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Regulation of Delayed Prostaglandin Production in Activated P388D$_1$ Macrophages by Group IV Cytosolic and Group V Secretory Phospholipase A$_2$s*

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Group V secretory phospholipase A$_2$ (sPLA$_2$) rather than Group IIA sPLA$_2$ is involved in short term, immediate arachidonic acid mobilization and prostaglandin E$_2$ (PGE$_2$) production in the macrophage-like cell line P388D$_1$. When a new clone of these cells, P388D$_1$/MAB, selected on the basis of high responsivity to lipopolysaccharide plus platelet-activating factor, was studied, delayed PGE$_2$ production (6–24 h) in response to lipopolysaccharide alone occurred in parallel with the induction of Group V sPLA$_2$ and cyclooxygenase-2 (COX-2). No changes in the level of cytosolic phospholipase A$_2$ (cPLA$_2$) or COX-1 were observed, and Group IIA sPLA$_2$ was not detectable. Use of a potent and selective sPLA$_2$ inhibitor, 3-(3-acetamide 1-benzyl-2-ethylindolyl-5-oxy)-propanesulfonic acid (LY311727), and an antisense oligonucleotide specific for Group V sPLA$_2$ revealed that delayed PGE$_2$ was largely dependent on the induction of Group V sPLA$_2$. Also, COX-2, not COX-1, was found to mediate delayed PGE$_2$ production because the response was completely blocked by the specific COX-2 inhibitor NS-398. Delayed PGE$_2$ production and Group V sPLA$_2$ expression were also found to be blunted by the inhibitor methylarachidonyl fluorophosphonate. Because inhibition of Ca^{2+}-independent PLA$_2$ by an antisense technique did not have any effect on the arachidonic acid release, the data using methylarachidonyl fluorophosphonate suggest a key role for the cPLA$_2$ in the response as well. Collectively, the results suggest a model whereby cPLA$_2$ activation regulates Group V sPLA$_2$ expression, which in turn is responsible for delayed PGE$_2$ production via COX-2.

Arachidonic acid (AA)[1] mobilization and the generation of prostaglandins by major immunoinflammatory cells such as macrophages and mast cells usually occur in two phases. The immediate phase, which takes minutes and is elicited by Ca^{2+}-mobilizing agonists such as platelet-activating factor (PAF), is characterized by a burst of AA liberation. In some cells such as P388D$_1$ macrophages (1, 2) and MMC-34 mast cells (3), this burst is mainly produced by a secretory phospholipase A$_2$ (sPLA$_2$) but is strikingly regulated by the cytosolic Group IV phospholipase A$_2$ (cPLA$_2$).

The delayed phase of prostaglandin production is accompanied by the continuous supply of AA over long incubation periods spanning several hours. There is some discrepancy about the identity of the PLA$_2$ isoform(s) involved in the delayed phase. Despite this phase being independent of a Ca^{2+} increase, the cPLA$_2$ has often been suggested to be critically involved (3–5). However, other studies have suggested the quantitatively more important role of the sPLA$_2$, an enzyme that is dramatically induced during long term incubation of the cells with a variety of stimuli (4–6). There is, however, agreement that COX-2, another enzyme whose expression is augmented dramatically after long term stimulation, is absolutely required for long term PGE$_2$ production, irrespective of the constitutive presence of COX-1 (7–9).

Using a new clone of the P388D$_1$ macrophage-like cells termed P388D$_1$/MAB, we provide herein evidence for the involvement of Group V sPLA$_2$ in delayed PGE$_2$ production. Furthermore, our results suggest that Group V sPLA$_2$ expression is dependent upon the activation of Group IV cPLA$_2$.

EXPERIMENTAL PROCEDURES

Materials—Mouse P388D$_1$ macrophage-like cells were obtained from the American Type Culture Collection (Rockville, MD). Iscove's modified Dulbecco's medium (endotoxin <0.05 ng/ml) was from Whittaker Bioproducts (Walkersville, MD). Fetal bovine serum was from Hyclone Laboratories (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) was from NEN Life Science Products, and 1-palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (specific activity, 54 mCi/mmol) was from Amersham Pharmacia Biotech. PAF, LPS (*Escherichia coli* 0111:B4), and actinomycin D were from Sigma. Methylarachidonyl fluorophosphonate (MAFP) and NS-398 were from Biomol (Plymouth Meeting, PA). Antibodies against murine COX isoforms were generously provided by Dr. W. L. Smith (Department of Biochemistry, Michigan State University, East Lansing, MI). The antibody against Group IV cPLA$_2$ was generously provided by Dr. Ruth Kramer (Lilly). The sPLA$_2$ inhibitor, 3-(3-acetamide 1-benzyl-2-ethylindolyl-5-oxy)propanesulfonic acid (LY311727), was generously provided by Dr. Edward Mihelich (Lilly). cDNA probes for Groups V and IIA sPLA$_2$s were synthesized as described previously (11). cDNA probes for murine glyceraldehyde-3-phosphate dehydrogenase were from Cayman (Ann Arbor, 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/well, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free Iscove's

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[1] The abbreviations used are: AA, arachidonic acid; PAF, platelet-activating factor; LPS, bacterial lipopolysaccharide; cPLA$_2$, Group IV cytosolic phospholipase A$_2$; sPLA$_2$, secretory phospholipase A$_2$; COX, cyclooxygenase (prostaglandin H$_2$ synthase); MAFP, methylarachidonyl fluorophosphonate; PGE$_2$, prostaglandin E$_2$; iPLA$_2$, Ca^{2+}-independent phospholipase A$_2$.

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 1 mg/ml albumin.

