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Effects of Local Anesthetics on Phospholipase D Activity in Differentiated Human Promyelocytic Leukemic HL60 Cells

Zhiming Tan, Shuji Dohi*§, Kenji Ohguchi,† Shigeru Nakashima,† Yoshiko Banno,† Yoshitaka Ono‡ and Yoshinori Nozawa†*

Departments of *Anesthesiology and Critical Care Medicine, and †Biochemistry, Gifu University School of Medicine, Gifu, Japan; and ‡Department of Biology, Faculty of Science, Kobe University, Kobe, Japan

ABSTRACT. Local anesthetics impair certain functions of neutrophils, and phospholipase D (PLD) is considered to play an important role in the regulation of these functions. To understand the mechanisms by which local anesthetics suppress the functions of neutrophils, we examined the effects of local anesthetics on PLD in neutrophil-like differentiated human promyelocytic leukemic HL60 cells. Tetracaine, a local anesthetic, inhibited formyl-methionyl-leucyl-phenylalanine (fMLP)- and 4β-phorbol 12-myristate 13-acetate (PMA)-induced PLD activation, but potentiated fMLP-stimulated phospholipase C activity. All four local anesthetics tested suppressed PMA-induced PLD activation to different extents, and the order of their potency was tetracaine > bupivacaine > lidocaine > procaine. In a cell-free system, tetracaine suppressed guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS)-induced PLD activation as well as PMA-induced PLD activation. Western blot analysis revealed that tetracaine prevented the membrane translocation of PLD-activating factors, ADP-ribosylation factor, RhoA, and protein kinase Cα. Tetracaine also inhibited the activity of recombinant hPLD1a *in vitro*. These results suggest that local anesthetics suppress PLD activation in differentiated HL60 cells by preventing the membrane translocation of PLD-activating factors, and/or by directly inhibiting the enzyme *per se*. Therefore, it could be assumed that local anesthetics would suppress the functions of neutrophils by inhibition of PLD activation. BIOCHEM PHARMACOL **58**;12:1881–1889, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. local anesthetic; tetracaine; phospholipase D; human promyelocytic leukemic HL60 cell; protein kinase C; small G protein

Several studies have indicated that local anesthetics interfere with neutrophil functions. Haines *et al.* [1] reported that cocaine, lidocaine, bupivacaine, and tetracaine blunt the responses of neutrophils, such as O_2^- generation and degranulation induced by fMLP‖ and PMA. Lidocaine has been reported to impair O_2^- generation and degranulation after stimulation by PMA in human neutrophils, and the activity of neutrophils from lidocaine-treated patients also has been reported to be suppressed [2]. In a previous study, we observed that eight local anesthetics suppressed O_2^- generation of human neutrophils and that the inhibitory effects were related to their local anesthetic potency [3].

HL60 cells can be differentiated into neutrophil-like cells upon the addition of dbcAMP [4]. Differentiated HL60 cells display functions similar to neutrophils and have been used to study the regulatory mechanisms of differentiation and functions of neutrophils [4]. In neutrophils and differentiated HL60 cells, PLD, which hydrolyzes membrane phospholipids, especially PC leading to the production of PA and subsequently DG via PA phosphohydrolase [5], is activated rapidly by chemoattractants [6, 7]. Evidence has been accumulating to indicate that PLD may play an important role in the regulation of neutrophil functions through PA and DG, as second messengers. In the presence of primary alcohols, phosphatidylalcohols are produced via the phosphatidylation of PLD at the expense of PA [8]. Several reports showed that primary alcohols could block the activation of NADPH oxidase by chemoattractants [9–11], resulting from the reduction of PA and DG accompanied by phosphatidylalcohol production. Treatment with exogenous PA stimulates NADPH oxidase in permeabilized neutrophils and isolated membranes [12, 13]. PA may activate a putative protein kinase that induces p47-*phox* phosphorylation and NADPH oxidase activation [14, 15]. It also has been shown that PA and DG synergis-

§ Corresponding author: Dr. Shuji Dohi, Department of Anesthesiology and Critical Care Medicine, Gifu University School of Medicine, Tsukasa-machi-40, Gifu City, Gifu 500-8705, Japan. Tel. (81) 58-267-2293; FAX (81) 58-267-2961.

‖ *Abbreviations*: fMLP, formyl-methionyl-leucyl-phenylalanine; PLD, phospholipase D; PLC, phospholipase C; PKC, protein kinase C; PMA, 4β-phorbol 12-myristate 13-acetate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PBut, phosphatidylbutanol; PC, phosphatidylcholine; PA, phosphatidic acid; DG, 1,2-diacylglycerol; Arf, ADP-ribosylation factor; dbcAMP, dibutyryl cyclic 3′,5′-AMP; PMSF, phenylmethylsulfonyl fluoride; and IP_3, inositol (1,4,5)-trisphosphate.

