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Molecular Mechanisms of *N*-Formyl-Methionyl-Leucyl-Phenylalanine-Induced Superoxide Generation and Degranulation in Mouse Neutrophils: Phospholipase D Is Dispensable

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Phospholipase D (PLD), which produces the lipid messenger phosphatidic acid (PA), has been implicated in superoxide generation and degranulation in neutrophils. The basis for this conclusion is the observation that primary alcohols, which interfere with PLD-catalyzed PA production, inhibit these neutrophil functions. However, off-target effects of primary alcohols cannot be totally excluded. Here, we generated *PLD*$^{-/-}$ mice in order to reevaluate the involvement of PLD in and investigate the molecular mechanisms of these neutrophil functions. Surprisingly, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) induced these functions in *PLD*$^{-/-}$ neutrophils, and these functions were suppressed by ethanol. These results indicate that PLD is dispensable for these neutrophil functions and that ethanol nonspecifically inhibits them, warning against the use of primary alcohols as specific inhibitors of PLD-mediated PA formation. The calcium ionophore ionomycin and the membrane-permeative compound 1-oleoyl-2-acetyl-*sn*-glycerol (OADG) synergistically induced superoxide generation. On the other hand, ionomycin alone induced degranulation, which was further augmented by OADG. These results demonstrate that conventional protein kinase C (cPKC) is crucial for superoxide generation, and a Ca^{2+}-dependent signaling pathway(s) and cPKC are involved in degranulation in mouse neutrophils.

Neutrophils play an important role in host defense against bacterial infections and are the major component of the nonspecific cell-mediated immune system (1). Upon activation of chemotactic factor receptors, neutrophils evoke multiple inflammatory responses, including phagocytosis, chemotaxis, generation of reactive oxygen species, and release of antimicrobial molecules from granules to attack invading microorganisms (2, 3, 4). *N*-Formyl-methionyl-leucyl-phenylalanine (fMLP) is one of the potent leukocyte chemotactic factors which bind to cell surface receptors coupled to the heterotrimeric G protein G_{i2} (5). fMLP stimulation of neutrophils activates a wide variety of intracellular signaling pathways mediated by phosphatidylinositol-specific phospholipase C (PLC), phospholipase A_2, phospholipase D (PLD), phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinases (MAPKs) to induce various cellular functions (6, 7).

PLC has been reported to be involved in superoxide generation and degranulation in neutrophils (8). In response to fMLP stimulation, PLC is rapidly activated and hydrolyzes phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) to generate diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3) (9), the former being an activator of protein kinase C (PKC) (10) and the latter triggering the release of Ca^{2+} from the endoplasmic reticulum (ER) to increase the cytosolic Ca^{2+} concentration (11). Conventional PKC (cPKC), which is synergistically activated by DG and Ca^{2+} in conjunction with phosphatidylserine (PS) (12, 13), has been suggested to play a role in superoxide generation and degranulation (14, 15). In addition, PI3K and MAPK are also suggested to be involved in these neutrophil events (16–20). Thus, the molecular mechanisms which regulate superoxide generation and degranulation are very complicated.

A number of studies have shown that PLD also plays essential roles in fMLP-induced superoxide generation and degranulation in neutrophils (21–23). PLD hydrolyzes the membrane phospholipid phosphatidylcholine (PC) to generate the lipid messenger phosphatidic acid (PA) (24, 25) and is believed to exert its functions via its product PA (26, 27). To date, two major classical mammalian PLD isozymes, PLD1 and PLD2, have been identified (28, 29). They are activated by different mechanisms and involved in a wide variety of cell functions. PLD1 has a low basal activity *in vitro*, is directly activated by small G proteins, such as Arf, Rho, Ral, and cPKC, in conjunction with $PI4,5P_2$ (30–32), and regulates several cellular events, including vesicular trafficking (33, 34) and exocytosis (35–37). In contrast, PLD2 has a constitutively high activity in the presence of $PI4,5P_2$ and is insensitive to PLD1 activators (28, 32). PLD2 has been implicated in various cellular events, such as antiapoptosis (38), endocytic recycling (39), endocytosis (40), differentiation of PC12 cells (41), actin-based membrane dynamics (42), and L1-dependent neurite outgrowth (43). Conclusions about some of these PLD functions are based on observations from experiments using primary alcohols, such as ethanol (EtOH) and 1-butanol. Since specific inhibitors for PLD were unavailable till recently, primary alcohols were widely utilized as inhibitors of the PLD-mediated signaling pathway to investigate PLD functions. In the presence of primary alcohols, PLD preferentially catalyzes the transphosphatidylation reaction, in which the phosphatidyl group of PC is transferred to the hydroxyl group of primary alcohols to yield the nonphysiological products

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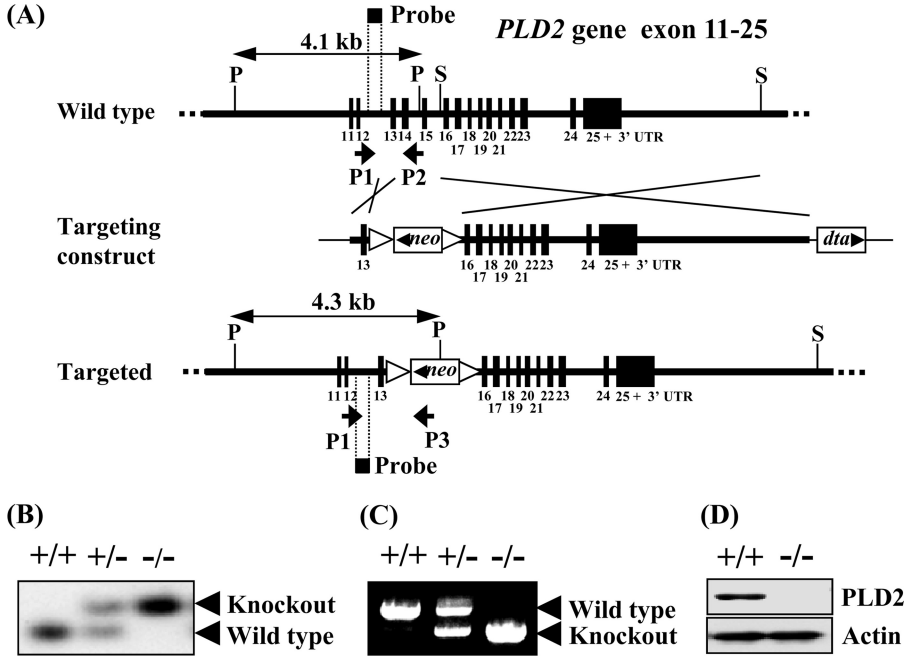

