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Distinct Roles in Signal Transduction for Each of the Phospholipase A_2 Enzymes Present in P388D_1 Macrophages*

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Receptor-stimulated arachidonic acid (AA) mobilization in P388D_1 macrophages consists of a transient phase in which AA accumulates in the cell and a sustained phase in which AA accumulates in the incubation medium. We have shown previously that a secretory group II phospholipase A_2 ($sPLA_2$) is the enzyme responsible for most of the AA released to the incubation medium. By using selective inhibitors for each of the PLA_2s present in P388D_1 macrophages, we demonstrate herein that the cytosolic group IV PLA_2 ($cPLA_2$) mediates accumulation of cell-associated AA during the early steps of P388D_1 cell activation. The contribution of both $cPLA_2$ and $sPLA_2$ to AA release can be distinguished on the basis of the different spatial and temporal characteristics of activation and substrate preferences of the two phospholipase A_2s (PLA_2s). Furthermore, the results suggest the possibility that a functionally active $cPLA_2$ may be necessary for $sPLA_2$ to act. $cPLA_2$ action precedes that of $sPLA_2$, and overcoming $cPLA_2$ inhibition by artificially increasing intracellular free AA levels restores extracellular AA release. Although this suggests cross-talk between $cPLA_2$ and $sPLA_2$, selective inhibition of one other PLA_2 present in these cells, namely the Ca^{2+}-independent PLA_2, does not block, but instead enhances receptor-coupled AA release. These data indicate that Ca^{2+}-independent PLA_2 does not mediate AA mobilization in P388D_1 macrophages. Collectively, the results of this work suggest that each of the PLA_2s present in P388D_1 macrophages serves a distinct role in cell activation and signal transduction.

Phospholipase A_2 (PLA_2)[1] enzymes play a fundamental role in numerous cellular processes by generating an array of metabolites with various biological functions. PLA_2-mediated hydrolysis of glycerophospholipids results in the release of arachidonic acid (AA) and lysophospholipids, which may either exert direct effects or serve as substrates for the generation of other lipid messengers such as the eicosanoids or platelet-activating factor (PAF) (1).

Mammalian cells contain multiple PLA_2 forms (1), and there is considerable interest in determining the role that each PLA_2 plays in mediating cellular functions. At least three different cellular PLA_2s have been proposed to play a role in the mobilization of AA from phospholipids. These are the cytosolic group IV PLA_2 ($cPLA_2$) (2–4), the secretory group II PLA_2 ($sPLA_2$) (5–7), and a cytosolic Ca^{2+}-independent PLA_2 ($iPLA_2$) (8, 9). Involvement of one or another PLA_2 form appears to depend on the cell type and agonist involved.

Our laboratory has been examining the molecular mechanisms involved in AA mobilization in murine P388D_1 macrophage-like cells (6, 10–12). Stimulation of these cells with nanomolar quantities of the receptor agonist PAF results in a very modest mobilization of free AA. However, preincubation of the cells with bacterial lipopolysaccharide (LPS) prior to stimulation with PAF increases the release of AA by these cells by about 2–3-fold (10). Recently, we have demonstrated that AA mobilization in response to LPS/PAF involves participation of a $sPLA_2$ localized at the outer surface of the cell and that this enzyme accounts for the majority of the AA released to the extracellular medium (6, 12). In the current study, we have obtained further evidence using chemical inhibitors for the role of $sPLA_2$ and have aimed at defining the roles played by the other two PLA_2s present in P388D_1 macrophages, namely $cPLA_2$ and $iPLA_2$.

EXPERIMENTAL PROCEDURES

Materials—P388D_1 cells were obtained from the American Type Culture Collection (Rockville, MD). LPS Re595 was the kind gift of Dr. Richard Ulevitch (Scripps Clinic and Research Foundation, La Jolla, CA). Iscove's modified Dulbecco's medium (endotoxin <0.05 ng/ml) was from Whittaker Bioproducts (Walkersville, MD). Fetal bovine serum was from Hyclone Labs. (Logan, UT). Nonessential amino acids were from Irvine Scientific (Santa Ana, CA). (5,6,8,9,11,12,14,15-^{3}H)Arachidonic acid (specific activity, 100 Ci/mmol), (1-^{14}C)arachidonic acid (specific activity, 57 mCi/mmol), and [methyl-^{3}H]choline chloride (specific activity, 79 Ci/mmol) were from New England Nuclear (Boston, MA). PAF, unlabeled fatty acids, and lysophospholipids were from Sigma. Okadaic acid was from Calbiochem (San Diego, CA) or Biomol (Plymouth Meeting, PA). The $sPLA_2$ inhibitor 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propane phosphonic acid (LY311727) was kindly provided by Dr. Edward Mihelich (Lilly Research Laboratories, Indianapolis, IN). Methyl arachidonyl fluorophosphonate (MAFP) was from Cayman (Ann Arbor, MI). (*E*)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (bromoenol lactone, BEL), and 1-hexylthio-2-hexanoylamino-1,2-dideoxy-*sn*-glycero-3-phosphoethanolamine (diC_6SNPE) were synthesized in our laboratory by Kilian Conde-Frieboes and Scott Boegeman, respectively, following previously published procedures (13, 14). Silicagel G-60 TLC plates were from Analtech (Newark, DE). Organic solvents (analytical grade) were from Baker (Phillipsburg, NJ) or Fisher.

Cell Culture and Labeling Conditions—P388D_1 cells were maintained at 37 °C in a humidified atmosphere at 90% air and 10% CO_2 in

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[1] The abbreviations used are: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic group IV phospholipase A_2; $iPLA_2$, cytosolic Ca^{2+}-independent phospholipase A_2; $sPLA_2$, secretory group II phospholipase A_2; AA, arachidonic acid; PAF, platelet-activating factor; LPS, bacterial lipopolysaccharide; BEL, (*E*)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one or bromoenol lactone; LY311727, 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propane phosphonic acid; diC_6SNPE, 1-hexylthio-2-hexanoylamino-1,2-dideoxy-*sn*-glycero-3-phosphoethanolamine; MAFP, methyl arachidonyl fluorophosphonate; PC, choline-containing glycerophospholipids; PE, ethanolamine-containing glycerophospholipids; PI, phosphatidylinositol.

Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids. Cells were plated at 10^6 per well, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free Iscove's modified Dulbecco's medium. Radiolabeling of the cells with [^{3}H]AA was achieved by including 0.5 μCi/ml [^{3}H]AA during the overnight adherence period (20 h) (12). When double-labeled cells were used, the cells were first labeled with [^{3}H]AA for 20 h as described above and then were incubated in serum-free medium with [^{14}C]AA (0.1 μCi/ml) for 10 min. Labeled AA that had not been incorporated into cellular lipids was removed by washing the cells four times with serum-free medium containing 5 mg/ml albumin.

Stimulation of P388D_1 Cells—The standard regimen for activating cells with LPS and PAF has been described previously (12). Briefly, P388D_1 cells were placed in serum-free medium containing 1 mg/ml bovine serum albumin for 30–60 min before the addition of LPS (200 ng/ml) for 1 h. After the LPS incubation, cells were overlaid with serum-free medium containing 1 mg/ml albumin for 5–15 min, after which they were challenged with 100 nM PAF for the time indicated. More than 99% of released radioactive material remains as unmetabolized AA under these experimental conditions (12).

Measurement of Extracellular AA Release and Cell-associated Free AA—LPS-treated cells labeled with either [^{3}H]AA alone or [^{3}H]AA plus [^{14}C]AA were stimulated with 100 nM PAF for the times indicated. The supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting. For analysis of cell-associated, free AA, the cell monolayers were scraped in 1 ml of 0.5% Triton X-100. Lipids were extracted according to Bligh and Dyer (15) and separated by thin-layer chromatography using *n*-hexane/diethyl ether/acetic acid (70:30:1, v/v/v) as a solvent system. Authentic AA was co-chromatographed and visualized by exposing the plates to iodine vapors. Areas containing AA were scraped into scintillation vials, and the amount of radioactivity was measured by liquid scintillation counting.

Measurement of lyso-PC Levels—For the measurement of lyso-PC, cells were labeled with 0.5 μCi/ml [^{3}H]choline for 3 days. The cells were activated with LPS and PAF as described above. After the indicated times, supernatants were discarded, and the cell monolayers were scraped in 1 ml of 0.5% Triton X-100. Lipids were extracted with ice-cold *n*-butanol and separated by thin-layer chromatography, using chloroform/methanol/acetic acid/water (50:40:6:0.6, v/v/v/v) as a solvent system (16). Spots corresponding to lyso-PC were scraped into scintillation vials, and the amount of radioactivity was measured by liquid scintillation counting.

Data Presentation—Except for the data shown in Fig. 1, agonist-stimulated AA release is expressed by subtracting the basal rate observed in the absence of agonist and inhibitor. These background values were in the range of 2000–3000 cpm for extracellular [^{3}H]AA and 500-1000 cpm for cell-associated free [^{3}H]AA. Assays were carried out in duplicate or triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data presented are from representative experiments.

RESULTS

Two Different AA Pools Contribute to AA Release during Activation of P388D_1 Cells—Our previous work established that AA release in LPS-primed, PAF-stimulated P388D_1 cells is composed of two events: a transient phase in which AA accumulates in the cell and a sustained phase in which the fatty acid accumulates in the extracellular medium (12). We began the current study by determining whether the fatty acid liberated during these two phases arises from separate phospholipid pools. To this end, we used the methodology described by Fonteh and Chilton (17) to selectively label the AA-containing phospholipids. The cells were first labeled with [^{3}H]AA for 20 h, a time frame long enough to allow [^{3}H]AA to equilibrate among phospholipids (12). Under these conditions, PE accounts for the majority of esterified [^{3}H]AA in phospholipids, the remainder being esterified in PC and PI/phosphatidylserine (12). After the 20-h incubation with [^{3}H]AA, the cells were primed with LPS for 1 h, washed, and pulse-labeled with [^{14}C]AA for 10 min. At these short labeling times, the distribution of [^{14}C]AA esterified in phospholipids dramatically differs from

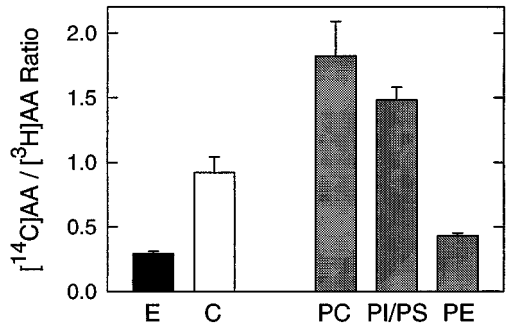

FIG. 1. **Different AA pools in P388D_1 cells.** LPS-treated cells labeled with both [^{3}H]AA and [^{14}C]AA were stimulated with 100 nM PAF for 10 min to measure extracellular AA or for 1.5 min to measure cell-associated AA. The ratio ^{14}C/^{3}H of extracellular AA (*E*) or cell-associated AA (*C*) was quantitated and is shown. The ^{14}C/^{3}H ratio for the major AA-containing phospholipid classes in these cells is also shown for comparison. *PS*, phosphatidylserine.

that seen at long incubation times in that PC, not PE, is the phospholipid that incorporates most of the radiolabeled AA (12). Treating the cells with LPS alone did not raise the levels of extracellular or cell-associated free AA.

Subsequent to the labeling, the cells were activated with PAF, and the [^{14}C]/[^{3}H] ratios were determined in the phospholipid classes as well as in the AA liberated at the two different locations. Cell-associated free AA and extracellular free AA had very different [^{14}C]/[^{3}H] ratios, indicating that the AA released at these two locations was derived from different pools (Fig. 1). The [^{14}C]/[^{3}H] ratio for extracellular free AA had a ratio well below those of PC and PI/phosphatidylserine, but close to that of PE (Fig. 1). This suggested that PE may be a major source for the AA released to the extracellular medium. Consistent with this view, the ^{14}C/^{3}H ratio for extracellular free AA in unstimulated cells was 0.7 ± 0.1, that is, slightly higher than that observed in PAF-activated cells (0.4 ± 0.1). In contrast, the [^{14}C]/[^{3}H] ratio for cell-associated free AA was intermediate between that of PE and those of PC and PI/phosphatidylserine (Fig. 1), suggesting that cell-associated free AA has been derived from all of these phospholipid classes. Within error, there was no difference between the ^{14}C/^{3}H ratio for cell-associated AA in unstimulated cells (1.0 ± 0.2) *versus* PAF-stimulated cells (0.8 ± 0.1), suggesting that intracellular resting levels of AA may also derive from all major phospholipid classes.