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tically cause NADPH oxidase activation in a reconstitution system of neutrophils [16]. Moreover, PLD is involved in degranulation and phagocytosis of neutrophils and differentiated HL60 cells. When these cells were stimulated by fMLP, PA formation was closely associated with granule secretion [7, 17, 18]. The addition of exogenous PA also induces the release of lysosomal enzymes from permeabilized neutrophils [19]. Furthermore, PLD activation has been reported to occur during phagocytosis of opsonized zymosan by neutrophils or macrophages [20, 21]. The purpose of the present study was to investigate the effects of local anesthetics on PLD and then to determine the mechanisms by which local anesthetics impair the functions of neutrophils.

MATERIALS AND METHODS

Materials

The HL60 human promyelocytic leukemic cell line was supplied by Dr. T. Okazaki (Kyoto University). RPMI 1640 medium was obtained from the Gibco BRL Co. Fetal bovine serum was purchased from Irvine Scientific. [^{3}H]Oleic acid, [*palmitoyl*-^{3}H]dipalmitoyl phosphatidylcholine, and *myo*-[^{3}H]inositol were obtained from DuPont New England Nuclear. dbcAMP, fMLP, PMA, cytochalasin B, BSA, phosphatidylethanolamine, phosphatidylinositol 4,5-bisphosphate, egg PC, tetracaine hydrochloride, procaine hydrochloride, lidocaine hydrochloride, and bupivacaine hydrochloride were purchased from the Sigma Chemical Co. GTPγS was obtained from Boehringer Mannheim. Antibodies against PKCα and RhoA were purchased from Santa Cruz Biotechnology Inc. Monoclonal antibody against Arf and *Escherichia coli* bearing Arf1 plasmid were gifts from Dr. Joel Moss (National Institutes of Health). Recombinant Arf1 was purified as described previously [22]. The recombinant hPLD1a transfer vectors infecting Sf9 cells were supplied by Dr. Michael Frohman (Institute for Cell and Developmental Biology). Protein determination reagents were obtained from Bio-Rad Laboratories. The enhanced chemiluminescence system used for western blot analysis was from Amersham International plc. Other reagents were of the highest quality available.

Culture and Neutrophil-Like Differentiation of HL60 Cells

HL60 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin in a humidified atmosphere containing 5% CO_2 at 37°. For neutrophil-like differentiation, cells were transferred to serum-free RPMI 1640 medium supplemented with 5 μg/mL of transferrin and 5 μg/mL of insulin, and cultured for 24 hr. Differentiation was initiated by the addition of 0.5 mM dbcAMP. For the assay of PLD activity, cells were labeled with [^{3}H]Oleic acid (0.5 μCi/mL) for the last 12–15 hr.

Assay of PLD Activity in Intact Differentiated HL60 Cells

[^{3}H]Oleic acid-labeled differentiated HL60 cells were washed twice with buffer A (25 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and 1 mg/mL of BSA). The cells (0.5×10^6/300 μL) were preincubated in buffer A with or without local anesthetics, plus 10 μM cytochalasin B for the last 5 min for 30 min and then stimulated with 1 μM fMLP or 100 nM PMA for the indicated duration at 37° in the presence of 0.3% butanol (v/v). The reaction was terminated by the addition of chloroform:methanol (1:2, v/v). Lipids were extracted according to the method of Bligh and Dyer [23] and separated on Silica Gel 60 TLC plates in a solvent system of the upper phase of ethyl acetate:2,2,4-trimethylpentane:acetic acid:water (13:2:3:10, by vol.) as described previously [24]. The plates were exposed to iodine vapor, and [^{3}H]PBut was identified by comigration with PBut standard. The spots scraped off the plates were mixed with a scintillation solution, and the radioactivity was counted in a liquid scintillation counter (Beckman LS-6500).

Assay of PLD Activity in Lysates from Differentiated HL60 Cells

[^{3}H]Oleic acid-labeled cells were washed twice with buffer B (25 mM HEPES, pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM $MgCl_2$, 1 mM MgATP, 1 mM EGTA, 5 mM dithiothreitol, 0.5 mM PMSF, 10 μg/mL of leupeptin) and suspended in buffer B. Cells were disrupted by N_2 cavitation (600 psi at 4° for 30 min). Then unbroken cells and nuclei were removed by centrifugation at 900 *g* for 5 min, and the resulting supernatant was used as differentiated HL60 lysate for experiments.

[^{3}H]Oleic acid-labeled lysates were incubated in buffer B containing $CaCl_2$ to give a final free Ca^{2+} concentration of 1 μM with or without local anesthetics and stimulated with 10 μM GTPγS or 100 nM PMA for indicated times at 37° in the presence of 0.3% butanol (v/v). The reactions were terminated by the addition of cold chloroform:methanol (1:2, v/v). Lipids were extracted, and [^{3}H]PBut formation was measured as stated above.

