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Authors

Balsinde, Jesús
Balboa, María A
Dennis, Edward A

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Functional coupling between secretory phospholipase A_2 and cyclooxygenase-2 and its regulation by cytosolic group IV phospholipase A_2

JESÚS BALSINDE, MARÍA A. BALBOA, AND EDWARD A. DENNIS*

Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA 92093-0601

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ABSTRACT **Secretory phospholipase A_2 ($sPLA_2$) is the major effector involved in arachidonic acid (AA) mobilization and prostaglandin E_2 (PGE_2) production during stimulation of $P388D_1$ macrophages with the inflammatory stimuli bacterial lipopolysaccharide and platelet-activating factor. We herein demonstrate that PGE_2 in stimulated $P388D_1$ cells is accounted for by the inducible cyclooxygenase (COX)-2. COX-1, though present, appears not to participate significantly in stimulus-induced PGE_2 production in $P388D_1$ macrophages. Reconstitution experiments utilizing exogenous recombinant $sPLA_2$ demonstrate that activation of the $sPLA_2$ at the plasma membrane is highly dependent on previous activation of the cytosolic phospholipase A_2 ($cPLA_2$). Collectively these results demonstrate (*i*) that functional coupling exists between $sPLA_2$ and COX-2 in activated cells, (*ii*) the critical role that $cPLA_2$ plays in lipid mediator production, and (*iii*) that there is crosstalk between $cPLA_2$ and $sPLA_2$ in the cell.**

The phospholipase A_2 (PLA_2) superfamily is composed of a number of lipolytic enzymes whose common feature is to hydrolyze the fatty acid present in the *sn*-2 position of glycerophospholipids (1). At least three different PLA_2s exist in mammalian cells, namely a secretory PLA_2 ($sPLA_2$), the 85-kDa cytosolic group IV PLA_2 ($cPLA_2$), and a Ca^{2+}-independent group VI PLA_2 ($iPLA_2$) (1, 2). Increasing evidence suggests that all three PLA_2 forms may participate in some manner in arachidonic acid (AA) turnover and mobilization with their relative contribution being dependent on the cell type and agonist used.

$cPLA_2$ has been shown to act on intracellular membranes—i.e., those of the perinuclear envelope and endoplasmic reticulum (3, 4). The plasma membrane of the cells appears to be the major site of action of the $sPLA_2$, which is consistent with the fact that $sPLA_2$ requires millimolar Ca^{2+} for activity (5–11). $sPLA_2$ appears to be responsible for the majority of free AA produced by stimulated $P388D_1$ cells (7, 12–14).

Once liberated from phospholipids, the AA is converted to prostaglandins by the cyclooxygenases (COXes). Two different COX enzymes have been identified in cells (15). The first one, COX-1, is constitutively expressed by most cell types, whereas the other one, COX-2, is generally induced after cell stimulation (15). As is the case for the PLA_2s, the relative contribution of each of these COX isoenzymes to overall prostaglandin synthesis appears to be strikingly dependent on cell type and stimulus used (16). However the question remains as to whether a specific coupling of each COX isoenzyme to a particular PLA_2 exists. Although supportive evidence for that view is emerging (15, 16), identification of the particular COX and PLA_2 isoenzymes involved has proven to be difficult and often contradictory. For example, Herschman and colleagues (11, 17–19) have suggested a coupling between $sPLA_2$ and COX-1 for the immediate response of mast cells; however, Arm and colleagues (20, 21) have suggested that the $sPLA_2$ is coupled to COX-2 in the same cells. In a recent report, Murakami *et al.* (22) have shown that the $cPLA_2$ couples to both COX-1 and COX-2 for the two phases of prostaglandin production in mouse osteoblasts.

There is a second important unanswered question regarding the cellular biochemistry of $sPLA_2$. This enzyme appears to act poorly against intact cellular membranes. Thus, some sort of physical perturbation or "membrane rearrangement" is required for $sPLA_2$ to act on a cellular membrane (5, 6). However, the biochemical signals that cause such a perturbation are largely unknown. Fourcade *et al.* (23) have suggested that loss of membrane asymmetry due to phospholipid flip-flop could be one of these signals. We have recently found that accumulation of membrane destabilizers such as diacylglycerol or ceramide may also serve to perturb plasma membrane packing and hence favor $sPLA_2$ action (24). More importantly, we found as well that increasing the intracellular levels of AA by exogenous addition of unlabeled fatty acid to [^{3}H]AA-labeled cells (which mimics $cPLA_2$ activation) was able to trigger release of [^{3}H]AA by a mechanism dependent on $sPLA_2$. These data suggested that $cPLA_2$ activation may be another key event that leads to perturbation of the membrane, rendering it susceptible to $sPLA_2$ attack (13).

