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Localization of Group V Phospholipase A_2 in Caveolin-enriched Granules in Activated $P388D_1$ Macrophage-like Cells*

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In murine $P388D_1$ macrophages, the generation of prostaglandin E_2 in response to long term stimulation by lipopolysaccharide involves the action of Group V secreted phospholipase A_2 (PLA_2), Group IV cytosolic PLA_2 ($cPLA_2$), and cyclooxygenase-2 (COX-2). There is an initial activation of $cPLA_2$ that induces expression of Group V PLA_2, which in turn induces both the expression of COX-2 and most of the arachidonic acid substrate for COX-2-dependent prostaglandin E_2 generation. Because Group V PLA_2 is a secreted enzyme, it has been assumed that after cellular stimulation, it must be released to the extracellular medium and re-associates with the outer membrane to release arachidonic acid from phospholipids. In the present study, confocal laser scanning microscopy experiments utilizing both immunofluorescence and green fluorescent protein-labeled Group V PLA_2 shows that chronic exposure of the macrophages to lipopolysaccharide results in Group V PLA_2 being associated with caveolin-2-containing granules close to the perinuclear region. Heparin, a cell-impermeable complex carbohydrate with high affinity for Group V PLA_2, blocks that association, suggesting that the granules are formed by internalization of the Group V $sPLA_2$ previously associated with the outer cellular surface. Localization of Group V PLA_2 in perinuclear granules is not observed if the cells are treated with the Group IV PLA_2 inhibitor methyl arachidonyl fluorophosphonate, confirming the important role for Group IV PLA_2 in the activation process. Cellular staining with antibodies against COX-2 reveals the presence of COX-2-rich granules in close proximity to those containing Group V PLA_2. Collectively, these results suggest that encapsulation of Group V PLA_2 into granules brings the enzyme to the perinuclear envelope during cell activation where it may be closer to Group IV PLA_2 and COX-2 for efficient prostaglandin synthesis.

The phospholipase A_2 (PLA_2)[1] superfamily includes a large group of enzymes that catalyze the hydrolysis of fatty acids located at the sn-2 position of phospholipids (1). PLA_2 constitutes the main metabolic route by which fatty acids such as arachidonic acid (AA) are liberated from their lipid storage sites for the synthesis of eicosanoids, a family of compounds with important pathophysiological roles. Controlling the production of eicosanoids has been found to be of great benefit for the treatment of inflammatory diseases. The PLA_2-catalyzed release of AA constitutes a new alternative pharmacological target for the development of anti-inflammatory therapies, and it is for this reason that mammalian PLA_2s have been so extensively studied in the recent years (2). However, the signaling cascades and the identification of mechanisms for PLA_2 activation is a very complicated issue because of the presence of multiple PLA_2 forms within a single cell (3).

Using the murine macrophage-like cell line $P388D_1$, we have shown that AA mobilization and prostaglandin production in response to bacterial lipopolysaccharide (LPS) occurs in two temporally distinct manners. In an immediate phase, the LPS does not act as a trigger of the response itself but rather enables the cells to rapidly respond to a second pro-inflammatory stimulus such as platelet-activating factor (4–7). This immediate phase (LPS-primed phase) is completed within minutes and appears to occur at the expense of pre-existing effectors. Prostaglandin E_2 (PGE_2) production is detected within 10 min of cellular exposure to platelet-activating factor (after LPS priming), and depends on the coupling of three different enzymes: the cytosolic Group IV PLA_2 ($cPLA_2$), the Group V PLA_2, and COX-2. The elevation of the intracellular Ca^{2+} generated by occupancy of the platelet-activating factor receptor appears to be a key signaling event for $cPLA_2$ activation. The transient elevation of intracellular AA (or of one of its metabolites) would then help increase the activity of preexisting Group V PLA_2, generating a second wave of AA liberation that would be metabolized to PGE_2 by COX-2, which exists at low levels under these conditions (4–7).

