maximal activity at around 1 mM (Fig. 1A). We also tested the concentration-dependency of LPA on Ca^{2+} influx. As shown in Fig. 1B, Ca^{2+} influx was significantly induced at 1 μM LPA in a concentration-dependent manner, with maximal activity at 5–10 μM. The addition of 20 μM LPA caused more sustained Ca^{2+} influx (Fig. 1B). When U937 cells were stimulated with <1 μM, such as 500 or 100 nM, no significant Ca^{2+} influx was induced (Fig. 1B). These results indicate that LPA stimulates Ca^{2+} influx without inducing Ca^{2+} release in U937 cells, and that for adequate Ca^{2+} influx, over 1 μM LPA is required.

Since we observed that LPA induces Ca^{2+} influx without Ca^{2+} release, we investigated the effects of lipids structurally analogous to LPA. The stimulation of U937 cells with 20 μM lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylserine (LPS), or phytosphingosine-1-phosphate (Ph-S1P) did not induce significant Ca^{2+} influx (Fig. 1C). Under the same experimental conditions, LPA-stimulated U937 cells and resulted in dramatic Ca^{2+} influx (Fig. 1C). We also found that sphingosine-1-phosphate (S1P) also induced Ca^{2+} influx in U937 cells at 20 μM (Fig. 1C). These results indicate that Ca^{2+} influx is specifically induced by LPA and S1P but not by other structural analogues of LPA.

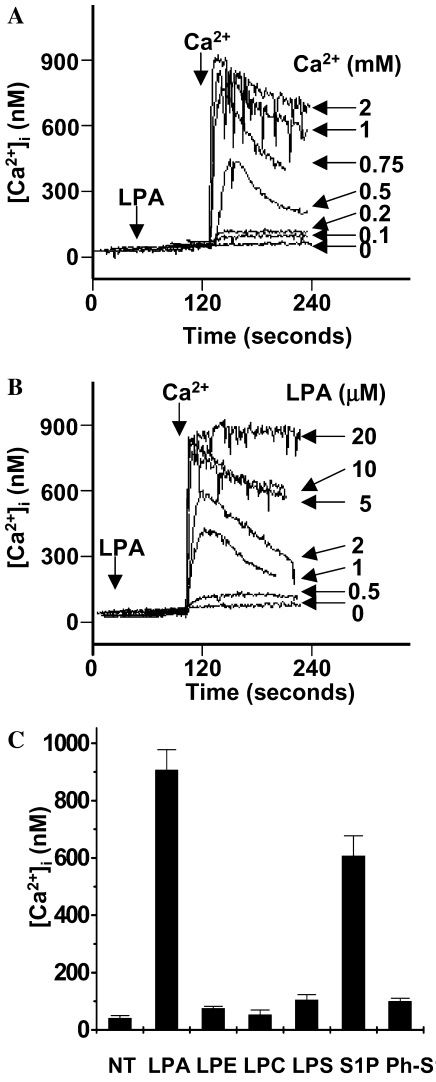

Fig. 1. The effects of LPA and its analogues on $[Ca^{2+}]_i$ in U937 cells. U937 cells were stimulated with 5 μM LPA in the absence of extracellular Ca^{2+}, and then different concentrations of Ca^{2+} were added at the indicated time points (A). U937 cells were stimulated with various concentrations of LPA in the absence of extracellular Ca^{2+}, and then 2 mM Ca^{2+} was added at the indicated time (B). The peak level of $[Ca^{2+}]_i$ was recorded. Results are representative of at least three independent experiments (A,B). U937 cells were stimulated with 20 μM LPA, LPE, LPC, LPS, S1P, or Ph-S1P in the absence of extracellular Ca^{2+}, and then 2 mM Ca^{2+} was added (C). The peak level of $[Ca^{2+}]_i$ was recorded. Data are presented as means ± SE of five independent experiments (C).

LPA stimulates Ca^{2+} influx without ER store depletion in U937 cells

To confirm that LPA does not stimulate ER store depletion in U937 cells, we stimulated cells with 5 μM LPA and then subsequently treated the cells with 1 μM WKYMVm, to asses the status of IP_3-releasable ER Ca^{2+} stores. As shown in Fig. 2, no significant evidence of LPA-induced ER store depletion was detected and WKYMVm-induced store release was not affected

