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Ceramide-1-phosphate activates cytosolic phospholipase A2α directly and by PKC pathway

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ABSTRACT

Ceramide-1-phosphate (C1P), a novel bioactive sphingolipid, is implicated in the vital cellular processes such as cell proliferation and inflammation. The role of C1P on activity of cytosolic phospholipase A2α (cPLA2α), a key enzyme for the release of arachidonic acid (AA) and prostanoids, has not been well elucidated. In this study, we investigated the effect of C1P on the release of AA from L929 cells and a variant, which lacks cPLA2α expression, C12 cells. C1P at 30 μM alone induced AA release from L929 cells without an increase in intracellular Ca^{2+} concentration. C1P-induced AA release was marginal in C12 cells, and treatment with an intracellular Ca^{2+} chelator (BAPTA-AM) or an inhibitor of cPLA2α (2 μM pyrrophenone) decreased C1P-induced AA release in L929 cells. C1P increased the enzymatic activity of cPLA2α over two-fold in the presence of Ca^{2+}. C1P triggered the translocation of cPLA2α and its C2 domain from the cytosol to the perinuclear region in CHO-K1 cells. Interestingly, C1P at 10 μM synergistically enhanced ionomycin-induced AA release from L929 cells. The AA release induced by C1P with and without ionomycin decreased by treatment with protein kinase C (PKC) inhibitor (10 μM GF109203X) and in the PKC-depleted cells. C1P at 10 μM stimulated the translocation of PKC (α and δ) from the soluble to the membrane fractions. We propose that C1P stimulates AA release via two mechanisms; direct activation of cPLA2α, and the PKC-dependent pathway.

1. Introduction

Arachidonic acid (AA) is a precursor of eicosanoids, including prostaglandins and thromboxanes, playing an important role in several physiological functions [1]. The biosynthesis of these AA metabolites occurs mainly by activation of phospholipase A2 (PLA2) in response to a wide variety of stimuli [2]. PLA2 hydrolyzes the *sn*-2 position of glycerophospholipids and release AA. Mammalian cells have structurally diverse forms of PLA2 including secretory PLA2, Ca^{2+}-independent PLA2, and cytosolic PLA2 (cPLA2) [3,4]. Among these PLA2s, the 85-kDa cPLA2, specifically cPLA2α, is highly selective for glycerophospholipids containing AA.

cPLA2α is regulated mainly by an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and by phosphorylation on serine residues [4]. It has been well established that Ca^{2+} binds to the amino-terminal C2 domain, and serves to localize cPLA2α to membranes. The C2 domain is necessary for the translocation of cPLA2α from the cytosol to the perinuclear region following an increase in $[Ca^{2+}]_i$. cPLA2α can be phosphorylated at Ser^{505}, Ser^{515}, and Ser^{727}, which increases its intrinsic enzymatic activity two- to three-fold in vitro [5–8].

Ceramide-1-phosphate (C1P) is formed by phosphorylation of ceramide catalyzed by ceramide kinase, and has been implicated in the regulation of vital cellular processes, such

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as cell proliferation, apoptosis, phagocytosis, and inflammation [9]. Ceramide kinase mediates calcium ionophore- and interleukin-1β-induced AA release [10]. We previously reported that sphingosine directly inhibited cPLA2α activity in vitro [11], and suspected a possibility that cPLA2α is responsible for C1P-induced AA release. To address this issue, we examined the effect of C1P on the release of AA from L929 cells and a variant, which lacks cPLA2α expression, C12 cells. We found that C1P directly activated cPLA2α and induced AA release through the activation of protein kinase C (PKC) in L929 cells.

2. Materials and methods

2.1. Materials

The sources of materials used in this study were as follows: [5,6,8,9,11,12,14,15-^{3}H]AA (215 Ci/mmol, 7.96 TBq/mmol) from Amersham (Buckinghamshire, UK); 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidyl-choline (48 mCi/mmol, 1776 MBq/mmol) from Perkin-Elmer (Boston, MA, USA); C1P (from bovine brain, Lot: 063K4094), GF109203X and SB203580 from Sigma (St. Louis, MO, USA); ionomycin and phorbol-12-myristate-13-acetate (PMA) from Calbichem (La Jolla, CA, USA); C8-C1P from Biomol Res. Lab. (Plymouth Meeting, PA, USA); U0126 from Promega (Woods Hallow, WI, USA). Pyrrophenone was a generous gift from Shionogi. Co. Ltd. (Osaka, Japan). C1P and C8-C1P were dissolved in a solvent mixture of chloroform/methanol (2:1, v/v). The final concentration of organic solvents in treatment was under 0.1%. The vehicle did not have any effects on AA release and calcium imaging.