FIG 1 Generation of *PLD2*-null mice. (A) Schematic representation of *PLD2* targeting showing the partial genomic structure of the *PLD2* locus (wild-type allele), a linearized *PLD2* targeting vector (targeting construct), and a targeted *PLD2* gene allele (targeted). A neomycin resistance cassette (*neo*), *loxP* sites (triangles), and exons (filled rectangles with the exon number) are shown. Primers used for genomic PCR (P1 to P3) and a probe fragment for Southern blotting are represented by arrows and black boxes, respectively. The relevant restriction fragments for Southern blotting are shown by horizontal lines with fragment sizes. "S" and "P" represent SmaI and PstI sites, respectively. Deletion of the *PLD2* gene was verified by Southern blotting (B), genomic PCR (C), and Western blotting (D).

phosphatidyl alcohols at the expense of PA (44). Thus, primary alcohols interfere with PLD-catalyzed PA production, being believed to thereby inhibit the PLD-mediated signaling pathway and consequently cellular functions (45, 46). However, more recent studies raised concern about off-target effects of primary alcohols, even when the tertiary alcohol is used as a control, and emphasized that role of PLD in cell functions should be reevaluated (47, 48).

In the present study, we generated *PLD* knockout ($PLD^{-/-}$) mice. Using neutrophils prepared from these mice, we reevaluated the involvement of PLD in fMLP-induced superoxide generation and degranulation and also examined molecular mechanisms of these neutrophil functions. The results obtained in this study demonstrate that PLD is dispensable for these neutrophil functions and that primary alcohols have the off-target effect(s) of inhibiting them, warning against the use of primary alcohols as the specific inhibitor for PLD-catalyzed PA formation. Furthermore, we provide evidence that cPKC is crucial for superoxide generation and that a Ca^{2+}-dependent signaling pathway(s), cPKC, and additional factors coordinately regulate degranulation.

MATERIALS AND METHODS

Targeted disruption of PLD1 and *PLD2* genes. $PLD1^{-/-}$ mice were generated as previously described (49). A schematic representation of *PLD2* targeting is shown in Fig. 1A. For targeting *PLD2*, a targeting vector which replaces exon 14 and 15 of *PLD2* with a *neo* cassette was constructed. This targeting construct was linearized and electroporated into TT2 embryonic stem (ES) cells. ES cell colonies resistant to G418 were screened for homologous recombination with the targeting vector homology arms by PCR using primers specific to *PLD* and *neo*. The selected colonies were confirmed for recombination by Southern blotting. Chimeric male mice were obtained by injection of the selected ES clones into blastocysts, and the mice were crossed with C57BL/6J females. Germ line transmission of the targeted allele was confirmed by Southern blotting of tail DNA (Fig. 1B) using the DNA probes shown in Fig. 1A. Genotyping was performed by PCR amplification (Fig. 1C) with primers P1 to P3 (Fig. 1A) (5′-GGGAGATAGAAACAGGAAGACTAGG-3′, 5′-AGTAGCTTGGAGCCTCTGAAAGGCT-3′, and 5′-CACGGGTGTTGGGTCGTTTGTTCGG-3′, respectively) for *PLD2*. Protein expression of PLD2 in the mice was assessed by Western blotting (Fig. 1D).

All experiments with mice were conducted according to the Guidelines for Proper Conduct of Animal Experiments, Science Council of Japan, and protocols were approved by the Animal Care and Use Committee, University of Tsukuba.

Antibodies and chemicals. Anti-PLD1 and -PLD2 antibodies were prepared as previously described (43). Rabbit anti-sheep IgG, antiactin antibody, cytochalasin B (CB), cytochrome *c*, and fMLP were purchased from Sigma, anti-$p47^{Phox}$ and anti-phospho-$p47^{Phox}$ (Ser-370) antibodies were from Assay Biotech, 1-oleoyl-2-acetyl-*sn*-glycerol (OADG) was from Avanti Polar Lipids, and R59022, calphostin C (UCN-1028c), and ionomycin were from Calbiochem. Percoll and sheep red blood cells (SRBCs) in Alsever's solution were obtained from GE Healthcare and Nippon Biotest Laboratories Inc., respectively. 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine ([^{3}H]lyso-PAF) was purchased from Muromachi Yakuhin, and $^{32}P_i$ was from PerkinElmer Japan. 5-Fluoro-2-iodoyl deschlorohalopemide (FIPI) was a generous gift from M. A. Frohman.

Southern blot analysis. Genomic DNA extracted from mouse tails was digested with PstI for genotyping of $PLD2^{-/-}$ mice. The digested DNAs were separated by agarose electrophoresis, denatured with 0.5 M NaOH–1.5 M NaCl, transferred onto Hybond-N+ membranes (Amersham Pharmacia), anchored to the membrane by UV cross-linker (BM Equipment), and detected with DNA probes, which were generated by PCR amplification using the oligonucleotides 5′-GGGACGTTATTGGAAGGACTGCGGC-3′ and 5′-CCTAGTCTTCCTGTTTCTATCTCCC-3′. DNA probes were labeled with digoxigenin (DIG) using a DIG High Prime II labeling and detection starter kit (Roche Diagnostics) and a High Pure PCR product purification kit (Roche Diagnostics).

RT-PCR. Total RNA was extracted from the mouse brains and neutrophils using TRIzol (Invitrogen) and reverse transcribed using Super Script III (Invitrogen). The products were amplified by PCR using the primers 5′-AGATAGCGGGGCTATTGCCTTCG-3′ and 5′-CTTTCCGT TTGAGGATGCAGTCCAG-3′ for *PLD1* and 5′-AAATTTACCTGAAGC GTCCAGCC-3′ and 5′-AGTCCTTGGTGATGAGGTTGC-3′ for *PLD2*.

Western blotting. For PLD detection, mouse brain and neutrophils were lysed in urea buffer (8 M urea, 50 mM Tris-HCl [pH 6.8], 0.01% SDS, 0.01% β-mercaptoethanol, and 0.001% bromophenol blue) and sonicated. Proteins in the lysate were heat denatured, separated by SDS-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride (PVDF) membranes (Nippon Genetics Co., Ltd., Japan). PLD1, PLD2, and actin were detected using antibodies specific to each protein as previously described (43). $p47^{Phox}$ and phospho-$p47^{Phox}$ (Ser-370) in neutrophils were also detected as described above using antibodies specific to each protein, except that neutrophils were lysed in lysis buffer (25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgCl_2$, 10 mM KCl, and 1% Triton X-100).

Preparation of mouse neutrophils. To prepare peritoneal neutrophils, 8- to 12-week-old mice were injected intraperitoneally with 2% casein–phosphate-buffered saline (PBS). After 4 h, peritoneal exudate cells were collected, spun down, and suspended in Hanks balanced salt solution (HBSS; 20 mM HEPES-NaOH [pH 7.4], 138 mM NaCl, 5.3 mM KCl, 0.34 mM NaH_2PO_4, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.26 mM $CaCl_2$, 0.82 mM $MgSO_4$, and 5.56 mM D-glucose) supplemented with 0.1% bovine serum albumin (HBSS–0.1% BSA). The purity of neutrophils thus obtained was more than 90%.