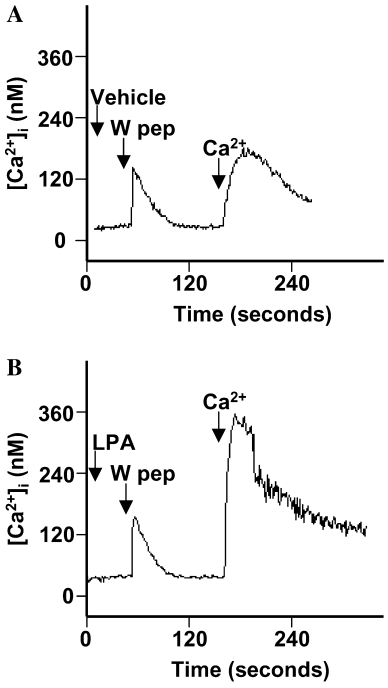

Fig. 2. The effects of LPA on WKYMVm-induced $[Ca^{2+}]_i$ release and Ca^{2+} influx in U937 cells. U937 cells were stimulated with vehicle (A) or 5 μM LPA (B) for 1 min prior to adding 1 μM WKYMVm (W pep) in the absence of extracellular Ca^{2+}, and then 2 mM Ca^{2+} was added at the indicated time. $[Ca^{2+}]_i$ was determined fluorometrically using fura-2/AM, as described in Materials and methods and the peak level of $[Ca^{2+}]_i$ was recorded. Results are representative of four independent experiments (A,B).

by LPA pretreatment. Moreover, we found that LPA pretreatment prior to U937 stimulation with WKYMVm caused a greater Ca^{2+} influx increase compared with stimulation by WKYMVm alone (Fig. 2). The results indicate that LPA induces Ca^{2+} influx in an ER store depletion-independent manner.

LPA induces Ca^{2+} influx independently of G-protein and PLC

Several previous reports have demonstrated that LPA act, on cell surface receptors, such as LPA_1, LPA_2, and LPA_3, to cause intracellular Ca^{2+} release, and Ca^{2+} influx [20]. Moreover, pertussis toxin (PTX)-sensitive G-proteins have been reported to play a role in the process of LPA receptor-mediated Ca^{2+} signaling [21]. In this study, we found that LPA induced Ca^{2+} influx without intracellular Ca^{2+} release (Fig. 1), and thus we investigated the role of PTX-sensitive G-protein on the LPA-induced Ca^{2+} influx in U937 cells. Previously we demonstrated that WKYMVm stimulates FPRL1 to cause intracellular Ca^{2+} release and Ca^{2+} influx, and that these events are completely inhibited by PTX [22]. When U937 cells were preincubated with 100 ng/ml PTX for 24 h we found that LPA-induced Ca^{2+} influx was not affected by preincubating the cells with 100 ng/ml PTX for

24 h (Fig. 3A). However, WKYMVm-induced intracellular Ca^{2+} release and Ca^{2+} influx were almost completely inhibited by PTX (Fig. 3A). Thus, Ca^{2+} influx in response to LPA is independent of PTX-sensitive G-protein-linked LPA receptors.