PLA_2 Inhibition Studies—A useful approach to study the involvement of distinct PLA_2s in AA mobilization is the use of selective inhibitors for each of these enzymes. Because each PLA_2 group exhibits different catalytic properties and substrate preferences (1), inhibitors based on these characteristics should allow one to distinguish among the different cellular PLA_2s. The PLA_2 inhibitors used in this work are all based on the aforementioned properties.

Using antisense RNA technology, we have previously demonstrated that group II sPLA_2 is responsible for at least 60–70% of the AA released to the incubation medium but is not involved in raising cellular AA levels shortly after cell activation with PAF (12). In the current study, pharmacological inhibition of sPLA_2 was accomplished by incubating the cells either with the water-soluble phospholipid analog, diC_6SNPE (14), or the indole derivative LY311727, which is an indomethacin analogue (18). diC_6SNPE inhibits human synovial group II PLA_2 with an IC_{50} of 27 μM when assayed in a spectrophotometric assay with 2 mM substrate.[2] At concentrations up to 100 μM, diC_6SNPE has no effect on pure human group IV cPLA_2,

[2] S. C. Boegeman, and E. A. Dennis, unpublished data.

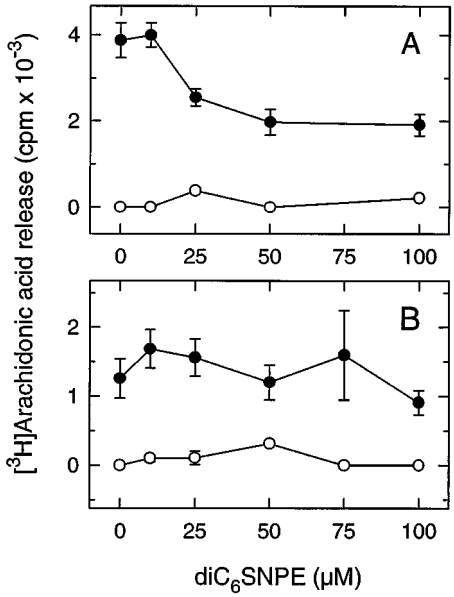

FIG. 2. **Effect of diC$_6$SNPE on PAF-stimulated [^{3}H]AA mobilization in P388D$_1$ cells.** [^{3}H]AA-labeled LPS-treated cells were incubated with the indicated concentrations of diC$_6$SNPE for 15 min. Subsequently, the cells were incubated with (●) or without (○) 100 nM PAF for either 10 (*A*) or 1.5 (*B*) min. Extracellular [^{3}H]AA release (*A*) and cell-associated [^{3}H]AA (*B*) were quantitated as described under "Experimental Procedures."

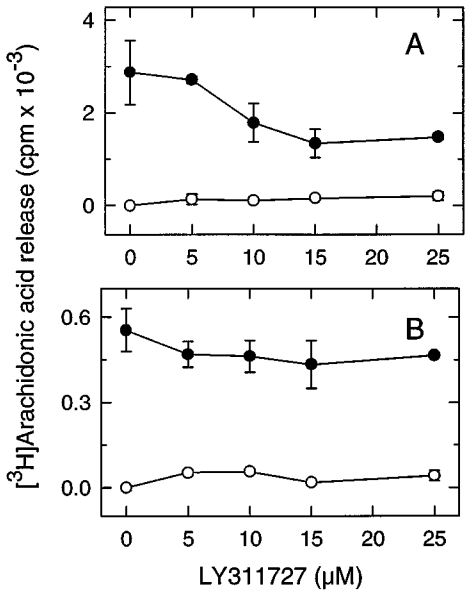

FIG. 3. **Effect of LY311727 on PAF-stimulated [^{3}H]AA mobilization in P388D$_1$ cells.** [^{3}H]AA-labeled LPS-treated cells were incubated with the indicated concentrations of LY311727 for 15 min. Subsequently, the cells were incubated with (●) or without (○) 100 nM PAF for either 10 (*A*) or 1.5 (*B*) min. Extracellular [^{3}H]AA release (*A*) and cell-associated [^{3}H]AA (*B*) were quantitated as described under "Experimental Procedures."

nor does it affect PLA$_2$ activity from P388D$_1$ cell homogenates as measured toward 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine vesicles in the presence of Ca^{2+} and β-mercaptoethanol.[3] In addition, diC$_6$SNPE does not inhibit pure Ca^{2+}-independent PLA$_2$ from P388D$_1$ cells (13). The properties of the indole derivative LY311727 as a potent and selective inhibitor of sPLA$_2$ have recently been reported (18).

sPLA$_2$ inhibition by either diC$_6$SNPE or LY311727 markedly decreased the extracellular release of [^{3}H]AA from prelabeled P388D$_1$ cells (Figs. 2*A* and 3*A*). No effect of these inhibitors was detected on the accumulation of cell-associated free [^{3}H]AA (Figs. 2*B* and 3*B*). These data are fully consistent with our previous data using antisense RNA technology to block sPLA$_2$ activity (12).

Involvement of group IV cPLA$_2$ was initially investigated by using MAFP (19). This compound is an irreversible inhibitor of the cPLA$_2$ and has no effect on the sPLA$_2$ (19). We have confirmed in our laboratory these findings and in addition have found that MAFP does not appreciably affect arachidonoyl-CoA synthetase, lysophosphatidylcholine:arachidonoyl-CoA acyltransferase, or CoA-independent transacylase activities in homogenates from MAFP-treated cells. Fig. 4 shows that MAFP strongly inhibited AA mobilization in PAF-activated cells. Whereas MAFP inhibited the extracellular release of [^{3}H]AA from prelabeled cells by about 75% (Fig. 4*A*), the PAF-induced accumulation of cellular [^{3}H]AA was almost completely blocked by the inhibitor (Fig. 4*B*).

