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Journal

Journal of Biological Chemistry, 275(29)

ISSN

0021-9258

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Publication Date

2000-07-01

DOI

10.1074/jbc.m910163199

Peer reviewed

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 29, Issue of July 21, pp. 22544–22549, 2000

Printed in U.S.A.

Identification of a Third Pathway for Arachidonic Acid Mobilization and Prostaglandin Production in Activated P388D$_1$ Macrophage-like Cells*

Received for publication, December 21, 1999, and in revised form, March 29, 2000
Published, JBC Papers in Press, May 15, 2000, DOI 10.1074/jbc.M910163199

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Previous studies have demonstrated that P388D$_1$ macrophages are able to mobilize arachidonic acid (AA) and synthesize prostaglandins in two temporally distinct phases. The first phase is triggered by platelet-activating factor within minutes, but needs the cells to be previously exposed to bacterial lipopolysaccharide (LPS) for periods up to 1 h. It is thus a primed immediate phase. The second, delayed phase occurs in response to LPS alone over long incubation periods spanning several hours. Strikingly, the effector enzymes involved in both of these phases are the same, namely the cytosolic group IV phospholipase A$_2$ (cPLA$_2$), the secretory group V phospholipase A$_2$, and cyclooxygenase-2, although the regulatory mechanisms differ. Here we report that P388D$_1$ macrophages mobilize AA and produce prostaglandins in response to zymosan particles in a manner that is clearly different from the two described above. Zymosan triggers an immediate AA mobilization response from the macrophages that neither involves the group v phospholipase A$_2$ nor requires the cells to be primed by LPS. The group VI Ca^{2+}-independent phospholipase A$_2$ is also not involved. Zymosan appears to signal exclusively through activation of the cPLA$_2$, which is coupled to the cyclooxygenase-2. These results define a secretory PLA$_2$-independent pathway for AA mobilization in the P388D$_1$ macrophages, and demonstrate that, under certain experimental settings, stimulation of the cPLA$_2$ is sufficient to generate a prostaglandin biosynthetic response in the P388D$_1$ macrophages.

Phospholipase A$_2$ (PLA$_2$)[1] enzymes play key roles in a variety of cellular processes by generating a number of bioactive mediators. PLA$_2$-mediated hydrolysis of phospholipids results in the release of arachidonic (AA) and lysophospholipids, both of which may possess biological activity or serve as substrates for the generation of other bioactive lipid mediators such as the eicosanoids or platelet-activating factor (PAF) (1, 2).

In major eicosanoid-producing immunoinflammatory cells such as macrophages and mast cells, prostaglandin production usually occurs in two phases (1, 2). The first phase takes place in minutes and is strikingly characterized by its dependence on Ca^{2+} mobilization from internal stores; whereas, the second, delayed phase, spanning several hours, takes place in the absence of Ca^{2+} elevations (2). Substantial evidence suggests that specific coupling between certain PLA$_2$ and cyclooxygenase (COX) forms accounts for the differential regulation of the immediate and delayed responses (3–14). Thus, depending on whether group IV cPLA$_2$ or sPLA$_2$ (group IIA or group V) is the provider of free AA, either COX-1 or COX-2 would be responsible for prostaglandin production. Which PLA$_2$ form couples to which COX isoform appears also to depend critically on cell type.

We have shown that the rapid, PAF receptor-mediated phase of PGE$_2$ production in lipopolysaccharide (LPS)-primed P388D$_1$ macrophages involves group V sPLA$_2$ coupling to COX-2 (4). It is important to emphasize here that this response to PAF will not occur if the cells have not been first exposed to bacterial LPS for 1 h (15, 16). Thus the P388D$_1$ cell response to PAF is not, strictly speaking, an immediate response but rather a primed immediate one.

Interestingly, we have recently discovered that group V sPLA$_2$ also couples to COX-2 for the delayed PGE$_2$ biosynthetic response of P388D$_1$ macrophages exposed to LPS alone (5, 17). Under those conditions, expression of both group V sPLA$_2$ and COX-2 is markedly induced and correlates with ongoing AA release and prostaglandin biosynthesis, respectively (5), indicating that the AA produced by group V sPLA$_2$ is used by COX-2 to produce PGE$_2$. Importantly, expression of both group V sPLA$_2$ and COX-2 can be abolished by pretreating the cells with the cPLA$_2$ inhibitor methyl arachidonyl fluorophosphonate, implying that a functionally active cPLA$_2$ is essential for the delayed PGE$_2$ response to occur (5, 17).