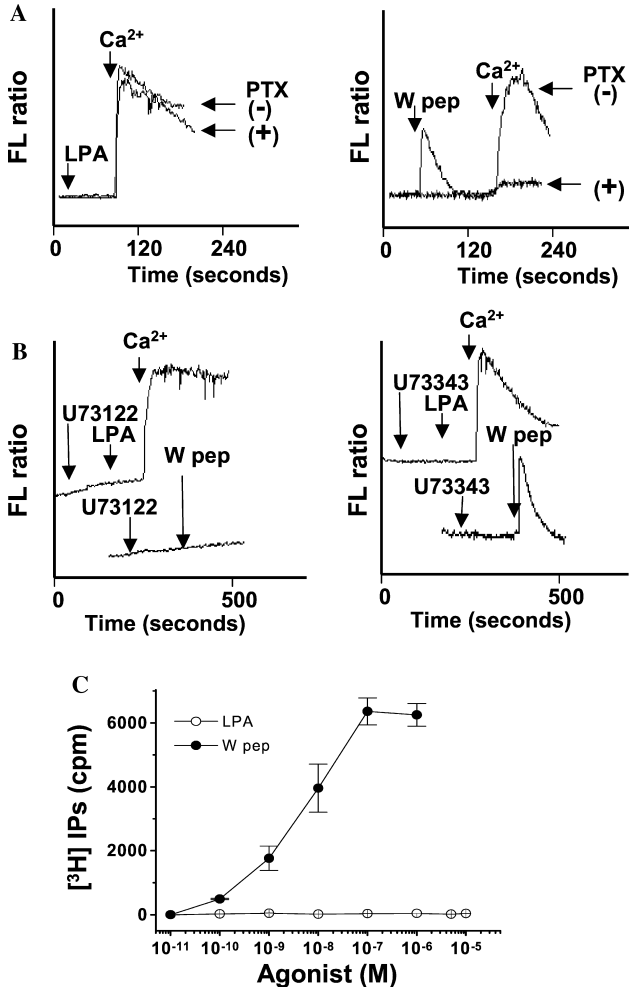

Fig. 3. The effects of PTX or PLC inhibitor on LPA- or WKYMVm-induced $[Ca^{2+}]_i$ signaling in U937 cells. U937 cells were cultured in the absence or presence of PTX (100 ng/ml) for 24 h, and then the cells were loaded with fura-2/AM. Cells were stimulated with 5 μM LPA in the absence of extracellular Ca^{2+}, and then 2 mM Ca^{2+} was added at the indicated time (A). Cells were stimulated with 1 μM WKYMVm (W pep) in the absence of extracellular Ca^{2+}, and then 2 mM of Ca^{2+} was added at the indicated time (A). The peak level of $[Ca^{2+}]_i$ was recorded. Results are representative of three independent experiments (A). Fura-2 loaded U937 cells were pretreated with U73122 (4 μM) or U73343 (4 μM) for 3 min prior to being stimulated with 5 μM LPA, in the absence of extracellular Ca^{2+}, and then 2 mM Ca^{2+} was added at the indicated time (B). Cells were pretreated with U73122 (4 μM) or U73343 (4 μM) for 3 min prior to being stimulated with 1 μM WKYMVm (W pep) in the absence of extracellular Ca^{2+} (B). $[Ca^{2+}]_i$ was determined fluorometrically using fura-2/AM, as described in Materials and methods. The peak level of $[Ca^{2+}]_i$ was recorded. Results are representative of three independent experiments (B). U937 Cells were labeled with *myo*[^{3}H]inositol (1 μCi/10^6 cells) for 24 h at 37 °C and then treated with various concentrations of LPA or WKYMVm (W pep) (C). Total inositol phosphates were eluted with a solution containing 1 M ammonium formate and 0.1 M formic acid. [^{3}H]Inositol phosphate radioactivity was determined by counting in a scintillation counter. Data are presented as means ± SE of five independent experiments (C).

To further confirm that LPA induces Ca^{2+} influx without activating IP_3 release-dependent Ca^{2+} release, we examined the effect of PLC inhibitor (U73122) on LPA-induced Ca^{2+} influx. As shown in Fig. 3B, the preincubation of U937 cells with a PLC-specific inhibitor (U73122) did not affect LPA-induced Ca^{2+} influx. Previously we demonstrated that WKYMVm stimulates intracellular Ca^{2+} release and subsequent Ca^{2+} influx in a PLC-dependent manner [23]. When U937 cells were preincubated with 4 μM U73122 for 3 min, WKYMVm-induced intracellular Ca^{2+} release was almost completely inhibited (Fig. 3B), supporting our previous report [23]. However, the inactive analogue of U73122, U73343, did not affect WKYMVm-induced Ca^{2+} signaling (Fig. 3B). We also investigated the effect of LPA and WKYMVm on PI hydrolysis-dependent inositol phosphates formation. Stimulation of U937 cells with various concentrations of WKYMVm caused inositol phosphates formation in a concentration-dependent manner, showing the maximal activity at 100 nM (Fig. 3C). However, no significant change of inositol phosphates formation was found after adding various concentrations of LPA in U937 cells (Fig. 3C). Up to 20 μM LPA was added and no change in inositol phosphates formation was observed. This result concurs with the failure of LPA to induce intracellular Ca^{2+} release in U937 cells (Fig. 1A). Taken together, LPA did not stimulate PLC activity in U937 cells, but did induce Ca^{2+} influx.

Stimulation of FPRL1 by WKYMVm elicits PLA_2 and phospholipase D activation in U937 cells

We found that LPA directly induces Ca^{2+} influx without eliciting ER store depletion in U937 cells (Figs. 1 and 2). To investigate the role of LPA on GPCR-mediated Ca^{2+} influx, we examined the effects of the inhibitors of enzymes involved in the generation of LPA as a downstream event of GPCR activation in U937 cells, particularly, WKYMVm a potent FPRL1 agonist [14,15]. At first, we verified the effect of WKYMVm on PLA_2 activity in U937 cells by measuring AA release, as described previously [19]. When stimulated with various concentrations of WKYMVm, U937 cells responded with a concentration-dependent increase in AA release. This effect was highest at around 100 nM, which coincided with its maximal effect on Ca^{2+} mobilization (Fig. 4A). At 100 nM, WKYMVm caused a rapid release of AA from U937 cells, which peaked after 5 min (Fig. 4B). To identify the PLA_2 isoform responsible for the WKYMVm-induced increase in AA release, we introduced several isoform-specific inhibitors of PLA_2. Pretreatment of the cells with the $cPLA_2$-specific inhibitors, MAFP and $AACOCF_3$, blocked the

