Group V Phospholipase A2 Induces Leukotriene Biosynthesis in Human Neutrophils through the Activation of Group IVA Phospholipase A2*

We reported previously that exogenously added human group V phospholipase A2 (hVPLA2) could elicit leukotriene B4 (LTB4) biosynthesis in human neutrophils (Han, S. K., Kim, K. P., Koduri, R., Bittova, L., Munoz, N. M., Leff, A. R., Wilton, D. C., Gelb, M. H., and Cho, W. (1999) J. Biol. Chem. 274, 11881–11888). To determine the mechanism of the hVPLA2-induced LTB4 biosynthesis in neutrophils, we thoroughly examined the effects of hVPLA2 and their lipid products on the activity of group IVA cytosolic PLA2 (cPLA2) and LTB4 biosynthesis under different conditions. As low as 1 nM exogenous hVPLA2 was able to induce the release of arachidonic acid (AA) and LTB4. Typically, AA and LTB4 were released in two phases, which were synchronized with a rise in intracellular calcium concentration ([Ca2+]i) near the perinuclear region and cPLA2 phosphorylation. A cellular PLA2 assay showed that hVPLA2 acted primarily on the outer plasma membrane, liberating fatty acids and lysophosphatidylcholine (lyso-PC), whereas cPLA2 acted on the perinuclear membrane. Lyso-PC and polyunsaturated fatty acids including AA activated cPLA2 and 5-lipoxygenase by increasing [Ca2+]i, and inducing cPLA2 phosphorylation, which then led to LTB4 biosynthesis. The delayed phase was triggered by the binding of secreted LTB4 to the cell surface LTB4 receptor, which resulted in a rise in [Ca2+]i, and cPLA2 phosphorylation through the activation of mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2. These results indicate that a main role of exogenous hVPLA2 in neutrophil activation and LTB4 biosynthesis is to activate cPLA2 and 5-lipoxygenase primarily by liberating from the outer plasma membrane lyso-PC that induces [Ca2+]i increase and cPLA2 phosphorylation and that hVPLA2-induced LTB4 production is augmented by the positive feedback activation of cPLA2 by LTB4.

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Phospholipase A2 (PLA2) catalyzes the release from the sn-2 position of certain membrane phospholipids of arachidonic acid (AA) that can be transformed into potent inflammatory lipid mediators, including prostaglandins, leukotrienes, and thromboxanes. Multiple forms of mammalian PLA2 have been identified from mammalian tissues, which include several forms of secretory PLA2 (sPLA2) (1), group IVA Ca2+-dependent cytosolic PLA2 (cPLA2) (2), and group VI Ca2+-independent PLA2 (iPLA2) (3). Recent studies have indicated that sPLA2s work in concert with cPLA2 to induce eicosanoid formation in different cells (4–6). Neutrophils are inflammatory cells that release AA and 5-lipoxygenase products, most notably leukotriene B4 (LTB4), upon activation by various agonists, including a bacterial peptide, formyl-Met-Leu-Phe (fMLP). It was reported that human neutrophils contain several forms of endogenous PLA2s, including cPLA2, iPLA2, and group V and group X sPLA2s (7). However, roles of these PLA2s in inflammatory actions of neutrophils, biosynthesis and release of LTB4 in particular, have not been elucidated fully. Based on the effects of exogenously added sPLA2, AA, and 5-lipoxygenase (5-LO) products on cPLA2 activity, it was postulated earlier that stimulus-induced AA release or exocytosis of sPLA2 activates cPLA2 by initiating the formation of LTB4 which leads to the phosphorylation of cPLA2 (8). Similarly, both sPLA2 and cPLA2 were shown to be involved in the fMLP-stimulated AA release from human neutrophils (9). Recently, however, it was reported that fMLP-induced secretion of group V sPLA2 from human neutrophils did not lead to LTB4 biosynthesis (7). Instead, cPLA2 was reported to be entirely responsible for the fMLP-stimulated LTB4 release from human neutrophils (7). Independently, we showed that exogenously added human group V PLA2 (hVPLA2) could induce AA and LTB4 release from unprimed human neutrophils half as effectively as fMLP (10)

1 The abbreviation used are: PLA2, phospholipase A2; AA, arachidonic acid; AOCOP, acetylsalicylic acid; BSA, bovine serum albumin; cPLA2, group IVA cytosolic PLA2; DiIC12, 1',1'-diododecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; ERK, extracellular signal-regulated kinase; FMLP, formyl-Met-Leu-Phe; FRET, fluorescence resonance energy transfer; HBSS, Hanks' balanced salt solution; hIIaPLA2, human group IIa PLA2; hVPLA2, human group V PLA2; HPLC, high performance liquid chromatography; iPLA2, group VI Ca2+-independent PLA2; 5-LO, 5-lipoxygenase; LTB4, leukotriene B4; LTB4DMA, LTB4 dimethyl amide; lyso-PC, 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; OA, oleic acid; PED6, N-(6-(2,4-dinitrophenylamino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-1-bora-5A, 4A-diaza-s-indacene-8-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt; PDBG, prostaglandin B2; SACP, 1-stearyoyl-2-arachidonoyl-sn-glycero-3-phosphocholine; sPLA2, secretory PLA2.
and that hVPLA2 bound to cell surface heparan sulfate proteoglycans was eventually internalized and degraded (11). To understand better the interplay between sPLA2 and cPLA2 in glycan biosynthesis in neutrophils, we thoroughly examined the effects of sPLA2s and their lipid products on cPLA2 activity and leukotriene biosynthesis under different conditions. Results not only account for the discrepancy in previous reports but also provide new insights into the mechanism by which sPLA2 and cPLA2 work in concert to achieve effective and controlled leukotriene biosynthesis in neutrophils.