2.2. Cell cultures

L929 (murine fibroblast) cells and its variant C12 cells (provided by Dr. M. Tsujimoto, Setsunan University, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS). Human embryonic kidney (HEK) 293T cells were cultured in DMEM supplemented with 10% FBS. Chinese hamster ovary (CHO)-K1 cells were cultured in Ham's F12 medium supplemented with 10% FBS.

2.3. AA release assay

L929 and C12 cells were seeded onto 12-well culture plates at a density of 4×10^4 cells/well in DMEM supplemented with 5% FBS. After 24 h incubation, the medium was removed, and the cells were labeled by incubation for 24 h in 0.75 mL of serum-free medium containing 0.1 μCi [^{3}H]AA and 0.1% fatty acid-free bovine serum albumin (BSA). The cells were then washed and stimulated with reagents in DMEM containing 0.1% BSA at 37 °C. The radioactivity of supernatants and cell lysates (in 1% Triton X-100) was measured by liquid scintillation counting. The amount of radioactivity released into the supernatant was expressed as a percentage of the total amount of radioactivity incorporated.

2.4. Single cell calcium imaging

L929 cells (4×10^4) were seeded on glass bottom dishes in DMEM and incubated for 24 h. After another 24 h incubation in serum-free medium containing 0.1% fatty acid-free BSA, the cells were washed and incubated at 37 °C with 10 μM fura-2 acetoxymethyl ester and 0.01% cremophore EL for 1 h in HBSS buffer containing 0.1% BSA. After loading, cells were washed with and maintained in the same buffer in the dark at room temperature. The intensity of fura-2 fluorescence was measured by alternating excitation at 340 and 380 nm and detecting emission at 505 nm with a 40× objective lens (Nikon UV-fluor), an SIT camera, and an ARGUS-50/CA imaging processor (Hamamatsu Photonics, Japan). Since measurement of $[Ca^{2+}]_i$ at lower than physiological temperatures was recommended in fura-2 method [12,13], cells were stimulated with reagents at room temperature in this study. Stimulation with C1P had no effect on $[Ca^{2+}]_i$ in L929 cells when measured at 37 °C (data not shown).

2.5. PLA2 assay

HEK293T cells were transfected with an expression vector for human cPLA2α (pcDNA4/HisMax A-human cPLA2α) or a control vector (pcDNA4) by LiopofectAMINE PLUS (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Following transfection, the cells were homogenized with a Potter homogenizer in the lysis buffer (0.34 M sucrose, 100 μM dithiothreitol, Complete tablet mini (protease inhibitor cocktail; 1 tablet per 10 mL), 0.2% CHAPS, 10 mM HEPES, pH 7.4). PLA2 activity was measured using 1-palmitoyl-2-[^{14}C]-arachidonyl phosphatidylcholine as a substrate as described previously [11].

2.6. Plasmid construction, transfection and confocal microscopy

The plasmid for a chimeric protein containing enhanced green fluorescent protein (EGFP) at the N-terminus of cPLA2α (EGFP-cPLA2α) was prepared as described previously [14]. For a chimeric protein containing EGFP at the C2 domain i cPLA2α (EGFP-C2), cDNA encoding human cPLA2α was amplified by polymerase chain reaction with primers upstream (GGAAGATCTATGTCATTTATAGATCCTT) and downstream (GGTCTGCAGTCAGTCTGGGCATGAGCAAAC) using pcDNA3.1/Zeo(+)-cPLA2α. The resulting polymerase chain reaction product was cloned into the Bglll-Pstlsites of the vector pEGFP-C1 (Clontech) to obtain a EGFP-C2 fusion construct. The orientation of cPLA2α and the integrity of the reading frame were verified by restriction analysis and DNA sequencing.

For EGFP-cPLA2α or EGFP-C2 expression, cells were seeded at a density of 2×10^5 cells/60-mm dish and transiently transfected with 2 μg of the expression vector with LipofectAMINE PLUS (Invitrogen) according to the manufacturer's protocol. After 24 h incubation, transfected cells were seeded on coverslips (12 mm in diameter) of glass-bottomed dishes (IWAKI, Japan) at a density of 1×10^4. After another 24 h incubation, the culture medium was replaced and the cells were washed with HBSS buffer containing 10 mM HEPES and

0.1% BSA and stimulated with reagents in the same buffer. Fluorescence images were taken with a FLUOVIEW confocal laser scanning microscope system (Olympus, Japan).

