博士学位論文

ヒト末梢血好酸球のロイコトリエン合成と脱顆粒におけるホスファチジルコリン特異的ホスホリパーゼCの役割

近畿大学大学院医学研究科
医学系呼吸器・アレルギー病態制御学

佐野安希子

Doctoral Dissertation

Functional role of phosphatidylcholine-specific phospholipase C in regulating leukotriene synthesis and degranulation in human eosinophils

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Department of Respiratory Medicine and Allergology,
Major in Medical Sciences
Kindai University Graduate School of Medical Sciences

Akiko Sano

論博

同　意　書

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近畿大学大学院
医学研究科長　殿

共著者 佐野博幸 ㊞　　共著者 ＿＿＿＿＿＿＿＿ ㊞

共著者 安永賢司 ㊞　　共著者 ＿＿＿＿＿＿＿＿ ㊞

共著者 東田有智 ㊞　　共著者 ＿＿＿＿＿＿＿＿ ㊞

共著者 ＿＿＿＿＿＿＿＿ ㊞　　共著者 ＿＿＿＿＿＿＿＿ ㊞

共著者 ＿＿＿＿＿＿＿＿ ㊞　　共著者 ＿＿＿＿＿＿＿＿ ㊞

論文題目

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1．学位論文提出者氏名　　佐野　安希子

2．専攻分野　医学系　　呼吸器・アレルギー病態制御学

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Functional role of phosphatidylcholine-specific phospholipase C in regulating leukotriene synthesis and degranulation in human eosinophils

Akiko Sano *, Hiroyuki Sano, Takashi Iwanaga, Yuji Tohda

Department of Respiratory Medicine and Allergology, Kindai University Faculty of Medicine, 377-2, Ohnohigashi Osakasayama, Osaka, 589-8511, Japan

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ABSTRACT

Phosphatidylinositol-specific phospholipase C (PI-PLC) and cytosolic phospholipase A_2 ($cPLA_2$) regulate both eosinophil degranulation and leukotriene (LT) synthesis via PI-PLC-mediated calcium influx and $cPLA_2$ activation. Phosphatidylcholine-specific phospholipase C (PC-PLC) likely plays a key role in cellular signaling, including the eosinophilic allergic inflammatory response. This study examined the role of PC-PLC in eosinophil LT synthesis and degranulation using tricyclodecan-9-yl-xanthogenate (D609), a PC-specific PLC inhibitor. D609 inhibited *N*-formyl-met-leu-phe + cytochalasin B (fMLP/B)-induced arachidonic acid (AA) release and leukotriene C_4 (LTC_4) secretion. However, at concentrations that blocked both AA release and LTC_4 secretion, D609 had no significant inhibitory effect on stimulated $cPLA_2$ activity. D609 also partially blocked fMLP/B-induced calcium influx, indicating that inhibition of AA release and LTC_4 secretion by D609 is due to inhibition of calcium-mediated $cPLA_2$ translocation to intracellular membranes, not inhibition of $cPLA_2$ activity. In addition, D609 inhibited fMLP/B-stimulated eosinophil peroxidase release, indicating that PC-PLC regulates fMLP/B-induced eosinophil degranulation by increasing the intracellular calcium concentration ($[Ca^{2+}]_i$). Overall, our results showed that PC-PLC is critical for fMLP/B-stimulated eosinophil LT synthesis and degranulation. In addition, degranulation requires calcium influx, while PC-PLC regulates LTC_4 synthesis through calcium-mediated $cPLA_2$ activation.

1. Introduction

Phospholipid-mediated signaling involves the production of bioactive lipid metabolites in response to agonist receptor interactions at the plasma membrane level (Farooqui and Horrocks, 2005). Several enzymes involved in phospholipid metabolism, such as phospholipase A_2 (PLA_2), phospholipase C (PLC), and phospholipase D (PLD), generate second messengers and regulate their levels. PLA_2 isoforms hydrolyze nuclear membrane phospholipids at the sn-2 position, producing lysophospholipids and free fatty acids, such as arachidonic acid (AA) (Farooqui and Horrocks, 2005).

The intracellular 85 kDa cytosolic phospholipase A_2 ($cPLA_2$) is an important enzyme that mediates agonist-induced AA release and eicosanoid production (Leslie, 2015; Murakami et al., 2011). $cPLA_2$ plays a major role in AA hydrolysis and LTC_4 secretion in formyl-Met-Leu-Phe + cytochalasin B (fMLP/B)-activated human eosinophils (Zhu et al., 2001). However, although the role of $cPLA_2$ in agonist-induced AA release and eicosanoid production is widely recognized, the role of other phospholipases in regulating eosinophil function is unclear.

PLCs also hydrolyze a membrane phospholipid substrate and are key in regulating cellular functions. Phosphatidylinositol (PI)-specific PLC is a critical enzyme for both eosinophil degranulation and synthesis of LTC_4 (Sano et al., 2001). Eosinophils are key effector cells involved in the pathogenesis of allergic diseases, including asthma. They contribute to tissue injury and inflammation after the production of lipid mediators, such as leukotrienes (LTs) and granular proteins. PI-PLC-mediated calcium influx and protein kinase C (PKC) activation are required for eosinophil degranulation, while PI-PLC-mediated calcium influx and $cPLA_2$ activation are involved in LTC_4 synthesis and secretion from human eosinophils (Sano et al., 2001).

Phosphatidylcholines (PCs) are another abundant class of glycerophospholipids in the mammalian cell membrane that act as substrates for the production of second messengers involved in receptor-mediated cell signal transduction. PC-PLC activation hydrolyzes cell membrane PC into diacylglycerol (DAG) and phosphocholine, and DAG enhances PKC activity (Singh et al., 2015).

This study examined the mechanisms by which PC-PLC causes

* Corresponding author.
E-mail address: sanoa@med.kindai.ac.jp (A. Sano).