**EXPERIMENTAL PROCEDURES**

**Materials**—Arachidonitrifluorenyl methyl ketone (AACOCF3) was purchased from Biomol (Plymouth Meeting, PA). Surfactin was a generous gift from Dr. C. H. Lee of CheilJedang Co. (Incheon, Korea). The 38 mM mitogen-activated (MAP) kinase inhibitor SB203580 was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-cPLA2 monoclonal antibody was generously provided by Dr. James Clark of the Genetics Institute (Cambridge, MA). [3H]AA and [14C]-labeled oleic acid (OA) were purchased from American Radiochemical Co. (St. Louis, MO). 1-Stearoyl-2-([N-acetoxy]sn-glycero-3-phosphatriyl)choline ([NACAPC]) was obtained from Avanti Polar Lipids (Alabaster, AL). LTB4, LTB4 dimethyl amide (LTB4DMA) and [14C]OA were purchased from Molecular Probes (Eugene, OR). Anti-Active MAP kinase antibody, anti-Active-p38 MAP kinase antibody, and a mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK)1/2 antibody, Anti-Active-ERK1/2, or Anti-Active-p38 MAP kinase antibody were obtained from Cell Signaling Technology (Beverly, MA). anti-cPLA2, Anti-Active-cPLA2, Anti-active MAPK antibody, and ionomycin were purchased from Sigma.

**sPLA2-induced cPLA2 Activation in Neutrophils**—A neutrophil purity of 80% was achieved by density gradient centrifugation. Cells were seeded in 12-well plates (3 × 10⁶/sample) and grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 50 μg/ml streptomycin, and 5 μg/ml of deoxycholic acid. Cells were maintained in a humidified incubator at 37 °C and 5% CO₂ for 24 h before treatment with either sPLA2 or the substrate solution.

**Expression and Purification of sPLA2**—Recombinant human group IIa PLA2 (hIIaPLA2) was prepared as described (12). Recombinant hVPLA2 and mutants were expressed in Escherichia coli (Ann Arbor, MI). N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (PED6), Fluo-4-AM, and 1,1′-diododecyl-3,3′,3′-tetramethylindocarbocyanine perchlorate (DiIC1(2)) were purchased from Molecular Probes (Eugene, OR). Anti-1-nitro-2-hydroxy-5-succinimido-3-phosphocholesterol (lyso-PC) was purchased from Avanti Polar Lipids (Alabaster, AL). AA and ionomycin were purchased from Sigma.

**Fatty Acid and LTB4 Release from Human Neutrophils**—Dual radio-labeling of neutrophils was achieved by incubating 2 × 10⁶ cells with [3H]AA and [14C]OA (0.1 μCi/ml each) in calcium-free Hank’s balanced salt solution (HBSS) for 3 h. [3H]AA and [14C]OA that had not been incorporated into cellular lipids were removed by washing the cells three times with HBSS containing 0.2% bovine serum albumin (BSA). Radiolabeled cells (10⁶) were resuspended in 180 μl of HBSS containing 1.2 mM CaCl₂ and 0.2% BSA, preincubated with a selected inhibitor for 20 min at 37 °C if necessary, and then stimulated with hVPLA2. The reaction was quenched by centrifugation and the radioactivity in the cell pellet and the medium was measured separately by a two-channel liquid scintillation counter. LTB4 levels were determined using a LTB4 enzyme immunoassay kit from Cayman and then corrected for background signals from control cells that were not treated with hVPLA2.

**Measurement of cPLA2 Activity**—Neutrophils (2 × 10⁶ cells) were stimulated with varying concentrations of hIIaPLA2. The reaction was quenched by adding 1 ml of ice-cold water, and the reaction mixture was centrifuged. The pellet was resuspended in 70 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, containing 2.5 mM EDTA, 10 μM leupeptin, 5 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride, 2 mM VO₂, 50 mM NaF, and 5 μg/ml pepstatin) and sonicated briefly. The resulting cell lysate was pretreated with 10 μl of dithiothreitol (final concentration 10 mM) on ice for 5 min to inactivate sPLA2, and 10 μl of 1 M CaCl₂ (final concentration 0.1 mM) was then added to each sample. The cPLA2 substrate solution was prepared by drying a chloroform solution of [14C]SAIPC under a stream of N₂ and suspending the film in 100 μl of 10% aqueous ethanol by vortexing. The reaction was initiated by adding a 10 μl-portion of the substrate solution (final concentration 9 μM) to each cell lysate. The reaction was carried out for 30 min at 37 °C and quenched by adding 500 μl of Dole’s reagent (heptane, 2-propanol, 1 N H₂SO₄, water/acetic acid, 10:10:80:0.2, v/v/v/v, pH 5.5, adjusted with ammonium hydroxide), injected into a C18 reverse phase HPLC column (5 × 250 mm; Waters, Milford, MA). Water was introduced at the electrospray source of a triple quadrupole mass spectrometer (Micromass Quattro II). The column was eluted with a linear gradient of solvent B (acetonitrile/methanol, 65:35) from 40 to 100% at a flow rate of 100 μl/min over 30 min. Each leukotriene peak was monitored in the negative ion mode.
bars

hVPLA2. As reported previously (10, 11), hIIaPLA2 up to 100 nM hVPLA2 was shown to have little effect on LTB4 release. Together, these data suggest that the release of AA and LTB4 biosynthesis in human neutrophils have disparate dependence on hVPLA2 concentration acting on their cell surfaces.