2.7. Western blot analyses

L929 cells were scraped and homogenated using a potter homogenizer with ice-cold buffer containing 20 mM Tris–HCl, 250 mM sucrose, 1 mM EDTA, 100 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride. Membrane and soluble fractions were then separated by centrifugation at 17,400 × *g* at 4 °C for 30 min. Membrane fraction was lysed with the same buffer supplemented with 1% Triton X-100. Protein concentration was determined by Bio-Rad Protein Assay. Laemmli electrophoresis sample buffer (5×) was added to the membrane and soluble fractions, and SDS-polyacrylamide gel electrophoresis was performed using 10 μg of lysate protein. After electrophoresis, proteins were electro-blotted onto polyvinyldifluoride membrane. Detection of PKC or β-tubulin were performed using anti-PKCα polyclonal antibody, anti-PKCδ polyclonal antibody (Santa Cruz Biotechnology), or anti-β-tubulin antibody (Sigma) subsequently followed by incubation with anti-rabbit IgG horseradish peroxidase antibody (Amersham). The immunoreactive bands were visualized by Enhanced Chemiluminescence.

2.8. Statistics

Values are the mean ± S.E.M. of three to four independent experiments performed in triplicate. In some cases, data are shown as the mean ± S.D. of two or three observations in a typical representative experiment. In case of multiple comparisons, the significance of differences was determined using one-way analysis of variance by Dunnett's or Tukey's test. For pairwise comparisons, the Student's two-tailed *t*-test was used. *P* values <0.05 were considered to be significant.

3. Results

3.1. Effect of C1P on AA release in L929 and C12 cells

Stimulation for 30 min with 30 μM C1P increased AA release from L929 cells (Fig. 1A). Stimulation with C1P at 2.5–10 μM for 30 min or 6 h did not cause AA release (data not shown). Treatment with 4 mM EGTA did not inhibit the C1P-induced AA release, indicating that the release was not dependent on extracellular Ca^{2+}. Treatment with BAPTA-AM (an intracellular Ca^{2+} chelator, 50 μM) decreased C1P-stimulated AA release from L929 cells in the presence of 4 mM EGTA. C8-C1P has been reported to induce Ca^{2+} mobilization in GH4C1 rat pituitary cells [15]. Stimulation with 30 μM C8-C1P did not increase AA release from L929 cells; 0.46 ± 0.04% in the vehicle-treated cells and 0.50 ± 0.03% in the C8-C1P-treated cells in a typical experiment.

To examine whether cPLA2α is responsible for C1P-induced AA release, we used the L929 variant C12 cells which lacks cPLA2α expression [16]. Stimulation of C12 cells with 30 μM C1P slightly increased AA release. In the control cells and EGTA treated cells, the responses in L929 cells were significantly greater than those in C12 cells (Fig. 1A). In addition, pretreatment of L929 cells for 15 min with 2 μM pyrrophenone, a selective inhibitor of cPLA2α [17], reduced C1P-stimulated AA release (Fig. 1B). These results suggest that cPLA2α mediates C1P-induced AA release from L929 cells.