P388D$_1$ macrophages possess a third PLA$_2$ enzyme, namely a cytosolic iPLA$_2$ that shows no preference for AA-containing phospholipids; in fact, it prefers palmitoyl over arachidonoyl residues (20). Recent evidence from our laboratory indicates that MAFP also inhibits pure iPLA$_2$ from P388D$_1$ cells.[4] Therefore, at least part of the MAFP-sensitive AA mobilization could be mediated by the iPLA$_2$ in addition to the cPLA$_2$. The iPLA$_2$

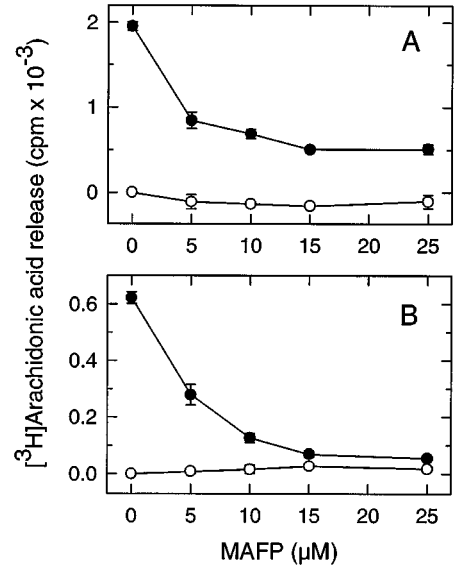

FIG. 4. **Effect of MAFP on PAF-stimulated [^{3}H]AA mobilization in P388D$_1$ cells.** [^{3}H]AA-labeled LPS-treated cells were incubated with the indicated concentrations of MAFP for 15 min. Subsequently, the cells were incubated with (●) or without (○) 100 nM PAF for either 10 (*A*) or 1.5 (*B*) min. Extracellular [^{3}H]AA release (*A*) and cell-associated [^{3}H]AA (*B*) were quantitated as described under "Experimental Procedures."

from P388D$_1$ macrophages is potently and irreversibly inhibited by the mechanism-based inhibitor (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (bromoenol lactone, BEL) (13). This compound manifests over a 1000-fold selectivity for inhibition of the iPLA$_2$s *versus* the Ca^{2+}-dependent sPLA$_2$s (21) and has previously been used to investigate the role of iPLA$_2$ in AA release in certain cell types (8, 9). In our laboratory, we have found that BEL is a poor inhibitor of pure cPLA$_2$.[5] This inhibitor does not affect any of the following

[3] J. Balsinde, I. D., Bianco, and E. A. Dennis, unpublished data.

[4] Lio, Y.-C., Reynolds, L. J., Balsinde, J., and Dennis, E. A. (1996) *Biochim. Biophys. Acta*, in press.

[5] L. J. Reynolds, and E. A. Dennis, unpublished data.

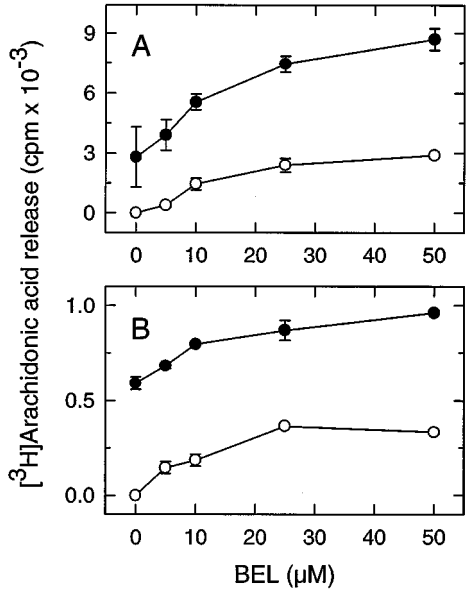

FIG. 5. **Effect of BEL on PAF-stimulated [^{3}H]AA mobilization in P388D_1 cells.** [^{3}H]AA-labeled LPS-treated cells were incubated with the indicated concentrations of BEL for 30 min. Subsequently, the cells were incubated with (●) or without (○) 100 nM PAF for either 10 (*A*) or 1.5 (*B*) min. Extracellular [^{3}H]AA release (*A*) and cell-associated [^{3}H]AA (*B*) were quantitated as described under "Experimental Procedures."

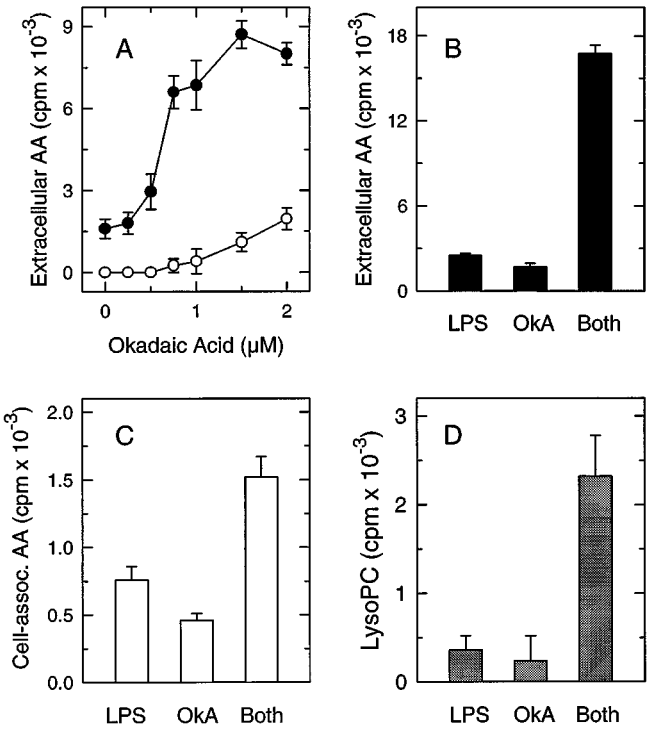

FIG. 6. **Effect of okadaic acid (*OkA*) on [^{3}H]AA mobilization in P388D_1 cells.** *A*, [^{3}H]AA-labeled cells were exposed to the indicated amounts of okadaic acid for 30 min, washed, and incubated for an additional 10-min period with (●) or without (○) 100 nM PAF. *B* and *C*, [^{3}H]AA-labeled cells were incubated with either 200 ng/ml LPS for 1 h, 1 μM okadaic acid for 30 min, or both. In the LPS plus okadaic acid incubations, okadaic acid was present only during the last 30 min of incubation. Subsequently, the cells were washed and stimulated with 100 nM PAF. *D*, cells labeled with [^{3}H]choline were preincubated with LPS, okadaic acid, or both as described above and then stimulated with PAF for 1.5 min. Lyso-PC accumulation was determined as described under "Experimental Procedures."

activities, measured in homogenates from BEL-treated cells: $cPLA_2$, $sPLA_2$, arachidonoyl-CoA synthetase, lysophosphatidylcholine:arachidonoyl-CoA acyltransferase, and CoA-independent transacylase (22).