In the current study we have uncovered a third pathway for AA mobilization and PGE$_2$ production in activated P388D$_1$ macrophages that appears not to require the group V sPLA$_2$. We show here that exposure of the cells to zymosan particles triggers the immediate release of both AA and PGE$_2$ in a process that depends only on cPLA$_2$ and COX-2. Thus in P388D$_1$ macrophages there are at least three different phases for AA release: an immediate phase (zymosan), which does not require the participation of the sPLA$_2$, a primed immediate phase (LPS/PAF), which does require the sPLA$_2$, and a delayed phase (LPS), which also requires the sPLA$_2$. All of the three phases, however, require the cPLA$_2$.

* This work was supported by Grants HD26171 and GM20501 from the National Institutes of Health and Grant S96-08 from the University of California Biostar Project/Lilly Research Laboratories. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This paper is dedicated to the memory of Belén Fernández-Boya.

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[1] The abbreviations used are: PLA$_2$, phospholipase A$_2$; AA, arachidonic acid; PAF, platelet-activating factor; COX, cyclooxygenase; PGE$_2$, prostaglandin E$_2$; LPS, bacterial lipopolysaccharide; MAFP, methyl arachidonyl fluorophosphonate; BEL, bromoenol lactone; DAG, diacylglycerol; cPLA$_2$, group IV cytosolic PLA$_2$; sPLA$_2$, secretory PLA$_2$; iPLA$_2$, group VI Ca^{2+}-independent PLA$_2$.

EXPERIMENTAL PROCEDURES

Materials—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) was from New England Nuclear (Boston, MA). PAF, LPS (*Escherichia coli* 0111:B4), and yeast-derived zymosan were from Sigma. Methyl arachidonyl fluorophosphonate (MAFP), bromoenol lactone (BEL), and NS-398 were from Biomol (Plymouth Meeting, PA). The sPLA$_2$ inhibitor LY311727 was generously provided by Dr. Edward Mihelich (Lilly Research Labs, Indianapolis, IN). iPLA$_2$ antiserum was generously provided by Dr. Simon Jones (Genetics Institute, Cambridge, MA). Human recombinant group V sPLA$_2$ was produced in our laboratory utilizing the *Pichia pastoris* expression system (17).

Cell Culture and Labeling Conditions—P388D$_1$ cells (MAB clone) (5, 17) 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 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 of Extracellular [^{3}H]AA Release—The cells were placed in serum-free medium for 30 min before the addition of zymosan 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.

Preparation of Zymosan—Zymosan was prepared exactly as described (18). Briefly, zymosan particles were suspended in phosphate-buffered saline, boiled for 60 min, and washed three times. The final pellet was resuspended in phosphate-buffered saline at 20 mg/ml and stored frozen. Zymosan aliquots were diluted in serum-free medium and sonicated before addition to the cells. No PLA$_2$ activity was detected in the zymosan batches used in this study, as assessed by *in vitro* activity assays (19).

iPLA$_2$ Antisense Inhibition Studies—A 20-base-long antisense corresponding to nucleotides 59–78 in the murine group VI iPLA$_2$ sequence (20) was utilized (ASGVI-18, 5′-CTC CTT CAC CCG GAA TGG GT). As a control, the sense complement of ASGVI-18 was used (SGV-18, 5′-ACC CAT TCC GGG TGA AGG AG). Both ASGVI-18 and SGVI-18 contained phosphorothioate linkages to limit degradation. We have previously described a procedure of transfection that involves long incubation periods of the cells with the oligonucleotides (21). In the current study, we have employed the procedure recently described by Akiba *et al.* (22) in which the oligonucleotides are presented to the cells in a complex with a lipophilic carrier. The antisense and sense oligonucleotides were mixed with LipofectAMINE, and complexes were allowed to form at room temperature for 10–15 min. The complexes were then added to the cells, and the incubations were allowed to proceed for 24 h under standard cell culture conditions. The final concentrations of oligonucleotide and LipofectAMINE in the incubation medium were 1 μM and 10 μg/ml, respectively. Oligonucleotide treatment and culture conditions were not toxic for the cells as assessed by the Trypan blue dye exclusion assay and by quantitating adherent cell protein.