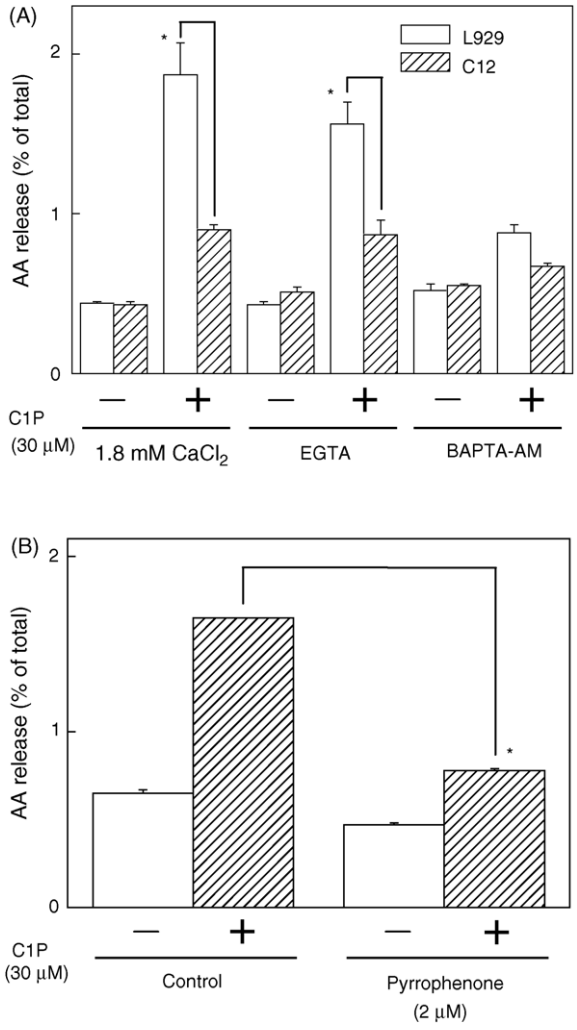

Fig. 1 – C1P stimulates AA release from L929 cells. (A) Effect of C1P on AA release from L929 cells and C12 cells. [^{3}H]AA-labeled cells were treated with 30 μM C1P for 30 min. BAPTA-AM (50 μM) was pretreated for 15 min prior to stimulation with vehicle or C1P. (B) Effect of pyrrophenone on C1P-stimulated AA release from L929 cells. L929 cells were pretreated with vehicle (control) or 3 μM pyrrophenone for 15 min then stimulated with vehicle (white column) or 30 μM C1P (hatched column) for 30 min. Data represent mean ± S.E.M. of three independent experiments and each performed in duplicate. $^*P < 0.05$, significantly different from the C1P-treated C12 cells.

3.2. C1P activates cPLA2α in vitro

We examined the effect of C1P on the various concentrations of $CaCl_2$-induced enzymatic activity of cPLA2α. C1P increased the activity of cPLA2α over two-fold in the presence of 0.1 mM

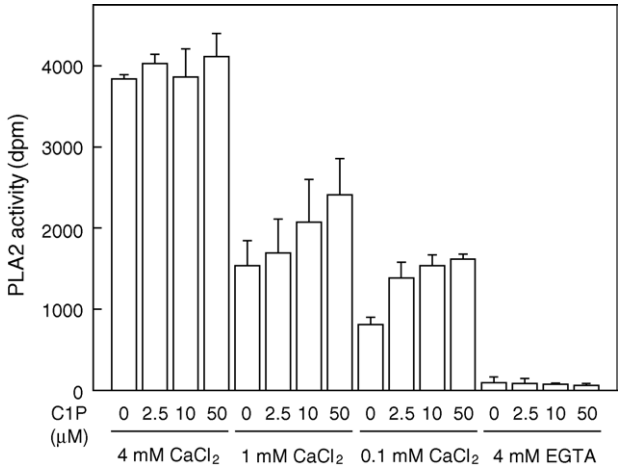

Fig. 2 – C1P activates cPLA2α in vitro. The PLA2 activity in the cytosol fraction from HEK293T cells expressing human cPLA2α was measured as described in Section 2. The assays were performed in the absence or presence of the indicated calcium concentrations and various concentrations of C1P. Data represent mean ± S.D. of two determinations in a typical experiment.

$CaCl_2$, and these responses were slightly increased in the presence of 1 mM $CaCl_2$. In the presence of 4 mM $CaCl_2$, C1P did not further increase the PLA2 activity (Fig. 2). C1P failed to increase the enzymatic activity when Ca^{2+} was chelated by 4 mM EGTA.

3.3. *C1P-induced translocation of cPLA2α in cells*

To examine whether C1P-induced AA release is accompanied by the translocation of cPLA2α, we investigated the effect of C1P on the localization of the chimeric protein EGFP-cPLA2α in living cells. Because the efficiency of transfection of EGFP-cPLA2α into L929 cells was low, we used CHO-K1 cells. In the resting state, the EGFP-cPLA2α expressed in CHO-K1 cells was almost homogeneously present in the cytosol. Stimulation of CHO-K1 cells expressing EGFP-cPLA2α with 5 μM ionomycin or 30 μM C1P triggered the translocation of EGFP-cPLA2α from the cytosol to the perinuclear region within 1–2 min (Fig. 3, Panels A and B), and the translocated EGFP-cPLA2α returned to the cytosol within 20 min. In the presence of 4 mM EGTA, C1P-induced the translocation of EGFP-cPLA2α was not inhibited (Fig. 3C).