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eosinophil inflammatory responses, such as LT synthesis and degranulation. However, mammalian PC-PLC has not yet been purified and characterized. Therefore, we used tricyclodecan-9-yl-xanthogenate (D609), a specific PC-PLC inhibitor, to study the roles of PC-PLC in cellular responses (Adibhatla et al., 2012). The results will provide evidence for (1) PC-PLC activation induced by fMLP/B, indicating the presence of PC-PLC in human isolated eosinophils; (2) a functional role of PC-PLC in $cPLA_2$ activation by regulating $[Ca^{2+}]_i$; and (3) involvement of PC-PLC in eosinophil degranulation through calcium influx.

2. Materials and methods

2.1. Reagents

N-formyl-methionine-leucine-phenylalanine (fMLP), cytochalasin B, and ethylene glycol-bis (β-aminoethyl ether) -*N,N,N′,N′*-tetraacetic acid (EGTA; an intracellular calcium chelator) were obtained from Sigma Chemical Co. (St. Louis, MO, USA); A23187 (a calcium ionophore that allows entry of calcium), D609, edelfosine (ET-18-OCH_3), and 1,2-bis(2-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid tetrakis (acetoxymethyl ester) (BAPTA/AM) from Calbiochem (San Diego, CA, USA); 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U73122; a broad PLC inhibitor) from Biomol (Plymouth Meeting, PA, USA), and Fluo 3-AM from Dojindo (Kumamoto, Japan).

We obtained LTC_4 enzyme immunoassay (EIA) kits from Cayman Chemical Company (Ann Arbor, MI, USA), Amplex® Red Phosphatidylcholine-Specific Phospholipase C Assay Kits from Molecular Probes (Eugene, OR, USA), AA-[5,6,8,9,11,12,14,15-^{3}H] and donkey anti–rabbit-Ig conjugated with horseradish peroxidase (HRP) from Amersham Life Science (Arlington Heights, IL, USA), and 1-palmitoyl-2-[^{14}C] arachidonylphosphatidylcholine from NEN Life Science Products (Boston, MA, USA) for use in various assays.

All supplies for eosinophil isolation were purchased from Miltenyi Biotech (Sunnyvale, CA, USA). Anti-phospho–extracellular signal–regulated kinase (ERK)1/2 Ab and U0126 (a mitogen-activated protein kinase [MAPK]/ERK kinase [MEK] inhibitor) were obtained from Promega (Madison, WI, USA); anti-ERK1/2, anti-phospho-p38 MAPK, and anti-p38 MAPK Abs from New England Biolabs (Beverly, MA, USA); SB203580 (a specific p38 MAPK inhibitor) from Upstate Biotechnology (Lake Placid, NY, USA); and anti-$cPLA_2$ Ab and anti-phospho-$cPLA_2$ Ab from Cell Signaling Technology (Danvers, MA, USA).

2.2. Isolation of human peripheral blood eosinophils

We isolated eosinophils from mildly atopic human volunteers using negative selection immunomagnetic separation (Hansel et al., 1991) with modifications. The treated granulocyte cell suspension was passed through a 1 × 10 cm steel wool column within a 0.6 Tesla magnetic-activated cell sorting (MACS) magnet (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). A hemocytometer was used to take the total cell count, and cell purity was assessed by differential counts from Wright-Giemsa stained cytocentrifuge preparations. Viability of the eosinophils isolated was confirmed using trypan blue exclusion assay. Cell preparations contained >99% eosinophils and were kept on ice until further use.

The study protocol was approved by the ethics committee of the Tottori University Hospital ethics committee (Permission number: 341), and all subjects provided written informed consent.

2.3. Measurement of eosinophil peroxidase release and LTC_4 secretion

Eosinophil peroxidase (EPO) and LTC_4 release was determined in eosinophils from the same experiments. First, 2.5 × 10^5/250 μl of eosinophils were treated with buffer or D609 prior to activation with 1 μM fMLP +5 μg/ml of cytochalasin B. Next, 30 min later, the reaction was terminated by centrifugation, and the supernatant was collected and stored at −80 °C until further analysis.

Briefly, 50 μl of the sample or EPO standard was mixed with 100 μl of a substrate (final concentration: 0.01% hydrogen peroxide, 6 mM o-phenylenediamine, and 0.06% Triton X-100 dissolved in 60 mM Tris; pH 8.0) in a polystyrene 96-well microplate and incubated for 30 min at room temperature. The reaction was stopped by adding 50 μl of 4M H_2SO_4. Absorbance was measured using a Thermomax thermoregulating microplate absorbance spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA) at a wavelength of 490 nm. The final EPO concentrations were calculated from standard curves fitted by four-parameter analysis. The stimulated LTC_4 secretion was measured using an EIA kit with a separate aliquot of the same eosinophils.

2.4. PC-PLC activity assay

PC-PLC activity was determined using the Amplex® Red Phosphatidylcholine-Specific Phospholipase C Assay Kit.

2.5. Measurement of eosinophil AA release

We incubated10^7 eosinophils/ml with 0.5 μCi/ml of [^{3}H]AA overnight. Next, they were washed thrice with Hank's Balanced Salt Solution (HBSS) containing 0.2% bovine serum albumin (BSA) and then aliquoted (10^6 eosinophils/100 μl final volume) and treated with D609 for 20 min at 37 °C prior to activation using fMLP/B. After centrifugation, a 90 μl aliquot of the supernatant was counted in 2 ml of a scintillation solvent using a Beckman scintillation counter (PerkinElmer, Inc., Waltham, MA, USA).