Immunoblot Analysis of iPLA$_2$—The cells were lysed in a buffer consisting of 150 mM NaCl, 20 mM Tris-HCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 20 μM aprotinin, 100 mM sodium vanadate, pH 7.5. The homogenates were centrifuged at 500 × *g* for 5 min at 4 °C to separate nuclei. Samples (50 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide gel) and transferred to Immobilon-P (Millipore). Nonspecific binding was blocked by incubating the membranes with a buffer consisting of 5% nonfat milk, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100 for 60 min. Membranes were then incubated with anti-iPLA$_2$ antiserum at a 1:1000 dilution for 30 min and then treated with horseradish peroxidase-conjugated protein A (Amersham Pharmacia Biotech). Bands were detected by enhanced chemiluminiscence (ECL, Amersham Pharmacia Biotech).

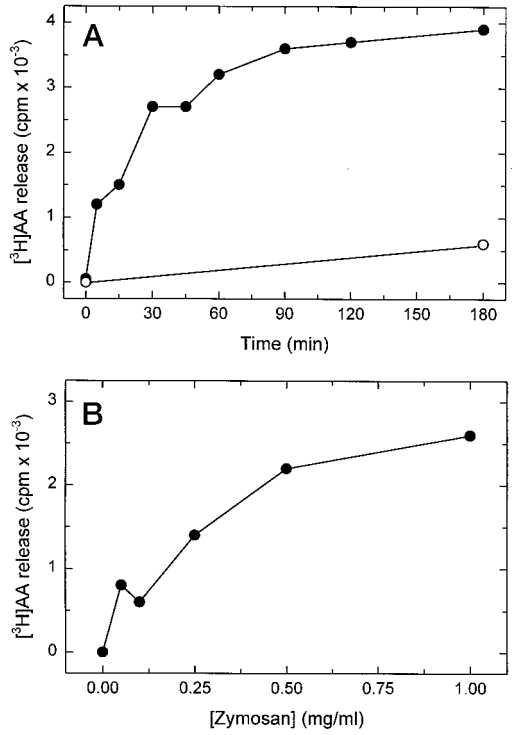

FIG. 1. **Zymosan-stimulated [^{3}H]AA release in P388D$_1$ macrophages.** *A*, time-course of [^{3}H]AA release upon stimulation with 0.5 mg/ml zymosan (*closed circles*) and in the absence of stimulation (*open circles*). *B*, concentration response of the zymosan effect (1-h incubation).

Measurement of DAG Levels—After the stimulations, the cell supernatants were taken off, and the cell monolayers were scraped with 0.5% Triton X-100. Total lipids were extracted according to the method of Bligh and Dyer (23). Lipids were separated by thin-layer chromatography with *n*-hexane/diethyl ether/acetic acid (70:30:1, by vol). This system allows a good resolution among phospholipids, monoacylglycerol, DAG, free fatty acids, and triacylglycerol (24). The plates were revealed by exposing them to iodine vapors, and the spot corresponding to DAG was cut out and assayed for radioactivity by liquid scintillation counting.

Data Presentation—Except for the data in Fig. 4, zymosan-stimulated AA release is expressed by subtracting the basal rate in the absence of agonist and inhibitor. These background values were in the range 2000–3000 cpm. 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

Zymosan-induced [^{3}H]AA Release in P388D$_1$/MAB Cells—We have recently reported on the use of a P388D$_1$ cell subclone, termed MAB, which manifests considerably higher [^{3}H]AA release responses to LPS and PAF than the ATCC cell batch from which the MAB clone was derived (5). We have repeatedly been unable to detect AA release in response to yeast-derived zymosan from P388D$_1$ cell batches directly obtained from the ATCC (15, 25). Highlighting another striking difference between the MAB cells and their parent ATCC cells, Fig. 1*A* shows that the MAB cells do respond to yeast-derived zymosan by rapidly releasing [^{3}H]AA to the extracellular medium. The concentration dependence of the effect of zymosan on [^{3}H]AA release is shown in Fig. 1*B*. Maximal effects were observed at zymosan concentrations between 0.25–0.5 mg/ml. From these data, a zymosan concentration of 0.5 mg/ml was chosen to be employed in all subsequent experiments.