In the second pathway of activation, called the delayed pathway, LPS induces AA release on its own in a process that takes several hours to develop and involves the *de novo* synthesis of two of the effector enzymes involved, namely Group V PLA_2 and COX-2 (8, 9). Importantly, the elevated expression of these two effectors can be prevented by inhibiting the $cPLA_2$ with methyl arachidonyl fluorophosphonate (MAFP) (6, 8, 9), which indicates that a functionally active $cPLA_2$ is a key regulator of Group V PLA_2 and COX-2 expression in this system. Moreover,

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[1] The abbreviations used are: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secreted phospholipase A_2; AA, arachidonic acid; LPS, bacterial lipopolysaccharide; COX-2, cyclooxygenase-2; MAFP, methyl arachidonoyl flourophosphonate; PG, prostaglandin; PGE_2, prostaglandin E_2; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GFP-GV, GFP-Group V $sPLA_2$.

inhibition of Group V PLA_2, by antisense technology or by chemical inhibitors, abolishes COX-2 expression in the LPS-treated cells (8, 9). Thus Group V PLA_2 plays two separate roles in the process: on one hand, it provides the bulk of AA release; on the other, Group V PLA_2 controls the induction of the enzyme that metabolizes the free AA *i.e.* COX-2. It is interesting to note that, despite the marked differences between the two pathways of AA release involving LPS, both of them appear to utilize the same PLA_2 effectors, *i.e.* $cPLA_2$, and Group V $sPLA_2$. The $cPLA_2$ fundamentally plays a regulatory role, whereas the $sPLA_2$ plays an augmentative role by providing most of the AA metabolized by COX-2 (6, 8, 9).

Although it is has been clearly demonstrated that $cPLA_2$ primarily acts on perinuclear membranes (10), the precise site of action of secreted PLA_2s such as the Group V PLA_2, is still the subject of intense research. Recent work using transfected cell lines (11) or exogenously added enzymes (12–15) has revealed that some secreted PLA_2 can re-associate with the outer cellular surface and, subsequently, be re-internalized. Although some of these studies have suggested that this internalization may serve as a means to terminate signaling (*i.e.* the internalized protein is degraded) (12), other studies have suggested the possibility that the internalization process serves to bring the secreted PLA_2 in close proximity to COX-2 in the perinuclear area for efficient conversion of AA into PGs (11).

In the current work we have sought to study the subcellular localization of the *de novo* Group V $sPLA_2$ in $P388D_1$ cells treated with LPS to release AA and generate PGs in a delayed phase. We demonstrate that Group V $sPLA_2$ localizes into perinuclear granules of the cell, which strongly correlates with the delayed onset of PG production (8).

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). Non-essential amino acids were from Irvine Scientific (Santa Ana, CA). LPS (*Escherichia coli* 0111:B4) and heparin were from Sigma. Rabbit anti-Group V $sPLA_2$ polyclonal antibody was generously provided by Dr. Jonathan Arm (Harvard Medical School) (16). Anti-caveolin-2 and COX-2 mouse monoclonal antibodies were from Transduction Laboratories (Lexington, KY). FITC-conjugated anti-rabbit IgG and Red X-conjugated anti-mouse IgG were from Jackson ImmunoResearch (West Grove, PA).

Cell Culture Conditions—$P388D_1$ cells (MAB clone) (8, 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 non-essential amino acids. $P388D_1$ cells were plated onto coverslips, allowed to adhere overnight, and used for experiments the following day. For stimulation, the cells were washed and placed in serum-free medium. Where indicated, the cells were incubated with 100 ng/ml LPS for the indicated periods of time. When heparin was used, it was added at 1 mg/ml. All experiments were conducted in serum-free medium.

Immunofluorescence—After each treatment, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 min at room temperature. Cells were then permeabilized with 0.3% Triton X-100/phosphate-buffered saline for 5 min at room temperature and blocked for 30 min in 0.5% bovine serum albumin, 2% normal donkey serum, and 50 mM glycine in phosphate-buffered saline (blocking buffer). Incubations with primary and secondary antibodies were performed at a 1:500 dilution in working buffer (1:5 dilution of blocking buffer) for 1–2 h at room temperature. Secondary antibodies were FITC-conjugated anti-rabbit IgG for Group V $sPLA_2$ antibody and Red X-conjugated anti-mouse IgG for caveolin-2 and COX-2 antibodies. Extensive washes in between incubations with antibodies were performed in phosphate-buffered saline. Specimens were mounted in anti-fade medium and viewed with a Bio-Rad MRC-1024 laser-scanning confocal system coupled to a Zeiss Axiovert 35M microscope. The objective was a Zeiss Plan-Apochromat, 63×, 1.4 numerical aperture, oil immersion. The fluorescence of FITC was monitored at 488-nm argon excitation using a 522–535 nm band pass barrier filter whereas that of Red-X was monitored at 568-nm argon excitation using a 605–632 nm band pass barrier filter.