In the current study, we have studied the expression of COX isoforms in $P388D_1$ cells, and we have found that a functional link exists between $sPLA_2$ and COX-2 for eliciting prostaglandin production in activated cells. Furthermore, reconstitution experiments using recombinant $sPLA_2$ further support the notion that crosstalk exists between the $cPLA_2$ and the $sPLA_2$ during stimulation of AA release in $P388D_1$ cells. Thus the $cPLA_2$ plays a dual role in arachidonate signaling as it (*i*) participates in an intermediate signaling step that is required for $sPLA_2$ activation and (*ii*) acts as a producer of AA itself.

EXPERIMENTAL PROCEDURES

Materials. Mouse $P388D_1$ macrophage-like cells were obtained from the American Type Culture Collection. Iscove's modified Dulbecco's medium (endotoxin <0.05 ng/ml) was from BioWhittaker. Fetal bovine serum was from HyClone. Nonessential amino acids were from Irvine Scientific. [5,6,8,9,11,12,14,15-^{3}H]AA (specific activity 100 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. Platelet-activating factor (PAF), bacterial lipopolysaccharide (LPS; Re

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Abbreviations: PLA_2, phospholipase A_2; $cPLA_2$, 85-kDa group IV cytosolic phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; AA, arachidonic acid; COX, cyclooxygenase; PGE_2, prostaglandin E_2; LPS, bacterial lipopolysaccharide; PAF, platelet-activating factor; MAFP, methyl arachidonyl fluorophosphonate.

*To whom reprint requests should be addressed. e-mail: edennis@ucsd.edu.

595 from *Salmonella minnesota*), actinomycin D, cycloheximide, and AA were from Sigma. Okadaic acid was from Calbiochem or Biomol (Plymouth Meeting, PA). Methyl arachidonyl fluorophosphonate (MAFP), NS-398, indomethacin, and aspirin were from Cayman Chemicals (Ann Arbor, MI). Human recombinant synovial PLA_2 and purified IgG from anti-human synovial PLA_2 rabbit antiserum (BQY-113A) were generously provided by J. L. Bobbitt and R. M. Kramer (Eli Lilly, Indianapolis, IN). Antibodies against murine COX isoforms were generously provided by W. L. Smith (Department of Biochemistry, Michigan State University, East Lansing, MI).

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 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. $P388D_1$ 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. When required, radiolabeling of the $P388D_1$ cells with [^{3}H]AA was achieved by including 0.5 μCi/ml [^{3}H]AA during the overnight adherence period (20 h). 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 the Cells. The standard regimen for activating $P388D_1$ cells with LPS and PAF has been described previously (7, 13, 25). Briefly, $P388D_1$ cells were placed in serum-free medium for 30–60 min before the addition of LPS (200 ng/ml) for 1 h, after which they were challenged with 100 nM PAF for the time indicated.

Western Blot Analyses. The cells were washed twice with serum-free medium and homogenized by 25 strokes in a Dounce homogenizer in a buffer consisting of 20 mM Tris·HCl, 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 20 μM aprotinin, and 0.1% 2-mercaptoethanol, pH 7.5. The homogenates were centrifuged at 500 $\times$ *g* for 5 min at 4°C to separate nuclei. The resulting supernatant was centrifuged at 100,000 $\times$ *g* for 90 min at 4°C to separate cytosolic and membrane fractions. Samples from cell homogenates (50 μg) and their equivalent cytosolic and membrane fractions were separated by SDS/PAGE (10% acrylamide gel) and transferred to Immobilon-P (Millipore). Nonspecific binding was blocked by incubating the membranes with 5% nonfat milk in phosphate-buffered saline for 18 h. Membranes were incubated with anti-COX-1 or anti-COX-2 antiserum and then treated with horseradish peroxidase-conjugated protein A (Amersham). Bands were detected by enhanced chemiluminiscence (ECL; Amersham).