Role of cPLA$_2$ and iPLA$_2$ in Zymosan-stimulated AA Release—Fig. 2 shows that the zymosan-induced AA release was

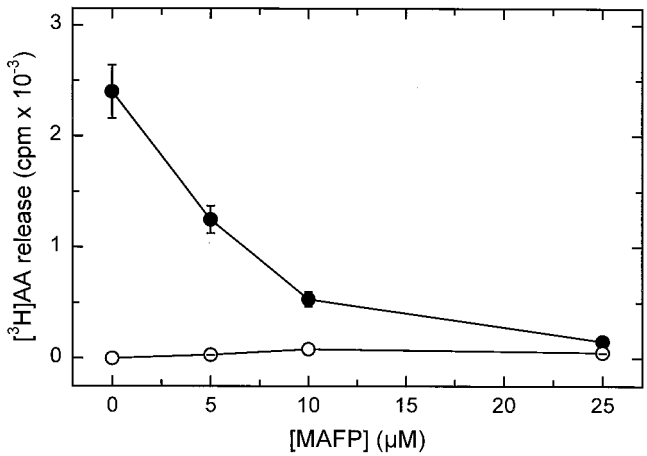

FIG. 2. **Effect of MAFP on zymosan-induced [^{3}H]AA release.** The cells were treated with the indicated concentrations of MAFP for 30 min before the addition of zymosan (*closed symbols*), and the incubations proceeded for 1 h. *Open circles* denote control incubations, *i.e.* those that did not receive zymosan.

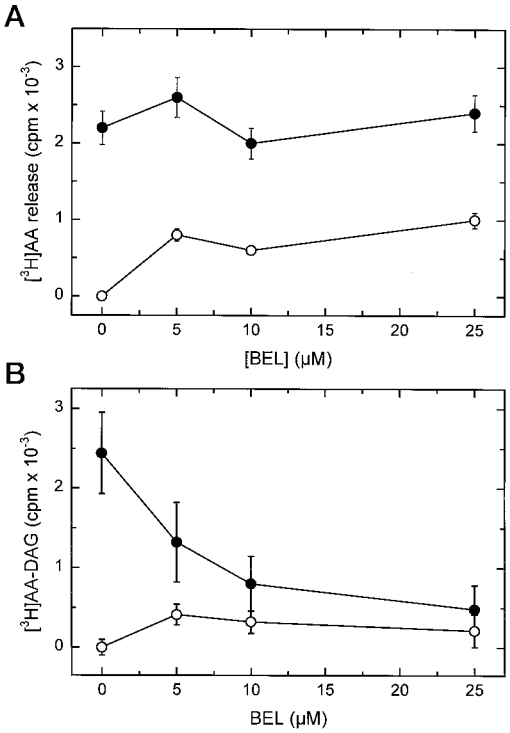

FIG. 3. **Effect of BEL on zymosan-induced [^{3}H]AA release (*A*) and [^{3}H]DAG accumulation (*B*).** The cells were treated with the indicated concentrations of BEL for 30 min before the addition of zymosan (*closed symbols*), and the incubations proceeded for 1 h. *Open circles* denote control incubations, *i.e.* those that did not receive LPS. Afterward, AA release (*A*) was determined in the supernatants and DAG accumulation (*B*) was determined in the cell monolayers as described under "Experimental Procedures."

strongly inhibited by MAFP a dual cPLA$_2$/iPLA$_2$ inhibitor. Complete inhibition of the response was observed at a MAFP concentration of 25 μM. To distinguish whether the inhibition of MAFP of AA release was because of either cPLA$_2$ or iPLA$_2$, we conducted studies with BEL, a compound that manifests a marked selectivity for inhibition of the iPLA$_2$ *versus* the cPLA$_2$ (3). BEL, at concentrations that we have previously shown to block P388D$_1$ cell iPLA$_2$ *in vitro* (26), had no significant effect on zymosan-stimulated AA release (Fig. 3*A*). Failure of BEL to inhibit the AA release was not because of the inhibitor not being able to cross the plasma membrane, because under identical experimental conditions BEL did inhibit zymosan-induced DAG production in a concentration-dependent manner (Fig. 3*B*). The latter effect most likely reflects the inhibition by BEL of the Mg^{2+}-dependent phosphatidate phosphohydrolase (27). In turn, the absence of an effect of BEL on zymosan-induced AA release indicates that the MAFP effects shown in Fig. 2 are because of inhibition of the cPLA$_2$, not the iPLA$_2$.