Measurement of PGE_2 Production and Extracellular [^{3}H]AA Release—The cells were placed in serum-free medium for 30 min before the addition of LPS for different periods of time. Afterward, the supernatants were removed and cleared of detached cells by centrifugation, and PGE_2 was quantitated using a specific radioimmunoassay (PerSpective Biosystems, Framingham, MA). For [^{3}H]AA release experiments, cells labeled with [^{3}H]AA were used, and the incubations were performed in the presence of 0.5 mg/ml bovine serum albumin. The supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting. The standard LPS/PAF stimulation protocol for immediate responses has been described previously (1). Briefly, the cells were incubated for 1 h with 200 ng/ml LPS followed by a 10-min incubation with 100 nM PAF.

Western Blot Analyses—The cells were overlaid with a buffer consisting of 10 mM Hepes, 0.5% Triton X-100, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 20 μM aprotinin, pH 7.5. Samples from cell extracts (10 μg for $cPLA_2$, 200 μg for COX) were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide gel) and transferred to Immobilon-P (Millipore). For $cPLA_2$ mobility shift studies, 24-cm acrylamide gels were run. Nonspecific binding was blocked by incubating the membranes with 5% nonfat milk in phosphate-buffered saline for 1 h. Membranes were then incubated with anti-$cPLA_2$, anti-COX-1, or anti-COX-2 antisera and treated with horseradish peroxidase-conjugated protein A (Amersham Pharmacia Biotech). Bands were detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Northern Blot Analyses—Total RNA was isolated from unstimulated or LPS-stimulated cells by the TriZOL reagent method (Life Technologies, Inc.), exactly as indicated by the manufacturer. Fifteen μg of RNA were electrophoresed in a 1% formaldehyde/agarose gel and transferred to nylon filters (Hybond, Amersham Pharmacia Biotech) in 10× SSC buffer. Hybridizations were performed in QuickHyb solution (Stratagene) following the manufacturer's instructions. ^{32}P-Labeled probes for Group IIA or glyceraldehyde-3-phosphate dehydrogenase were co-incubated with the filters for 1 h at 66 °C followed by three washes with 2× 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× SSC containing 0.1% SDS. For Group V $sPLA_2$, hybridizations were performed in ExpressHyb solution (CLONTECH) following the manufacturer's instructions. The ^{32}P-labeled probes were co-incubated with the filters for 1 h at 66 °C followed by two washes with 2× SSC containing 0.05% SDS for 15 min: the first at room temperature and the second at 40 °C. Afterward the filters were washed twice more with 0.1× SSC containing 0.1% SDS for 15 min at room temperature. Bands were visualized by autoradiography.

Phospholipase A_2 Assay—Aliquots (50–100 μl) of supernatants from LPS-treated cells were assayed for PLA_2 activity as follows. The assay mixture (500 μl) consisted of 100 μM 1-palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine substrate (2000 cpm/nmol), 10 mM $CaCl_2$, 100 mM KCl, 25 mM Tris-HCl, pH 8.5. Reactions proceeded at 40 °C for 30 min, after which [^{14}C]palmitate release was determined by a modified Dole procedure (10).

Antisense Inhibition Studies in P388D_1 Cells—Transient transfection of P388D_1 cells with antisense oligonucleotide, ASGV-2, or its sense counterpart, SGV-2, plus LipofectAMINE was carried out as described (11). Briefly, P388D_1 cells were transfected with oligonucleotide (125 nM) in the presence of 5 μg/ml LipofectAMINE (Life Technologies, Inc.) under serum-free conditions for 8 h prior to treating the cells with or without 100 ng/ml LPS for 10 h after transfection (11). Antisense oligonucleotide ASGV-2 (5′-GGA CUU GAG UUC UAG CAA GCC-3′) is complementary to nucleotides 64–84 of the mouse Group V PLA_2 gene. SGV-2 (5′-GGC UUG CUA GAA CUC AAG UCC-3′) is the sense complement of ASGV-2.

For Group VI $iPLA_2$ antisense experiments, a protocol identical to that reported elsewhere was followed (12).

Data Presentation—Assays were carried out in duplicate or triplicate. Each set of experiments was repeated three times with similar results. Unless otherwise indicated, the data presented are from representative experiments.

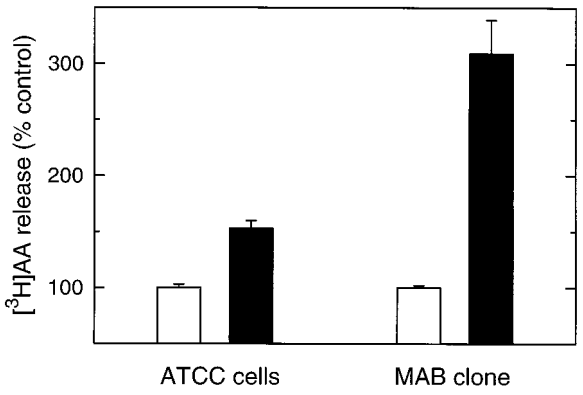

FIG. 1. **AA release in a new P388D_1 cell clone, MAB.** [^{3}H]AA release in LPS/PAF-treated (*closed bars*) or untreated (*open bars*) was assayed in cells from the ATCC or the MAB clone as indicated. The cells were incubated with 200 ng/ml LPS for 1 h followed by a 10-min incubation with 100 nM PAF.