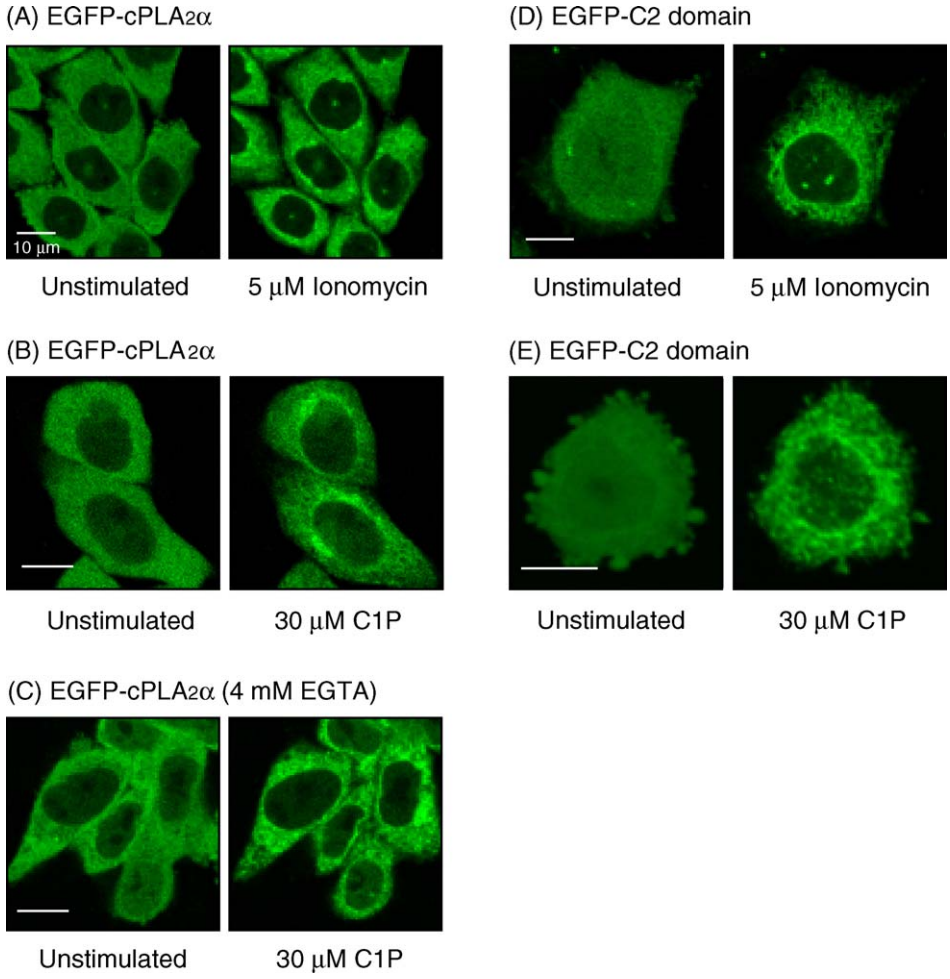

Fig. 3 – cPLA2α is translocated by stimulation with C1P. CHO-K1 cells transiently transfected with the expression vector for EGFP-cPLA2α (A, B, and C) or EGFP-C2 (D and E) were stimulated with 5 μM ionomycin (A and D) or 30 μM C1P (B, C and E) for 1 min. The cells were stimulated in the presence of 4 mM EGTA (C). Scale bars, 10 μm.