We then monitored the kinetics of fatty acid release from dual labeled neutrophils by 10 nm hVPLA2. As shown in Fig. 2A, the [14C]OA release showed simple saturation kinetics, whereas the [3H]AA release exhibited more complex two-phase kinetics under the same conditions, suggesting that at least two distinct pathways are involved in the latter case. The early phase of [3H]AA release reached a plateau in 10–20 min, as was the case with the [14C]OA release; however, the delayed phase of [3H]AA release followed after ~20 min and extended for about 1 h. When neutrophils were incubated with a cPLA2 inhibitor, AACOCF3 (25 μM) (18) or surfactin (10 μM) (19), prior to the addition of hVPLA2, the delayed phase AA release was abrogated, whereas the early phase AA release was modestly (about 30%) reduced. On the other hand, OA release remained essentially unchanged after treatment with cPLA2 inhibitors. An iPLA2 inhibitor, bromoelolactone (10 μM) had little effect on the time course of fatty acid release (data not shown).

In conjunction with the data shown in Fig. 1, these data suggest that in the presence of 10 nm exogenous hVPLA2, both hVPLA2 and cPLA2 are involved in the early phase of [3H]AA release, whereas cPLA2 is primarily responsible for the delayed phase of [3H]AA release. The involvement of hVPLA2 only in the early phase of AA release is also consistent with our previous finding that the exogenously added hVPLA2 is internalized and degraded in neutrophils within the first 10 min under similar experimental conditions (11).

We also monitored the time course of LTB4 release by 1–100 nm exogenous hVPLA2 under the same experimental conditions (Fig. 2B). With 10 nm hVPLA2, the LTB4 release reached a saturation in 5–10 min, consistent with the early phase AA release curve, and started to decline until it increased again at 15–20 min, which is approximately synchronized with the delayed phase AA release. The delayed phase LTB4 release was

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\caption{Dependence of fatty acid and LTB4 release on hVPLA2 concentration. A, human neutrophils were labeled for 3 h with [3H]AA and [14C]OA and incubated for 20 min at 37 °C with different concentrations (1–100 nm) of hVPLA2 or hIIaPLA2 in HBSS containing 1.2 mM Ca2+ and 0.2% BSA. Black and white bars indicate [3H]AA and [14C]OA release, respectively, by hVPLA2. Gray bars indicate [3H]AA release by hIIaPLA2. B, human neutrophils were incubated for 30 min at 37 °C with different concentrations (1–100 nm) of hIIaPLA2 or hVPLA2 in HBSS containing 1.2 mM Ca2+. The supernatants were collected, and the LTB4 level was assayed as described under "Experimental Procedures." Black and white bars indicate LTB4 secretion by hVPLA2 and hIIaPLA2, respectively. Data represent the mean ± S.E. from triplicate measurements.}
\end{figure}

RESULTS

hVPLA2-induced Fatty Acid and LTB4 Release from Neutrophils—We showed previously that exogenously added hVPLA2 triggers AA release and LTB4 secretion from unprimed human neutrophils (10). To understand better the mechanism of hVPLA2-induced LTB4 biosynthesis, we carefully examined the time course of the fatty acid and LTB4 release from neutrophils in the presence of varying concentrations of exogenously added hVPLA2. First, we measured the hVPLA2 concentration dependence of fatty acid release from neutrophils double labeled with [3H]AA and [14C]OA. Because of the high AA specificity of cPLA2 and lack of fatty acid selectivity of sPLA2s, the [3H]AA release in this system would reflect both sPLA2 and cPLA2 activities, whereas the [14C]OA release would largely represent sPLA2 activity. As illustrated in Fig. 1A, the [3H]AA release was detected at a lower hVPLA2 concentration than was [14C]OA release. For instance, at 1 nm hVPLA2, the [3H]AA release was twice larger than the control, whereas the [14C]OA release was essentially the same as the control. At 100 nm hVPLA2, however, both [3H]AA and [14C]OA releases were about three times higher than the control. This suggests that at lower concentrations of exogenously added hVPLA2, cPLA2 is mainly responsible for the AA release, whereas at higher concentrations (i.e. ≥10 nm) of exogenously added hVPLA2, the amount of secreted endogenous hVPLA2 (~1.5 nm) (7) should be much smaller than that of exogenous hVPLA2. As reported previously (10, 11), hIIaPLA2, up to 100 nm had little effect on fatty acid release under the same conditions, underscoring the unique ability of hVPLA2 to release fatty acid from unprimed neutrophils. We then measured the LTB4 release from neutrophils as a function of hVPLA2 concentration (Fig. 1B). Unlike the AA release that occurred with as low as 1 nm exogenously added hVPLA2, the LTB4 release was not detectable at 1 nm hVPLA2. However, the LTB4 release was clearly seen with ≥10 nm hVPLA2. As reported earlier (10, 11), 100 nm hVPLA2 was about half as effective as 1 μM fMLP + 5 μg/ml cytochalasin B in inducing the LTB4 release. As a negative control, hIIaPLA2, up to 100 nm was shown to have little effect on LTB4 release. Together, these data suggest that the release of AA and LTB4 biosynthesis in human neutrophils have disparate dependence on hVPLA2 concentration acting on their cell surfaces.

sPLA2-induced cPLA2 Activation in Neutrophils

6-trans-LTB4 isomers (m/z 335 → 195), 20-hydroxy-LTB4 (m/z 351 → 195), 20-carboxy-LTB4 (m/z 365 → 195). Quantitation of individual peaks was carried out using their mass ion abundance relative to that of PGB2.