RESULTS

AA Release in a Novel P388D_1 Cell Clone (MAB)—Stimulation of murine P388D_1 macrophages with nanomolar amounts of the receptor agonist PAF results in very little AA mobilization. However, preincubation of the cells with LPS prior to stimulation with PAF increases the release of AA by these cells well above unstimulated levels, the relative magnitude of the response being dependent on the cell batch (13, 14). We have now selected by limit dilution a clone of the P388D_1 cells termed MAB, which shows a remarkably higher [^{3}H]AA release response to LPS/PAF when compared with the ATCC batch of P388D_1 cells from which the MAB clone was obtained (Fig. 1). More interestingly, in addition to an immediate response to LPS/PAF (Fig. 2*A*), cells from the MAB clone also exhibited a delayed [^{3}H]AA release response, spanning several hours, to LPS alone (Fig. 2*A*). The dose response of the effect of LPS on long term [^{3}H]AA release is shown in Fig. 2*B*. The maximal effect was observed at a LPS dose as low as 10 ng/ml.

Prostaglandin Production by P388D_1/MAB Cells—Fig. 2*C* shows the time course of PGE_2 production by LPS in these cells as measured by radioimmunoassay, which corresponded well with the [^{3}H]AA mobilization response. Thus, LPS-induced PGE_2 barely increased above controls within the first 3 h of treatment, rising afterward, and reaching a plateau after 12 h.

The effect of LPS on the protein levels of the two COX isoenzymes these cells express (2) was assessed by immunoblot. Expression of COX-1 did not change along the time course of LPS activation (data not shown), whereas COX-2 levels noticeably increased with maximal expression between 12 and 18 h (Fig. 3*A*). Interestingly, LPS-induced COX-2 expression almost parallels PGE_2 generation (*cf.* Figs. 2*C* and 3*A*), suggesting that COX-2 is the enzyme responsible for LPS-induced PGE_2 synthesis. Indeed, the COX-2-specific inhibitor NS-398 (15) completely blocked LPS-induced PGE_2 production (Fig. 3*B*). Therefore, LPS-delayed PGE_2 generation depends exclusively on COX-2, irrespective of the continued presence of COX-1.

$cPLA_2$ Involvement in LPS-induced Long Term Responses—Expression of the Group IV $cPLA_2$ protein in P388D_1/MAB cells was constitutive and did not change after exposure to LPS. To address the possible involvement of $cPLA_2$ in LPS-induced AA mobilization in P388D_1/MAB cells, experiments were conducted with MAFP, an inhibitor that has previously been shown to block the immediate, $cPLA_2$-dependent [^{3}H]AA release in LPS/PAF-treated macrophages (1). As shown in Fig. 4, MAFP strongly blocked the LPS-induced long term [^{3}H]AA release response. MAFP has recently been observed to inhibit the Group VI $iPLA_2$ in addition to the $cPLA_2$ (10). Therefore, it

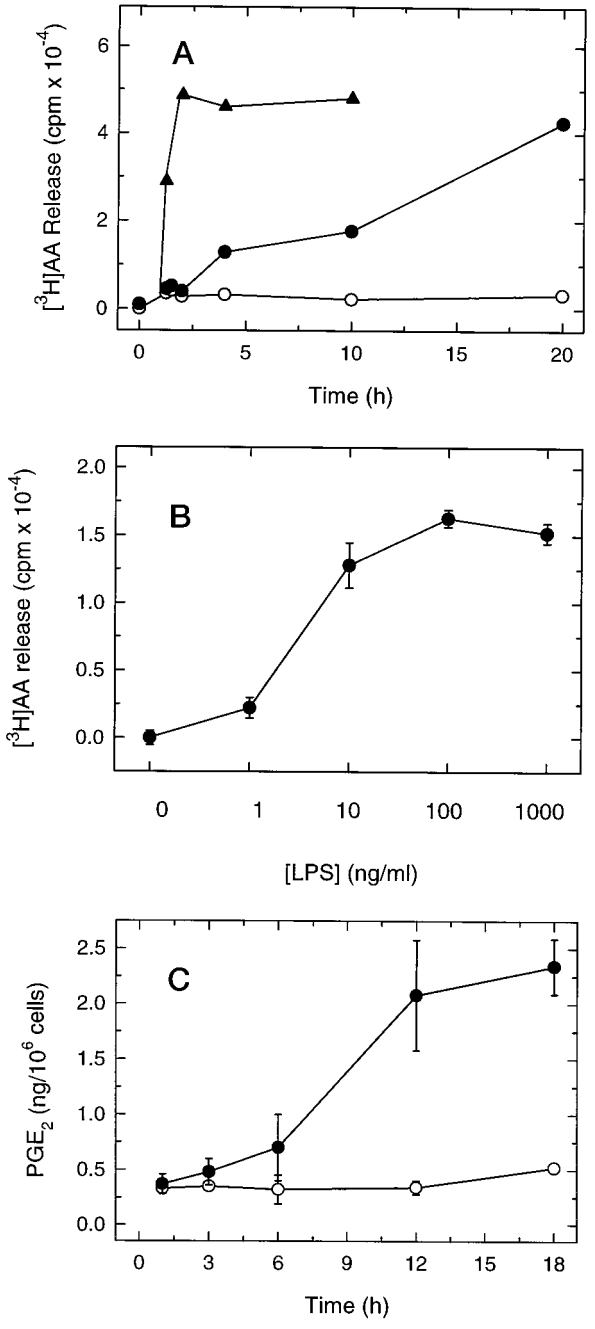

FIG. 2. **LPS-stimulated long term [^{3}H]AA metabolism in P388D$_1$ macrophages.** *A,* time course of [^{3}H]AA release in response to LPS/PAF (the latter was added 1 h after the former, and the incubations proceeded for the time indicated) (*closed triangles*), LPS alone (*closed circles*), or neither (*open circles*). *B,* dose response of the LPS effect (20-h incubation). *C,* the time course of PGE_2 production by cells treated with (*closed circles*) or without (*open circles*) 100 ng/ml LPS.