The effect of BEL on PAF-induced AA mobilization from LPS-primed P388D_1 cells is shown in Fig. 5. At concentrations up to 50 μM, which totally block cellular $iPLA_2$ (22), BEL was ineffective in inhibiting either the extracellular release of [^{3}H]AA (Fig. 5*A*) or the accumulation of cellular free fatty acid (Fig. 5*B*). Instead, BEL enhanced both basal and PAF-stimulated [^{3}H]AA mobilization, although the ratio of stimulated *versus* unstimulated release remained the same at all BEL concentrations. The enhancing effect of BEL on P388D_1 macrophage AA release is probably related to its inhibitory action on cellular fatty acid incorporation into phospholipid (22). The lack of any inhibitory effect of BEL on [^{3}H]AA release demonstrates that the $iPLA_2$ does not significantly contribute to this release. Therefore, the MAFP-sensitive [^{3}H]AA release should be ascribed to the $cPLA_2$.

Priming of AA Release by Okadaic Acid—It is well established that cellular $cPLA_2$ activity is regulated by phosphorylation (2–4). Okadaic acid, a protein phosphatase inhibitor, also induces activation of $cPLA_2$ by preventing dephosphorylation of the enzyme (4). Therefore, this reagent was used to further evaluate the involvement of $cPLA_2$ in the PAF activation. As shown in Fig. 6*A*, pretreatment with okadaic acid resulted in the cells becoming sensitized for an enhanced [^{3}H]AA release in response to PAF. Importantly, when okadaic acid was added along with LPS during priming, a strong potentiation of the AA release response was observed. Okadaic acid not only increased both extracellular (Fig. 6*B*) and cell-associated [^{3}H]AA (Fig. 6*C*), but it also augmented cellular lyso-PC levels in cells prelabeled with [^{3}H]choline (Fig. 6*D*). These data stress the ability of okadaic acid to amplify LPS/PAF activation of AA mobilization, thus supporting the involvement of the phosphorylation-regulated $cPLA_2$ in this process. Interestingly, when the experiments depicted in Fig. 6 were carried out in the presence of the $sPLA_2$ inhibitor diC_6SNPE (50 μM), extracellular [^{3}H]AA release was still inhibited by 45 ± 11% (mean ± S.E., n = 3), indicating that augmentation of $cPLA_2$ activity by okadaic acid does not result in a full response unless a functionally active $sPLA_2$ is present. diC_6SNPE did not affect cell-associated free AA levels in okadaic acid-treated cells.

$cPLA_2$ Activation Precedes That of $sPLA_2$—From our previous results using antisense RNA technology (6, 12) as well as our current data using the $sPLA_2$ inhibitors diC_6SNPE and LY311727, it is apparent that this enzyme accounts for a major portion of the [^{3}H]AA released into the incubation medium after 10 min of activation with PAF. However, our data using MAFP clearly show that selective inhibition of $cPLA_2$ results in at least 75% inhibition of extracellular [^{3}H]AA release (Fig. 4*A*). Inasmuch as MAFP does not directly inhibit $sPLA_2$ (see above), an explanation for these data could be that activation of $cPLA_2$ is necessary for $sPLA_2$ to act. Should this be the case, $cPLA_2$ activation must precede that of $sPLA_2$. Evidence in support of this view was obtained by investigating the time course of total [^{3}H]AA mobilization (*i.e.* cell-associated plus extracellularly released [^{3}H]AA) in cells treated with either MAFP or diC_6SNPE to selectively block $cPLA_2$ or $sPLA_2$, respectively. As shown in Fig. 7, inhibition of [^{3}H]AA release by diC_6SNPE was only apparent after 2–3 min of cell activation, a time frame at which cell-associated AA nearly drops to levels occurring in unstimulated cells (12). On the other hand, inhibition of AA release by MAFP was already observed at the earliest point measured, *i.e.* 1 min (Fig. 7), demonstrating that the action of $cPLA_2$ on cellular phospholipids precedes that of $sPLA_2$.

We next explored whether the addition of metabolites result-

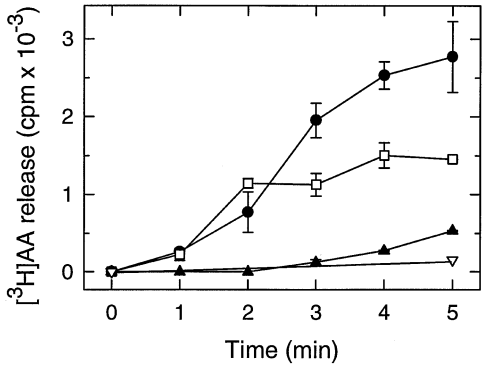

FIG. 7. **Effect of inhibiting either cPLA$_2$ or sPLA$_2$ on the time course of total [^{3}H]AA release from P388D$_1$ cells.** [^{3}H]AA-labeled LPS-treated cells were preincubated with MAFP (25 μM) (▲), diC$_6$SNPE (50 μM) (□), or neither (●) for 15 min. Subsequently, the cells were incubated with 100 nM PAF for the times indicated, except for the control (▽), which also lacked inhibitor. Afterwards, the supernatants were mixed with the cellular homogenates obtained from scraping the cell monolayers with 0.5% Triton X-100, and the resulting mix was subjected to lipid extraction. Free [^{3}H]AA was separated by thin-layer chromatography, and radioactivity was determined by scintillation counting.