2.6. Measurement of eosinophil $cPLA_2$ activity

We next assessed the effect of D609 on fMLP/B-stimulated activation of $cPLA_2$. $cPLA_2$ phosphorylation determined by immunoblotting or $cPLA_2$ activity assay is commonly used as an indicator of activation. In this study, $cPLA_2$ activity was measured as described previously (Kim et al., 1998). Briefly, 2 × 10^6 eosinophils were preincubated with or without D609 for 20 min prior to activation with a buffer alone or fMLP/B for 5 min. Next, the pellet was resuspended in 70 μl of a disruption buffer (20 mM Tris, pH 8.0, 2.5 mM ethylenediaminetetraacetic acid [EDTA], 10 μg/ml of leupeptin, 5 μg/ml of aprotinin, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM Na_3VO_4, 50 mM NaF, and 5 μg/ml of pepstatin) and treated with 5 mM dithiothreitol on ice for 5 min to inactivate soluble PLA_2. To initiate the reaction, 10 μl of a substrate ([^{14}C]-PAPC; final concentration: 9 μM) was added to the cell lysate for 30 min at 37 °C. Extraction was performed by adding 560 μl of Dole's reagent (400:390:10 v/v heptane:isopropyl alcohol:1 N H_2SO_4) to the sample tube, followed by 110 μl of H_2O. The upper layer was transferred to 800 μl of hexane containing 25 mg of silica gel, and an aliquot (upper layer) was used to measure the radioactivity with a liquid scintillation counter.

2.7. Immunoblotting

To examine the effect of PC-PLC on fMLP/B-induced MAPK activation, we investigated the effect of D609 on fMLP/B-induced ERK1/2 and p38 phosphorylation in eosinophils. We incubated an aliquot of treated eosinophils (2 × 10^6 eosinophils/intervention) on ice with ice-cold lysis buffer (20 mM Tris-HCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 40 mM NaCl, 5 mM EDTA; pH 7.4) containing 1% Nonidet P-40, 10 μg/ml of leupeptin, 5 μg/ml of aprotinin, 1 mM PMSF, 2 mM Na_3VO_4, and 0.5% deoxycholic acid for 20 min. Next, the sample was centrifuged to remove nuclear and cellular debris, and the supernatants were mixed with 14 μl of 6 × sample buffer and boiled for 5 min. The samples were collected and kept at −70 °C.

Proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 10% acrylamide gel under

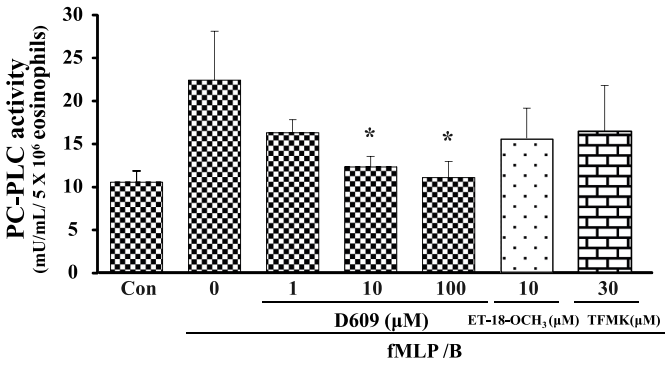

Fig. 1. Inhibition of fMLP/B-induced PC-PLC activation. D609 inhibited PC-PLC activity dose-dependently, while neither ET-18-OCH_3 (a PI-specific PLC inhibitor) nor TFMK (a $cPLA_2$ inhibitor) had an inhibitory effect. Data are represented as the mean ± S.E.M. from five independent experiments. *P < 0.05 versus fMLP/B induction. fMLP/B, formyl-Met-Leu-Phe + cytochalasin B; PC-PLC, phosphatidylcholine-specific phospholipase C; D609, tricyclodecan-9-yl-xanthogenate; PI, phosphatidylinositol; $cPLA_2$, cytosolic phospholipase A_2; TFMK, arachidonyl trifluoromethylketone; S.E.M., standard error of the mean.

reducing conditions (15 mA/gel). A semidry system (400 mA, 60 min) was used to electrotransfer proteins from the gel to polyvinylidene fluoride (PVDF) membrane. Next, the PVDF membrane was blocked using 1% BSA for 60 min and then incubated with 1/5000 anti-phosphorylation-specific ERK1/2 Ab, 1/1000 anti-ERK1/2 Ab, 1/1000 anti-phosphorylation-specific p38 MAPK Ab, 1/1000 anti-p38 Ab, 1/1000 anti-phosphorylation-specific $cPLA_2$ Ab, or 1/1000 anti-$cPLA_2$ Ab diluted in Tris-buffered saline with Tween-20 (TBST) overnight. The PVDF membrane was washed thrice with TBST, and the bound antibody was detected using HRP-conjugated donkey anti–rabbit immunoglobulin G (IgG) as a secondary antibody and developed using an enhanced chemiluminescence (ECL) system (Amersham Life Science). Finally, western blots were scanned and band intensities quantified using ImageJ software (http://rsb.info.nih.gov/ij/), and relative protein levels were expressed in reference to the untreated sample.

2.8. Measurement of eosinophil $[Ca^{2+}]_i$

We examined whether calcium elevation is involved in fMLP/B-induced PC-PLC activation. Calcium influx was analyzed by flow cytometry with Fluo3-AM-loaded eosinophils. A23187 was used as a positive control, and EGTA was used as a negative control. We incubated eosinophils (10^7 eosinophils/ml) in Roswell Park Memorial Institute (RPMI) buffer with 4 μM Fluo-3-acetoxymethyl (Fluo-3-AM) for 30 min at 37 °C. After washing, Fluo3-AM-loaded eosinophils (3×10^6 eosinophils/sample) were dispensed in fluorescence-activated cell sorting (FACS) tubes and incubated with buffer, U73122 or D609. Twenty minutes later, we added fMLP/B, and analyzed $[Ca^{2+}]_i$ using a FACS Calibur HG flow cytometer (Becton, Dickinson and Company). Finally, to evaluate kinetic flow cytometry data, we used the kinetic module of FlowJo software (FlowJo LLC, OR, USA) based on a smoothing method of median fluorescence values. Since the area under the curve (AUC) is believed to be the most representative parameter describing the calcium influx magnitude, we also calculated this parameter using the kinetic module of FlowJo software.