Northern Blot Analyses. Total RNA was isolated from unstimulated or LPS/PAF-stimulated cells by the Trizol reagent method (Life Technologies), exactly as indicated by the manufacturer. Five micrograms of RNA was electrophoresed in an 1% formaldehyde/agarose gel and transferred to nylon filters (Hybond; Amersham) in 10$\times$ SSC buffer (1$\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Hybridizations were performed in QuickHyb solution (Stratagene) by following the manufacturer's instructions. ^{32}P-labeled probes for COX-1 or COX-2 (Cayman Chemicals) were coincubated with the filters for 1 h at 66°C, followed by three washes with 2$\times$ SSC containing 0.1% SDS at room temperature for 30 min. A final wash was carried out at 60°C for 30 min with 1$\times$ SSC containing 0.1% SDS. Bands were visualized by autoradiography.

Measurement of Prostaglandin E_2 (PGE_2) Production and of Extracellular [^{3}H]AA Release. For PGE_2 production, LPS-treated cells were stimulated with 100 nM PAF for 10 min. Afterward, the supernatants were removed and cleared of detached cells by centrifugation, and PGE_2 was quantitated by using a specific radioimmunoassay (PerSeptive Biosystems, Framingham, MA). For [^{3}H]AA release experiments, the cells, labeled with [^{3}H]AA as described above, were stimulated with 100 nM PAF for 10 min in the presence of 0.1 mg/ml BSA. The supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

Data Presentation. 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 AND DISCUSSION

Expression of COX Isoforms. When $P388D_1$ macrophages are treated with 200 ng/ml LPS for 1 h and then stimulated with 100 nM PAF, a rapid burst of PGE_2 is generated, reaching a peak 3–5 min after PAF addition (12). Immediate PGE_2 production occurs concomitantly with activation of both $cPLA_2$ and $sPLA_2$, and no further PGE_2 is produced in a delayed phase, even after several hours of incubation with LPS/PAF (12, 25). Immunoblot analysis of the COX isoforms expressed by resting $P388D_1$ cells revealed that COX-2 protein was increased by LPS treatment of the cells (Fig. 1*A*). COX-1, on the contrary, did not appear to change (Fig. 1*A*). LPS-induced expression of COX-2 was blocked by both actinomycin D and cycloheximide, inhibitors of RNA transcription and protein synthesis, respectively (Fig. 1*A*). COX-1 expression was not affected by either actinomycin D or cycloheximide (Fig. 1*A*).

The above results were confirmed by analyzing RNA message levels for both COX-1 and COX-2. When total RNA from resting cells was analyzed by Northern blotting using probes for

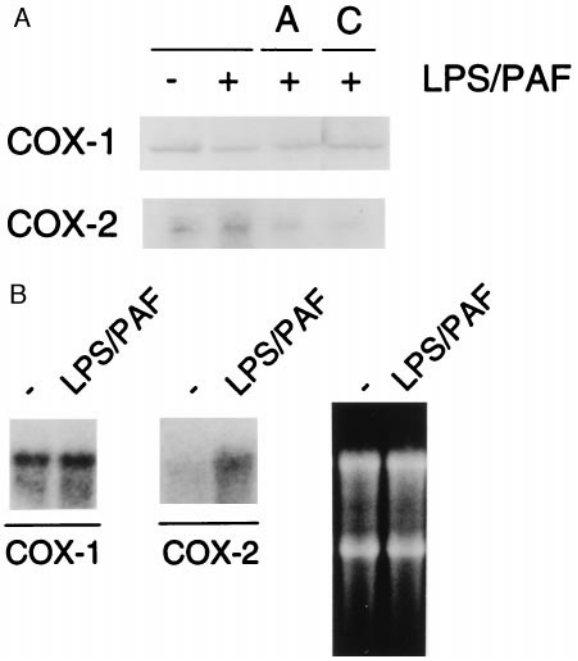

FIG. 1. (*A*) Expression of COX isoforms in $P388D_1$ cells. The cells were incubated with 5 μM actinomycin D (A) or 10 μM cycloheximide (C) for 30 min before the LPS/PAF treatment (1 h with LPS plus 10 min with PAF). Cells were then lysed as described in the text. Protein (100 μg) was separated by SDS/PAGE and analyzed by immunoblotting using specific antibodies against COX-1 or COX-2. (*B*) Northern blot of COX-1 and COX-2 from total $P388D_1$ RNA. Total RNA from LPS/PAF-stimulated cells or nonstimulated (−) was isolated and hybridized with probes for COX-1 and COX-2, as indicated. As a control for RNA loading, an "in-gel" RNA picture is shown on the right.