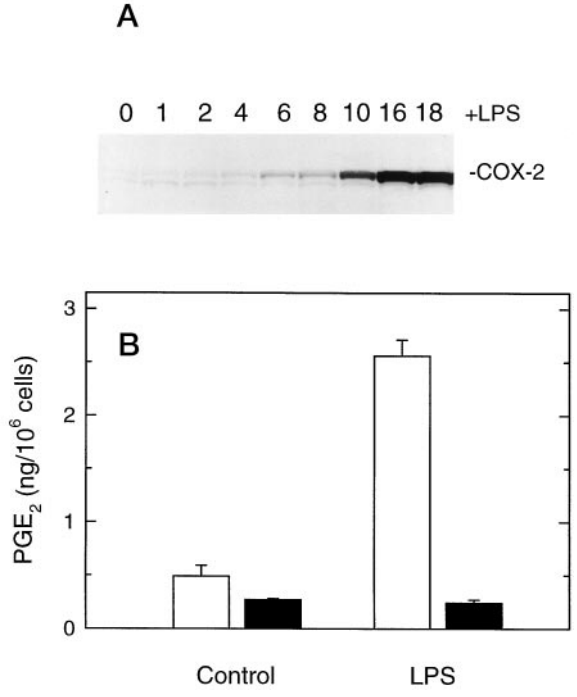

FIG. 3. **LPS-stimulated long term PGE_2 production.** *A,* the effect of 100 ng/ml LPS on COX-2 protein levels at the indicated times (h) as measured by immunoblot. *B,* the effect of NS-398 on LPS-induced PGE_2 production. The cells were treated with (*closed bars*) or without (*open bars*) 5 μM NS-398 for 20 min before the addition of LPS for 18 h.

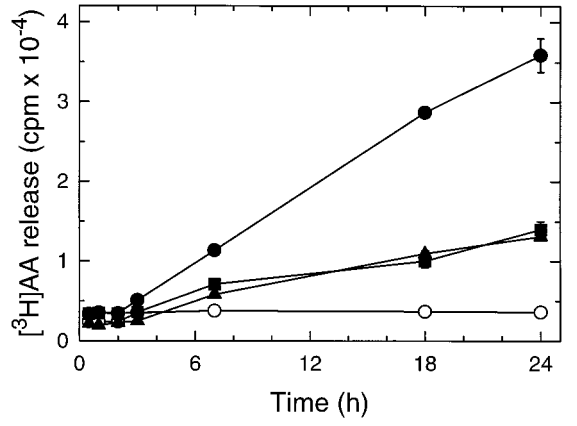

FIG. 4. **Effect of MAFP and LY311727 on LPS-induced AA release.** The cells were treated with 25 μM MAFP (*closed triangles*), 25 μM LY311727 (*closed squares*), or neither (*closed circles*) for 20 min before the addition of LPS. *Open circles* denote control incubations, *i.e.* those that received neither LPS nor inhibitors. The inhibitors alone did not change the control release.

could be possible that part of the MAFP effects reported herein resulted from inhibition of the $iPLA_2$ in addition to any effect on the $cPLA_2$. We have recently described the inhibition of $iPLA_2$ expression in P388D$_1$ cells by antisense RNA oligonucleotides (12). Using this technique, we have been able to significantly inhibit $iPLA_2$ expression, assayed both by protein content by immunoblot and activity by a specific *in vitro* assay (12). Antisense RNA inhibition of the $iPLA_2$ under identical conditions as those shown previously (12) showed no reduction in AA release in response to LPS (not shown). Therefore these data make it likely that the above reported effects of MAFP on the response are because of the inhibition of the $cPLA_2$.

Role of sPLA$_2$—LPS-induced long term [^{3}H]AA release was also noticeably blocked by the selective $sPLA_2$ inhibitor LY311727 (17), indicating that in addition to the $cPLA_2$, a $sPLA_2$ is also involved in the process (Fig. 4). PGE_2 production by LPS was also inhibited by LY311727 by about 90%. Although originally described as a selective Group II $sPLA_2$ inhibitor (17), we have recently shown that this compound is also a potent Group V $sPLA_2$ inhibitor (18).

Our previous work (11) has demonstrated that P388D$_1$ cells express measurable message levels for Group V $sPLA_2$, both under unstimulated and LPS/PAF-stimulated conditions. However, message levels for Groups IIA $sPLA_2$ or IIC $sPLA_2$ were undetectable even by reverse transcriptase-polymerase chain reaction (11). As shown in Fig. 5*A*, an antisense oligonucleotide specific for Group V $sPLA_2$ (ASGV-2) strongly blocked PGE_2