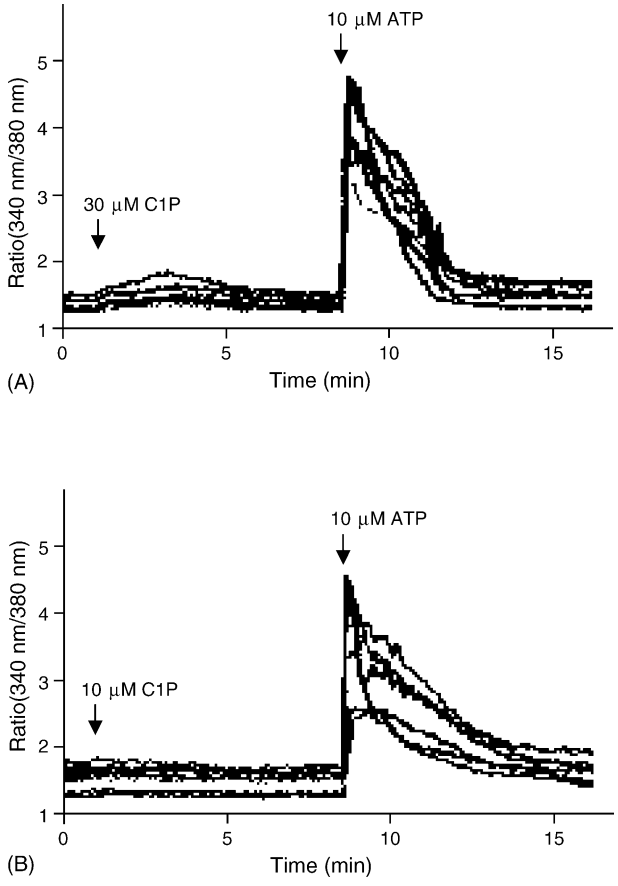

Fig. 4 – Effect of Ca^{2+} on response to C1P in L929 cells. Fura-2-loaded cells were stimulated with 30 μM (A) and 10 μM (B) C1P in the presence of 1.8 mM extracellular Ca^{2+}. ATP was used as a positive control. Each line represents the ratio of fluorescence at 340–380 nm in individual cells. Arrows indicate the point of stimulation.

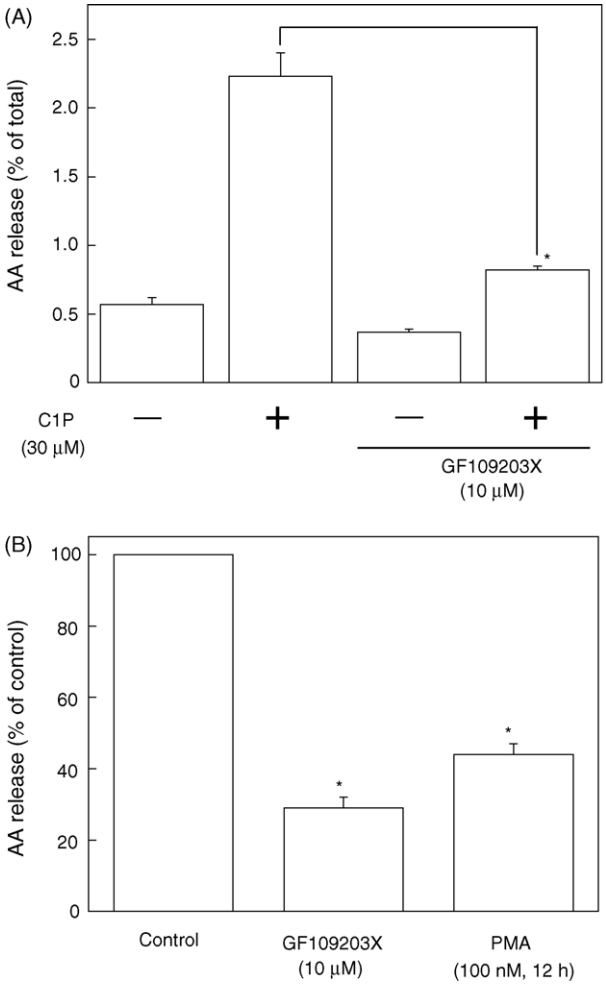

Fig. 5 – C1P induces AA release through a PKC-dependent pathway in L929 cells. (A) Effect of GF109203X on C1P-induced AA release from L929 cells. [^{3}H]AA-labeled L929 cells were pretreated with vehicle or 10 μM GF109203X for 15 min then stimulated with 30 μM C1P for 30 min. Data represent mean ± S.E.M. of three independent experiments and each performed in duplicate. (B) Effects of GF109203X or PKC down-regulation on 10 μM C1P plus 10 μM ionomycin-induced AA release from L929 cells. [^{3}H]AA-labeled cells were treated with or without GF109203X for 15 min or 100 nM PMA for 12 h and then costimulation with C1P and ionomycin for 30 min. Data represent mean ± S.E.M. of three independent experiments and each performed in duplicate. $^*P < 0.05$, significantly different from the value without GF109203X or PMA.