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\caption{Dependence of fatty acid and LTB4 release on hVPLA2 concentration. A, human neutrophils were labeled for 3 h with [3H]AA and [14C]OA and incubated for 20 min at 37 °C with different concentrations (1–100 nm) of hVPLA2 or hIIaPLA2 in HBSS containing 1.2 mM Ca2+ and 0.2% BSA. Black and white bars indicate [3H]AA and [14C]OA release, respectively, by hVPLA2. Gray bars indicate [3H]AA release by hIIaPLA2. B, human neutrophils were incubated for 30 min at 37 °C with different concentrations (1–100 nm) of hIIaPLA2 or hVPLA2 in HBSS containing 1.2 mM Ca2+. The supernatants were collected, and the LTB4 level was assayed as described under "Experimental Procedures." Black and white bars indicate LTB4 secretion by hVPLA2 and hIIaPLA2, respectively. Data represent the mean ± S.E. from triplicate measurements.}
\end{figure}
much more pronounced with 100 nM hVPLA2. Importantly, treatment of neutrophils with AACOCF3 abrogated the LTB4 release, indicating that cPLA2 is mainly responsible for LTB4 biosynthesis under these conditions. With 1 nM hVPLA2, the LTB4 release rapidly reached a maximal point at 5 min and then decreased to a basal level after 10 min, which is consistent with the lack of LTB4 release at 10 min shown in Fig. 1B. These data indicate, however, that as low as 1 nM exogenous hVPLA2 can stimulate the LTB4 biosynthesis.

In neutrophils AA is transformed into several different 5-LO products including LTB4 (16). Also, LTBlb is known to be degraded and inactivated by microsomal ω-oxidation and peroxisomal β-oxidation in myeloid cells (16). To understand better the fate of AA liberated in neutrophils, we analyzed the composition of lipid products that neutrophils released to the medium when they were challenged with 1 and 10 nM exogenous hVPLA2, respectively. The chromatogram in Fig. 3/A shows that at 1 nM hVPLA2, two main leukotriene products are 20-carboxy-LTB4 and 20-hydroxy-LTB4, which are produced as a consequence of LTB4 oxidation (16). These data thus confirm that even 1 nM exogenous hVPLA2 can induce the biosynthesis of a considerable amount of LTB4. It appears, however, that LTB4 is degraded relatively rapidly to oxidation products that do not cross-react with the LTB4 antibody used in the commercial LTB4 detection kit, hence there is no LTB4 signal with 1 nM hVPLA2 in Fig. 1B. With 10 nM hVPLA2, LTB4 was clearly seen along with other leukotrienes. As was the case with 1 nM hVPLA2, 20-carboxy-LTB4 was the most abundant component.

Fig. 2. Time courses of hVPLA2-induced fatty acid and LTB4 release from neutrophils. A, dual radiolabeled neutrophils were incubated with 10 nM hVPLA2, and the time courses of [3H]AA (□) and [14C]OA (△) releases were monitored. Also, the [3H]AA release was monitored for neutrophils preincubated for 30 min with 25 μM AACOCF3 before the addition of 10 nM hVPLA2 (○). B, time courses of LTB4 secretion were measured in the presence of 1 nM (□), 10 nM (○), and 100 nM (△) hVPLA2. Also, the LTB4 release from neutrophils preincubated for 30 min with 25 μM AACOCF3 before the addition of 10 nM hVPLA2 was measured (●). Data represent the mean ± S.E. from triplicate measurements.

hVPLA2-induced Activation of cPLA2 in Neutrophils—Accumulating evidence has indicated that cPLA2 plays a pivotal role in the receptor-mediated mobilization of AA and eicosanoid biosynthesis in neutrophils (7, 9, 20). Furthermore, several reports have indicated that exogenously added sPLA2s activate cPLA2 in neutrophils (8) and other mammalian cells (21, 22). Also, the occurrence of the delayed phase AA release in our studies implies that cPLA2 is activated during or after the early phase of AA release. We therefore measured the effect of hVPLA2 on cPLA2 activities in neutrophils. It has been established that cPLA2 can be activated by a rise in [Ca2+]i (23) and the phosphorylation of Ser residues, most notably Ser205 (24). In neutrophils, it was shown previously that exogenously added pancreatic sPLA2 phosphorylated and activated cPLA2 through the formation of 5-LO products, including LTBlb (8). To elucidate the mechanism by which hVPLA2 activates cPLA2, we monitored the time-dependent changes in cPLA2 activity and the phosphorylation by enzyme assay and electrophoretic mobility assay, respectively, upon incubating neutrophils with 10 nM hVPLA2. First, we measured the time course of cPLA2 activity from neutrophil lysates. To eliminate residual sPLA2 activities in the cell lysates, the lysates were incubated with 10 mM dithiothreitol before the addition of a cPLA2 substrate, [14C]SAPC. As shown in Fig. 4A, the cPLA2 activity of neutrophils was enhanced by exogenously added hVPLA2, but the time course of activation was rather complex. The cPLA2 activity increased about 2.3-fold in first 5 min but then started to decrease until it rose again at −10 min and reached a plateau in 20 min. As was the case with AA and LTB4 release, it thus appears that cPLA2 activation also occurs in two phases. Interestingly, preincubation of neutrophils with a LTBlb receptor antagonist, LTB4DMA (0.3 μM) abrogated the delayed phase activation of cPLA2, suggesting that it is mediated through the binding of LTBlb to its cell surface receptor. Because the cPLA2 assay of the lysates was done in the presence of a saturating concentration of calcium for cPLA2 (0.1 mM), the activity enhancement should reflect mainly the protein phosphorylation. Indeed, Fig. 4B shows that the extent of cPLA2 phosphorylation is synchronized with the change in cPLA2 activity shown in Fig. 4A.