Assay of Recombinant hPLD1a Activity

PLD activity was measured by using the substrate phospholipid vesicles prepared as described previously [24]. Mixed-lipid vesicles (phosphatidylethanolamine:phosphatidylinositol 4,5-bisphosphate:egg PC, 10:1.5:1 molar ratio) containing [*palmitoyl*-^{3}H]dipalmitoyl phosphatidylcholine (3 μCi/mL) were added to the membrane fraction from Sf9 cells infected with the recombinant hPLD1a transfer vectors in a reaction mixture containing 50 mM sodium-HEPES (pH 7.5), 80 mM KCl, 1 mM dithiothreitol, 3 mM $MgCl_2$, 3 mM EGTA, and 2 mM $CaCl_2$ to give a final free Ca^{2+} concentration of 300 nM (total, 0.1 mL)

and incubated with 10 μM recombinant Arf and indicated concentrations of tetracaine in the presence of 10 μM GTPγS and 0.3% butanol (v/v) at 37° for 30 min. The reaction was terminated by the addition of chloroform: methanol (1:2, v/v).

SDS–PAGE and Western Blot Analysis

After incubation and stimulation under the same conditions as those used for the PLD assay in lysates, the reaction mixture was centrifuged at 100,000 *g* for 30 min to obtain the membrane pellet. Membranes were washed twice with buffer B. The washed membranes were resuspended with buffer C (10 mM Tris–HCl, pH 7.4, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, 10 μg/mL of leupeptin, 1 mM Na_3VO_4, 10 mM NaF, and 0.1 mM Na_2MoO_4). After incubation on ice for 30 min, the suspension was centrifuged at 13,000 *g* for 20 min to obtain the membrane extract. Proteins were subjected to SDS–PAGE on 13 or 8% polyacrylamide gels for small G-proteins and PKCα. Proteins were transferred electrophoretically to nitrocellulose membrane, and then the membranes were blocked with TBS-T (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) containing 5% skim milk powder. Western blot analysis using specific antibodies was performed as described previously [24].

PLC Activity Assay in Intact Differentiated HL60 Cells

Cells were labeled for 48 hr with 10 μCi/mL of *myo*-[^{3}H]inositol and washed twice with buffer D (25 mM HEPES, pH 7.4, 125 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM LiCl, 5 mM glucose, and 1 mg/mL of BSA). The cells (10^6/300 μL) were preincubated in buffer D with or without local anesthetics for 30 min and then were stimulated with 2 μM fMLP for 30 sec at 37°. The reaction was terminated by the addition of 10% perchloric acid. After neutralizing with 1.53 M KOH/75 mM HEPES, the tubes were centrifuged (900 *g*, 5 min), and the aqueous phase was removed and diluted to 5 mL with H_2O. Then the diluted extract was applied to an AG1-X8 resin (formate) column. IP_3 was eluted sequentially using 1.0 M ammonium formate in 0.1 M formic acid as eluent, and the radioactivity was counted in a liquid scintillation counter (Beckman LS-6500).

Statistical Analyses

Data are expressed as means ± SD. Differences between values were analyzed using ANOVA; when $P < 0.05$, differences were considered significant. The IC_{50} values were determined by computerized regression analysis of log concentrations versus enzyme activity.

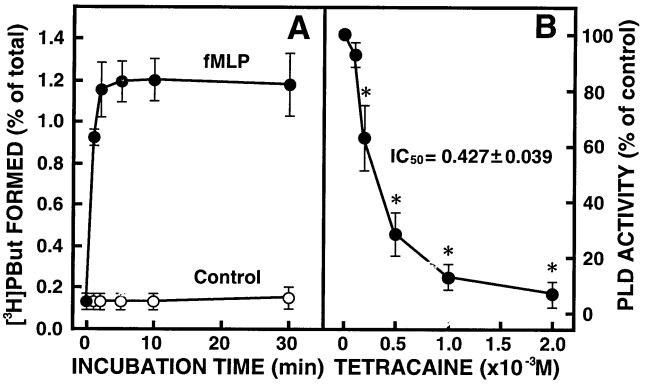

FIG. 1. Time course of fMLP-induced PLD activation (A) and concentration dependency of tetracaine in fMLP-induced PLD activation (B) in differentiated HL60 cells. [^{3}H]Oleic acid-labeled HL60 cells (0.5×10^6/300 μL) were preincubated in buffer A with or without tetracaine at the indicated concentrations, in the presence of 10 μM cytochalasin B for the last 5 min for 30 min, and then stimulated with 1 μM fMLP for the indicated duration at 37° in the presence of 0.3% butanol. PLD activity was determined by measuring the formation of [^{3}H]PBut. Data represent the means ± SD of three different experiments, each carried out in duplicate. Key: (*) $P < 0.05$ vs 0×10^{-3} M. The absolute value for the total [^{3}H]-labeled PBut was 567,635 dpm (mean) in panel A. PLD activities in the absence of tetracaine (control) were expressed as 100%: fMLP-stimulated [^{3}H]PBut formed, 6755 dpm (mean), minus nonstimulated [^{3}H]PBut formed, 908 dpm (mean), at 5 min as expressed in panel B.