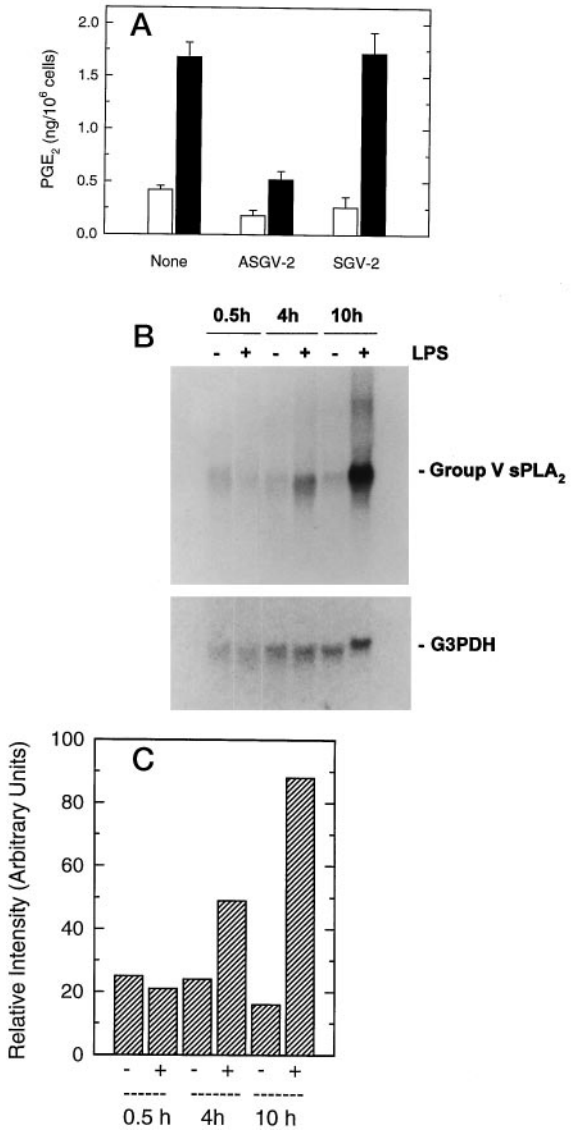

FIG. 5. **Group V $sPLA_2$ involvement in AA release.** *A,* the effect of a specific Group V antisense oligonucleotide (AGV-2) or its sense control (SGV-2) on PGE_2 production in cells treated without (*open bars*) or with (*closed bars*) 100 ng/ml LPS for 10 h. *None* denotes incubations that received no oligonucleotide. *B,* the effect of LPS on Group V $sPLA_2$ message levels as determined by Northern blot. Total RNA from cells incubated with (+) or without (−) 100 ng/ml LPS for the indicated periods of time was isolated and analyzed by Northern blot with probes specific for Group V $sPLA_2$ or glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*). *C,* densitometric analysis of the Group V $sPLA_2$ signals normalized for the glyceraldehyde-3-phosphate dehydrogenase signal in each lane.

production in LPS-treated cells, whereas its sense control (SGV-2) had no effect. Moreover, mRNA analyses by Northern blot at long times of stimulation with LPS confirmed the presence of mRNA for Group V $sPLA_2$ (Fig. 5, *B* and *C*) but not for Group IIA $sPLA_2$ (data not shown). The signal for Group V $sPLA_2$ markedly increased after LPS stimulation, reaching a peak at approximately 10 h.

PLA_2 activity measurements in the supernatants of LPS-stimulated cells revealed a time-dependent increase in activity (Fig. 6), which correlated well with the time course of Group V $sPLA_2$ mRNA induction (*cf.* Figs. 5*B* and 6). Extracellular PLA_2 activity was decreased if the experiments were conducted in the presence of the RNA synthesis inhibitor actinomycin D (Fig. 7*A*). This increased extracellular activity was found to correspond to that of Group V $sPLA_2$ by the following criteria:

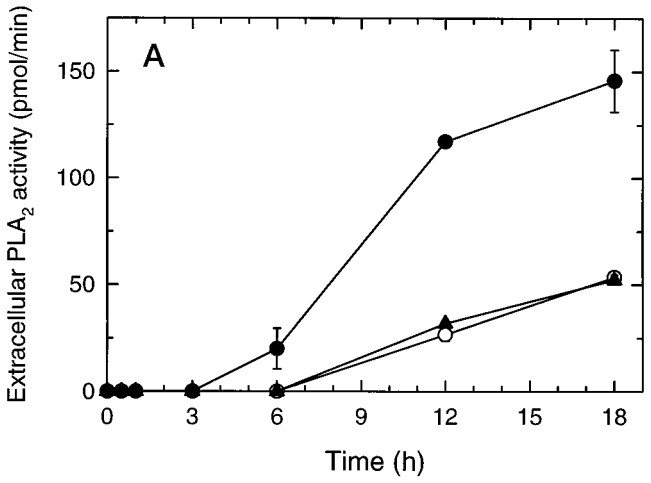

FIG. 6. **Effect of MAFP on the appearance of $sPLA_2$ activity in the supernatants of $P388D_1$ cells and the effect of MAFP.** The cells, pretreated with (*closed triangles*) or without (*closed circles*) 25 μM MAFP for 20 min, were challenged with (*closed symbols*) or without (*open circles*) 100 ng/ml LPS for the indicated times. Afterward, supernatants were collected and assayed for PLA_2 activity. The amount of PLA_2 activity detected in supernatants of cells not treated with LPS (*open circles*) was not changed whether the cells were pretreated or not with MAFP.

(i) it was completely inhibited by the $sPLA_2$ inhibitor LY311727 (Fig. 7*B*) and (ii) it was decreased in supernatants from cells treated with an antisense RNA oligonucleotide specific for Group V $sPLA_2$, ASGV-2 (11) (Fig. 7*C*).

Role of $cPLA_2$ in $sPLA_2$ Activation—Our previous studies have indicated that the immediate AA release triggered by LPS/PAF in these cells involves the sequential action of both $cPLA_2$ and $sPLA_2$, with the activity of the latter being dependent on previous activation of the former (1, 2). Thus we sought to investigate if a similar cross-talk exists between the two enzymes during long term stimulation conditions. We found that no increased PLA_2 activity beyond what was observed in the basal state could be found in supernatants from cells treated with MAFP (Fig. 6). In addition, the $cPLA_2$ inhibitor markedly decreased the LPS-induced expression of Group V $sPLA_2$ mRNA (Fig. 8).