To further substantiate the lack of a role for the iPLA$_2$ in zymosan-stimulated AA release, we also examined the effects of an iPLA$_2$ antisense oligonucleotide, which we have previously used to attenuate the levels of immunoreactive iPLA$_2$ in P388D$_1$ cells (21). Using this antisense, we detected a decrease in the immunoreactive iPLA$_2$ protein of about 60%, as judged by Western blot (Fig. 4*A*). iPLA$_2$-deficient cells, however, did not show any significant reduction of their capacity to release AA to the extracellular medium, either spontaneously or in response to zymosan (Fig. 4*B*). Collectively, the data of Figs. 3 and 4 strongly suggest that the iPLA$_2$ does not play a significant role in mediating agonist-induced AA mobilization in P388D$_1$ cells.

sPLA$_2$ Role—To investigate the involvement of the sPLA$_2$ in zymosan-stimulated AA release, we employed LY311727, a well known sPLA$_2$ inhibitor (1). Treatment of the cells with 25 μM LY311727 had no appreciable effect on the zymosan-stimulated AA release (Fig. 5). We have previously shown that this treatment leads to a marked reduction of the AA release response of the cells to LPS/PAF (3). Identical results were obtained with the use of CMPE (*N*-derivatized phosphatidylethanolamine covalently linked via the headgroup to carboxymethyl cellulose), another sPLA$_2$ inhibitor that is structurally unrelated to LY311727 (28), and that we have previously shown to strongly inhibit fatty acid release in LPS-activated P388D$_1$ macrophages (29). In keeping with these previous data, CMPE strongly inhibited the LPS/PAF-induced AA release but had no measurable effect on the response to zymosan (data not shown). These results, together with the finding that zymosan did not increase cellular group V sPLA$_2$ mRNA levels for periods of time up to 1 h, strongly suggest that sPLA$_2$ has no role in zymosan-stimulated AA release.

The priming effect of LPS on PAF-induced AA mobilization in P388D$_1$ cells is thought to involve the increased synthesis of group V sPLA$_2$ (4, 30). Interestingly LPS also primed the AA release in response to zymosan (Fig. 5). This effect was found to be inhibited by LY311727, implying the involvement of group V sPLA$_2$ in the priming effect (Fig. 5). Likewise, the addition of exogenous group V sPLA$_2$ to the zymosan-treated cells increased the AA release in a concentration-dependent manner (Fig. 6). Thus, when cellular sPLA$_2$ levels are increased because of the LPS treatment or to the addition of exogenous enzyme, the zymosan response is accordingly increased. Collectively the data indicate that the lack of involvement of sPLA$_2$ in the response triggered by zymosan alone is not because of zymosan suppression of endogenous sPLA$_2$ activity.

Role of COX-2 in the Zymosan Stimulation of P388D$_1$ Cells—We have previously observed that in addition to COX-1, P388D$_1$ macrophages constitutively express low levels of COX-2 (4, 17). Therefore it was of interest to examine which of these COX isoforms participates in the prostaglandin response of the cells to zymosan. Neither COX-1 nor COX-2 levels were changed during incubation of the cells for 1 h with zymosan, as assessed by immunoblot. We studied the effect of NS-398, a compound that inhibits COX-2 with an IC_{50} <5 μM, whereas COX-1 remains unaffected at concentrations higher than 100 μM (31). Fig. 7 shows complete inhibition of zymosan-induced PGE_2 production by NS-398, which indicates that COX-2 is responsible for the response.