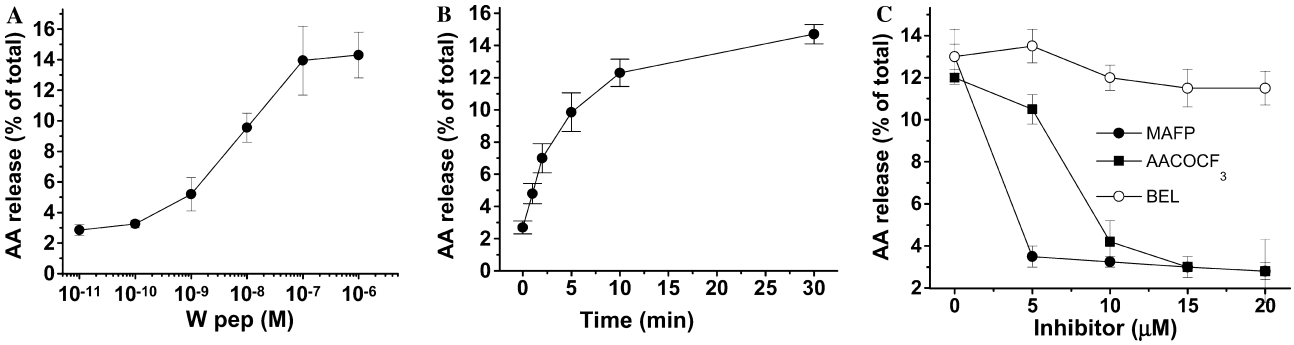

Fig. 4. WKYMVm-induced activation of AA release in U937 cells. U937 cells preloaded with [^{3}H]arachidonic acid were stimulated with various concentrations of WKYMVm (W pep) for 30 min (A), and for various times with 100 nM WKYMVm (W pep) (B) in the presence of 0.1% fatty acid-free BSA at 37 °C. U937 cells were suspended in HBSS containing 0.1% fatty acid-free BSA, incubated for 15 min in the presence or absence of MAFP, $AACOCF_3$, and BEL at the indicated concentrations at 37 °C, and stimulated for 30 min with 100 nM WKYMVm or vehicle (C). The release of [^{3}H]arachidonic acid into the extracellular medium was determined using a liquid scintillation counter. Data are presented as means ± SE of four independent experiments.

WKYMVm-induced liberation of AA in a concentration-dependent manner (Fig. 4C). At a concentration of 10 μM MAFP or $AACOCF_3$ WKYMVm-induced AA release was almost blocked, while another PLA_2 inhibitor, BEL, which is known to be specific for $iPLA_2$, did not interfere with WKYMVm-induced AA release (Fig. 4C). These results indicate that WKYMVm evokes AA release by stimulating $cPLA_2$, but not $iPLA_2$, in U937 cells.

We also examined the effect of WKYMVm on phospholipase D (PLD) activation in U937 cells using transphosphatidylation, a characteristic reaction of PLD in the presence of 0.5% *n*-butanol. WKYMVm stimulated PBtOH formation in a concentration-dependent manner with maximal activity at 100 nM within 1 min (Figs. 5A and B). The concentration dependency of WKYMVm-induced PBtOH formation was similar to that of the peptide-stimulated Ca^{2+} release and PLA_2 activation in U937 cells.

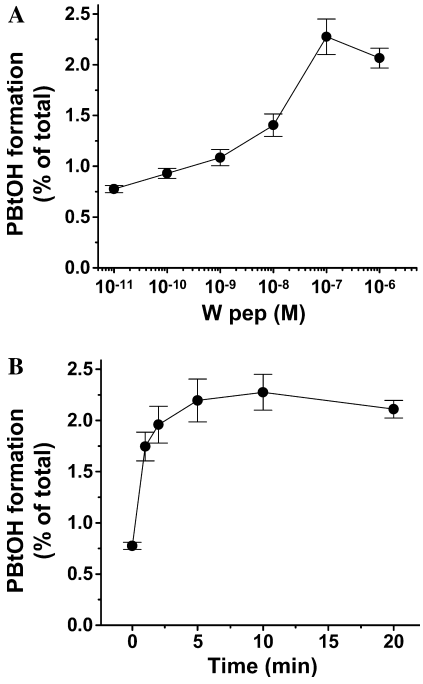

Fig. 5. The effect of WKYMVm on the formation of PBtOH in U937 cells. U937 cells were serum starved for 20 h and then labeled with [^{3}H]myristic acid for an additional 4 h at 37 °C in RPMI containing 2.5% FBS. After three washes with PBS the labeled cells were stimulated with various concentrations of WKYMVm in the presence of 0.5% butanol for 10 min (A) or with 100 nM WKYMVm in the presence of 0.5% *n*-butanol for various lengths of time (B). PBtOH was resolved from total lipids by thin layer chromatography. A Fuji BAS-2000 was used to quantify each lipid. Data are presented as means ± SE of three separate experiments.

LPA is involved in FPRL1-induced Ca^{2+} influx

We found that the stimulation of FPRL1 by WKYMVm induces not only intracellular Ca^{2+} release, but also PLA_2 and PLD activation in U937 cells (Figs. 2, 4, and 5). The activations of PLA_2 and PLD can generate LPA synthesis from lipid substrates, such as phosphatidylcholine, in cells [24,25]. In this study, we investigated the involvement of LPA on WKYMVm-induced Ca^{2+} influx. Thus, we introduced a PLA_2-specific inhibitor (MAFP) or a PA acceptor (*n*-butanol). When U937 cells were stimulated with 1 μM WKYMVm, potent and dramatic Ca^{2+} changes were observed. Because WKYMVm stimulates intracellular Ca^{2+} release and Ca^{2+} influx, Ca^{2+} change should be the sum of Ca^{2+} release and Ca^{2+} influx. Preincubation with 20 μM MAFP (a PLA_2-specific inhibitor) prior to stimulating the cells with 1 μM WKYMVm caused a significant inhibition in this peptide-induced Ca^{2+} change (Fig. 6A). This result suggests that PLA_2 activity is necessary for the WKYMVm-induced Ca^{2+} change in U937 cells. The morphology of WKYMVm-induced Ca^{2+} change in MAFP-pretreated U937 cells is similar to that of WKYMVm-induced Ca^{2+} change in the absence of