Neutrophils were also prepared from bone marrow (BM) of 8- to 12-week-old mice as previously described (47). Briefly, BM cells were suspended in HBSS–0.25% BSA and centrifuged over a discontinuous 62-to-55% Percoll gradient in HBSS. BM neutrophils, which were obtained from the upper layer, were centrifuged and resuspended in HBSS–0.1% BSA. Red blood cells were removed by lysis in Tris-buffered ammonium chloride (0.017 M Tris-HCl [pH 7.65], 0.75% ammonium chloride).

Opsonization of SRBCs. IgG-opsonized SRBC (IgG-SRBC) were prepared as previously reported (47). Briefly, 10 μl of SRBCs in Alsever's solution were washed with and resuspended in HBSS–0.1% BSA. SRBCs were opsonized with 1/1,000-diluted rabbit anti-sheep IgG by agitation at room temperature for 20 min. IgG-SRBCs were then washed with and resuspended in HBSS–0.1% BSA.

Assay for superoxide generation. Neutrophils suspended in HBSS–0.1% BSA at 3.0×10^6 cells/ml were pretreated with various inhibitors as described in the figure legends and primed with 5 μM CB at 37°C for 5 min in the presence of cytochrome *c* (80 μM). Neutrophils were then stimulated with various concentrations of fMLP for 10 min in the presence or absence of 1% EtOH and IgG-SRBC for various times or with ionomycin and/or OADG for 7 min. The reaction was stopped by chilling the cell suspension on ice, the mixture was centrifuged, and the absorbance of the supernatant was measured at 550 nm with a spectrophotometer (Beckman DU-640).

Assay for degranulation. Degranulation was assessed by measuring the activity of β-glucosaminidase released from neutrophils. Neutrophils at 1.0×10^7 cells/ml were pretreated with various inhibitors, primed with CB, and then stimulated with fMLP, IgG-SRBC, or ionomycin and/or OADG as described in the preceding paragraph. The reaction was stopped by adding ice-cold HBSS–0.1% BSA, and the mixture was centrifuged. The supernatant was collected, and the cell pellet was lysed with 0.12% Triton X-100. The substrate solution (5 mM *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside in 25 mM sodium citrate, pH 4.5) was added to the supernatant and the cell lysate and incubated at 37°C for 1 h. The reaction was stopped by adding 52 mM glycine-NaOH, pH 10.5, and absorbance at 410 nm was measured with a Varioscan system (Thermo Electron Co.). The amount of β-glucosaminidase released from neutrophils was expressed as a percentage of the total β-glucosaminidase.

Assay of PLD activity. Neutrophils were suspended in RPMI 1640–0.1% BSA at 1.0×10^7 cells/ml and incubated with [^{3}H]lyso-PAF (5 μCi/ml) at 37°C for 1 h to label PC in neutrophils. Cells were washed with PBS–0.1% BSA, suspended in HBSS–0.1% BSA plus 1% EtOH, and primed with 5 μM CB for 5 min, followed by incubation with various concentrations of fMLP for 10 min. Lipids of cells were extracted by the method of Bligh and Dyer (50) and subjected to thin-layer chromatography on a silica gel plate (silica gel 60 Å; Whatman) pretreated with 1.2% potassium oxalate. The plate was developed with chloroform-methanol-acetic acid (75:35:6, vol/vol/vol). The separated lipids were visualized by iodine staining, the spots were cut out, and their radioactivities were measured with a liquid scintillation counter to calculate the percentage of [^{3}H]phosphatidylethanol (PEt) in the total [^{3}H]phospholipids.

Measurement of PA and PEt production. Neutrophils were suspended in P_i-free minimal essential medium (Sigma)–0.1% BSA at 1.0×10^7 cells/ml and then incubated at 37°C with $^{32}P_i$ (5 μCi/ml) to label membrane phospholipids. Cells were washed with and suspended in PBS–0.1% BSA. The labeled neutrophils were pretreated with the appropriate inhibitors and primed with 5 μM CB for 5 min, followed by incubation with 10 μM fMLP in the presence or absence of EtOH for various times. Lipids of cells were extracted by the method of Bligh and Dyer (50) and subjected to two-dimensional thin-layer chromatography on a silica gel plate (silica gel, 60 Å; Whatman) treated with 1.2% potassium oxalate. The plate was first developed with chloroform–methanol–28% ammonium (65:34:6, vol/vol/vol), and then developed again vertically in the first direction by chloroform-acetone-methanol-acetic acid-H_2O (10:4:2:2:1). The separated ^{32}P-labeled lipids were visualized with a BAS2500 imaging analyzer (Fujifilm, Japan), and the percentages of [^{32}P]PA and [^{32}P]PEt in the total ^{32}P-labeled phospholipids were calculated.

RESULTS

fMLP-dependent PLD activation is completely abrogated in *PLD1*$^{-/-}$ neutrophils. To reevaluate the involvement of PLD in neutrophil functions, we generated *PLD1*$^{-/-}$ mice as previously described (49) and *PLD2*$^{-/-}$ mice, whose genotype was verified by Southern blotting, genomic PCR, and Western blotting (Fig. 1). Both *PLD1*$^{-/-}$ and *PLD2*$^{-/-}$ mice were born at the expected Mendelian ratio and were normally fertile, without any apparent mutant phenotypes (data not shown). We also generated *PLD1*$^{-/-}$/*PLD2*$^{-/-}$ double-knockout (*PLD1/2*$^{-/-}$) mice, which were also apparently healthy and fertile (data not shown).

Peritoneal neutrophils were prepared from these mice, and the expression of PLDs and fMLP-stimulated PLD activation was examined. In wild-type neutrophils, expression of PLD1, but not of PLD2, was detected by Western blotting (Fig. 2A), although lower expression of *PLD2* mRNA was detected by RT-PCR (Fig. 2B). When neutrophils were stimulated with fMLP, PLDs in wild-type and *PLD2*$^{-/-}$ neutrophils were activated by fMLP stimulation in a dose-dependent manner, while fMLP-induced PLD activation was completely abrogated in *PLD1*$^{-/-}$ and *PLD1/2*$^{-/-}$ neutrophils (Fig. 2C). These results demonstrate that PLD1 is the major isozyme responsible for fMLP-induced PLD activation in neutrophils.

Deletion and pharmacological inhibition of PLDs do not affect fMLP-evoked neutrophil functions. To reevaluate the involvement of PLD in neutrophil functions, we analyzed the effects of *PLD* deletion on fMLP-induced superoxide generation and degranulation, both of which have been reported to implicate PLD (22, 23). Surprisingly, these fMLP-induced neutrophil functions were observed in *PLD1*$^{-/-}$, *PLD2*$^{-/-}$, and *PLD1/2*$^{-/-}$ neutrophils as well as in wild-type neutrophils (Fig. 3), leading us to conclude that PLD is inessential for both superoxide generation and degranulation.