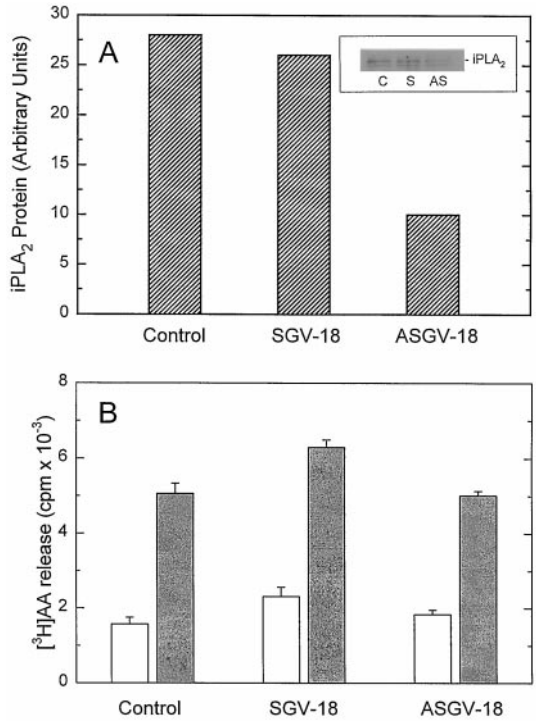

FIG. 4. **Effects of an iPLA$_2$ antisense oligonucleotide on iPLA$_2$ protein and zymosan-induced AA release.** The cells were treated for 24 h with 1 μM antisense (ASGV-18), sense (SGV-18) oligonucleotide, or vehicle (Control). *A*, effect on iPLA$_2$ protein. The *inset* shows the immunoblot from which the densitometry data were obtained. *B*, effect on zymosan-stimulated AA release. The cells, treated with ASGV-18, SGV-18, or neither as indicated, were incubated without (*open bars*) or with (*gray bars*) zymosan for 1 h. Extracellular [^{3}H]AA release was quantitated as described under "Experimental Procedures."

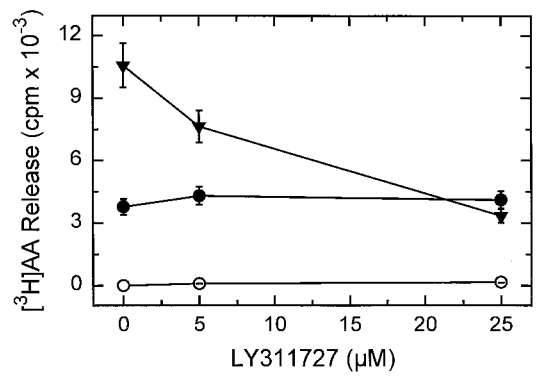

FIG. 5. **Effect of LY311727 on AA release.** The cells were treated with the indicated concentrations of LY311727 for 30 min before the addition of zymosan (*closed circles*), LPS for 1 h followed by zymosan (*inverted triangles*), or neither (*open circles*) as indicated.

DISCUSSION

We have recently identified in P388D$_1$ macrophages (MAB clone) the existence of two distinct pathways for AA mobilization and prostaglandin production. The first one, herein referred to as the "primed immediate phase" takes place in minutes and is elicited by the Ca^{2+}-mobilizing agonist PAF but requires the cells to be exposed first to LPS for 1 h (3, 4, 30, 32). The second pathway, or "delayed phase," is elicited by LPS for periods of time spanning several hours (4, 5, 33). Interestingly, both pathways utilize the same effectors, namely cPLA$_2$, sPLA$_2$, and COX-2, although the molecular mechanisms involved dramatically differ (3, 4, 5, 30, 32, 33). In both of these routes the cPLA$_2$ appears to behave primarily as an initiator of the response, whereas the sPLA$_2$ plays an augmentative role

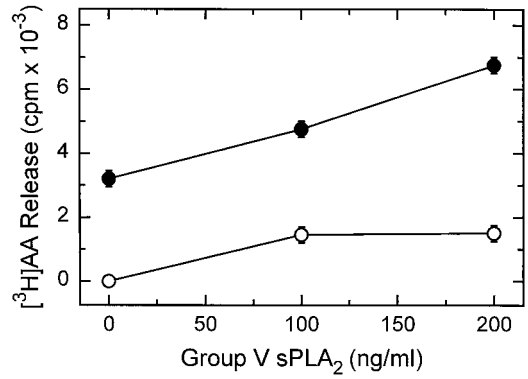

FIG. 6. **Effect of exogenous group V sPLA$_2$ on zymosan-stimulated AA release.** The cells were treated without (*open circles*) or with zymosan (*closed circles*) in the presence of the indicated concentrations of exogenous group V sPLA$_2$. Afterward, supernatants were assayed for [^{3}H]AA release.