DISCUSSION

A striking hallmark of the immunoinflammatory response is the generation of oxygenated derivatives of AA such as the prostaglandins. The response of major prostaglandin-secreting cells such as macrophages and mast cells to proinflammatory stimuli is generally biphasic (4). The first phase is completed within minutes after the addition of the stimulus, whereas the second phase usually takes several hours (4). Using the murine macrophage-like cell line $P388D_1$, we have been studying the molecular mechanisms responsible for AA mobilization and prostaglandin production in response to LPS/PAF. When primed by LPS, these cells will respond to Ca^{2+}-mobilizing stimuli such as PAF by generating a rapid burst of free AA, part of which is converted to prostaglandins such as PGE_2. Strikingly, this process is completed within a few minutes after the addition of PAF (19). No free AA or prostaglandins are produced after the immediate phase is completed, not even after several hours of cell exposure to LPS/PAF (13). Such a behavior, which is abnormal for a macrophage cell, has prevented us from studying the molecular mechanisms responsible for delayed prostaglandin production in macrophages. In an attempt to overcome this problem, we subcloned the $P388D_1$ cells by limit dilution, and selecting on the basis of high responsivity to LPS/PAF, we obtained a clone termed MAB,

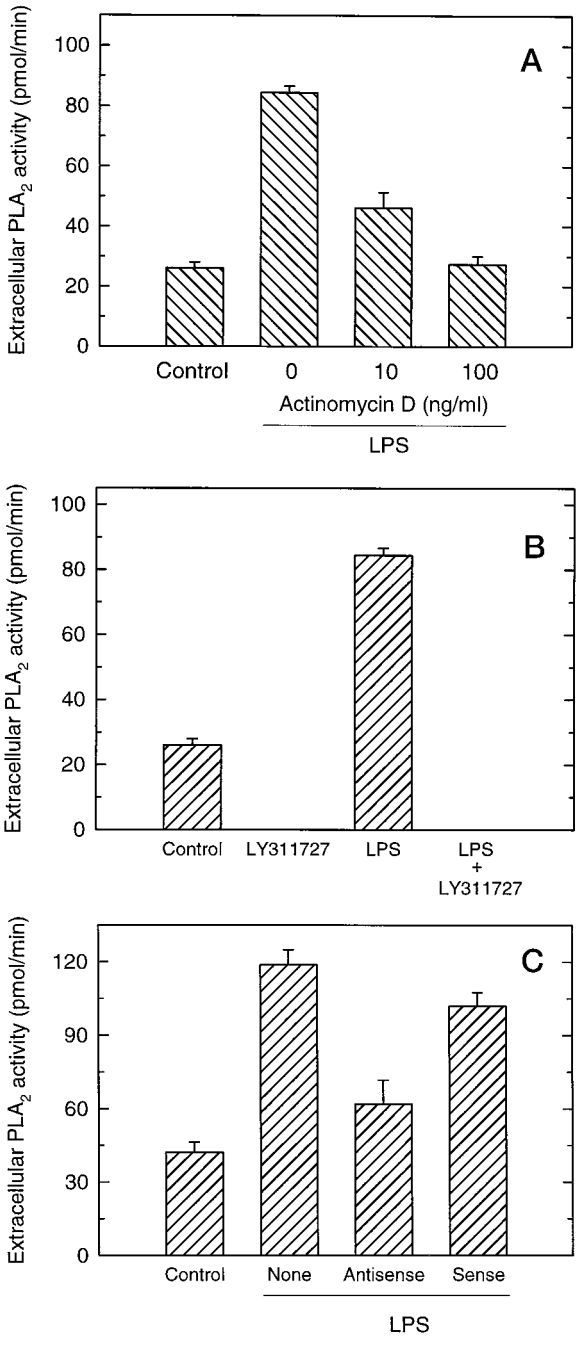

FIG. 7. **Effect of different treatments on the appearance of PLA_2 activity in supernatants from LPS-treated cells.** *A,* the effect of actinomycin D is shown. PLA_2 activity in the supernatants from cells treated with LPS plus the indicated concentrations of actinomycin D for 18 h is indicated. *Control* denotes incubations carried out without either LPS or actinomycin D. *B,* blockage by LY311727 of the PLA_2 activity of supernatants from LPS-treated or untreated cells. An aliquot of the culture medium of cells treated without (*Control*) or with LPS for 18 h was incubated with or without 25 μM LY311727 for 20 min at 40 °C and then assayed for PLA_2 activity. *C,* the effect of a specific Group V antisense oligonucleotide (AGV-2) or its sense control (SGV-2) on PLA_2 activity in the supernatants from LPS-treated or untreated cells.

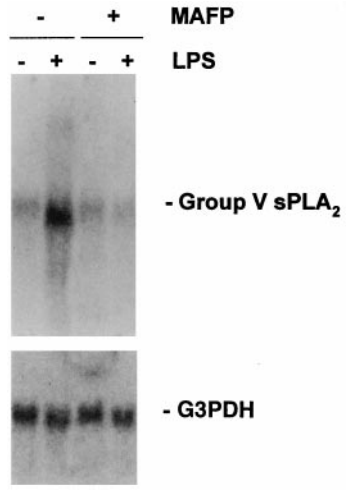

FIG. 8. **The effect of 25 μM MAFP on Group V $sPLA_2$ expression from LPS-treated (100 ng/ml, 18 h) or untreated cells.**

which shows enhanced sensitivity to LPS/PAF in the immediate phase (min) and exhibits a delayed response (h) to LPS alone.