To confirm the dispensability of PLD for these neutrophil

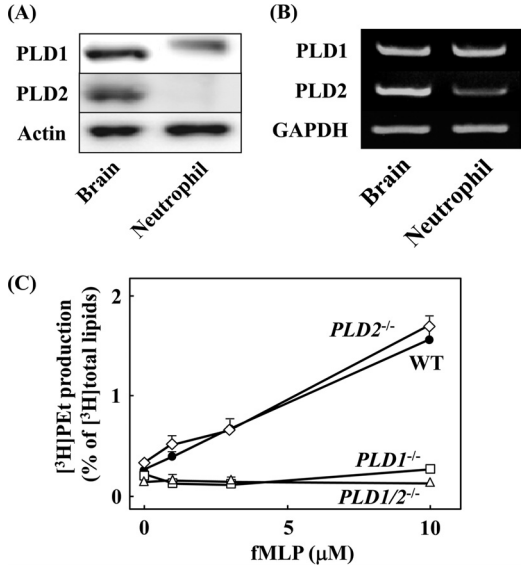

FIG 2 Expression of PLD isozymes in mouse neutrophils and their activation in response to fMLP. Expression of PLD1 and PLD2 in the mouse brains and neutrophils was analyzed by Western blotting (A) and RT-PCR (B). Actin (A) and GAPDH (B) were also analyzed as internal controls. (C) Wild-type, *PLD1*$^{-/-}$, *PLD2*$^{-/-}$, and *PLD1/2*$^{-/-}$ neutrophils labeled with [^{3}H]lyso-PAF (5 μCi/ml) were primed with CB and incubated with the indicated concentrations of fMLP in the presence of 1% EtOH at 37°C for 10 min, and then PLD activity was determined as described in Materials and Methods. Data are means ± standard errors of the means (SEM) from three independent experiments.

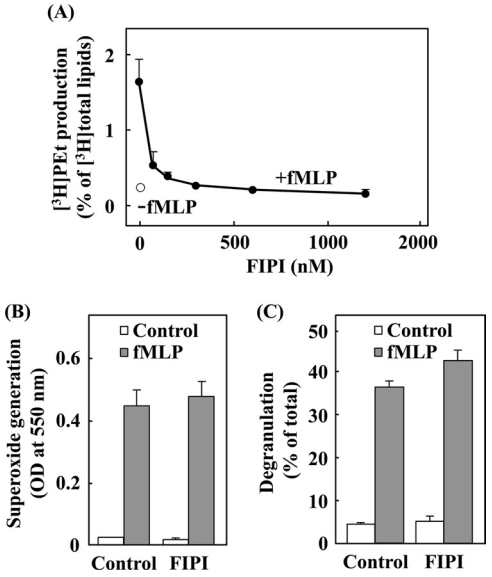

FIG 4 Pharmacological inhibition of PLDs does not affect fMLP-induced superoxide generation and degranulation. [^{3}H]lyso-PAF-labeled wild-type neutrophils pretreated with the indicated concentrations of FIPI in the presence of CB and EtOH were stimulated with 10 μM fMLP at 37°C for 10 min, and then PLD activity was determined (A). Wild-type neutrophils were pretreated with 750 nM FIPI in the presence of CB and stimulated with 10 μM fMLP, and then superoxide generation (B) and degranulation (C) were determined. Data are means ± SEM from at least three independent experiments.

functions, the potent PLD inhibitor FIPI, an analog of the dopamine receptor antagonist (51), was employed. This compound was initially identified as a PLD2 inhibitor (51), but Su et al. (52) showed that the compound inhibits both PLD1 and PLD2. When neutrophils were pretreated with FIPI, fMLP-induced PLD activation was strongly inhibited in a concentration-dependent manner (Fig. 4A), consistent with the previous study, which used other types of cells (52). On the other hand, FIPI failed to inhibit fMLP-evoked superoxide generation and degranulation (Fig. 4B and C), again supporting the notion that PLD is not essential for these neutrophil functions.

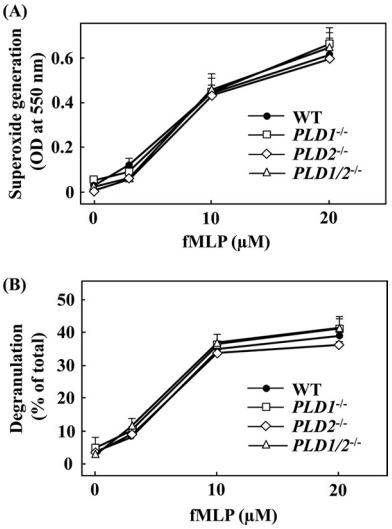

FIG 3 Deletion of *PLD* genes does not affect fMLP-induced superoxide generation and degranulation. Wild-type, *PLD1*$^{-/-}$, *PLD2*$^{-/-}$, and *PLD1/2*$^{-/-}$ neutrophils primed with CB were incubated with the indicated concentrations of fMLP at 37°C for 10 min. Superoxide generation (A) and degranulation (B) were then determined as described in Materials and Methods. Data are means ± SEM from three independent experiments.

It was recently reported that PLD1 regulates fMLP receptor- and Fcγ receptor-stimulated production of reactive oxygen species in neutrophils (47), which is inconsistent with the results obtained in this study. One of the differences in the experiments is the source of neutrophils: Norton et al. (47) used BM neutrophils, while we employed peritoneal neutrophils. However, this is not responsible for the differences in our results, since the extent of fMLP-induced superoxide generation in BM neutrophils prepared from *PLD1/2*$^{-/-}$ mice was almost the same as that in wild-type neutrophils (Fig. 5A). Another possible reason for the different results is the use of different agonists to stimulate neutrophils. However, this is also unlikely, since superoxide generation by Fcγ receptor stimulation in *PLD1/2*$^{-/-}$ peritoneal neutrophils was induced to almost the same level as that in wild-type neutrophils (Fig. 5B). Although at present we cannot explain why the results of our study are different from the observations by Norton et al. (47), it is plausible that difference in the priming manipulation of neutrophil is responsible for the different results: in the present study, we used the cytoskeleton-disrupting compound CB as a priming reagent, while Norton et al. (47) employed tumor necrosis factor alpha (TNF-α) and granulocyte-macrophage colony-stimulating factor, the former inducing mobilization of receptor-storing neutrophil organelles (53) and the latter partially phosphorylating p47^{Phox} (54). This point remains to be clarified.