RESULTS

Effects of Tetracaine on fMLP-Induced PLD Activation in Differentiated HL60 Cells

PLD catalyzes the transphosphatidylation reaction in addition to hydrolysis. In the presence of primary alcohols such as ethanol and butanol, metabolically stable phosphatidylalcohols are produced, which are specific parameters for PLD activity [25]. In the present study, the accumulation of [^{3}H]PBut was measured as the assay for PLD activity in [^{3}H]Oleic acid-labeled differentiated HL60 cells in the presence of 0.3% butanol. In differentiated HL60 cells, upon fMLP stimulation, the PBut formation increased, reaching a plateau within 5 min (Fig. 1A). However, when the cells were pretreated with tetracaine for 30 min, the fMLP-induced PLD activity was suppressed in a concentration-dependent manner with an IC_{50} value of approximately 0.4×10^{-3} M (Fig. 1B).

Effects of Tetracaine on PMA-Induced PLD Activation in Differentiated HL60 Cells

PMA, as a PKC activator, is known to activate PLD in various cell types [25]. In differentiated HL60 cells, 100 nM PMA also induced PBut formation in a time-dependent manner (Fig. 2A). PBut formation stimulated by PMA at 30 min was about 8.5-fold over the nonstimulated levels. PMA-induced PLD activation was inhibited concentration-dependently by tetracaine in differentiated HL60 cells. The IC_{50} value was about 0.5×10^{-3} M (Fig. 2B). When the

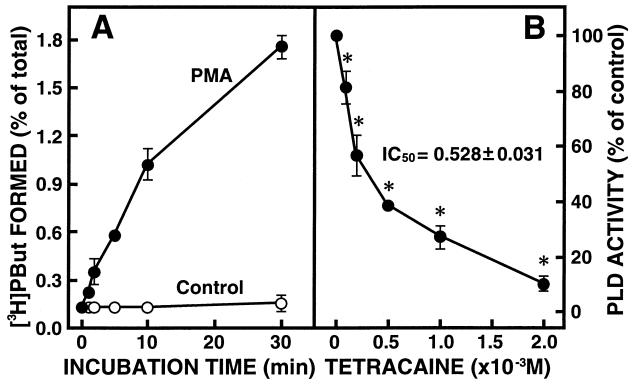

FIG. 2. Time course of PMA-induced PLD activation (A) and concentration dependency of tetracaine in PMA-induced PLD activation (B) in differentiated HL60 cells. [^{3}H]Oleic acid-labeled HL60 cells (0.5×10^6/300 μL) were preincubated in buffer A with or without tetracaine at the indicated concentrations for 30 min and then were stimulated with 100 nM PMA for the indicated duration at 37° in the presence of 0.3% butanol. PLD activity was determined by measuring the formation of [^{3}H]PBut. Data represent the means ± SD of three different experiments, each carried out in duplicate. Key: (*) $P < 0.05$ vs 0×10^{-3} M. The absolute value for the total [^{3}H]-labeled PBut was 531,716 dpm (mean) in panel A. PLD activities in the absence of tetracaine (control) were expressed as 100%: PMA-stimulated [^{3}H]PBut formation, 9251 dpm (mean), minus nonstimulated [^{3}H]PBut formation, 798 dpm (mean), at 30 min in panel B.

cells were preincubated with 0.5×10^{-3} M procaine, lidocaine, bupivacaine, and tetracaine, respectively, it was observed that these four local anesthetics all significantly suppressed PMA-induced PLD activation in the order of procaine < lidocaine < bupivacaine < tetracaine (Fig. 3).

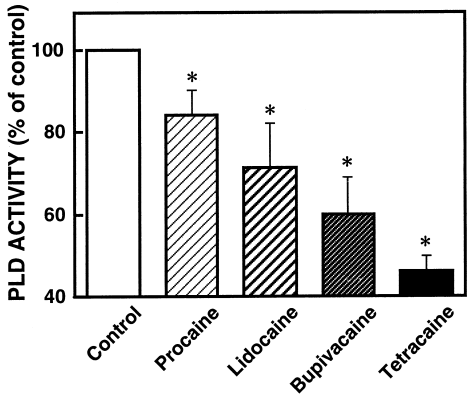

FIG. 3. Inhibitory effects of local anesthetics on PMA-induced PLD activation in differentiated HL60 cells. [^{3}H]Oleic acid-labeled HL60 cells (0.5×10^6/300 μL) were preincubated in buffer A with or without 0×10^{-3} M local anesthetics for 30 min, and then were stimulated with 100 nM PMA for the indicated duration at 37° in the presence of 0.3% butanol. PLD activity was determined. Data represent the means ± SD of three different experiments, each carried out in duplicate. Key: (*) $P < 0.05$ vs control. PLD activities in the absence of local anesthetics (control) were expressed as 100%: PMA-stimulated [^{3}H]PBut formation, 9093 dpm (mean), minus nonstimulated [^{3}H]PBut formation, 744 dpm (mean), at 30 min.