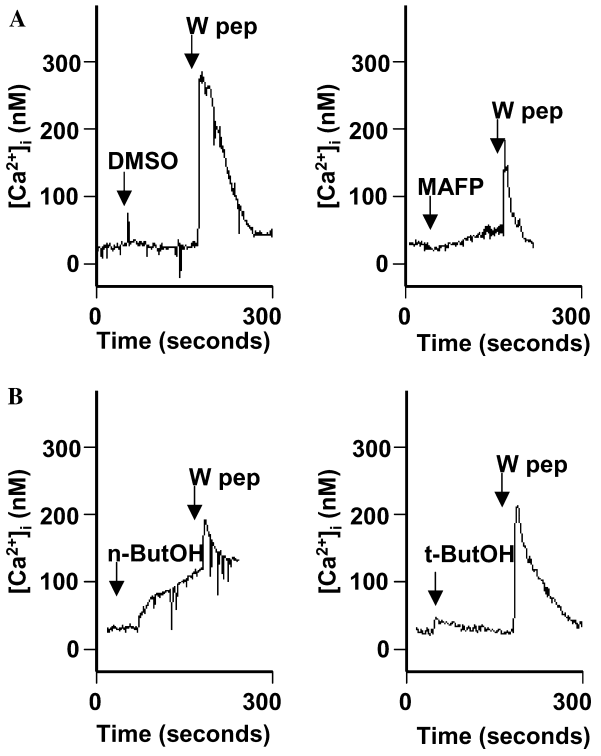

Fig. 6. The roles of PLA_2 and PLD activity on WKYMVm-induced $[Ca^{2+}]_i$ signaling in U937 cells. Fura-2 loaded U937 cells were pretreated with vehicle (DMSO) or MAFP (20 μM) (A) for 3 min prior to being stimulated with 1 μM WKYMV in the presence of 2 mM of extracellular Ca^{2+} (A). Cells were pretreated with *n*-butanol (final 1%) or *t*-butanol (final 1%) for 3 min prior to being stimulated with 1 μM of WKYMVm in the presence of 2 mM extracellular Ca^{2+} (B). $[Ca^{2+}]_i$ was determined fluorometrically using fura-2/AM, as described in Materials and methods. The peak $[Ca^{2+}]_i$ level was recorded. Results are representative of three independent experiments (A,B).

extracellular Ca^{2+}, with respect to the steepness of the response. This suggests that MAFP may specifically inhibit WKYMVm-induced Ca^{2+} influx but not intracellular Ca^{2+} release in U937 cells.

We also investigated the possible role of PLD activity on WKYMVm-induced Ca^{2+} change. When U937 cells were stimulated with 1 μM WKYMVm in the presence of 1% *n*-butanol, the WKYMVm-induced Ca^{2+} change was dramatically inhibited (Fig. 6B), and showed a pattern that was similar to that of MAFP-pretreated cells (Fig. 6A). However, the addition of 1% *t*-butanol did not affect the WKYMVm-induced Ca^{2+} change (Fig. 6B). From these results, we believe that PA generation after the activation of PLD by WKYMVm is required for Ca^{2+} influx in these cells.

Discussion

This study demonstrates that LPA plays a role in the regulation of Ca^{2+} influx in U937 cells. Moreover, the generation of LPA is crucially required for GPCR (FPRL1)-induced Ca^{2+} influx in U937 cells. Since many reports have demonstrated the activation of cell surface receptors, such as LPA_1, LPA_2, and LPA_3 by LPA, we also checked whether U937 cells express cell surface LPA receptors by RT-PCR analysis. We found that U937 cells express the mRNA transcript of LPA_2, but not those of LPA_1 and LPA_3 (data not shown). Many reports have demonstrated that the stimulation of LPA_1, LPA_2, and LPA_3 by LPA result in intracellular Ca^{2+} release via the activation of PLC [21,26,27]. In this study, we examined the effect of LPA on intracellular Ca^{2+} release, but we did not observe any significant change in intracellular Ca^{2+} release by stimulating the cells with LPA (Fig. 1). As a positive control, we found that the stimulation of U937 cells with a FPRL1-specific agonist (WKYMVm) caused a dramatic increase of intracellular Ca^{2+} release, and that this was PLC-dependent (Figs. 2 and 3). Furthermore, several reports have demonstrated that intracellular Ca^{2+} release and MAPK activation by LPA are inhibited by PTX, suggesting the role of cell surface receptor-mediated PTX-sensitive G-proteins [28,29]. However, in the present study, we found that LPA-induced Ca^{2+} influx was not inhibited by PTX (Fig. 3A), ruling out the involvement of PTX-sensitive G-proteins. Taken together, our results suggest that LPA modulates Ca^{2+} influx in its specific cell surface receptors (eg LPA_1, LPA_2, or LPA_3)-independent manner.