2.9. Assessment of eosinophil viability

To assess whether D609 affects eosinophil viability, in all experiments, we performed trypan blue exclusion assay. We incubated aliquots of 10^4 eosinophils with various concentrations of D609 for 20 min at 37 °C. Eosinophil viability was determined using a hemocytometer.

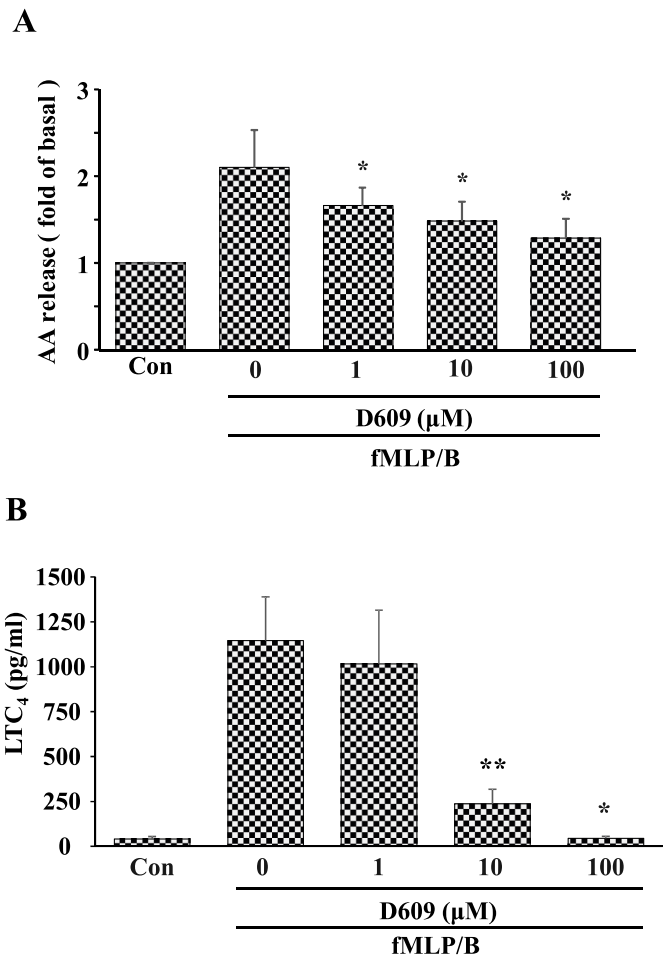

Fig. 2. Inhibition of fMLP/B-induced (A) AA release and (B) LTC_4 secretion by D609. (A) Inhibition of fMLP/B-induced AA release by D609. Data are represented as the mean ± S.E.M. from six independent experiments. *P < 0.05 versus induction by fMLP/B. (B) D609 blocked stimulated LTC_4 secretion. Data are represented as the mean ± S.E.M. from five independent experiments. **P < 0.01 versus induction by fMLP/B. fMLP/B, formyl-Met-Leu-Phe + cytochalasin B; AA, arachidonic acid; LTC_4, leukotriene C_4; D609, tricyclodecan-9-yl-xanthogenate; S.E.M., standard error of the mean.

2.10. Statistical analysis

Data were expressed as mean ± standard error of the mean (S.E.M.). Differences between treatment groups were assessed by analysis of variance (ANOVA), and statistical significance was determined using the Wilcoxon signed-rank test. P < 0.05 was considered statistically significant.

3. Results

3.1. Inhibition of stimulated PC-PLC activity by D609

We observed a significant increase in PC-PLC activity (10.6 ± 1.3 vs. 22.4 ± 5.7 mU/ml of total cell lysates; P < 0.05) after fMLP/B stimulation (Fig. 1). In D609-pretreated eosinophils, PC-PLC activity decreased to 12.3 ± 1.2 mU/ml of total cell lysates (P < 0.05) with 10 μM D609 and to 11.1 ± 1.9 mU/ml of total cell lysates (P < 0.05) with 100 μM D609. In contrast, neither ET-18-OCH_3 (a selective PI-PLC inhibitor) nor arachidonyl trifluoromethylketone (TFMK; a $cPLA_2$ inhibitor) inhibited PC-PLC activity after fMLP/B stimulation.

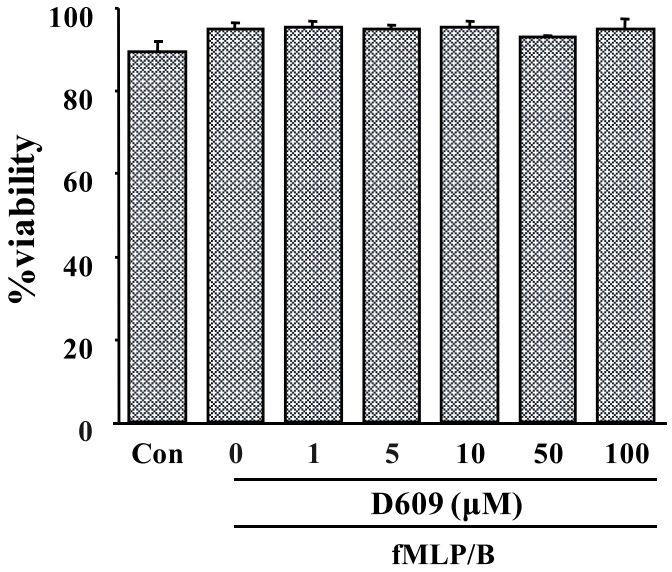

Fig. 3. Eosinophil viability evaluated by trypan blue exclusion assay. The D609 concentrations used in the study had no effect on eosinophil viability. Data are represented as the mean ± S.E.M. from five independent experiments. D609, tricyclodecan-9-yl-xanthogenate; S.E.M., standard error of the mean.