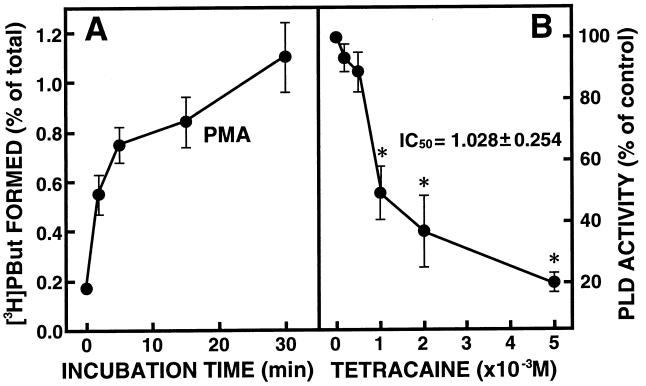

FIG. 4. Time course of PMA-induced PLD activation (A) and concentration dependency of tetracaine in PMA-induced PLD activation (B) in differentiated HL60 cell lysates. [^{3}H]Oleic acid-labeled differentiated HL60 lysates were incubated in buffer B with or without local anesthetics and were stimulated with 100 nM PMA for the indicated duration at 37° in the presence of 0.3% butanol (v/v). PLD activity was determined by measuring the formation of [^{3}H]PBut. Data represent the means ± SD of three different experiments, each carried out in duplicate. Key: (*) $P < 0.05$ vs 0×10^{-3} M. The absolute value for the total [^{3}H]-labeled PBut was 362,844 dpm (mean) in panel A. PLD activities in the absence of tetracaine (control) were expressed as 100%: PMA-stimulated [^{3}H]PBut formation, 4064 dpm (mean), minus nonstimulated [^{3}H]PBut formation, 653 dpm (mean), at 30 min in panel B.

Effects of Tetracaine on PMA-Induced PLD Activation in Lysates from Differentiated HL60 Cells

In lysates prepared from neutrophils and HL60 cells, PMA-induced PLD activation has been demonstrated to be mediated through PKC [26, 27]. In the present study, PMA-stimulated PBut formation was increased time-dependently to nearly 5-fold at 30 min in lysates from differentiated HL60 cells (Fig. 4A). Treatment of the cell lysates with tetracaine inhibited PLD activation induced by 100 nM PMA. Tetracaine at 5×10^3 M reduced PMA-PLD activition by about 80%. The IC_{50} value was approximately 1×10^{-3} M (Fig. 4B).

Effects of Tetracaine on GTPγS-Induced PLD Activition in Lysates from Differentiated HL60 Cells

PBut formation was induced by incubating lysates from differentiated HL60 cells with 10 μM GTPγS, in a time-dependent manner, up to 5- to 6-fold at 30 min (Fig. 5A). Tetracaine suppressed GTPγS-induced PLD activation in a concentration-dependent fashion (Fig. 5B). Tetracaine at only 0.2×10^{-3} M significantly inhibited GTPγS-PLD activation. The presence of the drug at 2×10^{-3} M decreased GTPγS-PLD activation maximally. Tetracaine had an IC_{50} value of approximately 1×10^{-3} M.

Effects of Tetracaine on the Membrane Translocation of PLD Activators

By using western blot analysis, we investigated the effects of tetracaine on the membrane translocation of Arf, RhoA, or

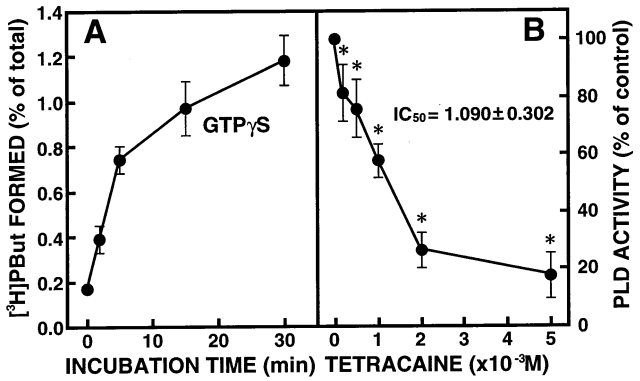

FIG. 5. Time course of GTPγS-induced PLD activation (A) and concentration dependency of tetracaine in GTPγS-induced PLD activation (B) in differentiated HL60 cell lysates. [^{3}H]Oleic acid-labeled differentiated HL60 lysates were incubated in buffer B with or without local anesthetics and were stimulated with 10 μM GTPγS for the indicated duration at 37° in the presence of 0.3% butanol (v/v). PLD activity was determined. Data represent the means ± SD of three different experiments, each carried out in duplicate. Key: (*) $P < 0.05$ vs 0×10^{-3} M. The absolute value for the total [^{3}H]-labeled PBut was 367,078 dpm (mean) in panel A. PLD activities in the absence of tetracaine (control) were expressed as 100%: GTPγS-stimulated [^{3}H]PBut formation, 4332 dpm (mean), minus nonstimulated [^{3}H]PBut formation, 624 dpm (mean), at 30 min in panel B.