COX-1, the signal detected was identical to that found when RNA from LPS/PAF-activated cells was used (Fig. 1*B*). Total RNA from resting cells also gave a positive signal when probes for COX-2 were used, albeit the signal was much weaker than that found for COX-1. A strong signal for COX-2 was detected, however, in RNA samples from LPS/PAF-activated cells (Fig. 1*B*).

LPS/PAF-induced PGE_2 production was completely abolished by NS-398, a specific COX-2 inhibitor (Fig. 2*A*). NS-398 completely inhibits COX-2 with an IC_{50} <5 μM, whereas COX-1 remains unaffected at concentrations higher than 100 μM (26). Thus the above data suggest that COX-2 is the only enzyme responsible for stimulus-induced PGE_2 in these cells. Our previous results have demonstrated that $sPLA_2$ is the enzyme that provides most of the AA to be used for PGE_2 production in activated conditions in $P388D_1$ cells (7, 12, 14). Thus these previous data coupled with the results reported herein formally establish the existence of a link between $sPLA_2$ and COX-2 in activated cells.

From the data shown in Fig. 2*A*, it is apparent that basal unstimulated PGE_2 levels were not affected by NS-398. This finding could suggest that basal PGE_2 is produced by COX-1 in the resting cells. However it could also be possible that COX-1 is not functional in $P388D_1$ cells and that the basal PGE_2 produced corresponds to the fraction of PGE_2 that was produced during preincubation, just before the COX-2 became inactivated by NS-398. To investigate these possibilities, experiments were performed wherein PGE_2 production was analyzed in cells exposed to 1 μM exogenous AA (Fig. 2*B*). As well as being physiologically sound (under normal circumstances, the $sPLA_2$ provides the AA from *outside*), this approach has the advantage that the total amount of PGE_2 produced by the cells can be controlled (by adding more or less exogenous AA). PGE_2 production under these conditions was strongly, but not completely, inhibited by NS-398, indicating that the majority of the COX activity to which exogenous AA is accessible arises from COX-2 ($\approx$80%). However, there is also an appreciable contribution by COX-1, as judged by the NS-398-resistant PGE_2 production ($\approx$20%) (Fig. 2*B*). Aspirin and indomethacin, two nonspecific COX inhibitors, abolished PGE_2 production (Fig. 2*B*). Collectively, the results from these experiments suggest that the AA released by the $sPLA_2$ after cellular stimulation is accessible only to COX-2 and not to COX-1, even though the latter is present in active form in the macrophages.