3.2. Inhibition of stimulated AA release and LTC_4 secretion by D609

D609-treated eosinophils showed concentration-dependent inhibition of fMLP/B-induced AA release and LTC_4 secretion (Fig. 2A and B). We observed a significant increase in AA release after fMLP/B stimulation (2.1 ± 0.4 times that in non-stimulated controls). However, AA release decreased by 1.7 ± 0.2 times ($P < 0.05$) with 1 μM D609, by a further 1.5 ± 0.2 times ($P < 0.05$) with 10 μM D609, and by 1.3 ± 0.2 times ($P < 0.05$) with 100 μM D609. In addition, LTC_4 release decreased from 1145.0 ± 243.8 to 237.0 ± 80.7 pg/ml ($P < 0.01$) with 10 μM D609 and to 44.8 ± 10.9 pg/ml ($P < 0.01$) with 100 μM D609.

Eosinophil viability was always >95%, even at the highest D609 concentration, as determined using a hemocytometer, indicating that the D609 concentration does not affect eosinophil viability (Fig. 3).

3.3. Effect of D609 on fMLP/B-stimulated $cPLA_2$ activation

Fig. 4A shows one representative immunoblot, and Fig. 4B and C shows a bar graph of the ImageJ quantitative analysis of the immunoblot. $cPLA_2$ phosphorylation increased by 3.45 ± 0.59-fold after fMLP/B stimulation and decreased by 1.59 ± 0.43-fold with 10 μM U0126 compared the control ($p < 0.05$) (Fig. 4C). However, D609 did not inhibit $cPLA_2$ phosphorylation. In addition, D609 concentrations that blocked fMLP/B-induced AA release and LTC_4 secretion had no significant inhibitory effect on stimulated $cPLA_2$ activity (Fig. 5). These results indicated that $cPLA_2$ activity inhibition does not inhibit AA release and LTC_4 secretion.

3.4. Effect of D609 on fMLP/B-induced activation of MAPK isoforms

Figs. 6A and 7A show representative immunoblots, Fig. 6B and C and 7B, C show bar graphs of the ImageJ quantitative analysis of the immunoblots. ERK1/2 phosphorylation increased by 8.31 ± 2.16-fold after fMLP/B stimulation and decreased by 2.12 ± 0.89-fold with 10 μM U0126 compared to the control group ($P < 0.05$) (Fig. 6C). In contrast, D609 did not inhibit fMLP/B-induced ERK1/2 phosphorylation. In addition, p38 phosphorylation increased by 4.87 ± 1.76-fold after fMLP/B stimulation and decreased by 1.12 ± 0.23-fold with 30 μM SB203580. Similar to ERK1/2 phosphorylation, D609 did not inhibit p38 MAPK phosphorylation (Fig. 7C).

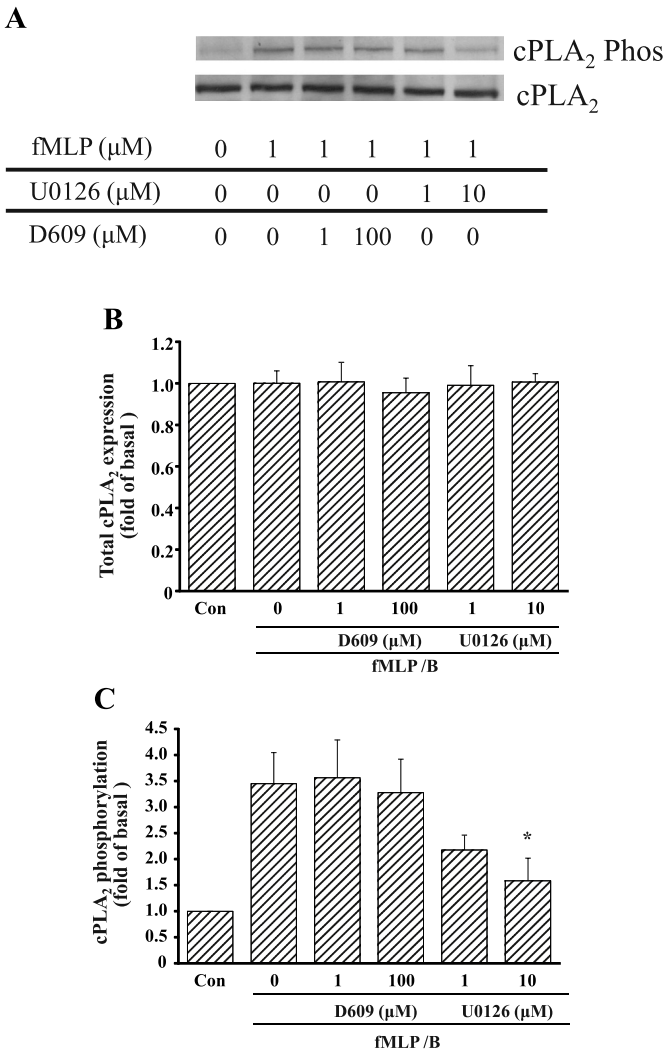

Fig. 4. Effect of D609 on fMLP/B-induced $cPLA_2$ phosphorylation. (A) One representative immunoblot. (B, C) A bar graph showing ImageJ quantitative analysis of the immunoblot. $cPLA_2$ phosphorylation was partially blocked by 10 μM U0126, while D609 had no inhibitory effect. Data are represented as the mean ± S.E.M. from six independent experiments. *$P < 0.05$ versus induction by fMLP/B. D609, tricyclodecan-9-yl-xanthogenate; fMLP/B, formyl-Met-Leu-Phe + cytochalasin B; $cPLA_2$, cytosolic phospholipase A_2; S.E.M., standard error of the mean.

3.5. Inhibition of stimulated EPO release by D609

As shown in Fig. 8, EPO release decreased from 3970.6 ± 1074.8 to 2440.7 ± 475.0 pg/ml ($P < 0.01$) with 10 μM D609 and to 86.1 ± 20.6 ng/ml ($P < 0.01$) with 100 μM D609. Eosinophils remained viable throughout the experiments (data not shown).