We then measured the effect of exogenously added hVPLA2 on [Ca2+]i. We monitored the fluorescence of [Ca2+]i, with a fluorescence indicator, Fluo-4. Although UV-excitable Ca2+ indicators, such as Indo-1 and Fura-2, allow more accurate [Ca2+]i measurement by a ratiometric analysis, we used Fluo-4 in our studies because the UV irradiation severely damages human neutrophils. We monitored the fluorescence intensity changes of Fluo-4 in the perinuclear region by confocal microscopy. As shown in Fig. 5, the addition of 10 nM hVPLA2 evoked an immediate increase in [Ca2+]i (to ~500 nm) in the perinuclear region. Interestingly, a second [Ca2+]i peak was seen in the perinuclear region, which was about 50% of the first one in magnitude. The timing of the second spike varied between 10 and 15 min among different cells. As seen with the progress curve of cPLA2 activation (see Fig. 4A), the second [Ca2+]i peak was completely abrogated when the cells were pretreated with a LTBlb receptor antagonist, LTB4DMA. In conjunction with cPLA2 phosphorylation data, these data suggest that the addi-
tion of hVPLA₂ activates cPLA₂ by increasing the [Ca²⁺], and inducing cPLA₂ phosphorylation in both the early and the delayed phases and that the delayed phase activation is mediated through the binding of LTB₄ to its cell surface receptor.

Sites of hVPLA₂ and cPLA₂ Actions in Neutrophils—To determine the exact site of actions for hVPLA₂ and cPLA₂ in neutrophils, we performed a cellular PLA₂ activity assay using a fluorescent phospholipid, PED6. We recently reported the use of PED6 in the real-time activity assay for hVPLA₂ internalized into human embryonic kidney 293 cells (25). Because cPLA₂ has much lower specific activity than sPLA₂ for this phospholipid (25, 26), the cellular cPLA₂ activity would yield only a low fluorescence signal from PED6 hydrolysis. To improve the sensitivity of assay for cPLA₂, we double labeled neutrophil membranes with DiIC₁₂ and PED6. DiIC₁₂ is a nonhydrolyzable fluorescent lipid that shows a greatly enhanced fluorescent signal at 585 nm by FRET from hydrolyzed PED6. Indeed, the in vitro FRET assay using POPS/cholesterol/POPG/PED6/DiIC₁₂ (107:31:20:1:1) vesicles in the presence of the same concentration of cPLA₂ (10 nM) (data not shown). When neutrophils double labeled with DiIC₁₂ and PED6 were incubated with 10 nM exogenous hVPLA₂, prominent signals appeared at the plasma membrane and the perinuclear region after 5 min (Fig. 6). Because of the high laser power necessary for visualization of the signal change, real-time monitoring was not attempted in this case because it would lead to serious photo-bleaching. Most importantly, the perinuclear signal was abrogated when the labeled neutrophils were pretreated with 25 μM AACOCF₃ before the addition of hVPLA₂. No change was observed, however, when the cells were treated with 10 μM bromo-eno-lactone. This clearly indicates that the perinuclear signal is the result of cPLA₂ activity and that hVPLA₂ primarily acts on the plasma membrane in neutrophils. Taken together, these results indicate that hVPLA₂-induced activation of cPLA₂ by calcium increase and phosphorylation results in the lipolytic action of cPLA₂ in the perinuclear region.

Effects of hVPLA₂ Products and LTB₄ on cPLA₂ Activity—To determine the mechanism by which hVPLA₂ activates cPLA₂, we first measured the effect of exogenously added lipid products of hVPLA₂, fatty acids (AA and OA) and lysophospholipids (lyso-PC) on the [³H]AA and LTB₄ release from neutrophils. Lyso-PC was selected as a representative lysophospholipid because the main phospholipid component of the outer plasma membrane of mammalian cells is phosphatidylcholine. As illustrated in Fig. 7A, 3 μM lyso-PC had the same potency as 10 nM hVPLA₂ in eliciting [³H]AA release from labeled neutrophils. Even 1 μM lyso-PC was able to induce significant [³H]AA release (data not shown). Although less potent than lyso-PC, AA was also able to induce [³H]AA release. In this case, 10 μM exogenous AA was as effective as 10 nM hVPLA₂. In contrast, OA up to 30 μM showed negligible effects on [³H]AA release. Lyso-PC and AA showed an additive effect when used in combination. A similar trend was seen with the LTB₄ release. Lyso-PC (3 μM) was nearly twice as effective as 10 nM hVPLA₂ in net LTB₄ release activity (see Fig. 7B), whereas the same concentration of AA was about 30% active. OA up to 30 μM
FIG. 5. Time lapse changes in [Ca\(^{2+}\)]\(_i\), of human neutrophils caused by exogenous hVPLA\(_2\). The fluorescence intensity of Fluo-4 near the perinuclear region of neutrophils was monitored upon adding 10 nM hVPLA\(_2\) (arrows), and [Ca\(^{2+}\)]\(_i\) values were calculated from the intensity values using a calibration curve (see “Experimental Procedures”). [Ca\(^{2+}\)]\(_i\) changes are shown for a single representative cell in the absence (A) and presence (B) of 0.3 mM LTB\(_4\)DMA (total number of cells = 12).