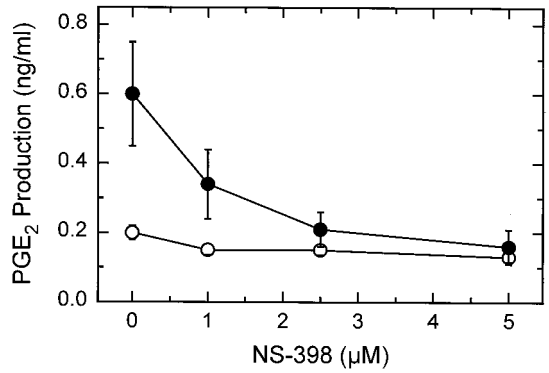

FIG. 7. **Effect of NS-398 on PGE$_2$ production.** The cells were treated with the indicated concentrations of NS-398 for 30 min before the addition of zymosan (*closed symbols*), and the incubations proceeded for 1 h. *Open circles* denote control incubations, *i.e.* those that did not receive zymosan.

by providing most of the AA to be converted to prostaglandins via COX-2.

In the current study we show that zymosan triggers AA mobilization and prostaglandin synthesis in the P388D$_1$ macrophages by a pathway that is clearly different from the ones identified for LPS and PAF. Thus, zymosan elicits an "immediate" AA response whose unique features are that (i) it does not cease after a few minutes but goes on for longer times, (ii) takes place in the absence of LPS priming, and (iii) utilizes only the cPLA$_2$ to effect the AA release. In common with the response to PAF, however, prostaglandin production in the zymosan-stimulated cells is also mediated by COX-2. Thus, in zymosan-stimulated cells cPLA$_2$ couples directly to COX-2 for prostaglandin production. This is strikingly different from the situation in the PAF-stimulated cells, where the bulk of the prostaglandins is produced by a sPLA$_2$/COX-2 coupling mechanism (4).

Exposure of the P388D$_1$ macrophages to LPS increases group V sPLA$_2$ levels in a concentration-dependent manner and this appears to constitute a key event of LPS priming (5, 30). We have found here that zymosan does not trigger the increased synthesis of new sPLA$_2$. However, resting cells still contain appreciable amounts of sPLA$_2$ (30). Thus it is not easy to envision the reasons for the zymosan not to signal to AA release via recruitment of the endogenous sPLA$_2$ already present in the cell. We have previously observed that the sPLA$_2$ pool located on the cell surface appears to be involved in the LPS/PAF-induced AA mobilization and prostaglandin production (19, 30). sPLA$_2$ levels on the surface of the LPS/PAF-activated

cells are higher than in resting unstimulated cells (4, 30). This increased sPLA$_2$ expression can be blocked by actinomycin D (4), indicating the involvement of *de novo* protein synthesis. We have failed to detect an increase in group V sPLA$_2$ mRNA levels in cells stimulated by zymosan for periods of time up to 1 h. Thus, a tempting but yet speculative idea to explain the lack of sPLA$_2$ involvement in the zymosan response would be that the stimulus fails to recruit the constitutive sPLA$_2$ to the appropriate cell compartment, *i.e.* the cell surface. Interestingly, a recent report has shown that a major portion of the group V sPLA$_2$ that murine mast cells constitutively express is found intracellularly located during the resting state (34).

P388D$_1$ macrophages contain a third PLA$_2$ type, the group VI iPLA$_2$. By using chemical inhibitors and antisense approaches, we have shown that this enzyme does not seem to participate in the stimulation of AA release by zymosan. In agreement with our data, Akiba *et al.* (22) recently reported that MAFP strongly inhibits the zymosan-stimulated AA release in P388D$_1$ cells. Interestingly, Akiba *et al.* (22) also showed that low BEL concentrations (2 μM) partially decreased the AA release (up to 40%). This finding led the authors to suggest that, in addition to the cPLA$_2$, the iPLA$_2$ is also involved in the response. At 2 μM, no effect of BEL on DAG levels was detected but an inhibitory effect on the response became evident at 5 μM, which is in agreement with the results of our study. The discrepancy between the data by Akiba *et al.* (22) and ours regarding BEL effects on AA release probably arises from the fact that Akiba *et al.* (22) have used a heterogeneous P388D$_1$ cell population for their studies, whereas we have employed a clone of these cells, termed MAB (5, 17). Closer examination of the results by Akiba *et al.* (22) reveals that the concentration-response curves for the inhibitory effects of BEL on zymosan-stimulated AA release and endogenous iPLA$_2$ activity do not correspond (*cf.* Fig. 2, *A* and *B,* in Ref. 22). Maximal effects of BEL on AA release are found at 2 μM, but at this concentration, endogenous iPLA$_2$ activity is only inhibited by 40%; BEL concentrations higher than 10 μM were found to be required for full iPLA$_2$ inhibition (22). Thus, the BEL effects on zymosan-induced AA release reported by Akiba *et al.* (22) are likely not because of inhibition of the iPLA$_2$ but of another unidentified effector.