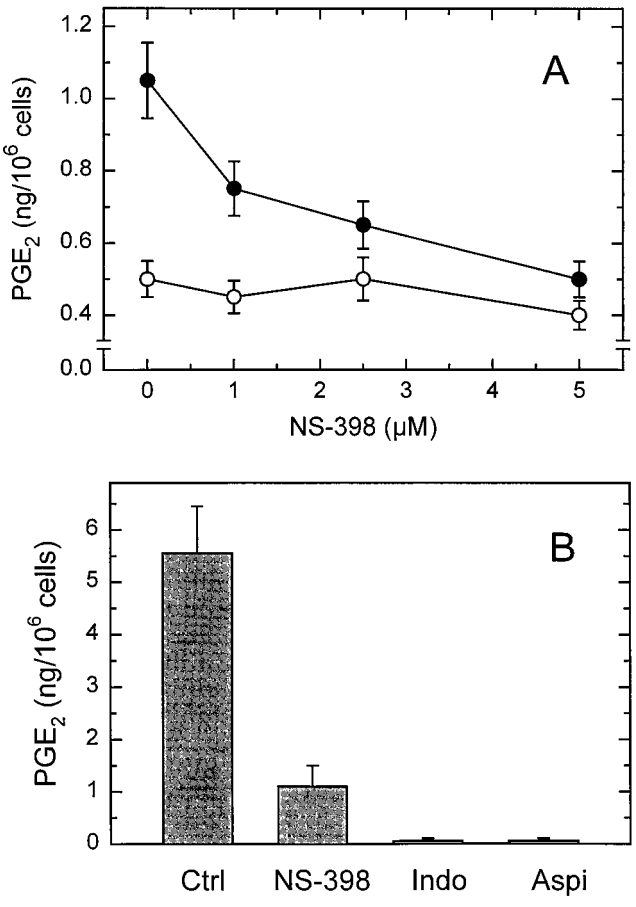

FIG. 2. A) Effect of NS-398 on PGE_2 production in $P388D_1$ cells. The cells were incubated with the indicated concentrations of NS-398 for 30 min before and during the LPS/PAF treatment. PGE_2 in the supernatants was quantitated by using a specific radioimmunoassay. Basal PGE_2 production—i.e., that observed in the absence of LPS/PAF at the indicated NS-398 concentrations—is indicated by the open circles. (*B*) Effect of COX inhibitors on PGE_2 production from exogenous AA. The cells were treated with 5 μM NS-398, 25 μM indomethacin (Indo), 25 μM aspirin (Aspi), or neither (Ctrl) for 30 min before and during the 1-h treatment with LPS. Afterward, 1 μM AA was added and the amount of PGE_2 produced after 10 min was quantitated by using a specific radioimmunoassay.

$cPLA_2$ Regulates $sPLA_2$-Dependent AA and PGE_2 Production in Activated $P388D_1$ Macrophages. To further assess the coupling of $sPLA_2$ to COX-2 in $P388D_1$ cells, a series of experiments was performed that involved adding exogenous

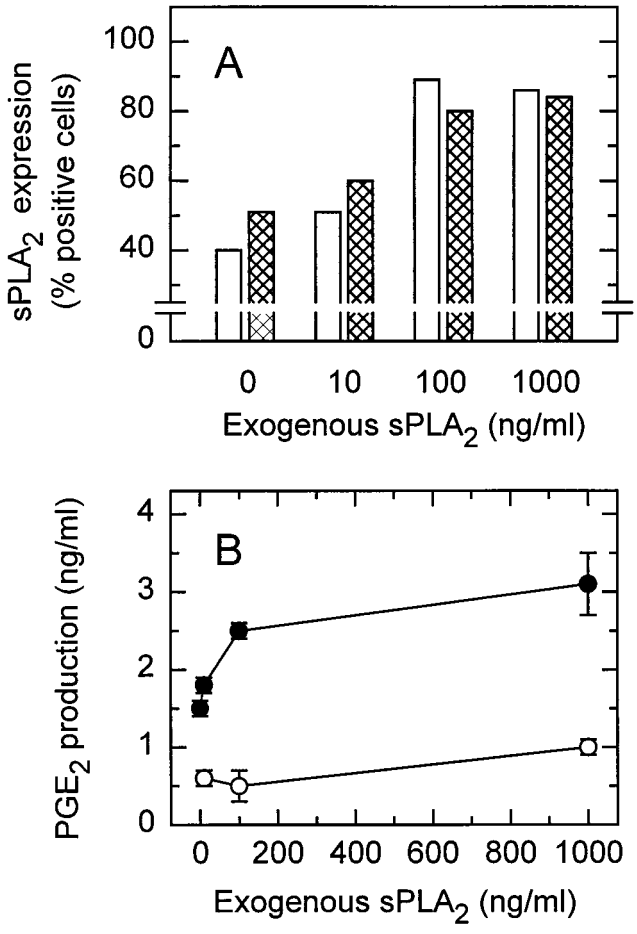

FIG. 3. Effect of adding exogenous $sPLA_2$ to $P388D_1$ cells. (*A*) Either resting (open bars) or LPS/PAF-activated (crosshatched bars) cells were incubated with the indicated concentrations of exogenous $sPLA_2$, and $sPLA_2$ surface expression was determined by flow cytometry. (*B*) PGE_2 production by resting (○) or LPS/PAF-activated (●) $P388D_1$ cells in the presence of the indicated concentrations of exogenous $sPLA_2$. Exogenous $sPLA_2$ was added at the time the cells were exposed to PAF or vehicle, and was present for 10 min.

recombinant $sPLA_2$ to the $P388D_1$ cells. The $sPLA_2$ present in $P388D_1$ cells belongs to group V (14). Unfortunately, no purification of group V $sPLA_2$ from either natural or recombinant sources has been reported. Thus for these experiments we used recombinant group IIA $sPLA_2$, which is structurally similar to the group V enzyme (16). Fig. 3*A* shows that exogenous $sPLA_2$ readily associated in a dose-dependent manner with the membrane of both resting and activated $P388D_1$ cells, as judged by flow cytometry under our previously described conditions (14). However, the ability of exogenous $sPLA_2$ to elicit lipid mediator production was strikingly dependent on the activation state of the cells. Exogenous $sPLA_2$ was almost inactive when added to nonstimulated cells, whereas the enzyme noticeably increased PGE_2 production when added to activated cells (Fig. 3*B*). These data are consistent with work by other laboratories in different cell types (8, 27, 28) and thus give additional support to the idea that, for the $sPLA_2$ to act on cellular membranes, membrane perturbation induced by a costimulator must occur first (5, 6).