Because the C2 domain of cPLA2α is sufficient for translocation and can interact with phospholipids, we monitored the cellular localization of the C2 domain of cPLA2α fused to EGFP (EGFP-C2). EGFP-C2 was distributed homogeneously both in the cytosol and in the nucleus in CHO-K1 cells. Stimulation with 30 μM C1P, like 5 μM ionomycin, triggered the translocation of EGFP-C2 from both the cytosol and the nucleus to the perinuclear region (Fig. 3, Panels D and E). These findings suggest that C1P induces translocation of EGFP-cPLA2α through the C2 domain.

3.4. *Effect of C1P on response to calcium in L929 cells*

An increase in $[Ca^{2+}]_i$ is an important mechanism for regulating cPLA2α by promoting its translocation and binding to the membrane. Because C1P induced an increase in $[Ca^{2+}]_i$ in several cells [15,18,19], we investigated whether C1P would induce a rise in $[Ca^{2+}]_i$ in L929 cells. Stimulation with 30 μM C1P caused a slow and slight $[Ca^{2+}]_i$ increase, but the effect was much less extensive than that by 10 μM ATP (Fig. 4A). However, stimulation with 10 μM C1P had no effect (Fig. 4B).

3.5. *C1P induces AA release in PKC-dependent manner in L929 cells*

Although C1P increased enzymatic activity of cPLA2α, the effect was limited within two-fold. Thus, we wondered whether phosphorylation of cPLA2α through the signal transduction is involved in C1P-induced AA release from L929 cells. It is known that PKC activator PMA induces phosphorylation of cPLA2α and AA release in various cells

Table 1 – Effect of costimulation of C1P and ionomycin on AA release from L929 cells

	[^{3}H]AA release (% of control)	
	Vehicle	10 μM Iono
Vehicle	100	190 ± 35
10 μM C1P	122 ± 48	1172 ± 167*
10 μM C8-C1P	93 ± 8	176 ± 10
100 nM PMA	166 ± 27	436 ± 89*

[^{3}H]AA-labeled L929 cells were treated with vehicle, 10 μM C1P, 10 μM C8-C1P or 100 nM PMA in the presence and absence of 10 μM ionomycin for 30 min. Data represent mean ± S.E.M. of three independent experiments performed in duplicate.
* $P < 0.05$, significantly different from the vehicle-treated cells.

[20]. In this study, stimulation with 100 nM PMA for 30 min increased AA release (1.5-fold) from L929 cells, and costimulation with ionomycin and PMA induced AA release significantly (four-fold) (Table 1). Similarly, stimulation of L929 cells with ionomycin plus 10 μM C1P induced AA release significantly, although the AA release induced by 10 μM C1P alone was limited. On the other hand, stimulation of 10 μM C8-C1P had no effect on ionomycin-induced AA release from L929 cells. Pretreatment of L929 cells with 10 μM GF109203X, a general PKC inhibitor, inhibited AA release induced by 30 μM C1P (Fig. 5A). In addition, AA release induced by costimulation of 10 μM C1P and 10 μM ionomycin was significantly inhibited by GF109203X treatment (Fig. 5B). Prolonged exposure of cells to PMA has been shown to cause a depletion of some PKC isoforms [21]. In our conditions, PMA plus ionomycin-induced AA release from L929 cells was inhibited 70 and 90% by pretreatment with 100 nM PMA for 6 and 12 h, respectively (data not shown). Furthermore, C1P plus ionomycin-induced AA release was inhibited by prolonged PMA treatment (Fig. 5B). These results suggest that C1P-induced AA release is dependent on the activation of PKC in L929 cells.

We further examined the ability of C1P to cause the translocation of PKC. C1P treatment resulted in a translocation of PKCα and δ from the soluble to the membrane fractions within 20 min (Fig. 6).

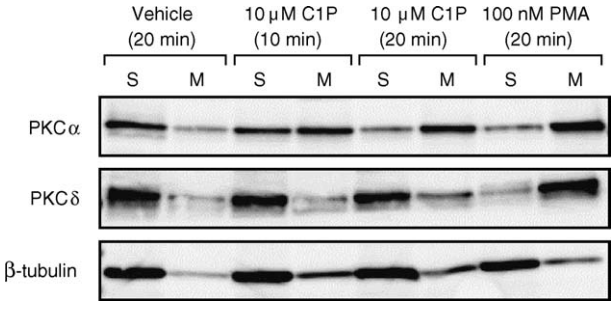

Fig. 6 – Translocation of PKC on C1P-stimulated L929 cells. L929 cells were treated with 10 μM C1P or 100 nM PMA for indicated time. The soluble (S) and the membrane (M) fractions were prepared above, and western blots for PKCα, δ and β-tubulin were performed. Data represent similar results from three independent experiments.