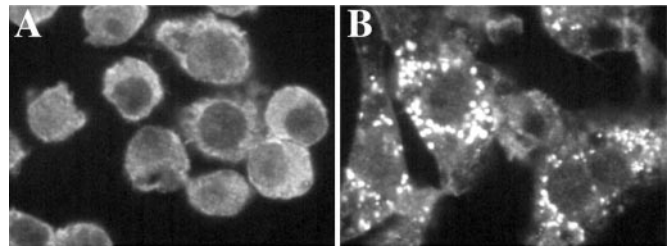

FIG. 1. **Localization of Group V PLA_2 in LPS-stimulated $P388D_1$ macrophages.** The cells were treated with vehicle (*A*) or 100 ng/ml LPS (*B*) for 18 h. After fixation and permeabilization, the cells were immuno-labeled with rabbit anti-mouse Group V PLA_2 antibodies followed by FITC-conjugated anti-rabbit IG antibody, and samples were analyzed by confocal microscopy.

The specificity of the anti-Group V PLA_2 antibody was tested by using the antibody absorbed with the Group V PLA_2 peptide used to obtain the rabbit anti-Group V PLA_2 serum: ELKSMIEKVTGKNAFKNYG (16). To this end the Group V PLA_2 peptide or a control peptide (CMETTPLNSQKVLSE) were conjugated to an agarose column (Hitrap affinity column, Amersham Biosciences), and the pass-through fraction of the Group V antibody (1:500 final dilution) was used for immunostaining.

Construction of the Plasmid Encoding GFP-Group V $sPLA_2$ (GFP-GV) and Production of Transfectants Stably Expressing GFP-GV—A cDNA fragment of human Group V $sPLA_2$ having a native signal peptide at the N terminus was produced by PCR with HPLA2–10 (18) as a template. The sense primer was 5′-TTGAGATCTGAGATGAAAGGC-CTCCTCCCA-3′, the antisense primer 5′-AAAGAGATCTGGGAGCA-GAGGATGTTGGG-3′. The PCR product of Group V PLA_2 was digested with *Bgl*II and then subcloned into the *Bgl*II site in pEGFPN1 (Clontech). Approximately 5.5 μg of plasmids encoding GFP-GV or GFP (pEGFPN1) was mixed with 15 μl of TransIT™-LT1 (Panvera Co., Madison, WI) in 1000 μl of Opti-MEM for 10 min at room temperature and then added to the $P388D_1$ cells (0.8×10^6 cells). After incubation at 37 °C for 4 h, the medium was replaced by the usual culture medium and incubated for an additional 12–18 h period. To obtain the GFP-GV or GFP stably expressing cells, the transfected cells were cloned by limiting dilution and kept in culture medium supplemented with 1 mg/ml Geneticin (Invitrogen) using 96-well plates. After 2 weeks, wells containing a single colony were chosen for further expansion, and the fluorescence of the cells was examined under an epifluorescence microscope. Among the cell clones exhibiting GFP fluorescence, stable-transfectants for GFP-GV or GFP were selected by immunoblotting and PLA_2 activity measurement. The stable cell lines were kept in culture in medium supplemented with 1 mg/ml Geneticin.

RESULTS

Subcellular Distribution of Group V $sPLA_2$ in LPS-treated Cells—Arm and co-workers (16) have recently produced antibodies that specifically recognize Group V $sPLA_2$ and Group IIA $sPLA_2$. These antibodies make it possible now to conduct ultrastructural studies aimed at determining the cellular/subcellular site of action of the $sPLA_2$ during cell activation. In unstimulated cells, confocal laser scanning microscopy revealed a diffuse pattern of cytoplasmic staining with anti-Group V $sPLA_2$ antibody (Fig. 1). However, after exposure to LPS for 18 h, a treatment that results in the generation of free AA and PGs such as PGE_2 (8, 9), a dramatic change in the subcellular localization of the Group V $sPLA_2$ was observed in that a more granular staining was now readily seen (Fig. 1). The staining was ablated when the primary antibody was first absorbed out with the Group V PLA_2 peptide antigen (see "Experimental Procedures") as shown in Fig. 2, but not with a control peptide. A magnification of those granules can be observed in Fig. 3, where the perinuclear localization is evident. Fig. 4 shows the kinetics of Group V $sPLA_2$ localization during exposure of the cells to LPS. An intense granular staining was already observed after 6 h of stimulation, increasing gradually with time. Staining was more prominent after 18 h of treatment (Fig. 4).