We recently proposed that a transient accumulation of intracellular free AA produced by $cPLA_2$ may constitute another important factor leading to $sPLA_2$ activation in stimulated cells (13). This suggestion was based on the finding that artificially increasing the intracellular free AA levels by exogenous addition of fatty acid enabled phospholipid hydrolysis, apparently mediated by $sPLA_2$ (13). Because transient elevations in the intracellular AA concentration are caused by the $cPLA_2$, these data suggested that crosstalk may exist between the two PLA_2s during $P388D_1$ cell activation by LPS/PAF (13). Further evidence for such crosstalk was obtained by experiments determining the effect of MAFP on PGE_2 production by LPS/PAF-treated cells. MAFP inhibits $cPLA_2$ but not $sPLA_2$ (13), yet it strongly inhibited PGE_2 production (Fig. 4), which is mostly an $sPLA_2$-dependent process (7, 14).

However, validation of the existence of crosstalk between the two PLA_2s requires proof that the AA is actually activating the $sPLA_2$ and not inducing phospholipid hydrolysis by some other, unexpected, mechanism. We have further addressed the specificity of AA in activating the $sPLA_2$ by carrying out AA-release reconstitution experiments utilizing exogenous $sPLA_2$. In agreement with our previous data (13), when [^{3}H]AA-prelabeled LPS-treated cells were incubated with PAF in the presence of the $cPLA_2$ inhibitor MAFP (25 μM), [^{3}H]AA release to the extracellular medium was markedly inhibited, but this inhibition was reversed when the cells were exposed to exogenous AA (1 μM) for 1 min before PAF addition (Fig. 5). Consistent with the data shown in Fig. 3*B*, addition of exogenous $sPLA_2$ (500 ng/ml) increased PAF-stimulated [^{3}H]AA release (Fig. 5). However, exogenous $sPLA_2$ did not have any effect on PAF-stimulated [^{3}H]AA release when MAFP-treated cells were used; in fact, the response still remained inhibited (Fig. 5).

The inability of exogenous $sPLA_2$ to enhance [^{3}H]AA release in MAFP-treated cells cannot be explained by lack of membrane perturbation, because a costimulator—i.e., PAF—is also present. Moreover because, as mentioned above, MAFP does not have any inhibitory effect on $sPLA_2$ (13), these results demonstrate that MAFP specifically blocks generation of a *signal* that is required for the *exogenous* $sPLA_2$ to attack the membrane. Direct proof that this signal is provided by elevation of the intracellular free AA levels was given by the finding that coincubation with this fatty acid not only reversed the MAFP inhibition but even restored the enhanced [^{3}H]AA release response to PAF plus exogenous $sPLA_2$ (Fig. 5). The enhancing effect of AA appears to be specific, because other fatty acids tested, whether saturated (palmitic acid, stearic acid, arachidic acid) or unsaturated (oleic acid, linoleic acid), failed to restore the [^{3}H]AA release (13).

$P388D_1$ cells manifest an extraordinarily high capacity to incorporate exogenous AA into membrane phospholipids (12, 29, 30). As much as 25% of the fatty acid at 1 μM—the concentration used in the experiment shown in Fig. 5—is incorporated into the phospholipids of 10^6 cells in 10 min in the absence of albumin (12). This amount gives a rate of incorporation of about 25 pmol of AA per min per 10^6 cells. However, in the presence of albumin—as employed in the experiment depicted in Fig. 5—the amount of available AA is reduced to one-fourth, as the majority is trapped by the albumin in the incubation medium (J.B. and E.A.D., unpublished data). Thus the rate of incorporation of exogenous