Using the MAB clone, we have characterized the LPS-induced delayed prostaglandin production in terms of the role played by distinct PLA_2 enzymes and their coupling with downstream COX enzymes during LPS signaling. Our previous work on the immediate response of the cells to LPS/PAF highlighted the very important role played by the novel Group V $sPLA_2$ as the provider of most of the free AA directed to PGE_2 biosynthesis (11). Herein, several lines of evidence suggest that Group V $sPLA_2$ also behaves as a major provider of AA for the delayed phase of PGE_2 production in LPS-treated cells. First, delayed [^{3}H]AA release and PGE_2 production correspond with the induction of Group V $sPLA_2$ mRNA and enhanced secretion of a $sPLA_2$-like activity to the supernatants, with no change in the constitutive levels of $cPLA_2$ and no detectable induction of Group IIA $sPLA_2$. Second, delayed PGE_2 production is strongly blunted by LY311727, a selective $sPLA_2$ inhibitor. Third, an antisense oligonucleotide specific for Group V $sPLA_2$ (11) suppresses Group V $sPLA_2$ activity and inhibits delayed PGE_2 production. Our conclusions in this regard fully agree with recent works by Kudo and co-workers (20, 21) that were published while this manuscript was under review. By using transfection techniques, Kudo and co-workers (20, 21) have also documented the importance of Group V $sPLA_2$ in delayed AA release and PGE_2 production.

Our data have also implicated the $cPLA_2$ as an important step in LPS signaling by enabling the subsequent action of the $sPLA_2$. Thus the $cPLA_2$ inhibitor MAFP (1) markedly blocked both long term [^{3}H]AA release and Group V $sPLA_2$ mRNA induction. Collectively, these results suggest an intriguing cross-talk between the $cPLA_2$ and the Group V $sPLA_2$ for the delayed phase of prostaglandin production in macrophages. This is a very interesting concept because cross-talk appears to exist as well between these two enzymes during the immediate phase of prostaglandin production (1, 2). In the immediate phase, $cPLA_2$ activation generates a rapid and early burst of free AA inside the cell that enables $sPLA_2$ activation by an as yet unidentified mechanism (1, 2). In the delayed phase, $cPLA_2$ activation influences $sPLA_2$ apparently by regulating $sPLA_2$ mRNA levels.

Cross-talk between $cPLA_2$ and $sPLA_2$ in the immediate phase of prostaglandin production was also found to take place in mast cells (3) when the same protocol originally used in macrophages (1) was employed. Furthermore, a recent study by Kuwata *et al.* (22) about fibroblasts suggests that cross-talk between $cPLA_2$ and $sPLA_2$ in the delayed phase could also constitute a general mechanism of activation. Using a different $cPLA_2$ inhibitor, arachidonyl trifluoromethyl ketone, Kuwata *et al.* (22) found that $cPLA_2$ inhibition blocked $sPLA_2$ expression in fibroblasts, leading to reduced PGE_2 generation. The

study by Kuwata *et al.* (22) is interesting not only because it supports the possible universality of cross-talk between $cPLA_2$ and $sPLA_2$ but because the $sPLA_2$ expressed by rat fibroblasts is a Group IIA enzyme, not Group V. This lends further support to the emerging notion that Group IIA and Group V $sPLA_2$ may be functionally redundant (23). In addition, Kuwata *et al.* (22) were able to show that overcoming $cPLA_2$ inhibition by exogenous AA partially restored the Group IIA $sPLA_2$ expression. These results suggest that the AA mobilized by $cPLA_2$ is responsible for cross-talk between $cPLA_2$ and $sPLA_2$ (22). This is again reminiscent of what happens in the immediate phase of activation, wherein the $cPLA_2$-derived AA is also responsible for cross-talk between $cPLA_2$ and $sPLA_2$, albeit by different mechanisms (1, 2). Unfortunately, inhibition by MAFP of Group V $sPLA_2$ expression and activity could not be reversed in our macrophage studies with LPS alone by supplementing the medium with exogenous AA (up to 100 μM). This was not unexpected because $P388D_1$ cells manifest an extraordinarily high capacity to import free AA from exogenous sources and incorporate it into membranes (19, 24, 25), which is much higher than that of most other cells (26). Thus, the half-life of the free AA in the cell would be too short to adequately mimic the low but continued production of AA-derived $cPLA_2$ upon long term LPS exposure.

A model has recently emerged suggesting differential actions of COX-1 and COX-2 by virtue of differential coupling to distinct PLA_2s (2, 3, 6, 8, 20, 21, 27). Thus, depending on whether $cPLA_2$ or $sPLA_2$ is the provider of free AA, either COX-1 or COX-2 would be responsible for PGE_2 release. However, which PLA_2 form couples to which COX isoform appears to depend strongly on cell type. We have recently demonstrated that the immediate, PAF receptor-mediated phase of PGE_2 production in LPS-primed macrophages involves $sPLA_2$ coupling to COX-2 (2). The current results support a similar kind of coupling for the delayed PGE_2 production in LPS-treated cells. Identical coupling has been suggested by Arm and co-workers (6) for the delayed phase of PGE_2 generation in mast cells. These results raise another interesting concept regarding the regulation of PGE_2 during both phases of activation. As is the case for AA release (Fig. 2*A*), we have observed that the amount of PGE_2 generated during the Ca^{2+}-dependent short term stimulation is comparable to the amount produced in the late phase. It follows from this comparison that although the effector enzymes involved in the response are the same (*i.e.* $cPLA_2$, $sPLA_2$, COX-2), the regulatory mechanisms differ. Thus, in the short phase at low levels of COX-2, it appears that the dramatic burst in AA release is what determines the amount of PGE_2 produced. In contrast, in the delayed phase at comparably lower AA availability, it appears that both the induction of large amounts of COX-2 protein and of the AA provider, Group V $sPLA_2$, determine the amount of PGE_2 produced.