PKCα in differentiated HL60 cell lysates. It was shown that tetracaine (5×10^{-3} M) markedly prevented the membrane translocation of GTPγS-induced Arf, RhoA, and PMA-induced PKCα (Fig. 6).

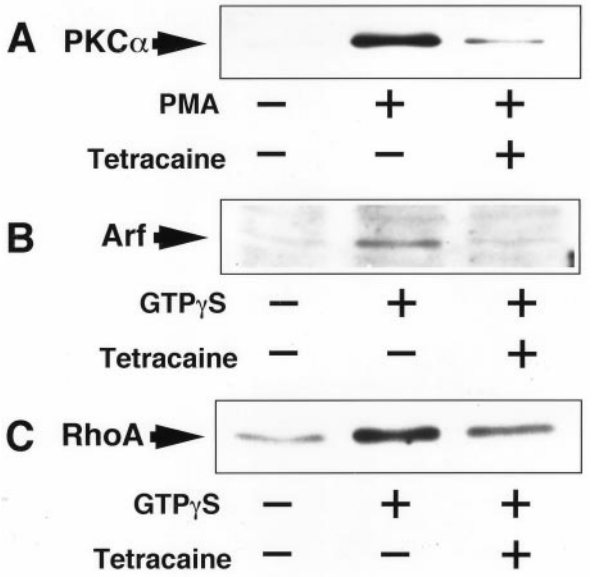

FIG. 6. Effects of tetracaine on membrane translocation of PKCα, Arf, and RhoA. After incubation with or without 5×10^{-3} M tetracaine and stimulation with 100 nM PMA or 10 μM GTPγS under the same conditions as those used for the PLD assay in lysates, HL60 membrane proteins were electrophoresed and transferred to the nitrocellulose membrane. Western blot analysis with anti-PKCα antibody (A), anti-Arf antibody (B), and anti-RhoA antibody (C) was performed.

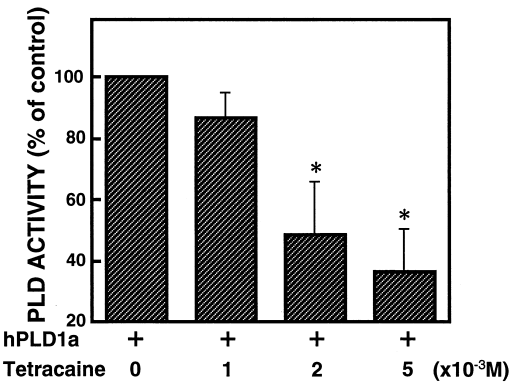

FIG. 7. Effects of tetracaine on hPLD1a activity. The recombinant hPLD1a fraction was incubated with the substrate phospholipid vesicles containing [*palmitoyl*-^{3}H]dipalmitoyl phosphatidylcholine at 37° for 30 min in the presence of 0.3% butanol. Ten micromolar recombinant Arf, 10 μM GTPγS, and the indicated concentration of tetracaine were included in the reaction mixture. PLD activity was determined by measuring the formation of [^{3}H]PBut as described under Materials and Methods. Data represent the means ± SD of two different experiments, each carried out in duplicate. Key: (*) $P < 0.05$ vs 0×10^{-3} M. The absolute value for the total [^{3}H]-labeled PBut was 621,108 dpm (mean), and PLD activities in the absence of tetracaine (control) were expressed as 100%: [^{3}H]PBut formation in the presence of the recombinant hPLD1a fraction, 26,521 dpm (mean), minus [^{3}H]PBut formation in the absence of the recombinant hPLD1a fraction by GTPγS, 1180 dpm (mean), at 30 min.

Inhibitory Effects of Tetracaine on Recombinant hPLD1 Activity

To exclude the possibility that local anesthetics directly affect the PLD enzyme, a recombinant hPLD1a from Sf9 cells was used. The presence of tetracaine in the reaction mixture, as described in Materials and Methods, significantly inhibited the hPLD1a activity in a concentration-dependent fashion (Fig. 7).

Potentiating Effects of Tetracaine on fMLP-Stimulated PLC Activity in Differentiated HL60 Cells

In response to fMLP stimulation, the IP_3 formation increased 2-fold in HL60 cells, and tetracaine treatment potentiated fMLP-induced IP_3 formation (Fig. 8). fMLP-IP_3 formation in the presence of 0.5×10^{-3} M tetracaine was 0.7 times more than in the absence of tetracaine. In response to 10^{-3} M tetracaine treatment, fMLP-induced IP_3 formation multiplied about 2-fold.