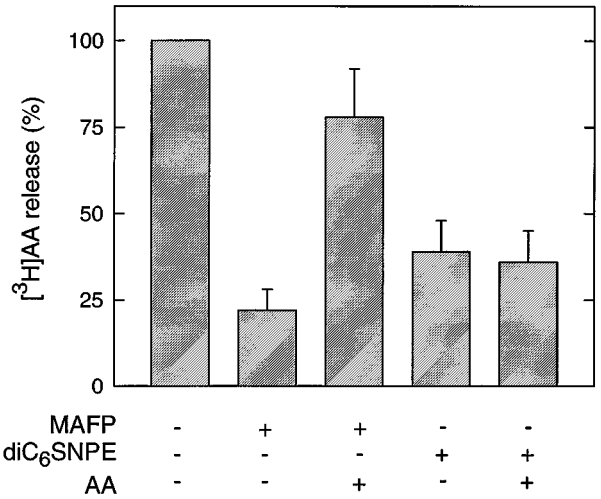

FIG. 8. **Exogenous AA overcomes the effect of MAFP on extracellular [^{3}H]AA release.** [^{3}H]AA-labeled LPS-treated cells were preincubated with MAFP (25 μM), diC$_6$SNPE (50 μM), or neither for 15 min, as indicated. Subsequently, 1 μM exogenous unlabeled AA was added 1 min before treatment with PAF (100 nM), as indicated. After 15 min, extracellular [^{3}H]AA release was quantitated as described under "Experimental Procedures." These data are the means ± S.E. of three experiments with duplicate incubations and are expressed as a percentage of the response observed in the absence of both inhibitor and exogenous AA. The 100% value corresponds to 2170 ± 520 cpm.

ing from cPLA$_2$ activity, *i.e.* free AA and lysophospholipids, could overcome the effect of MAFP on extracellular [^{3}H]AA release. As indicated earlier, when [^{3}H]AA-prelabeled LPS-treated cells were stimulated for 15 min with PAF in the presence of MAFP (25 μM), [^{3}H]AA release to the incubation medium was strongly decreased (Fig. 8). If, however, P388D$_1$ cells were exposed to exogenous AA (1 μM) for 1 min before PAF addition, the inhibitory effect of MAFP on extracellular [^{3}H]AA was greatly diminished (Fig. 8). Preincubating the cells with lysophospholipids (*i.e.* lyso-PC, lyso-PI, or lyso-PE) or other free fatty acids, whether saturated (*i.e.* palmitic, stearic, or arachidic acids) or unsaturated (oleic or linoleic acids) did not overcome the inhibitory effect of MAFP. Control experiments had shown that at the doses employed, none of the above mentioned fatty acids or lysophospholipids exerted cytotoxic effects or affected basal [^{3}H]AA release. On the other hand, preincubating the cells with exogenous AA prior to PAF addition did not overcome the inhibitory effect of diC$_6$SNPE on extracellular [^{3}H]AA release (Fig. 8).

DISCUSSION

Regulation of AA Mobilization by Two Different PLA$_2$ Enzymes—By using antisense RNA technology to block the expression of sPLA$_2$, we previously demonstrated that an extracellular pool of this enzyme is responsible for at least 60–70% of the AA released from P388D$_1$ macrophages after activation with LPS/PAF (6). By using an anti-sPLA$_2$ monoclonal antibody, Pfeilschifter *et al.* (7) have estimated a similar contribution of sPLA$_2$ to extracellular AA release during receptor stimulation of rat mesangial cells. In the current study, we have utilized a third and different strategy to block sPLA$_2$ activity, *i.e.* the use of diC$_6$SNPE and LY311727, two selective and structurally unrelated sPLA$_2$ inhibitors, and have confirmed that this enzyme mediates a major portion of the AA released to the incubation medium. Although these three different strategies emphasize the very important role of sPLA$_2$ in AA release, they also stress that another effector enzyme is involved as well. Moreover, both antisense inhibition of sPLA$_2$ and pharmacological inhibition of the enzyme by diC$_6$SNPE and LY311727 have highlighted the fact that sPLA$_2$ is not the enzyme that mediates the small burst of cell-associated free AA that occurs shortly after PAF stimulation. Use of multiple selective inhibitors in this study has provided evidence that the second effector enzyme in AA release in PAF-stimulated LPS-primed P388D$_1$ macrophages is the cPLA$_2$. Thus, the current work, along with our previous data (6, 12), establish that cPLA$_2$, acting intracellularly, and sPLA$_2$, acting on the outer surface of the cell, both mediate AA release in response to PAF receptor stimulation. It is important to note that inhibition of sPLA$_2$ by either antisense techniques (6, 12) or chemical inhibitors (this study) does not result in complete inhibition of AA release to the extracellular medium. This suggests that under PAF activation conditions, a portion of the AA released intracellularly by the cPLA$_2$ may exit the cell and mix with the fatty acid liberated by the sPLA$_2$.

The notion that both cPLA$_2$ and sPLA$_2$s mediate receptor activation of AA release may represent a signaling mechanism common to agonists that elicit short-term (*i.e.* PAF) or long-term responses (*i.e.* cytokines and growth factors). Work by Schalkwijk *et al.* (23, 24) in cytokine-stimulated rat mesangial cells and by Murakami *et al.* (5) in cytokine-stimulated human endothelial cells has also suggested that both PLA$_2$s may participate in regulating AA mobilization in these cells, their relative contribution being dependent on the agonist used. However, there are other cell systems such as platelets, in which a role for sPLA$_2$ in AA release could not be demonstrated (25). Moreover, AA release in thrombin-stimulated platelets could be completely blocked by inhibiting the cPLA$_2$, suggesting that in this system, cPLA$_2$ is perhaps the only effector involved in AA release (25).