EtOH inhibits fMLP-induced cell functions in *PLD*$^{-/-}$ neutrophils as well as in wild-type neutrophils. The results shown above are inconsistent with previous reports that PLD is required

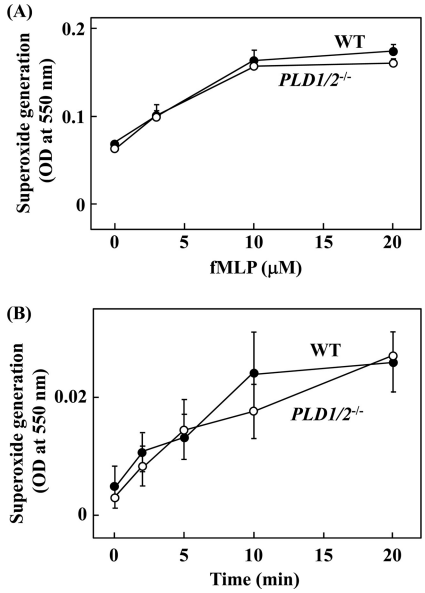

FIG 5 Deletion of *PLD* genes does not affect superoxide generation induced by fMLP in BM neutrophils and by IgG-SRBC in peritoneal neutrophils. Wild-type and $PLD1/2^{-/-}$ BM neutrophils primed with CB were incubated with the indicated concentrations of fMLP at 37°C for 10 min (A). Wild-type and $PLD1/2^{-/-}$ peritoneal neutrophils primed with CB were incubated with the IgG-SRBC at 37°C for the indicated times (B). Superoxide generation was then determined as described in Materials and Methods. Data are as means ± SEM from three independent experiments. *, $P < 0.05$ (Student's *t* test).

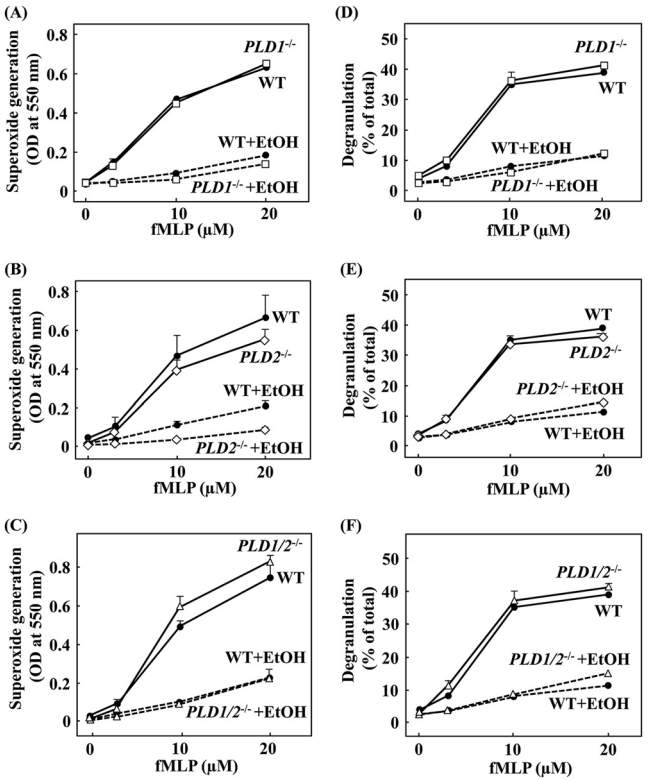

FIG 6 EtOH inhibits fMLP-induced cell functions in both wild-type and $PLD^{-/-}$ neutrophils. Wild-type, $PLD1^{-/-}$, $PLD2^{-/-}$, and $PLD1/2^{-/-}$ neutrophils primed with CB were stimulated with the indicated concentrations of fMLP at 37°C for 10 min in the presence or absence of 1% EtOH. Superoxide generation (A to C) and degranulation (D to F) were then measured as for Fig. 3. Data are means ± SEM from three independent experiments.

for fMLP-induced superoxide generation and degranulation (22, 23). Many previous studies utilized primary alcohols that inhibit PLD-catalyzed PA production. The results shown above raised the possibility that primary alcohols nonspecifically interfere with a signaling pathway(s) couple to these neutrophil functions, as was emphasized previously (47, 48). Consistent with this assumption, EtOH suppressed both fMLP-induced superoxide generation and degranulation in $PLD1^{-/-}$, $PLD2^{-/-}$, and $PLD1/2^{-/-}$ neutrophils as well as in wild-type neutrophils (Fig. 6). Thus, inhibition of these fMLP-induced neutrophil functions by EtOH is independent of its inhibition of PLD-catalyzed PA production.

fMLP-stimulated PA production is independent of PLD. In general, PA is produced by the action of PLD and the combined action of PLC and DG kinase in mammalian cells. To investigate whether PLD contributes to fMLP-stimulated PA production in neutrophils, the PA level in $PLD1/2^{-/-}$ neutrophils was compared with that in wild-type neutrophils. Upon fMLP stimulation, the PA level was rapidly and transiently increased in wild-type neutrophils (Fig. 7A). Unexpectedly, the pattern of the PA level in $PLD1/2^{-/-}$ neutrophils upon fMLP stimulation was almost the same as that in wild-type neutrophils (Fig. 7A). When fMLP-induced PEt formation was determined under the same conditions but in the presence of EtOH, the level of PEt produced upon fMLP stimulation was very low compared with that of PA produced in the absence of EtOH (Fig. 7A and B). Furthermore, it was found that pharmacological inhibition of PLDs did not affect fMLP-stimulated PA production (Fig. 7C). These results indicate that fMLP-dependent PA production is predominantly mediated by a signaling pathway(s) independent of PLD, e.g., the PLC/DG kinase pathway (55). To address this issue, the effect of the DG kinase inhibitor R59022 (56) was examined. As shown in Fig. 7D, R59022 markedly inhibited the fMLP-stimulated PA production in a dose-dependent fashion, supporting the notion described above. Interestingly, EtOH significantly inhibited fMLP-stimulated PA production in wild-type and $PLD1/2^{-/-}$ neutrophils to almost the same extent (Fig. 7E), suggesting that EtOH inhibits PLC and/or DG kinase.

Inhibition of DG kinase augments fMLP-induced superoxide generation and degranulation. It has been reported that fMLP-induced PA production is involved in superoxide generation and degranulation in neutrophils (21–23, 57). The results shown in Fig. 7D suggest that inhibition of DG kinase by R59022 could result in an accumulation of DG, which in turn activates PKC. To investigate whether PA or DG is involved in superoxide generation and degranulation, the effects of the compound on these neutrophil functions were examined. Inhibition of DG kinase by R59022 augmented these fMLP-induced neutrophil functions (Fig. 8A and B), although the extent of augmentation in degranulation was much smaller than that in superoxide generation. Similar results were obtained by using another DG kinase inhibitor, R59949 (data not shown). These results are consistent with the notion that DG, but not PA, is important for fMLP-induced neutrophil functions, especially for superoxide generation.