4. Discussion

In this study, we provided evidences that: (i) C1P stimulates AA release via activation of cPLA2α and (ii) stimulation of AA release by C1P involves a PKC-dependent pathway in L929 cells. C1P-induced AA release from C12 cells lacking cPLA2α expression was much less than that from L929 cells, and C1P response in L929 cells was inhibited by pyrrophenone. In addition, C1P directly increases the enzymatic activity of cPLA2α in the presence of Ca^{2+}, and C1P causes the translocation of cPLA2α in cells via its C2 domain. During the preparation of this manuscript, Pettus et al. [22] have been reported that C1P directly activates cPLA2α through interaction with the C2 domain, and enhances AA release. In our conditions, C1P increased cPLA2α activity in the presence of 0.1 mM $CaCl_2$, but not 4 mM $CaCl_2$. Our results are consisting with their studies [22] showing that C1P binding to cPLA2α increases the affinity of the enzyme for Ca^{2+}. However, C1P increases the Ca^{2+}-induced maximal activity of cPLA2α in their studies [22], but not in our conditions. The reason is not clear at the present. Since anionic phospholipids are reported to stabilize the association of cPLA2α with lipid vesicles and resulting increases its activity [22–25], the difference of experimental conditions such as detergents and reagents may explain the differences.

In the present study, we show for the first time the role of PKC pathway on C1P-induced AA release via cPLA2α activation in cells. An increase in $[Ca^{2+}]_i$ and phosphorylation of cPLA2α in cells synergistically activates the enzyme activity and/or AA release in many cell types [26]. Activation of PKC can trigger several kinase cascades including ERK, resulting in the phosphorylation of cPLA2α. In L929 cells, PMA, which alone showed marginal effect, significantly enhanced ionomycin-stimulated AA release. An increase in $[Ca^{2+}]_i$ may stimulate PKC activity in cells. However, contribution of the PKC pathway to ionomycin-stimulated AA release in L929 cells appeared to be limited. Thus, additional signaling including the $[Ca^{2+}]_i$-mediated phosphorylation signaling appears to be required for substantial AA release from L929 cells. C1P at 10 μM, which alone had no effect on increase in $[Ca^{2+}]_i$ and AA release, significantly stimulated AA release from L929 cells in the presence of ionomycin. In addition, the effect of C1P on cPLA2α activity in vitro was limited, within about two-fold, in our conditions. Thus, it is probable that C1P activates some phosphorylation signaling(s) such as the PKC pathway in cells. Treatment with an inhibitor of PKC (GF109203X) or depletion of PKC inhibited C1P-induced AA release from L929 cells. Expression of conventional PKC (α, β), novel PKC (δ, ε) and atypical PKC (ζ) has been confirmed in L929 cells [27], and we showed that C1P causes the translocation of PKCα and δ from the soluble to the membrane fractions in the cells. These findings suggest that C1P-induced AA release from L929 cells involves in the PKC-dependent pathway. Recently, Tornquist et al. [15] has been reported that C1P activates voltage-operated Ca^{2+} channels by a PKC-dependent mechanism in GH4C1 rat pituitary cells.

It should be determined which subtypes of PKC isoforms are involved in C1P response and the mechanism of C1P-induced activation. GF109203X is shown to be a general inhibitor of PKC and inhibit atypical PKC [28]. The inhibitory

effect of GF109203X was over 70%, but the inhibitory effect induced by down-regulation of conventional and novel PKC by PMA treatment was about 50%. In addition to PMA-sensitive PKCs, PMA-insensitive atypical PKCζ may be involved in C1P response. It is reported that ceramide directly interacts with some PKCs [29–32]. Like ceramide, C1P may activate PKC directly via its hydrophobic domains including C1 or C2 domain. We could not exclude the possibility that C1P interacts with un-identified receptors on membranes, as proposed [15].

In conclusion, this study provided novel information on activation mechanism of cPLA2α and AA release in response to C1P. C1P stimulates cPLA2α-mediated AA release through the PKC dependent pathway, while C1P is a direct activator of cPLA2α. Because stimulation of cPLA2α and subsequent conversion of AA to prostaglandins and leukotrienes have been implicated in the pathophysiology of asthma, arthritis, and other inflammatory diseases [1], these described pathways may provide some means for widespread therapeutic strategies.

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