DISCUSSION

The present study demonstrated that local anesthetics inhibit both fMLP-mediated and PMA-induced PLD activations in differentiated HL60 cells. Tetracaine suppressed PMA-PLD and GTPγS-PLD activations in the cell lysates from differentiated HL60 cells and exerted inhibitory effects on the recombinant hPLD1a activity *in vitro*. In

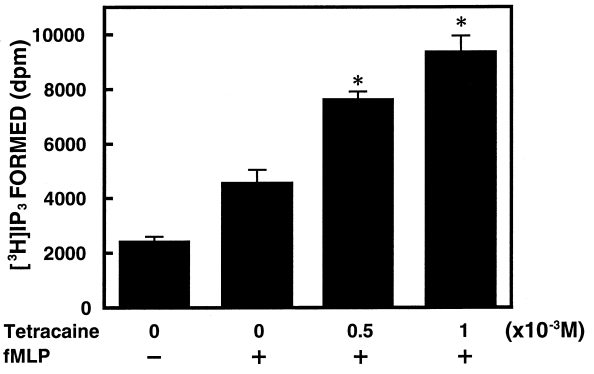

FIG. 8. Potentiating effect of tetracaine on fMLP-induced IP_3 formation in differentiated HL60 cells. *myo*-[^{3}H]Inositol-labeled HL60 cells (10^6/300 μL) were preincubated in buffer D with or without tetracaine at the indicated concentrations for 30 min and then were stimulated with 2 μM fMLP for 30 sec at 37°. IP_3 formation was measured as described in Materials and Methods. Data represent the means ± SD of two different experiments, each carried out in duplicate. Key: (*) $P < 0.001$ vs 0×10^{-3} M tetracaine with fMLP stimulation.

addition, tetracaine prevented the membrane translocation of PLD activators such as Arf, RhoA, and PKCα. Although local anesthetics have been shown to interfere with cellular signaling molecules, such as phospholipase C [28], PKC [29], and Ca^{2+} [30], which are thought to be involved in fMLP-induced PLD or PMA-induced PLD activation, the results obtained here suggested that local anesthetics probably affected activators of PLD or PLD enzymes *per se*.

In neutrophils, PLD activation by the receptor-mediated agonist fMLP has been reported to be calcium- and tyrosine kinase-dependent, but independent of PKC [31]. However, PMA, a membrane-bypassing PKC activator, also induced PLD activation, and calcium played a minor role in PMA-induced PLD activation [31]. In the cell-free system (lysate) from neutrophils or HL60 cells, it was demonstrated that RhoA, a small molecular weight GTP-binding protein, is a direct activator of GTPγS-stimulated PLD. Recombinant RhoGDI (GDP dissociation inhibitor) inhibited GTPγS-induced PLD activation, indicating the regulatory role of the Rho family [32]. When RhoA was depleted from both the plasma membrane and cytosol, the addition of GTPγS did not stimulate PLD activity. However, the addition of recombinant RhoA can restore the activity in a neutrophil cell-free system [33]; glucosylation of RhoA by *Clostridium difficile* toxin A and toxin B seems to prevent the GTPγS-induced PLD activation in HL60 cell lysates [34]. Several reports have suggested that RhoA translocation to membrane fractions is required for effective interaction with PLD [35, 36]. Additionally, Arf has been established as another primary activating factor of PLD [37]. Recent evidence indicated that Arf stimulates PLD activation synergistically with a 50-kDa cytosol factor in neutrophils [38] and differentiated HL60 cells [39]. Houle *et al.* [40] reported that treatment of HL60 granulocytes with fMLP also causes an increase in the membrane-associated Arf content and the GTPγS-induced PLD activity. The finding supports the notion that membrane translocation of Arf mediates fMLP-induced PLD activation.

PKC has been thought to play a key role in the regulation of PLD activity in neutrophils and HL60 cells. The addition of purified PKC to isolated plasma membranes from neutrophils [26] or HL60 cells [27, 41] enhanced the membrane-bound PLD activity. Our previous study indicated that PKC as a cytosolic factor is required for the activation of PLD in HL60 membranes by PMA or GTPγS [27]. That work also demonstrated that PKCα is more potent in activating PLD activity than PKCβ, whereas PKCγ is not able to affect PLD activation [27]. It was suggested recently that PKCα directly interacts with the recombinant PLD1 [42], supporting the notion that PKCα acts as a direct activator for PLD. It is worth noting that these three activators, RhoA, Arf and PKCα, exert a synergistic action on PLD activation. In HL60 cells, the combination of PKCα plus PMA was found to enhance markedly the stimulation of PLD by GTPγS plus RhoA [27]. PMA-induced PLD activation was partially abolished by pretreating with *C. difficile* toxin B, which inhibits Rho family proteins in HL60 cell lysates [34]. These synergistic effects were also observed in purified recombinant PLD1, supporting evidence for the suggestion that PKCα, RhoA, and Arf bind to different sites on a single PLD1 enzyme [42]. It has been established that both membrane and cytosol fractions are essential for PMA- and GTPγS-induced PLD activation in neutrophils [43] and HL60 cells [27, 44]. Furthermore, small GTP-binding proteins (Arf, RhoA) and PKCα are required to translocate from cytosol to membranes for activation of PLD [27, 37, 45]. In the present study, tetracaine suppressed PMA-PLD and GTPγS-PLD activation in the cell lysates, and this suppression was thought to be, at least in part, responsible for the prevention of membrane translocation of PKCα, Arf, and RhoA.