3.6. Inhibition of stimulated calcium influx by D609

Fig. 9A shows one representative result from seven independent experiments. fMLP/B addition caused a transient increase in $[Ca^{2+}]_i$, which decreased to the baseline level in <300 s. The $[Ca^{2+}]_i$ increase was almost completely inhibited by 10 μM U73122 and partially inhibited by 100 μM D609.

Fig. 9B shows a bar graph of the effects of D609 on AUC values of calcium influx kinetics in fMLP/B-stimulated eosinophils. Similarly to kinetic data, AUC values of calcium influx were inhibited by 10 μM U73122 ($P < 0.05$) and 100 μM D609 ($P < 0.05$).

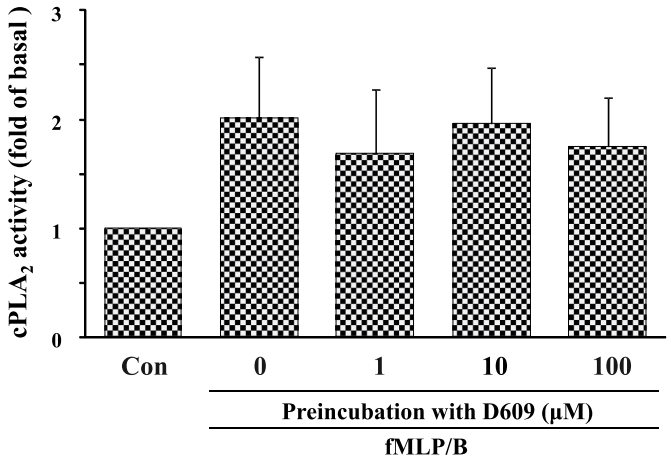

Fig. 5. Effect of D609 on fMLP/B-induced cPLA$_2$ activity. D609 had no inhibitory effect on fMLP/B-induced cPLA$_2$ activity. Data are represented as the mean ± S.E.M. from five independent experiments. D609, tricyclodecan-9-yl-xanthogenate; fMLP/B, formyl-Met-Leu-Phe + cytochalasin B; cPLA$_2$, cytosolic phospholipase A$_2$; S.E.M., standard error of the mean.

4. Discussion

Schütze et al. (1992) first reported that PC-PLC is involved in the activation of nuclear factor kappa B (NF-κB) signaling. Later studies showed multifunctional activities of D609, including antioxidative (Kalluri and Dempsey, 2014), anti-inflammatory (Anjum et al., 2012; Lee et al., 2013; Tzeng et al., 2010), antiproliferative (Kalluri et al., 2017; Mercurio et al., 2017), and neuroprotective (Adibhatla and Hatcher, 2010) activities. D609 is a specific and selective PC-PLC inhibitor that does not inhibit other phospholipases, including PC-PLD, PLA$_2$, and PI-PLC (Amtmann, 1996; Machleidt et al., 1996; Müller-Decker, 1989). The majority of studies correlating PC-PLC activation in mammalian cells relied on using D609 and revealed various aspects of PC-PLC signaling, but the underlying molecular mechanisms were unclear. D609 has a toxic effect on tumor cells but does not affect normal cells (Bai et al., 2004). This excludes nonspecific cytotoxicity as an inhibitory mechanism.

No studies have reported the presence and possible functional role of PC-PLC in human eosinophils. However, several studies have shown that the cellular responses involved in the PC-PLC pathway using isolated human blood cells or cell lines. Sternfeld et al. (2000) reported that PC-PLC activation is involved in fMLP-induced AA production in neutrophil-like db-cAMP-differentiated HL-60 cells. In addition, peptidoglycan activates the PC-PLC pathway, inducing PKC activation and ultimately cyclooxygenase-2 (COX-2) expression in RAW264.7 macrophages (Tzeng et al., 2010). Several studies have also reported that PC-PLC activation is required for increasing the $[Ca^{2+}]_i$ in human monocytes (Andrei et al., 2004; Willmott et al., 1996). To our knowledge, this is the first study that provides evidence of the key role played by PC-PLC in the eosinophilic allergic inflammatory response.

ERK1/2 and p38 MAPK phosphorylation precedes cPLA$_2$ activation (Zhu et al., 2001). cPLA$_2$ activation requires two signals, serine phosphorylation by MAPKs and calcium influx (Das et al., 2003; Pavicevic et al., 2008). Phosphorylation of cPLA$_2$ increases cPLA$_2$ activity, while calcium influx causes cPLA$_2$ translocation to intracellular membranes (Evans et al., 2001). PC-PLC is involved in fMLP/B-induced increase of cPLA$_2$ activation, at least in part, by regulating $[Ca^{2+}]_i$ increase.

PC-PLC is an upstream molecule involved in the cPLA$_2$ activation pathway in human eosinophils. PI-PLC regulates fMLP/B-induced AA release and LTC$_4$ secretion through calcium-mediated cPLA$_2$ activation (Sano et al., 2001). PC-PLC is also critical for AA release and LTC$_4$ secretion in fMLP/B-stimulated human eosinophils. In addition, PC-PLC is involved in human eosinophil degranulation. Studies have reported