It is important to note, however, that our results have not excluded that a minor fraction of the long term PGE_2 produced in response to LPS could arise from the AA generated by the $cPLA_2$. Should this be the case, some $cPLA_2$/COX-2 coupling may exist as well, similar to what has been suggested by Reddy and Herschman (3) for delayed PGD_2 production in mast cells and by Murakami *et al.* (5) in cells derived from Group IIA-deficient mice. The striking feature of the current work is that although COX-1 is present in active form in the $P388D_1$ cells (2), it appears to be spared from the process of long term PGE_2 production. This finding remains unexplained but has recently been recognized in other cell types as well (6, 8, 22). Recent work by Spencer *et al.* (16) showed no differences in the distribution of COX-1 *versus* COX-2 among subcellular fractions in a variety of cells. Thus subcellular compartmentalization may not be the cause for COX-1 not being utilized during LPS signaling. Other putative explanations may include the existence of COX-selective regulatory components, selective coupling to terminal PG synthases, or kinetic differences in AA utilization by the two isoforms.

In summary, we have established a subclone of $P388D_1$ cells, MAB, that displays long term responsiveness to LPS in terms of PGE_2 generation. We have confirmed (11) that these cells express Group V $sPLA_2$, not Group IIA $sPLA_2$, and found that (i) Group V $sPLA_2$ is a key enzyme in long term AA mobilization as well and (ii) Group V $sPLA_2$ is functionally coupled to COX-2. Furthermore, our results have suggested that $cPLA_2$ plays a key role in long term AA mobilization, at least partly by regulating the expression of Group V $sPLA_2$.

REFERENCES

1. Balsinde, J., and Dennis, E. A. (1996) *J. Biol. Chem.* **271,** 6758–6765
2. Balsinde, J., Balboa, M. A., and Dennis, E. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 7951–7956
3. Reddy, S. T., and Herschman, H. R. (1997) *J. Biol. Chem.* **272,** 3231–3237
4. Murakami, M., Nakatani, Y., Atsumi, G., Inoue, K., and Kudo, I. (1997) *Crit. Rev. Immunol.* **17,** 225–284
5. Murakami, M., Kuwata, H., Amakasu, Y., Shimbara, S., Nakatani, Y., Atsumi, G., and Kudo, I. (1997) *J. Biol. Chem.* **272,** 19891–19897
6. Bingham, C. O., III, Murakami, M., Fujishima, H., Hunt, J. E., Austen, K. F., and Arm, J. P. (1996) *J. Biol. Chem.* **271,** 25936–25944
7. Herschman, H. R. (1996) *Biochim. Biophys. Acta* **1299,** 125–140
8. Murakami, M., Matsumoto, R., Urade, Y., Austen, K. F., and Arm, J. P. (1995) *J. Biol. Chem.* **270,** 3239–3246
9. Langenbach, R., Morham, S. G., Tiano, H. F., Loftin, C. D., Ghanayem, B. I., Chulada, P. C., Mahler, J. F., Lee, C. A., Goulding, E. H., Kluckman, K. D., Kim, H. S., and Smithies, O. (1995) *Cell* **83,** 483–492
10. Lio, Y. C., Reynolds, L. J., Balsinde, J., and Dennis, E. A. (1996) *Biochim. Biophys. Acta* **1302,** 55–60
11. Balboa, M. A., Balsinde, J., Winstead, M. V., Tischfield, J. A., and Dennis, E. A. (1996) *J. Biol. Chem.* **271,** 32381–32384
12. Balsinde, J., Balboa, M. A., and Dennis, E. A. (1997) *J. Biol. Chem.* **272,** 29317–29321
13. Glaser, K. B., Asmis, R., and Dennis, E. A. (1990) *J. Biol. Chem.* **265,** 8658–8664
14. Balsinde, J., Balboa, M. A., Insel, P. A., and Dennis, E. A. (1997) *Biochem. J.* **321,** 805–809
15. Futaki, N., Takahashi, S., Yokoyama, M., Arai, I., Higuchi, S., and Otomo, S. (1994) *Prostaglandins* **47,** 55–59
16. Spencer, A. G., Woods, J. W., Arakawa, T., Singer, I. I., and Smith, W. L. (1998) *J. Biol. Chem.* **273,** 9886–9893
17. Schevitz, R. W., Bach, N. J., Carlson, D. G., Chirgadze, N. Y., Clawson, D. K., Dillard, R. D., Draheim, S. E., Hartley, R. W., Jones, N. D., Mihelich, E. D., Olkowski, J. L., Snyder, D. W., Sommers, C., and Wery, J. P. (1996) *Nat. Struct. Biol.* **2,** 458–464
18. Chen, Y., and Dennis, E. A. (1998) *Biochim. Biophys. Acta* **1394,** 57–64
19. Balsinde, J., Barbour, S. E., Bianco, I. D., and Dennis, E. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11060–11064
20. Murakami, M., Shimbara, S., Kambe, T., Kuwata, H., Winstead, M. V., Tischfield, J. A., and Kudo, I. (1998) *J. Biol. Chem.* **273,** 14411–14423
21. Murakami, M., Kambe, T., Shimbara, S., and Kudo, I. (1999) *J. Biol. Chem.* **274,** 3103–3115
22. Kuwata, H., Nakatani, Y., Murakami, M., and Kudo, I. (1998) *J. Biol. Chem.* **273,** 1733–1740
23. Tischfield, J. A. (1997) *J. Biol. Chem.* **272,** 17247–17250
24. Balsinde, J., Bianco, I. D., Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8527–8531
25. Balsinde, J., and Dennis, E. A. (1996) *Eur. J. Biochem.* **235,** 480–485
26. Surette, M. E., and Chilton, F. H. (1998) *Biochem. J.* **330,** 915–921
27. Murakami, M., Nakatani, Y., and Kudo, I. (1996) *J. Biol. Chem.* **271,** 30041–30051

Regulation of Delayed Prostaglandin Production in Activated P388D$_1$ Macrophages by Group IV Cytosolic and Group V Secretory Phospholipase A$_2$s

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