FIG. 6. Confocal microscopic imaging of PLA\(_2\) activities in human neutrophils. A, imaging was performed after adding 10 nM hVPLA\(_2\) to human neutrophils that were incubated with POPS/cholesterol/POPC/PE/PC/DHPC (107:31:20:1:1 in mol ratio) for 50 min at 37°C. B, neutrophils were treated with 25 μM AACOCF\(_3\) before the addition of hVPLA\(_2\).}

failed to induce LTB\(_4\) release. Given that AA constitutes only a small fraction (~5%) of fatty acids incorporated into the phospholipids in the outer plasma membrane, these data indicate that the cellular effect of hVPLA\(_2\) is mediated largely through the formation of lyso-PC.

Interestingly, the exogenous addition of 0.3 μM LTB\(_4\) enhanced the [\(^3\)H]AA release as much as 10 nM hVPLA\(_2\) (Fig. 7A). Furthermore, preincubation of labeled neutrophils with 0.3 μM LTB\(_4\)DMA significantly reduced the positive effects of hVPLA\(_2\), lyso-PC, and AA on [\(^3\)H]AA release, suggesting that a large part of their effects is mediated through the activation of LT\(_4\) receptors on the neutrophil surfaces. To investigate this aspect further, we measured the time course of cPLA\(_2\) activation by lyso-PC in the presence and absence of LTB\(_4\)DMA. As described above, the cPLA\(_2\) activity assay of the lysates was performed in the presence of 0.1 mM calcium and 10 mM dithiothreitol, and the activity enhancement should mainly reflect cPLA\(_2\) phosphorylation. Fig. 8 shows that lyso-PC increased the cPLA\(_2\) activity (and phosphorylation) in two phases, which is reminiscent of two-phase cPLA\(_2\) activation and phosphorylation by exogenous hVPLA\(_2\). Again, the delayed phase activation was abrogated by preincubation of neutrophils with LTB\(_4\)DMA. This indicates that lyso-PC induces the phosphorylation of cPLA\(_2\) in both phases of cPLA\(_2\) activation and that the delayed phase phosphorylation takes place via LTB\(_4\) formation and its receptor binding.

We also measured the effects of lyso-PC, fatty acids, and LT\(_4\) on the change of [Ca\(^{2+}\)]\(_i\) in the perinuclear region. It has been reported that AA (27) and LT\(_4\) (28) can increase [Ca\(^{2+}\)]\(_i\) in human neutrophils. We also observed that 3 μM AA or 0.3 μM LT\(_4\) rapidly enhanced [Ca\(^{2+}\)]\(_i\) in the perinuclear region to 400–600 nM. Similarly, 3 μM lyso-PC spontaneously raised [Ca\(^{2+}\)]\(_i\) to 500 nM in the perinuclear region as shown in Fig. 9. These effects on [Ca\(^{2+}\)]\(_i\) are reminiscent of the effect of hVPLA\(_2\) illustrated in Fig. 5. Finally, OA up to 10 μM had no effect on [Ca\(^{2+}\)]\(_i\) (data not shown).

MAP Kinases Involved in cPLA\(_2\) Phosphorylation—It has been reported that cPLA\(_2\) is phosphorylated and activated by different kinases in mammalian cells (29–35). In neutrophils, cPLA\(_2\) was shown to be phosphorylated by p38 MAP kinase, ERK1/2 MAP kinase, or both, depending on how neutrophils are activated (20, 36). To determine how these MAP kinases are involved in the hVPLA\(_2\)-induced cPLA\(_2\) activation and LT\(_4\) biosynthesis in neutrophils, we first measured the effect of hVPLA\(_2\) on p38 and ERK1/2 MAP kinase activation. Phosphorylation of these MAP kinases is commonly used as an indicator of their activation. As shown in Fig. 10A, hVPLA\(_2\) caused a time-dependent phosphorylation of p38 and ERK1/2 MAP kinases. The phosphorylation of ERK1/2 exhibited a biphasic pattern, peaking at 5 and 20 min, respectively, but the delayed phase phosphorylation was more pronounced. In contrast, p38 phosphorylation peaked at 10 min and declined thereafter. This suggests that both p38 and ERK1/2 MAP kinases are involved in the early phase cPLA\(_2\) phosphorylation, whereas ERK1/2 plays a predominant role in the delayed phase cPLA\(_2\) phosphorylation. To test this notion, we measured the effects on the time course of cPLA\(_2\) activation of specific inhibitors of two MAP kinase pathways: SB203580, which specifically inhibits p38 MAP kinase (37), and U0126, which specifically inhibits MEK, which is an upstream kinase of ERK1/2 (38). As shown in Fig. 10B, 30 μM SB203580 significantly inhibited the early phase cPLA\(_2\) activation with a lesser effect on the delayed phase. 10 μM U0126, however, had a much more pronounced effect on the delayed phase cPLA\(_2\) activation while also showing a significant effect on the early phase. Together,
these results indicate that both ERK1/2 and p38 MAP kinases are involved in the early phase of the hVPLA2-induced cPLA2 activation in neutrophils, whereas ERK1/2 is involved primarily in the delayed phase.