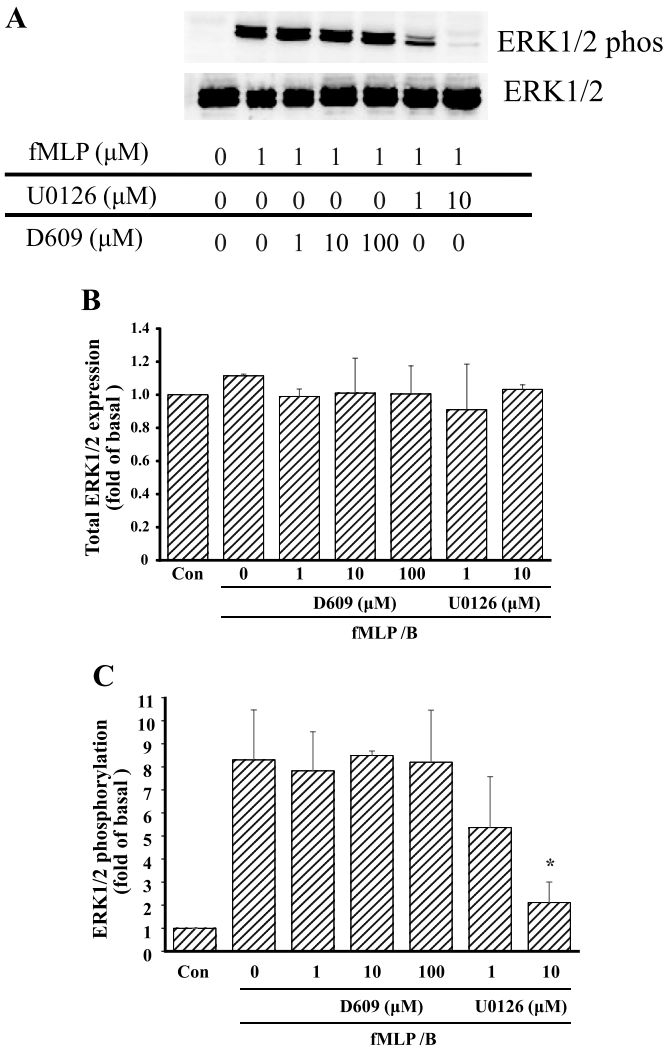

Fig. 6. Effect of D609 on fMLP/B-induced ERK1/2 phosphorylation. (A) One representative immunoblot. (B, C) A bar graph showing ImageJ quantitative analysis of the immunoblot. ERK1/2 phosphorylation was blocked by 10 μM U0126, while D609 had no inhibitory effect on fMLP/B-induced ERK1/2 phosphorylation. Data are represented as the mean ± S.E.M. from six independent experiments. *P < 0.05 versus induction by fMLP/B. D609, tricyclodecan-9-yl-xanthogenate; fMLP/B, formyl-Met-Leu-Phe + cytochalasin B; ERK, extracellular signal-regulated protein kinase; S.E.M., standard error of the mean.

that calcium influx is involved in PC-PLC activation (Liu et al., 2007; Suzuki et al., 2012) and that calcium influx is required for stimuli-induced eosinophil degranulation (Logan et al., 2003), indicating that PC-PLC regulates fMLP/B-induced eosinophil degranulation through $[Ca^{2+}]_i$ increase.

This study had a few limitations. First, we did not examine the effect of D609 on fMLP/B-induced DAG production or PKC activity. Activated PC-PLC hydrolyzes cell membrane PC into phosphocholine and DAG, enhancing PKC activity. Several PKC isozymes play an important role in coordinating ras-raf-MAPK activation, leading to an allergic inflammatory response. The interactions between PC-PLC and DAG-PKC need to be investigated in the future. Second, we did not fully investigate pathways that regulate calcium signaling in the eosinophilic allergic inflammatory response. PC-PLC and PI-PLC both are linked to the human eosinophilic allergic inflammatory response by regulating $[Ca^{2+}]_i$. However, other factors might also affect calcium signaling regulation, and further studies are required in order to determine how.

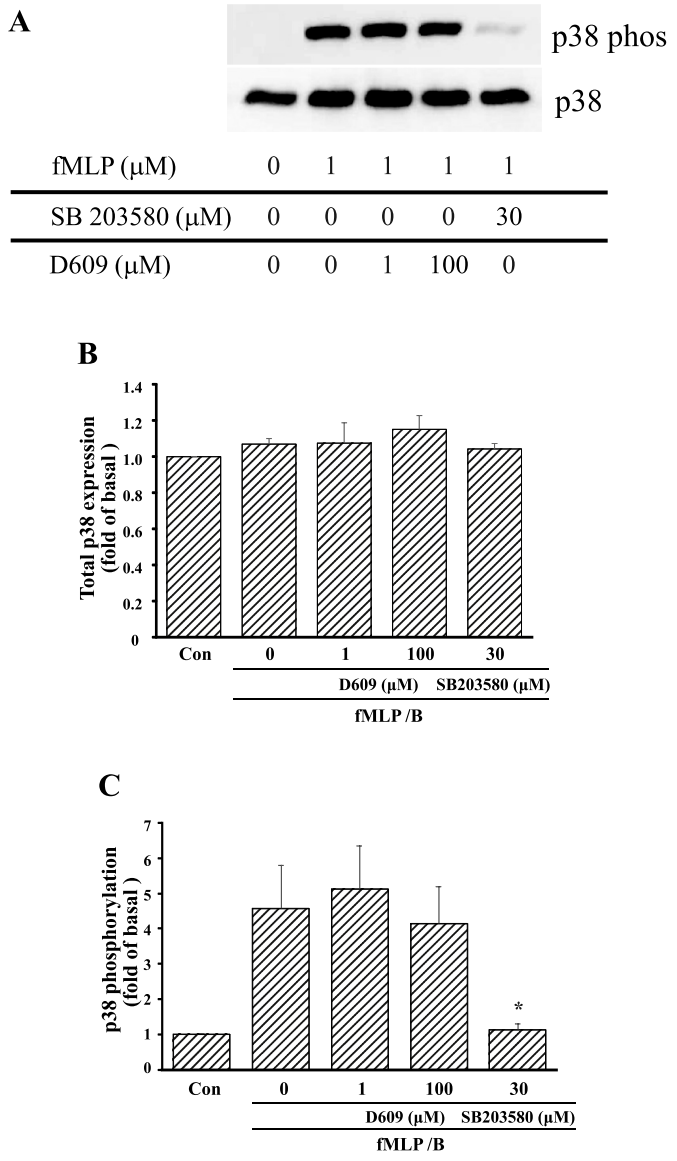

Fig. 7. Effect of D609 on p38 MAPK phosphorylation. (A) One representative immunoblot. (B, C) A bar graph showing ImageJ quantitative analysis of the immunoblot. p38 MAPK phosphorylation was almost completely blocked by 30 μM SB203580, while D609 had no inhibitory effect on fMLP/B-induced p38 MAPK phosphorylation. Data are represented as the mean ± S.E.M. from six independent experiments. *$P < 0.05$ versus induction by fMLP/B. D609, tricyclodecan-9-yl-xanthogenate; MAPK, mitogen-activated protein kinase; fMLP/B, formyl-Met-Leu-Phe + cytochalasin B; S.E.M., standard error of the mean.