Requirement of PKC activation for superoxide generation and of a Ca^{2+} signaling pathway(s) and PKC for degranulation. DG is a potent activator of cPKC and novel PKC (nPKC) (10), which have been suggested to be involved in superoxide genera-

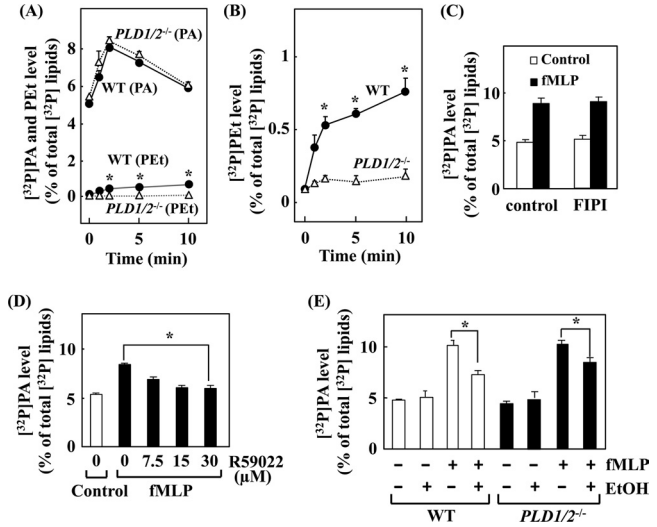

FIG 7 fMLP-induced PA formation is independent of the PLD-mediated signaling pathway. Wild-type and *PLD1/2*$^{-/-}$ neutrophils prelabeled with $^{32}P_i$ were primed with CB and stimulated with 10 μM fMLP in the presence or absence of 1% EtOH at 37°C for the indicated times, and then production of [^{32}P]PA in the absence of EtOH and [^{32}P]PEt in the presence of EtOH were measured (A and B) as described in Materials and Methods. [^{32}P]PEt formation (A) is shown in panel B on an enlarged scale. Wild-type neutrophils pretreated with 750 nM FIPI at 37°C for 10 min were primed with CB and stimulated with or without 10 μM fMLP at 37°C for 2 min, and then the production of [^{32}P]PA was measured (C). Wild-type neutrophils pretreated with the indicated concentrations of R59022 were primed with CB and stimulated with or without 10 μM fMLP at 37°C for 2 min, and then the production of [^{32}P]PA was measured (D). Wild-type and *PLD1/2*$^{-/-}$ neutrophils primed with CB were incubated with or without 10 μM fMLP in the presence or absence of 1% EtOH at 37°C for 2 min, and then the production of [^{32}P]PA was measured (E). Means ± SEM from at least three independent experiments are shown in all panels. *, $P < 0.05$ (Student's *t* test).

tion and degranulation in neutrophils (58, 59). The results shown above, taken together with these reports, indicate that cPKC or nPKC rather than PLD is a key molecule regulating these neutrophil functions. To test this assumption, we employed the PKC

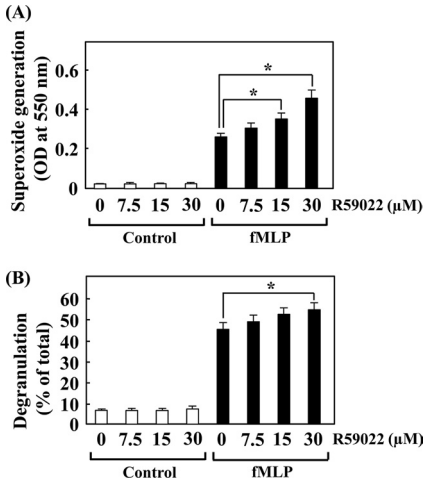

FIG 8 The DG kinase inhibitor R59022 enhances fMLP-induced superoxide generation and degranulation. Wild-type neutrophils pretreated with R59022 in the presence of CB were stimulated with or without 10 μM fMLP at 37°C for 10 min, and superoxide generation (A) and degranulation (B) were measured. Data are means ± SEM from three independent experiments. *, $P < 0.05$ (Student's *t* test).

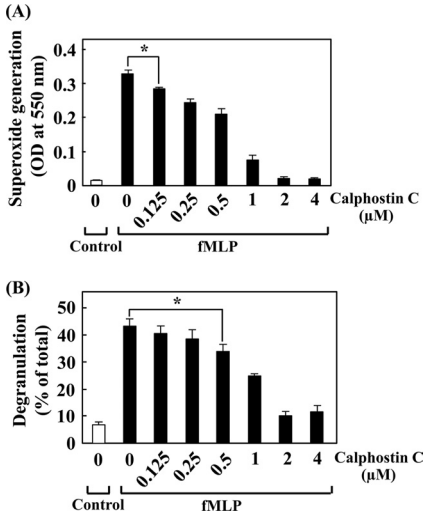

FIG 9 Pharmacological inhibition of PKC interferes with fMLP-induced superoxide generation and degranulation. Wild-type neutrophils were preincubated with the indicated concentrations of calphostin C at 37°C for 15 min. After calphostin C was photoactivated by fluorescent light at room temperature for 10 min, neutrophils were primed with CB and stimulated with 10 μM fMLP for 10 min, and then superoxide generation (A) and degranulation (B) were measured. Data are means ± SEM from three independent experiments. *, $P < 0.05$ (Student's *t* test).

inhibitor calphostin C, which competes with DG for binding to PKC (60). As was expected, calphostin C efficiently blocked both fMLP-induced superoxide generation and degranulation in a dose-dependent manner (Fig. 9), although its ability to inhibit superoxide generation was much greater than its ability to inhibit degranulation. Nevertheless, these results are consistent with the notion that cPKC or nPKC is required for these fMLP-induced neutrophil functions.

DG and Ca^{2+} are both required to fully activate cPKC, while nPKC and atypical PKC are insensitive to Ca^{2+} and DG/Ca^{2+}, respectively (10). To investigate which subfamily of PKC is required for superoxide generation and degranulation, the membrane-permeative DG, OADG, and the Ca^{2+} ionophore ionomycin were employed. When wild-type neutrophils were treated with OADG or ionomycin alone, superoxide generation was not induced at all (Fig. 10A). Application of both OADG and ionomycin synergistically induced superoxide generation in an OADG concentration-dependent manner (Fig. 10A), consistent with a previous study using differentiated HL-60 cells (14), which suggested that the cPKC isozyme PKCα is implicated in fMLP-induced superoxide generation. A drastic increase of OADG- and ionomycin-induced superoxide generation by the DG kinase inhibitor R59022 (Fig. 10B) also supports this assumption. Similar results for synergistic induction of superoxide generation by ionomycin and OADG and its augmentation by R59022 were obtained with *PLD1/2*$^{-/-}$ neutrophils (Fig. 10C). On the other hand, ionomycin alone evidently induced degranulation, and OADG augmented ionomycin-induced degranulation (Fig. 11A). These results suggest that a Ca^{2+}-dependent signaling pathway(s) and cPKC are both at least partly involved in degranulation. OADG/ionomycin-induced degranulation was slightly augmented by R59022 (Fig. 11B). However, the extent of degranulation induced by OADG/ionomycin in the presence of R59022 was much lower than that induced by fMLP, indicating that an additional signaling path-