Domin and Rozengurt (26) have recently demonstrated that AA mobilization in Swiss 3T3 cells treated with platelet-derived growth factor follows a bimodal kinetics. In this system, cPLA$_2$ activation appears to be responsible for the small burst of AA mobilization that occurs during the first 20 min following agonist stimulation. However, the major component of agonist-induced AA release was found to be due to another unidentified effector. Because these data are very reminiscent of the situation in PAF-stimulated LPS-primed P388D$_1$ cells, it is tempting to speculate that the second effector involved in the system studied by Domin and Rozengurt (26) is the sPLA$_2$. Although it is certain that the time course of the AA release responses in

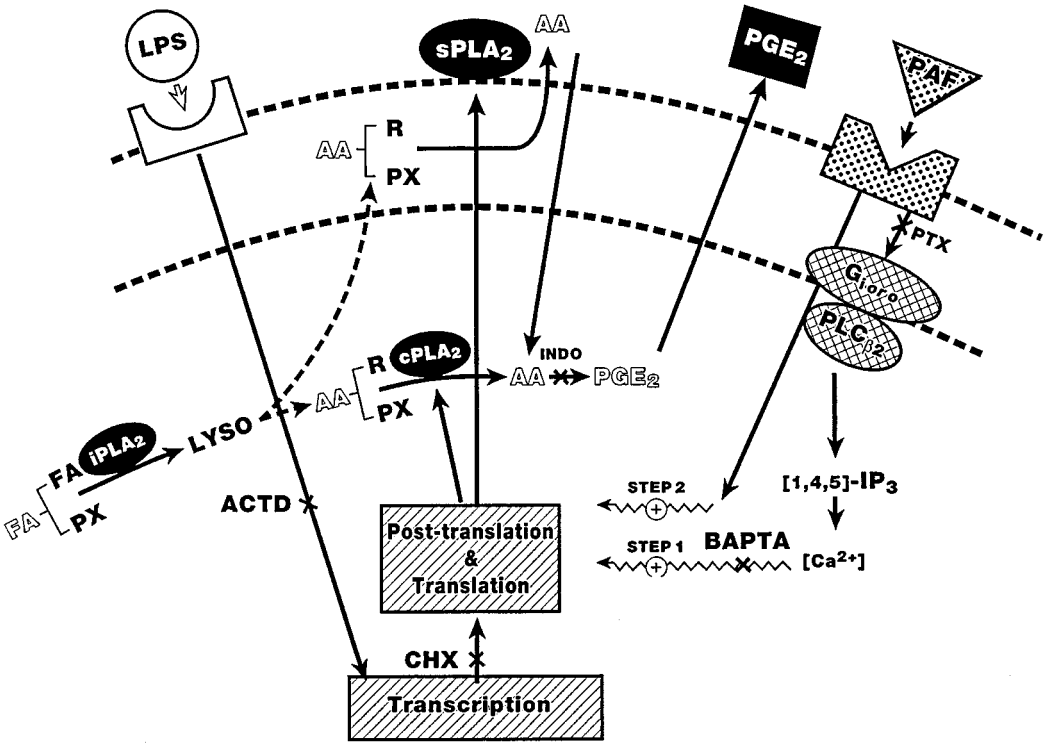

FIG. 9. **Signal transduction model for PAF-stimulated AA release in LPS-primed $P388D_1$ macrophages.** The different roles of the $iPLA_2$, $cPLA_2$, and $sPLA_2$ in AA incorporation and mobilization are indicated. Inhibition (×) by pertussis toxin (*PTX*), BAPTA (bis-(*O*-aminophenoxy)ethane-*NNN′N′*-tetracetic acid), actinomycin D (*ACTD*), cyclohexamide (*CHX*), and indomethacin (*INDO*) are also indicated. See text for further details.

platelet-derived growth factor-stimulated Swiss 3T3 cells and in PAF-stimulated $P388D_1$ cells are clearly distinct, this is most likely due to cell type differences and especially to the very distinct nature of the agonists employed. As a matter of fact, when 3T3 cells are challenged with a short burst agonist such as bombesin, a rapid and transient increase in cell-associated free AA is observed shortly after receptor occupancy (27).

Different Phospholipid Sources for the Two PLA_2s—Given the fact that $cPLA_2$ and $sPLA_2$ mobilize AA from activated $P388D_1$ cells with different spatial and temporal characteristics, it would seem possible that the two enzymes utilize different AA pools. We explored this issue by selectively labeling the different AA-containing phospholipids with [^{3}H]AA and [^{14}C]AA. In $P388D_1$ cells, two of the major AA-containing phospholipids, namely PC and PI, are labeled with exogenous radioactive AA very rapidly (within minutes), whereas PE is labeled more slowly (12). This phenomenon, along with the nonuniform distribution of arachidonoyl moieties in different phospholipid classes, allowed us to label the phospholipids with AA to different [^{14}C]/[^{3}H] ratios. Calculation of the [^{14}C]/[^{3}H] ratio for the AA released by PAF at two different locations, *i.e.* cell-associated and outside the cell, gives two very different values. This result strongly suggests that cell-associated free AA and extracellular free AA arise from different pools.

By comparing the [^{14}C]/[^{3}H] ratios in free AA with those in the phospholipids, we could delineate the origin of cell-associated AA and extracellular AA. Although the interpretation of these data may be complicated by the phenomenon of mixing AA pools as well as the molecular heterogeneity of each phospholipid class, some definite conclusions can be reached. The fact that the [^{14}C]/[^{3}H] ratio for extracellular AA is considerably lower than that of cell-associated AA suggests that most of the extracellular free AA arises from PE, but this is not the case for cell-associated AA. As a matter of fact, PE is the only phospholipid whose [^{14}C]/[^{3}H] ratio is comparable with that of extracellular AA. Following a similar rationale, it can be concluded that all major phospholipids contribute to the early burst in cell-associated AA, although their relative contribution cannot be estimated from our data. Because $cPLA_2$ is responsible for raising the levels of cell-associated free AA, involvement of all major phospholipid classes in this process is consistent with the notion that this enzyme does not distinguish among phospholipid head groups (28, 29).