5. Conclusions

Both PC-PLC and PI-PLC are involved in eosinophil LT synthesis and degranulation signaling pathways through $[Ca^{2+}]_i$ regulation in fMLP/B-stimulated human eosinophils. An understanding of the underlying regulatory mechanism should provide new insight into the allergic inflammatory response.

Author contributions

A.S. performed most of the experiments and wrote the manuscript. A. S and H.S conceived and designed the experiments. T.I. and Y.T. helped in conducting the experiments. All authors gave final approval for publication.

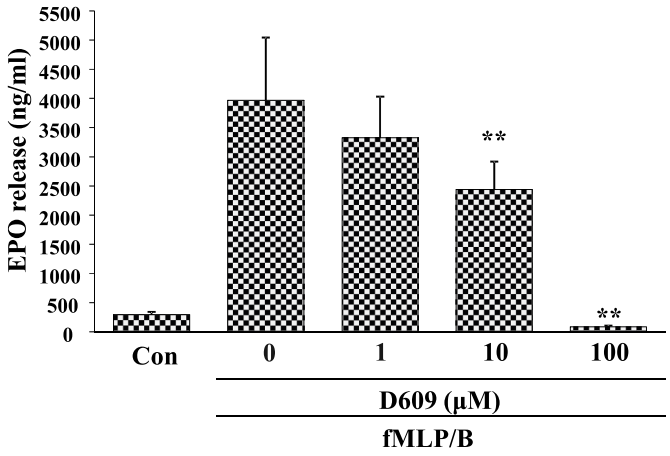

Fig. 8. Effect of D609 on fMLP/B-induced EPO release. D609 blocked fMLP/B-stimulated EPO release. Data are represented as the mean ± S.E.M. from eight independent experiments. **$P < 0.01$ versus induction by fMLP/B. D609, tricyclodecan-9-yl-xanthogenate; EPO, eosinophil peroxidase; fMLP/B, formyl-Met-Leu-Phe + cytochalasin B; S.E.M., standard error of the mean.

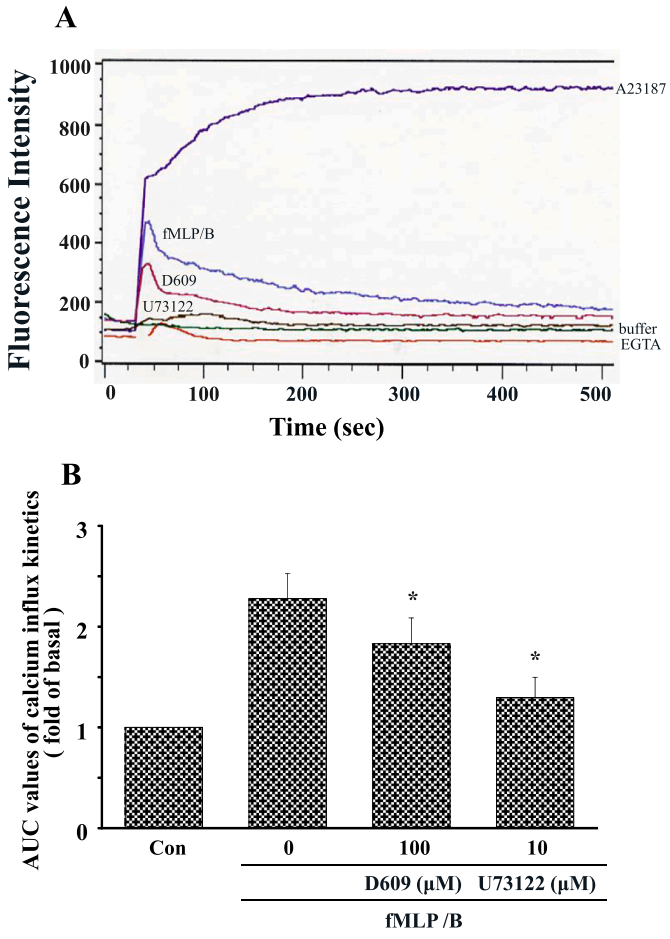

Fig. 9. Inhibition of fMLP/B-induced increase in $[Ca^{2+}]_i$ by D609. (A) One representative result of calcium influx kinetics. (B) A bar graph showing the effects of D609 on AUC values of calcium influx kinetics in fMLP/B-stimulated eosinophils. Data are represented as the mean ± S.E.M. from seven independent experiments. *$P < 0.05$ versus induction by fMLP/B. fMLP/B, formyl-Met-Leu-Phe + cytochalasin B; $[Ca^{2+}]_i$, intracellular calcium concentration; D609, tricyclodecan-9-yl-xanthogenate; AUC, area under the curve; S.E.M., standard error of the mean.

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CRediT authorship contribution statement

Akiko Sano: Conceptualization, Data curation, Writing - original draft. **Hiroyuki Sano:** Conceptualization, Methodology. **Takashi Iwanaga:** Visualization, Investigation. **Yuji Tohda:** Supervision.

Declaration of competing interest

The authors have no conflict of interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2020.173353.

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