Akiba *et al.* (22) also utilized antisense technology to study the role of iPLA$_2$ in their system. Surprisingly however, the functional consequences of iPLA$_2$ antisense depletion were investigated on prostaglandin D$_2$ production, not on the more direct analysis of AA release (22). A potential problem with measuring prostaglandin as a marker of phospholipase activation (22) is that any effect at step(s) distal from the phospholipolytic step cannot be distinguished from effects at the phospholipolytic step itself. We have confirmed in this study that the experimental conditions employed by Akiba *et al*. (22) result in a decrease in cellular iPLA$_2$, as assessed by immunoblot. We have also found that iPLA$_2$ depletion by antisense does not result in a decreased capacity of the cells to release AA to the incubation medium in response to zymosan. We previously observed that iPLA$_2$ depletion by the same antisense oligonucleotide also has no effect on the AA release response induced by LPS/PAF (21).

Our previous studies have suggested that the iPLA$_2$ serves in a phospholipid remodeling role that involves the generation of lysophospholipid precursors for incorporation of AA into phospholipids (35). Such a housekeeping function for the iPLA$_2$ was deduced from experiments involving inhibition of iPLA$_2$ activity with BEL (26, 36) or with an antisense oligonucleotide (21). Importantly, our data have now been confirmed and extended by other laboratories (37–40). Thus the role of iPLA$_2$ in regulating basal phospholipid deacylation/reacylation reactions appears not to be restricted to the P388D$_1$ cells but rather may represent a general homeostatic mechanism for the regulation of phospholipid levels. However, a recent study in pancreatic islets failed to detect a role for the iPLA$_2$ in basal AA incorporation into the phospholipids of these cells (41). It is important to note that, as the authors themselves acknowledge (41), pancreatic islets exhibit some atypical features of AA incorporation in that the basal levels of both esterified AA and lysophospholipid in these cells are substantially higher than in other tissues (41). Given the high levels and apparently slow turnover of lysophosphatidylcholine in these cells (41), the finding that lysophosphatidylcholine levels do not limit AA incorporation into islet phospholipids does not come as a surprise. Whether the observations with pancreatic islets represent another mechanism for incorporation of AA into phospholipids or merely reflect atypical features of a particular cell type is not yet certain.

In summary, we have described in this work the existence of a third, immediate pathway for AA mobilization and prostaglandin production that operates via activation of the cPLA$_2$ coupled to COX-2. Neither group V sPLA$_2$ nor group VI iPLA$_2$ appear to be involved.

Acknowledgments—We thank Dr. Satoshi Akiba (Kyoto Pharmaceutical University, Japan) for helpful discussions during the course of this work and Dr. Michelle Winstead for reading the manuscript.

Note Added in Proof—While this manuscript was under review, a report appeared (Gijón, M. A., Spencer, D. M., Siddiqi, A. R., Bonventre, J. V., and Leslie, C. C. (2000) *J. Biol. Chem.* **275,** 20146–20156) showing that in macrophages obtained from cPLA$_2$ knock-out mice, zymosan was unable to induce AA release. These results are fully consistent with the data presented in this manuscript and confirm the usefulness of the P388D$_1$ cells as a model for macrophage activation studies.

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Identification of a Third Pathway for Arachidonic Acid Mobilization and Prostaglandin Production in Activated P388D 1 Macrophage-like Cells

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J. Biol. Chem. 2000, 275:22544-22549.
doi: 10.1074/jbc.M910163199 originally published online May 15, 2000