In terms of GPCR-induced Ca^{2+} signaling, this study demonstrates that intracellularly synthesized LPA may play a role in the process. The stimulation of U937 cells with a FPRL1-specific agonist, WKYMVm, resulted in the activation of PLA_2 and PLD, indicating LPA formation (Figs. 4 and 5). To investigate the role of LPA on WKYMVm-induced Ca^{2+} signaling, we used a PLA_2-specific inhibitor, MAFP. The inhibition of LPA formation as a result of PLA_2 inhibition caused a dramatic reduction in WKYMVm-induced Ca^{2+} signaling (Fig. 6A). The morphology of WKYMVm-induced Ca^{2+} signaling in MAFP-pretreated cells is similar to that of WKYMVm-induced intracellular Ca^{2+} release (Fig. 6A). We believe that WKYMVm-induced Ca^{2+} influx is a PLA_2-dependent process. Since LPA formation can be induced by the sequential activation of PLD and PLA_2 or vice versa, we also confirmed that the inhibition of PA formation by the addition of a PA acceptor (*n*-butanol) dramatically inhibits WKYMVm-induced Ca^{2+} signaling in U937 cells (Fig. 6B). Recently Bolotina and colleagues [30] demonstrated the role of lysophospholipids on the activation of store-operated calcium channel. They proposed that depletion of calcium store triggers production of CIF, which cause the activation of membrane-bound calcium-independent PLA_2, resulting in the production of lysolipids which mediate calcium influx in a membrane-delimited fashion [30]. In this study we demonstrated that exogenous

addition of LPA in U937 cells elicited Ca^{2+} influx independently of calcium release, Gi protein, or PLC (Figs. 1 and 3). Taking together our and Bolotina's results, it will be reasonable to assume that LPA may modulate calcium influx in a membrane-delimited manner.

Previously Randriamampita and Tsien [6] reported that a soluble messenger molecule in cellular extracts could elicit store-operated Ca^{2+} entry. In their report, they suggested that CIF is a phosphorylated molecule with a molecular weight of less than 500 Da [6]. Recently, Itagaki and Hauser [31] reported that sphingosine-1-phosphate is a diffusible Ca^{2+} influx factor that mediates store-operated Ca^{2+} entry. They showed that the preincubation of neutrophils with a sphingosine kinase inhibitor (DMS) prior to platelet activating factor stimulation caused a dramatic inhibition in platelet activating factor-induced Ca^{2+} influx, suggesting that S1P is involved in the GPCR-mediated Ca^{2+} influx as a CIF [31]. Summarizing the findings of previous reports and those of this study, it is reasonable to presume the existence of additional Ca^{2+} influx factors that are involved in GPCR-induced Ca^{2+} influx. Further study is required to identify these factors.

Several reports have demonstrated that LPA modulates various cellular responses by acting on cell surface receptors, such as LPA_1, LPA_2, and LPA_3 [12]. However, reports on the cell surface receptor-independent role of LPA are limited. After suggesting a new cell surface receptor-independent mode of LPA action [13], PPARγ was reported to be an intracellular receptor for LPA [32]. Due to its hydrophobic character, some portion of LPA in the extracellular environment will be taken up by cells and combined with some intracellular or plasma membrane-bound targets [33]. Identification of PPARγ as an intracellular receptor for LPA supports the notion that extracellular LPA transports to intracellular part. In this study, we demonstrated that exogenously added LPA elicited Ca^{2+} influx (without Ca^{2+} release) (Fig. 1). We also suggested some evidences that intracellularly formed LPA can mediate WKYMVm-induced Ca^{2+} influx (Fig. 6). On the relationship between the roles of exogenously added LPA and intracellularly synthesized LPA by WKYMVm stimulation in calcium influx, it will be reasonable to assume that some portion of extracellularly added LPA, which act as a mimetic of intracellularly synthesized LPA by WKYMVm stimulation, can be transported into cells, where LPA can induce the activation of store-operated calcium channel, as reported previously [30]. Moreover, exogenous PA (a structural analogue of LPA) added to cell culture medium has been reported to incorporate rapidly into cellular membranes and subsequently participate in cellular functions [34,35]. It also supports our notion that exogenously added LPA transports to intracellular compartment and modulates certain target molecules. The mechanism involved in the LPA transport and delivery to the intracellular receptor should be investigated. Since Trepakova et al. [8] reported that CIF directly activates store-operated cation channels in vascular smooth muscle cells, it should be confirmed whether LPA directly stimulates Ca^{2+} channel. The results available to date indicate that unidentified intracellular target molecules modulated by LPA exist. It would be interesting to identify such unknown intracellular LPA receptors and to study their roles in the regulation of cellular responses.