Although it is not clear whether local anesthetics interfere with small GTP-binding proteins, several reports indicated that the drugs inhibited PKC activity *in vitro*. Either lidocaine, tetracaine, or dibucaine inhibits PKC activity in a competitive manner with phospholipids [29, 46]. Local anesthetics seem to reduce the activity of purified PKC, possibly by competing with DG or membrane phospholipids [47, 48]. Local anesthetics may interfere with PLD activators, such as PKCα, thereby preventing PLD activation. However, contrary to the *in vitro* systems mentioned above, local anesthetics did not affect PKC function in intact neuroblastoma cells [49]. If it was unlikely that local anesthetics inhibit PLD activation by their actions on PKC and other activators, there should be another mechanism(s) by which local anesthetics inhibit PLD in intact HL60 cells.

On the other hand, local anesthetics have been known to affect many membrane-associated proteins. Apart from Na^+ channels, they include adenylate cyclase, guanylate cyclase, and ion-pumping enzymes [50]. In addition, local anesthetics are thought to compete with Ca^{2+} for the binding sites in phospholipase A_2 and affect its activity [51]. The mechanism(s) of action of these drugs on mem-

brane-associated proteins is the conformational changes of proteins via hydrophobic interaction and/or the interaction with the lipids surrounding them [52]. For these reasons, the possibility could not be excluded that local anesthetics as membrane-disturbing agents [53] act on PLD and then affect the membrane translocation of their activators. The inhibition of PLD activity by tetracaine was demonstrated in intact differentiated HL60 cells and their cell lysates, and even in the recombinant hPLD1a activity, thus supporting the above possibility.

PLC acts on phosphatidylinositol 4,5-bisphosphate to produce IP_3 and DG, both of which serve as second messengers to mobilize calcium and activate PKC, respectively [54, 55]. Local anesthetics as anti-platelet drugs directly inhibit PLC in human platelets [28], and in guinea pig cortical synaptoneurosomes, local anesthetics inhibit PLC through the blockade of sodium channels or muscarinic receptors rather than by direct effects on PLC [56]. It could be considered that local anesthetics inhibit PLD activity, at least in part, by their effects on PLC in HL60 cells. However, in the present study, fMLP-induced PLC activity was potentiated by tetracaine, which suppressed fMLP-induced PLD activity at the same concentration. Thus, the effects of tetracaine on PLD are independent of its effects on PLC in differentiated HL60 cells. Exploring how local anesthetics affect fMLP-PLC will be another challenge for us.

Several reports have indicated that local anesthetics prevent the functions of neutrophils. Lidocaine has been demonstrated to inhibit phosphorylation of a 47-kDa cytoplasmic protein and O_2^- generation in guinea pig neutrophils [29]. Haines *et al.* [1] have shown that cocaine and other local anesthetics in the same order as their potency for local anesthesia inhibit neutrophil responses not only to fMLP, but also to PMA. Among the local anesthetics tested, tetracaine (1 mM) significantly blunted neutrophil functions. In another study, 0.25 to 1 mM tetracaine sufficiently reduced the lysosomal enzyme release and superoxide anion production of human neutrophils [57]. The concentrations of tetracaine at which it inhibited the functions of leukocytes were in agreement with that required to inhibit the PLD activation in the present study. In addition, local anesthetics inhibit neutrophil functions in the order of their anesthetic potency [1, 3]. This order was identical with that of their inhibitory potency for the PMA-induced PLD activation in differentiated HL60 cells. Therefore, inhibition of PLD activation by local anesthetics may be responsible for their inhibitory effects on neutrophil function.

In addition to the wide range of clinical uses as local anesthetics, these drugs also have systemic effects. Lidocaine and procainamide among them also are used for the treatment of acute cardiac arrhythmias. The neutrophil is a principal effector cell in inflammatory reactions. The attraction and activation of neutrophils are closely associated with neutrophil-dependent hyperalgesia [58] and with the amount of tissue damage in acute myocardial infarction [59]. Thus, studies of the effects of local anesthetics on PLD activation will be of great merit to clarify the mechanisms of their effects on neutrophils and also for better use of these drugs.

In conclusion, tetracaine not only inhibited fMLP- and PMA-induced PLD activation in intact differentiated HL60 cells, but also suppressed PMA-PLD and GTPγS-PLD in their cell lysates. The drug was also found to prevent the membrane translocation of PKCα, RhoA, and Arf. Furthermore, it suppressed recombinant hPLD1a activity. By contrast, fMLP-induced PLC activity was potentiated markedly by tetracaine. These results suggest that by preventing the membrane translocation of PLD activators and/or directly interfering with PLD itself, tetracaine suppresses PLD activation in differentiated HL60 cells. By generating PA and DG, PLD plays an important role in the regulation of the functions of the neutrophil. Therefore, it can be assumed that local anesthetics prevent neutrophil functions through their inhibition of PLD activation.

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