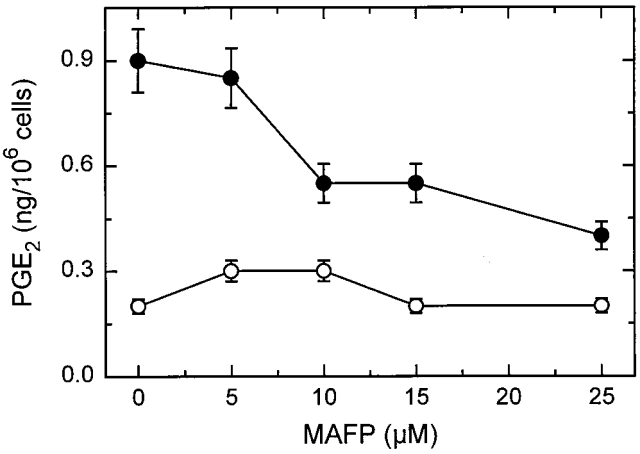

FIG. 4. Effect of MAFP on PGE_2 production by $P388D_1$ cells. The cells were incubated with the indicated concentrations of MAFP, which was added 15 min before PAF. PGE_2 in the supernatants was quantitated by using a specific radioimmunoassy. Basal PGE_2 production—i.e., that observed in the absence of LPS/PAF at the indicated MAFP concentrations—is indicated by ○.

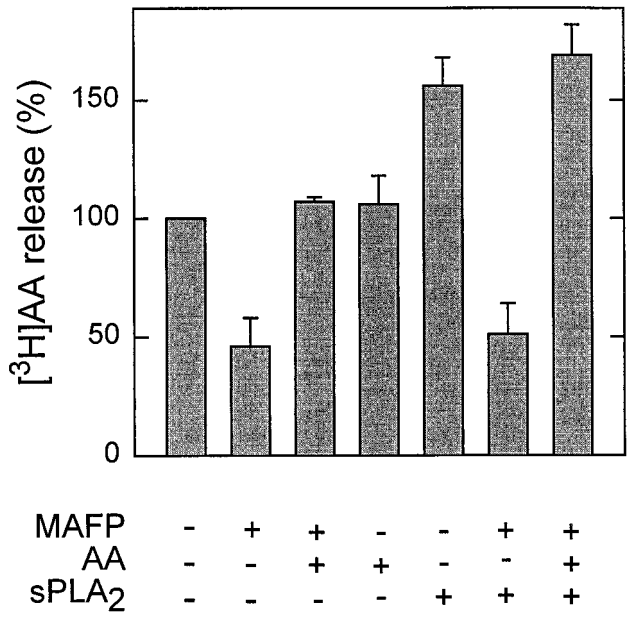

FIG. 5. AA restores the enhancing effect of exogenous $sPLA_2$ on [^{3}H]AA mobilization in activated $P388D_1$ cells. [^{3}H]AA-labeled LPS-treated cells were preincubated with MAFP (25 μM) for 15 min where indicated. Subsequently, 1 μM exogenous unlabeled AA was added 1 min before treatment with PAF (100 nM), as indicated. Where indicated, exogenous $sPLA_2$ (500 ng/ml) was added together with PAF. The data are expressed as a percentage of the response observed in activated cells in the absence of any addition, which was taken as 100%.

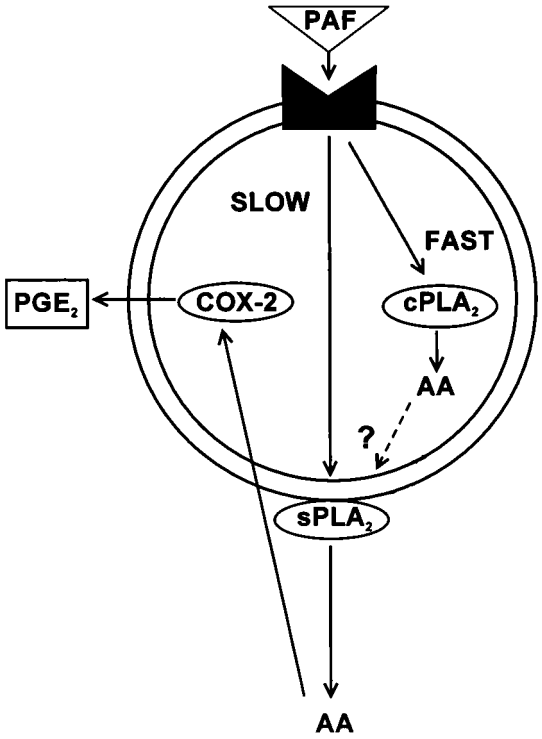

FIG. 6. Crosstalk between $cPLA_2$ and $sPLA_2$ in eliciting AA mobilization in activated $P388D_1$ macrophages. PAF activation of the LPS-primed macrophages triggers the "fast" activation of the $cPLA_2$ intracellularly, which in turn allows the "slow" action of $sPLA_2$ at the plasma membrane. The AA liberated by the $sPLA_2$ can be taken up and utilized by COX-2 to generate prostaglandins such as PGE_2. For further details see text.