Acknowledgments

This work was supported by Grant 02-PJ1-PG10-20706-0001 from the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (to Y.S.B.). We thank Dr. D.S. Im at Pusan National University College of Pharmacy for his helpful discussion.

References

[1] M.J. Berridge, Inositol trisphosphate and calcium signaling, Nature 361 (1993) 315–325.
[2] H.C. Lee, Mechanisms of calcium signaling by cyclic ADP–ribose and NAADP, Physiol. Rev. 77 (1997) 1133–1164.
[3] N. Burnashev, Calcium permeability of ligand-gated channels, Cell Calcium 24 (1998) 325–332.
[4] S.G. Rhee, Regulation of phosphoinositide-specific phospholipase C, Annu. Rev. Biochem. 70 (2001) 281–312.
[5] D.Y. Noh, S.H. Shin, S.G. Rhee, Phosphoinositide-specific phospholipase C and mitogenic signaling, Biochim. Biophys. Acta 1242 (1995) 99–113.
[6] C. Randriamampita, R.Y. Tsien, Emptying of intracellular Ca^{2+} stores releases a novel small messenger that stimulates Ca^{2+} influx, Nature 364 (1993) 809–814.
[7] P. Csutora, Z. Su, H.Y. Kim, A. Bugrim, K.W. Cunningham, R. Nuccitelli, J.E. Keizer, M.R. Hanley, J.E. Blalock, R.B. Marchase, Calcium influx factor is synthesized by yeast and mammalian cells depleted of organellar calcium stores, Proc. Natl. Acad. Sci. USA. 96 (1999) 121–126.
[8] E.S. Trepakova, P. Csutora, D.L. Hunton, R.B. Marchase, R.A. Cohen, V.M. Bolotina, Calcium influx factor directly activates store-operated cation channels in vascular smooth muscle cells, J. Biol. Chem. 275 (2000) 26158–26163.
[9] W.H. Moolenaar, Bioactive lysophospholipids and their G protein-coupled receptors, Exp. Cell Res. 253 (1999) 230–238.
[10] T. Yamada, K. Sato, M. Komachi, E. Malchinkhuu, M. Tobo, T. Kimura, A. Kuwabara, Y. Yanagita, T. Ikeya, Y. Tanahashi, T. Ogawa, S. Ohwada, Y. Morishita, H. Ohta, D.S. Im, K. Tamoto, H. Tomura, F. Okajima, Lysophosphatidic acid in malignant ascites stimulates motility of human pancreatic cancer cells through LPA1, J. Biol. Chem. 279 (2004) 6595–6605.
[11] X. Ye, N. Fukushima, M.A. Kingsbury, J. Chun, Lysophosphatidic acid in neural signaling, Neuroreport 13 (2002) 2169–2175.
[12] N. Fukushima, J. Chun, The LPA receptors, Prostaglandins 64 (2001) 21–32.
[13] S.B. Hooks, W.L. Santos, D.S. Im, C.E. Heise, T.L. Macdonald, K.R. Lynch, Lysophosphatidic acid-induced mitogenesis is regulated by lipid phosphate phosphatases and is Edg-receptor independent, J. Biol. Chem. 276 (2001) 4611–4621.