**DISCUSSION**

Neutrophils that play a key role in defense against microbial infection release AA, LTB4, and other 5-LO products in response to various stimuli, including bacterial peptides. Recent studies on fMLP-induced activation of human neutrophils have indicated that both sPLA2 and cPLA2 are involved in AA release (9), whereas cPLA2 is responsible primarily for LTB4 release (7). We showed previously that exogenously added hVPLA2 could also elicit the release of AA and LTB4 from unprimed human neutrophils almost as effectively as fMLP (10). This neutrophil activation involves the direct binding of hVPLA2 to the outer plasma membrane and the hydrolysis of phosphatidylcholine (10) and is terminated by the internalization and degradation of cell surface-bound hVPLA2 in a heparan sulfate proteoglycan-dependent manner (11). The present study shows that exogenous hVPLA2, as low as 1 nM is able to induce AA and LTB4 release from unprimed human neutrophils. Furthermore, the study reveals that the hVPLA2-induced formation of AA and leukotrienes in human neutrophils is a complex and dynamic process that involves cPLA2 activation by [Ca^{2+}], increase and phosphorylation. The most salient feature of hVPLA2-induced neutrophil activation is the two-phase kinetics. All phenomena associated with neutrophil activation, AA and LTB4 release, [Ca^{2+}], increase, and cPLA2 phosphorylation, follow similar two-phase kinetic patterns.

The time course of hVPLA2-induced OA release as well as the effect of cPLA2 inhibition on the time course of hVPLA2-induced AA release indicate that both hVPLA2 and cPLA2 contribute to the early phase AA release, whereas cPLA2 is responsible primarily for the delayed phase. Also, the primary sites of action for hVPLA2 and cPLA2 are the outer plasma membrane and the perinuclear region, respectively (see Fig. 6). Given that the AA composition of neutrophil plasma membrane is less than 5% (39) and that cell surface-bound hVPLA2 is readily internalized and degraded (11), a relatively high concentration of exogenous hVPLA2 would be necessary to liberate a significant amount of AA from the outer plasma membrane. Indeed, direct AA production by hVPLA2 becomes significant only when its concentration reaches 10 nM (see Fig. 1A). Importantly, the abrogation of LTB4 release in the presence of cPLA2 inhibitors points to the predominant role of cPLA2 in LTB4 biosynthesis under our experimental conditions. This in turn indicates that the AA liberated from the outer plasma membrane of neutrophils by direct lipolytic action of hVPLA2 in the early phase is not conducive to LTB4 biosynthesis by 5-LO. Thus, it would seem that the primary role of this hVPLA2-produced AA is to activate cPLA2. In fact, AA and other polyunsaturated fatty acids have been shown to activate cPLA2 in neutrophils (40). In this regard, it is noteworthy that lyso-PC is about three times more potent than AA in inducing LTB4 biosynthesis in neutrophils. Also, lyso-PC should be produced in a much larger amount than AA and polyunsaturated fatty acids from the outer plasma membrane of neutrophils because of the abundance of phosphatidylcholine. This and other results presented herein support the notion that hVPLA2-induced activation of neutrophils is largely mediated by lyso-PC. Lyso-PC species containing a saturated acyl chain in the sn-1 position, including the palmitoyl derivative employed in this study, have been shown to activate a cell surface G protein-coupled receptor (41) and thereby regulate a broad range of cell processes, including increases in cAMP (42) and [Ca^{2+}], (41) and the activation of...

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**Fig. 7.** Effects of hVPLA2 hydrolysis products and LTB4 on neutrophil activation. AA (A) and LTB4 (B) release was measured after neutrophils were treated with each agonist (10 nM hVPLA2, 3 μM AA, 3 μM lyso-PC, 0.3 μM LTB4, or 0.3 μM LTB4DMA) for 20 min. Data represent the mean ± S.E. from triplicate measurements.

**Fig. 8.** Time-dependent activation of neutrophil cPLA2 by exogenous lyso-PC. The effect of 3 μM lyso-PC was measured in the absence (○) and presence (●) of 0.3 μM LTB4DMA. Experimental conditions were the same as described for Fig. 4. Data represent the mean ± S.E. from triplicate measurements.
MAP kinase (41) and protein kinase C (43). In particular, 100 μM lyso-PC was shown to induce AA release and increase [Ca²⁺]ᵢ in rat heart myoblastic H9c2 cells (43). In our study, a few micromolar lyso-PC effectively simulated all activities of hVPLA₂ on human neutrophils, including AA and LTB₄ release, a rise in [Ca²⁺]ᵢ, and cPLA₂ phosphorylation. In particular, lyso-PC activates cPLA₂ by inducing both [Ca²⁺]ᵢ increase and cPLA₂ phosphorylation in both early and delayed phases. A rise in Ca²⁺ by lyso-PC would also activate 5-LO by inducing its translocation to the nuclear envelope (44), thereby promoting LTB₄ synthesis.