It is generally assumed that the phospholipids are asymmetrically distributed in cellular membranes, PC being localized primarily at the outer leaflet and PE at the inner leaflet of the plasma membrane (30). Thus the notion that the extracellular AA release arises primarily from PE would seem, at a first glance, unexpected. However, $sPLA_2$, the enzyme primarily responsible for mobilizing AA to the extracellular medium, has been reported to prefer PE over any other phospholipid when these are presented in a natural membrane system (29, 31). It is possible that the $sPLA_2$ preference for PE in these studies could be caused by a higher proportion of PE relative to other phospholipids in these membranes, because studies with vesicles containing various kinds of phospholipids have failed to detect any head group specificity (32). However, studies in platelets have demonstrated that during activation, a rapid translocation of AA-containing PE from the inner to the outer leaflet of the plasma membrane takes place (33). Such a translocation would permit the AA-containing PE to be readily accessible to the extracellular $sPLA_2$. Interestingly, recent work by Fourcade *et al.* (34) has suggested that loss of membrane asymmetry resulting from movement of phospholipids from the inner to the outer leaflet of the membrane may play a key role in regulating the activity of extracellular $sPLA_2$.

Cross-talk between the PLA_2s?—The results of this study raise the possibility that cross-talk may exist between the mechanisms of activation of $cPLA_2$ and $sPLA_2$. We have found that $cPLA_2$ becomes activated before $sPLA_2$ begins to act and

that inhibition of $cPLA_2$ by MAFP leads to a very marked inhibition of total AA release induced by PAF, higher than is expected if one considers that $sPLA_2$ is responsible for at least two-thirds of the AA released to the extracellular medium (6, 12). Thus the question arises as to whether $cPLA_2$-mediated events are required for the action of $sPLA_2$. We explored this issue by directly adding the PLA_2 by-products, namely free fatty acids and lysophospholipids, shortly before agonist addition to cells in which $cPLA_2$ had been inactivated by MAFP. Exogenous AA, but not other fatty acids or lysophospholipids, was able to restore the extracellular [^{3}H]AA release in response to PAF. Treating the cells with exogenous AA has the effect of increasing cell-associated free fatty acid levels well above those found in untreated cells, thereby mimicking $cPLA_2$ activation. No effect of was seen when exogenous AA was added in the absence of PAF or when a $sPLA_2$ inhibitor was used, suggesting that the effect may be specific.

A perturbation of the lipid bilayer or "membrane rearrangement," initiated by an agonist/receptor interaction, appears to be required to activate $sPLA_2$ at the outer surface of the cell (35) The data reported herein suggest that, in addition to phospholipid translocation (34), such a membrane rearrangement may involve a transient elevation of free AA mediated by receptor-activated $cPLA_2$. Thus, our results appear to suggest a new role for free AA in cellular signaling, *i.e.* to help regulate the accessibility of $sPLA_2$ to its substrate in the membrane.

However, such an intracellular elevation of free AA is not itself sufficient to elicit the cellular response. Therefore, other additional signals that occur at the earliest stages of PAF activation, such as inositol phospholipid turnover, Ca^{2+} mobilization, or protein phosphorylation (11) are also required for the AA release process to fully take place. When all of these signals are induced sufficiently, $sPLA_2$ begins to hydrolyze phospholipids at the outer surface of the cell, and this results in full AA mobilization. According to this model, augmentation of any of these early signals could result in an increased liberation of AA to the extracellular medium. This is what occurs in the experiments using okadaic acid, wherein augmentation of $cPLA_2$ activity and hence cell-associated free AA levels dramatically enhance the extracellular AA release, provided $sPLA_2$ is functional.

We should emphasize that the above model of cross-talk between $cPLA_2$ and $sPLA_2$ has to be regarded as a working model and not as an established one. Much of our evidence in favor of a causal relationship between $cPLA_2$ and $sPLA_2$ rests on the use of the phosphonylfluoride MAFP, a highly reactive compound. It cannot be ruled out at this time that MAFP is exerting some other undesired effects or that the $sPLA_2$-activating effect of exogenous AA is unrelated to $cPLA_2$.

$iPLA_2$ Role in AA Mobilization—Another striking feature of the current work is the role of the one other PLA_2 present in $P388D_1$ cells, *i.e.* the $iPLA_2$, during PAF activation. We have investigated this issue by conducting studies with BEL, a selective inhibitor of the $iPLA_2$. Our results clearly show that BEL does not inhibit AA release, ruling out a significant role for the $iPLA_2$ in this release. In a previous report, we demonstrated that the steady-state level of lysophospholipids in BEL-treated cells is decreased by about two-thirds and that this effect directly correlates with the inhibition of AA esterification into phospholipids as well as the inhibition of cellular $iPLA_2$ activity (22). Moreover, BEL does not have any effect on the AA reacylating enzymes arachidonoyl-CoA synthetase and lysophospholipid:arachidonoyl-CoA acyltransferase (22). Based on these previous data, our current finding that the unstimulated levels of [^{3}H]AA are increased in media from BEL-treated cells may be explained as the consequence of the diminished capacity of these cells to reacylate AA into membrane phospholipids. Thus, the basal level of AA, being produced by constitutively active enzymes different from the $iPLA_2$, increases because the $iPLA_2$ is blocked, thereby lowering the availability of acceptor.

The fact that the potentiating effect of BEL on extracellular AA release is still observed in activated cells further indicates that the receptor-regulated PLA_2s releasing AA during PAF stimulation are distinct from the BEL-sensitive $iPLA_2$. This view lends support to a model whereby each of the distinct PLA_2s present in $P388D_1$ cells may play different roles in regulating free AA availability during PAF-induced activation (Fig. 9). By interacting with its specific receptor at the plasma membrane, PAF initiates the stimulation process by increasing the intracellular Ca^{2+} concentration (step 1). PAF also triggers a second as yet unknown signal (step 2) (11). These signals act in concert to initiate translational/post-translational events that result in the activation of $cPLA_2$ and $sPLA_2$. The two enzymes, acting either intracellularly ($cPLA_2$) or extracellularly ($sPLA_2$), are responsible for mobilizing AA upon PAF receptor stimulation. On the other hand, the $iPLA_2$ allows reincorporation of part of the fatty acid previously liberated by its Ca^{2+}-dependent counterparts and in this manner helps replenish cellular AA pools. If the $iPLA_2$ were responsible for generating a significant portion of lyso acceptors under activation conditions, this enzyme might also play a role in eicosanoid metabolism by limiting the amount of AA available for eicosanoid biosynthesis.

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Distinct Roles in Signal Transduction for Each of the Phospholipase A Enzymes Present in P388D Macrophages

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