[14] Y. Le, W. Gong, B. Li, N.M. Dunlop, W. Shen, S.B. Su, R.D. Ye, J.M. Wang, Utilization of two seven-transmembrane, G protein-coupled receptors, formyl peptide receptor-like 1 and formyl peptide receptor, by the synthetic hexapeptide WKYMVm for human phagocyte activation, J. Immunol. 163 (1999) 6777–6784.
[15] Y.S. Bae, J.Y. Song, Y. Kim, R. He, R.D. Ye, J.Y. Kwak, P.G. Suh, S.H. Ryu, Differential activation of formyl peptide receptor signaling by peptide ligands, Mol. Pharmacol. 64 (2003) 841–847.
[16] Y.S. Bae, Y. Kim, J.C. Park, P.G. Suh, S.H. Ryu, The synthetic chemoattractant peptide, Trp-Lys-Tyr-Met-Val-D-Met, enhances monocyte survival via PKC-dependent Akt activation, J. Leukoc. Biol. 71 (2002) 329–338.
[17] Y.S. Bae, H. Bae, Y. Kim, T.G. Lee, P.G. Suh, S.H. Ryu, Identification of novel chemoattractant peptides for human leukocytes, Blood 97 (2001) 2854–2862.
[18] Y.S. Bae, T.G. Lee, J.C. Park, J.H. Hur, Y. Kim, K. Heo, J.Y. Kwak, P.G. Suh, S.H. Ryu, Identification of a compound that directly stimulates phospholipase C activity, Mol. Pharmacol. 63 (2003) 1043–1050.
[19] Y.S. Bae, Y. Kim, J.H. Kim, T.G. Lee, P.G. Suh, S.H. Ryu, Independent functioning of cytosolic phospholipase A2 and phospholipase D1 in Trp-Lys-Tyr-Met-Val-D-Met-induced superoxide generation in human monocytes, J. Immunol. 164 (2000) 4089–4096.
[20] H. Ohta, K. Sato, N. Murata, A. Damirin, E. Malchinkhuu, J. Kon, T. Kimura, M. Tobo, Y. Yamazaki, T. Watanabe, M. Yagi, M. Sato, R. Suzuki, H. Murooka, T. Sakai, T. Nishitoba, D.S. Im, H. Nochi, K. Tamoto, H. Tomura, F. Okajima, Ki16425, a subtype-selective antagonist for EDG-family lysophosphatidic acid receptors, Mol. Pharmacol. 64 (2003) 994–1005.
[21] M. Fueller, de.A. Wang, G. Tigyi, W. Siess, Activation of human monocytic cells by lysophosphatidic acid and sphingosine-1-phosphate, Cell. Signal. 15 (2003) 367–375.
[22] Y.S. Bae, J.C. Park, R. He, R.D. Ye, J.Y. Kwak, P.G. Suh, S.H. Ryu, Differential signaling of formyl peptide receptor-like 1 by Trp-Lys-Tyr-Met-Val-Met-CONH2 or lipoxin A4 in human neutrophils, Mol. Pharmacol. 64 (2003) 721–730.
[23] Y.S. Bae, S.A. Ju, J.Y. Kim, J.K. Seo, S.H. Baek, J.Y. Kwak, B.S. Kim, P.G. Suh, S.H. Ryu, Trp-Lys-Tyr-Met-Val-D-Met stimulates superoxide generation and killing of *Staphylococcus aureus* via phospholipase D activation in human monocytes, J. Leukoc. Biol. 65 (1999) 241–248.
[24] O. Fourcade, M.F. Simon, C. Viode, N. Rugani, F. Leballe, A. Ragab, B. Fournie, L. Sarda, H. Chap, Secretory phospholipase A2 generates the novel lipid mediator lysophosphatidic acid in membrane microvesicles shed from activated cells, Cell 80 (1995) 919–927.
[25] M.C. van Dijk, F. Postma, H. Hilkmann, K. Jalink, W.J. van Blitterswijk, W.H. Moolenaar, Exogenous phospholipase D generates lysophosphatidic acid and activates Ras, Rho and Ca^{2+} signaling pathways, Curr. Biol. 8 (1998) 386–392.
[26] F.S. Willard, L.A. Berven, M.F. Crouch, Lysophosphatidic acid activates the 70-kDa S6 kinase via the lipooxygenase pathway, Biochem. Biophys. Res. Commun. 287 (2001) 607–613.
[27] J.J. Contos, I. Ishii, N. Fukushima, M.A. Kingsbury, X. Ye, S. Kawamura, J.H. Brown, J. Chun, Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2), Mol. Cell. Biol. 22 (2002) 6921–6929.
[28] Y. Jin, E. Knudsen, L. Wang, A.A. Maghazachi, Lysophosphatidic acid induces human natural killer cell chemotaxis and intracellular calcium mobilization, Eur. J. Immunol. 33 (2003) 2083–2089.
[29] A. Grey, Q. Chen, K. Callon, X. Xu, I.R. Reid, J. Cornish, The phospholipids sphingosine-1-phosphate and lysophosphatidic acid prevent apoptosis in osteoblastic cells via a signaling pathway involving G(i) proteins and phosphatidylinositol-3 kinase, Endocrinology 143 (2002) 4755–4763.
[30] T. Smani, S.I. Zakharov, P. Csutora, E. Leno, E.S. Trepakova, V.M. Bolotina, A novel mechanism for the store-operated calcium influx pathway, Nat. Cell Biol. 6 (2004) 113–120.
[31] K. Itagaki, C.J. Hauser, Sphingosine 1-phosphate, a diffusible calcium influx factor mediating store-operated calcium entry, J. Biol. Chem. 278 (2003) 27540–27547.
[32] T.M. McIntyre, A.V. Pontsler, A.R. Silva, A. St Hilaire, Y. Xu, J.C. Hinshaw, G.A. Zimmerman, K. Hama, J. Aoki, H. Arai, G.D. Prestwich, Identification of an intracellular receptor for lysophosphatidic acid: LPA is a transcellular PPARgamma agonist, Proc. Natl. Acad. Sci. USA. 100 (2003) 131–136.
[33] G. Tigyi, A.L. Parrill, Molecular mechanisms of lysophosphatidic acid action, Prog. Lipid Res. 42 (2003) 498–526.
[34] K. Fukami, T. Takenawa, Phosphatidic acid that accumulates in platelet-derived growth factor-stimulated Balb/c 3T3 cells is a potential mitogenic signal, J. Biol. Chem. 267 (1992) 10988–10993.
[35] H.L. Reeves, M.G. Thompson, C.L. Dack, A.D. Burt, C.P. Day, Phosphatidic acid regulates systemic inflammatory responses by modulating the Akt-mammalian target of rapamycin-p70 S6 kinase 1 pathway, Hepatology. 31 (2000) 95–100.