unlabeled AA under the conditions of Fig. 5 is about 6 pmol per min per 10^6 cells. At 1 μM AA, little fatty acid is incorporated into triacylglycerols, which indicates that the steady-state lysophospholipid pool is sufficient to account for this rate of incorporation of free AA even in the absence of albumin (30). Moreover, the specific activity of the reacylating enzymes arachidonoyl-CoA synthetase and lysophospholipid:acyl-CoA acyltransferase in $P388D_1$ cells are 104 and 33 nmol per min per 10^6 cells (ref. 29; one million $P388D_1$ cells comprises $\approx$100 μg of protein). Therefore, because of the exceedingly high activity of the reacylating enzymes in $P388D_1$ cells it is highly unlikely that the restoring effect of exogenous unlabeled AA on [^{3}H]AA release shown in Fig. 5 is because of inhibition of [^{3}H]AA reacylation in the cells. Further experimental support for this interpretation is given by the finding that exogenous AA does not affect [^{3}H]AA release in cells not treated with MAFP (Fig. 5). If the enhancing effect of exogenous AA on [^{3}H]AA release in MAFP-treated cells was because of inhibition of reacylation, one would expect an enhanced [^{3}H]AA release in the non-MAFP-treated cells as well, which was clearly not the case (Fig. 5).

AA and PGE_2 Release Mechanism in $P388D_1$ Cells. Collectively, the data in this study establish that elevated levels of intracellular AA specifically lead to activation of $sPLA_2$ at the outer leaflet of the plasma membrane. Because elevated intracellular free AA levels are caused solely by $cPLA_2$ activation (13), the current results formally establish that crosstalk exists between $cPLA_2$ and $sPLA_2$ during $P388D_1$ cell activation by LPS/PAF. Therefore, the current data document a dual role for AA during macrophage activation by LPS/PAF. As well as serving itself as a substrate for the synthesis of other lipid mediators, the AA—or a metabolite—serves a signaling role by enabling activation of the $sPLA_2$ and thus allowing for a further amplification of the inflammatory response. These results, along with our previous data (12–14), support a mechanism for AA mobilization in $P388D_1$ cells whereby PAF receptor occupancy immediately leads to $cPLA_2$ activation, which, in turn, induces a transient elevation of intracellular free AA levels (Fig. 6). Elevated levels of free AA during the initial stages of $P388D_1$ cell activation may serve important metabolic and biochemical functions, one of which is to help activate the $sPLA_2$ that has appeared at the plasma membrane. Whether the activating effect of $cPLA_2$-derived AA on the $sPLA_2$ is a direct one or is mediated by accessory system(s) remains to be elucidated.

Once the $sPLA_2$ is activated at the plasma membrane, it starts to hydrolyze phospholipids and release AA to the extracellular medium. Part of this AA will be recaptured by the cell, and a minor portion of it (see below) will be made accessible to COX-2, the enzyme responsible for producing PGE_2 during activation conditions. COX-1, though present and active, is spared from the process. Thus, the $sPLA_2$ specifically feeds COX-2, not COX-1. One possibility for the COX-1 not to participate in PGE_2 production in activated cells could be that this enzyme is located farther away from the $sPLA_2$ than is COX-2. Because of the exceedingly high reacylating capacity of the $P388D_1$ cells (29, 30), only less than 5% of the liberated AA is converted into prostaglandins (31). If the $sPLA_2$—and/or the $cPLA_2$—releases AA not in the immediate vicinity of COX-1, the fatty acid might be impeded in reaching the enzyme, as it would be acylated back into phospholipids well before reaching COX-1. According to this scheme, it could be envisioned that the active COX-2 pool is located in close proximity to the intracellular compartment wherein cellular uptake of AA takes place. Further work will be necessary to explore this intriguing possibility.

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