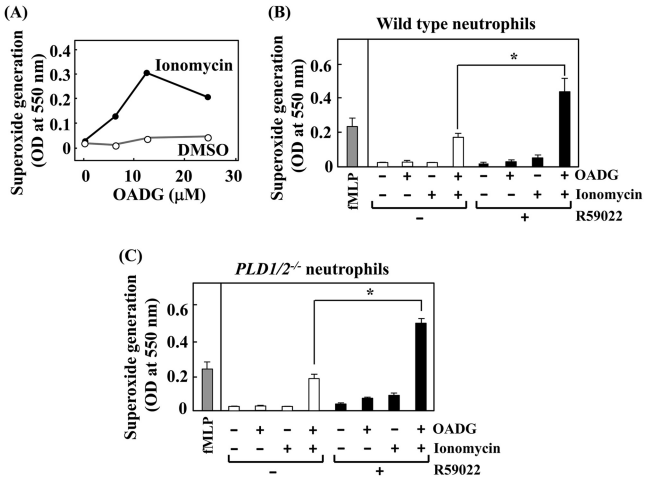

FIG 10 Induction of superoxide generation by ionomycin and OADG. Wild-type neutrophils primed with CB were incubated with the indicated concentrations of OADG in the presence or absence of 1 μM ionomycin at 37°C for 7 min, and superoxide generation was measured (A). Wild-type (B) or *PLD1/2*$^{-/-}$ (C) neutrophils primed with CB were incubated with or without 12.5 μM OADG and/or 1 μM ionomycin in the presence or absence of 30 μM R59022 at 37°C for 7 min. The cells were also stimulated with 10 μM fMLP at 37°C for 10 min. After treatment, superoxide generation was measured (B and C). Data are means ± SEM from three independent experiments. *, $P < 0.05$ (Student's *t* test).

way(s) is required to maximally induce degranulation. Similar effects of ionomycin, OADG, and R59022 on degranulation were observed in *PLD1/2*$^{-/-}$ neutrophils (Fig. 11C).

The regulation of fMLP-induced superoxide generation by PKC is at least in part mediated through the phosphorylation of p47^{Phox} at several serine residues (61, 62). Since Ser-370 of p47^{Phox} has been reported to be phosphorylated by PKCs, the effects of ionomycin and OADG on phosphorylation of p47^{Phox} at Ser-370 were investigated. As shown in Fig. 12, ionomycin and OADG

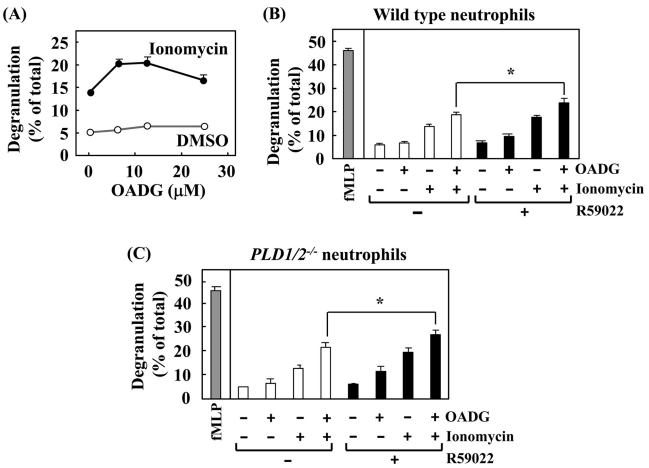

FIG 11 Induction of degranulation by ionomycin and OADG. Wild-type neutrophils primed with CB were incubated with ionomycin and/or OADG as in Fig. 10A, and degranulation was measured (A). Wild-type (B) and *PLD1/2*$^{-/-}$ (C) neutrophils primed with CB were incubated with ionomycin and/or OADG in the presence or absence of R59022 as for Fig. 10B and C. The cells were also stimulated with fMLP as for Fig. 10B and C. After treatment, degranulation was measured (B and C). Data are means ± SEM from three independent experiments. *, $P < 0.05$ (Student's *t* test).

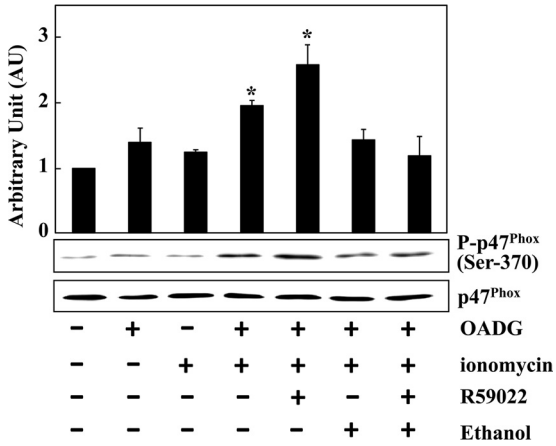

FIG 12 Phosphorylation of p47^{Phox} by OADG and ionomycin and its inhibition by EtOH. Wild-type neutrophils were pretreated with or without R59022 and incubated at 37°C with or without OADG and/or ionomycin in the presence or absence of EtOH for 7 min. Expression levels of phospho-p47^{Phox} (Ser-370) and p47^{Phox} were then determined by Western blot analysis as described in Materials and Methods. Data are means ± SEM from three independent experiments. *, $P < 0.05$ (Student's *t* test).

synergistically stimulated phosphorylation of p47^{Phox} at Ser-370. This ionomycin/OADG-dependent phosphorylation of p47^{Phox} was augmented by the DG kinase inhibitor R59022. Furthermore, we found that phosphorylation of p47^{Phox} stimulated by ionomycin/OADG in the presence or absence of R59022 was significantly inhibited by EtOH, suggesting that EtOH also inhibits PKCs, in addition to PLC and/or DG kinase. Thus, the augmentation of fMLP- and ionomycin/OADG-induced superoxide generation by the DG kinase inhibitor (Fig. 8A and 10B) might be mediated through the enhanced phosphorylation of p47^{Phox} by PKC.

DISCUSSION

PLD has been implicated in fMLP-induced superoxide generation and degranulation in neutrophils (22, 23, 63). This notion was based on observations from experiments using primary alcohols, which suppress PLD-catalyzed PA production by preferentially catalyzing the transphosphatidylation reaction. However, this is not conclusive, since it is not clear whether this effect of primary alcohols inhibiting PLD-catalyzed PA production is specific. Thus, the involvement of PLD in these neutrophil functions should be reevaluated using other reliable techniques or manipulations.