It has been shown that LTB₄ can activate neutrophils by an autocrine, positive feedback mechanism (49). Neutrophils contain a cell surface G protein-coupled LTB₄ receptor (50), and the binding of LTB₄ to the receptor leads to various cell activation, including a rise in [Ca²⁺]ᵢ, and the MAP kinase activation (50). It was shown previously that the agonist-induced biosynthesis of LTB₄ in neutrophils leads to cPLA₂ phosphorylation (8). Our results clearly show that the biosynthesis of LTB₄ and its binding to the cell surface receptor play a pivotal role in the delayed phase of hVPLA₂-induced cPLA₂ activation by causing both a rise in [Ca²⁺]ᵢ and cPLA₂ phosphorylation. Because blocking the LTB₄ receptor with LTB₄DMA abrogates the [Ca²⁺]ᵢ increase and the cPLA₂ phosphorylation only in the delayed phase, it is unlikely that LTB₄ is involved in the early phase cPLA₂ activation that is mediated primarily by lyso-PC (and polyunsaturated fatty acids). Our results also indicate that a certain threshold concentration of LTB₄ is required for its positive feedback effect because of the relatively rapid oxidative degradation of LTB₄ in neutrophils. In the case of neutrophil activation by exogenous hVPLA₂, this threshold concentration of LTB₄ is achieved by ~10 nM hVPLA₂. The threshold LTB₄ concentration was not determined directly in this study because of difficulties involved in distinguishing between exogenous and endogenous LTB₄.

In neutrophils, cPLA₂ is phosphorylated by p38 MAP kinase.
ERK1/2, or both, depending on the nature of agonists (20). Although the identification of the network of protein kinases involved in hVPLA2-induced cPLA2 phosphorylation and the site of cPLA2 phosphorylation are beyond the scope of this investigation, our results indicate that both ERK1/2 and p38 MAP kinases are involved in the early phase cPLA2 activation, whereas ERK1/2 is involved primarily in the delayed phase. The direct role of ERK1/2 in cPLA2 phosphorylation in neutrophils has been well documented. In particular, LTβ4 was shown to activate ERK1/2 (45) but not p38 MAP kinase (46). This is consistent with our finding that ERK1/2 is involved mainly in the delayed phase cPLA2 phosphorylation that is mediated by the binding of LTβ4 to its receptor. It has been shown that lyso-PC (43) and AA (and other polyunsaturated fatty acids) (47) can activate protein kinase C. Furthermore, the phorbol ester-induced activation of protein kinase C in neutrophils was shown to phosphorylate cPLA2 via ERK1/2 activation (20). Thus, it appears that at least one signaling pathway to cPLA2 phosphorylation in the early phase involves the protein kinase C activation that leads to ERK1/2 activation. A previous study reported that AA stimulated p38 phosphorylation in neutrophils (48). Thus, AA and polyunsaturated fatty acids released by hVPLA2 might be responsible for the p38 phosphorylation in the early phase of neutrophil activation by hVPLA2. It is not clear, however, whether the activated p38 is directly or indirectly involved in cPLA2 phosphorylation. Further studies are necessary to sort out the effects of different protein kinases in the activation of cPLA2 in neutrophils.

On the basis of our present and previous studies, we propose a mechanism by which hVPLA2 induces the LTβ4 biosynthesis in human neutrophils as shown in Fig. 11. In this model, hVPLA2 directly acts on the outer cell membranes of neutrophils to release fatty acids (including AA) and lysophospholipids, most likely lyso-PC. Both polyunsaturated fatty acids (including AA) and lyso-PC induce the immediate membrane translocation of 5-LO and cPLA2 with transient Ca\(^{2+}\) influx. Also, they activate cPLA2 via phosphorylation, which leads to the liberation of AA at the perinuclear region. cPLA2, activated by hVPLA2 products then returns to the resting state as cells internalize hVPLA2 via heparan sulfate proteoglycan binding and degrade them to avoid extensive lipolytic damage of the outer plasma membrane. In the meantime, activated 5-LO produces LTB4, which binds the cell surface LTB4 receptor in an autocrine manner and triggers a MAP kinase cascade to rephosphorylate and reactivate cPLA2 in the delayed phase. This delayed phase phosphorylation of cPLA2 will then lead to amplified and prolonged production of AA, LTβ4, and other eicosanoids.

It should be noted that this model focuses mainly on the action of exogenous hVPLA2 on neutrophils but not on the role of endogenous hVPLA2 in neutrophil activation. Based on the lack of LTβ4 release from human neutrophils stimulated with fMLP and cytochalasin B, it was postulated that the endogenous hVPLA2 in neutrophils is not involved in LTβ4 biosynthesis (7). In this report, the concentration of hVPLA2 released from neutrophils by fMLP and cytochalasin B was estimated to be in the low nanomolar range (7). Our study shows that even this concentration of hVPLA2 can induce the formation of a significant amount of LTβ4 but cannot trigger the receptor-mediated positive feedback effect because of rapid oxidative degradation. However, the amount of hVPLA2 in human neutrophils seems to vary to a large extent depending on the allergic state of donors (52), suggesting that higher concentrations of endogenous hVPLA2 could be secreted by activated neutrophils. Furthermore, the sPLA2 concentration in serum and inflammatory exudates was reported to be much higher (51). In particular, mast cells and macrophages release a significant amount of group V PLA2 in response to different stimuli. It is therefore likely that exogenous hVPLA2 is able to trigger LTB4 biosynthesis in neutrophils, either alone or in combination with other stimuli, under pathophysiological conditions. Undoubtedly, further studies are necessary to address this important question.

REFERENCES


\[N\] Munoz, A. Leff, and W. Cho, unpublished observation.
sPLA2-induced cPLA2 Activation in Neutrophils