To address this issue, we utilized a gene manipulation technique in this study. We showed that *PLD*$^{-/-}$ neutrophils, as well as wild-type neutrophils, evoked superoxide generation and degranulation in response to fMLP stimulation (Fig. 3), clearly demonstrating that PLD is not essential for these neutrophil functions. In addition, we showed that EtOH inhibited fMLP-induced superoxide generation and degranulation in *PLD*$^{-/-}$ neutrophils as well as in control cells (Fig. 6), suggesting that primary alcohols nonspecifically inhibit these fMLP-induced neutrophil functions. The inhibition of fMLP-stimulated, PLD-independent PA production by EtOH (Fig. 7E) also supports this notion. Thus, these observations warn against the use of primary alcohols as specific inhibitors for PLD-mediated PA formation.

What are the molecular mechanisms through which superoxide generation and degranulation are induced in response to fMLP

stimulation? PKC appears to play a pivotal role in fMLP-induced superoxide generation, as calphostin C strongly inhibited this neutrophil function at lower concentrations than those inhibiting degranulation (Fig. 9). Of PKC subfamilies, cPKC is most likely to be implicated in superoxide generation, since Ca^{2+} and DG, both of which are required to fully activate cPKC in the presence of the membrane phospholipid PS, synergistically induced superoxide generation to the same extent as fMLP (Fig. 10A and B). Involvement of cPKC is also supported by the observation that ionomycin/OADG-induced superoxide generation was further augmented by the pharmacological inhibition of DG kinase to a much greater extent than that induced by fMLP alone (Fig. 10B), probably due to the sustainable accumulation of DG. Although PKCα has been reported to be implicated in fMLP-induced superoxide generation in differentiated HL60 cells, it remains to be elucidated which isozyme of cPKC is involved in this function of neutrophils; it is very difficult to answer this question in the case of neutrophils, because the powerful technique of gene knockdown cannot be applied to neutrophils. To address this issue, neutrophils with knockouts of each cPKC isozyme are needed.

Under physiological conditions, DG production and elevation of the cytosolic Ca^{2+} concentration are elicited by the hydrolysis of the membrane phospholipid $PI4,5P_2$ by PLC: PLC hydrolyzes $PI4,5P_2$ to produce DG and IP_3, the latter triggering the release of Ca^{2+} from the ER to raise the cytosolic Ca^{2+} concentration (11). Thus, it is reasonable to speculate that PLC functions as the upstream molecule of cPKC in superoxide generation, consistent with the previous report that *PLC*β*2*-null neutrophils lose the ability to induce fMLP-dependent superoxide generation (64).

The regulatory mechanism of degranulation in mouse neutrophils is different from that of superoxide generation, although cPKC seems to be at least partly involved in it. Ca^{2+} appears to be essential for degranulation, as ionomycin by itself induced degranulation (Fig. 11A and B). In mammalian cells, the cytosolic Ca^{2+} concentration is raised by the influx of extracellular Ca^{2+} into cells or its release from the ER (65). In degranulation, Ca^{2+} release from the ER appears to be critical since the cytosolic Ca^{2+} chelator BAPTA-AM inhibited degranulation induced by fMLP in the absence of extracellular Ca^{2+} (data not shown). Although at present it is not clear how intracellular Ca^{2+} regulates degranulation, it may induce degranulation of releasable pools of granules as previously reported (66). Ca^{2+} may regulate its target molecules such as the Ca^{2+} sensor synaptotagmin, which regulates Ca^{2+}-sensitive vesicle fusion in neutrophils (67). In addition to Ca^{2+}, cPKC seems to be also required to further augment degranulation: ionomycin-induced degranulation was further increased by the addition of OADG (Fig. 11A and B). In this case, cPKC is likely involved, since application of OADG induced degranulation in the presence, but not in the absence, of ionomycin, again supporting the notion of the essential role of Ca^{2+} in degranulation. However, the extent of degranulation induced by Ca^{2+} and OADG was much lower than that induced by fMLP (Fig. 11B), suggesting that an additional signaling pathway(s) is required for the maximal induction of degranulation. It is plausible that the MAP kinase cascade and PI3K signaling pathways, which have been implicated in degranulation (16, 19, 68), may cooperate with Ca^{2+} and cPKC signaling pathways in degranulation.

It has been shown that the PA level in neutrophils increases in response to fMLP stimulation (22, 23), which was also observed in this study (Fig. 7A). The PA elevation has long been believed to result, at least in part, from the hydrolysis of the membrane phospholipid PC by the action of PLD. Inconsistent with this notion, however, fMLP-dependent elevation of the PA level was observed in *PLD1/2*$^{-/-}$ neutrophils as well as in wild-type neutrophils (Fig. 7A), indicating that PA is produced through another pathway(s) independent of the PLD-mediated signaling pathway under these conditions. The observation that PEt formation upon fMLP stimulation was significant but very small compared with PA formation (Fig. 7A and B) also supports this idea. PA can be generated through two alternative pathways: phosphorylation of PLC-generated DG by DG kinase (55) and acylation of lyso-PA (LPA) by LPA acyltransferase (69). It is most likely that the PLC/DG kinase pathway is responsible for PA production, as pharmacological inhibition of DG kinase almost completely suppressed the fMLP-induced PA production (Fig. 7D).

As shown in Fig. 7E, EtOH inhibited fMLP-dependent PA production in wild-type and *PLD1/2*$^{-/-}$ neutrophils. Since PA seemed to be exclusively produced through the PLC/DG kinase pathway in fMLP-stimulated neutrophils (Fig. 7A to D), it is reasonable to speculate that EtOH nonspecifically inhibits this pathway.

It is noteworthy that PLD1 was activated in response to fMLP stimulation of neutrophils (Fig. 2C), although a decrease in the total cellular PA level was not observed in *PLD1/2*$^{-/-}$ neutrophils under the conditions in this study (Fig. 7A). It is plausible, however, that PLD1 is activated at the limited area of cellular compartments to increase the local PA concentration. Our results raise the question of what kinds of neutrophil functions are regulated by PLD through the local PA production. In addition to superoxide generation and degranulation, fMLP induces a wide variety of cellular events, such as phagocytosis, chemotaxis, and cell adhesion. Nishikimi et al. (70) have reported that inhibition of PLD by FIPI attenuates fMLP-induced neutrophil chemotaxis. Furthermore, using more reliable techniques such as downregulation and overexpression, PLD has been revealed to be implicated in integrin-mediated cell spreading in ovarian cancer (71) and COS7 cells (72) and in phagocytosis in RAW 264.7 (73), and THP-1 and K562-CR3 (74) cells. Thus, it is of interest to investigate what kinds of neutrophil functions are regulated by PLD.

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