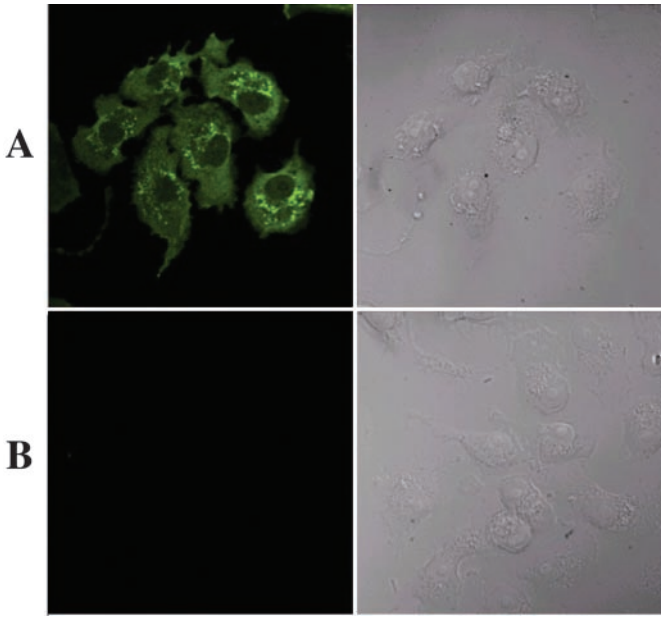

FIG. 2. **Group V PLA_2 antibody specificity.** The cells were treated with LPS 100 ng/ml for 18 h, fixed, permeabilized, and treated with the antibody anti-Group V PLA_2 absorbed to either a control peptide (*A*) or to the Group V PLA_2 peptide used for immunization (*B*).

As an alternative approach to further elucidate the localization of Group V $sPLA_2$ in $P388D_1$ cells, these cells were stably transfected with C-terminally GFP-tagged human Group V $sPLA_2$, and transfectants were examined by confocal laser microscopy (Fig. 5). The fusion protein was enzymatically active as judged by *in vitro* enzyme assay.[2] Control unstimulated cells (0 h stimulation) showed diffuse fluorescence in the cytoplasm, whereas exposure to LPS increased staining of cytoplasmic granular structures (Fig. 5*A*). These observations fully agree with the results shown in previous figures utilizing immunofluorescence. Control cells transfected only with GFP did not show those structures but a pattern of fluorescence evenly distributed across the cells, and no changes were observed after activation (Fig. 5*B*).

To investigate the Group V $sPLA_2$-containing granules in more detail, double antibody staining experiments were conducted in LPS-treated cells. Anti-Group V $sPLA_2$ and anti-caveolin-2 antibodies revealed co-localization of both proteins in granules close to the nuclear envelope (Fig. 6), although it should be noted that not all of the Group V $sPLA_2$ is associated with caveolin-2. In contrast to LPS-treated cells, unstimulated cells revealed a very poor staining with caveolin-2,[2] a finding that suggests up-regulation of caveolin-2 during cell activation, as previously reported for caveolin-1 in other macrophage cell lines (19). No cross-reactivity was found between the secondary antibody anti-rabbit IgG and the monoclonal antibody bound to caveolin-2, or between the secondary antibody anti-mouse IgG against the polyclonal antibody bound to Group V PLA_2. LysoTracker or cathepsin D, markers for acidic granules, did not co-localize with Group V $sPLA_2$, indicating that Group V-rich granules are not of lysosomal origin (data not shown).

The co-localization of Group V $sPLA_2$ and caveolin-2 into perinuclear granules suggests the possibility that the $sPLA_2$ present in these granules comes from the outside of the cell via a caveolae-mediated endocytotic event or potocytosis, as previously suggested by others (11). To test this possibility, cellular treatment with LPS was conducted in the presence of heparin in the extracellular medium. Heparin, a cell-impermeable polysaccharide, tightly binds Group V $sPLA_2$ in the incubation medium, thereby preventing the enzyme from interacting with the plasma membrane (8, 20). As a result of this action, heparin strongly blunts agonist-induced delayed AA mobilization in macrophages as well as other cells (20–22).

The presence of heparin in the incubation medium drasti-

[2] Y. Shirai and E. A. Dennis, unpublished data.

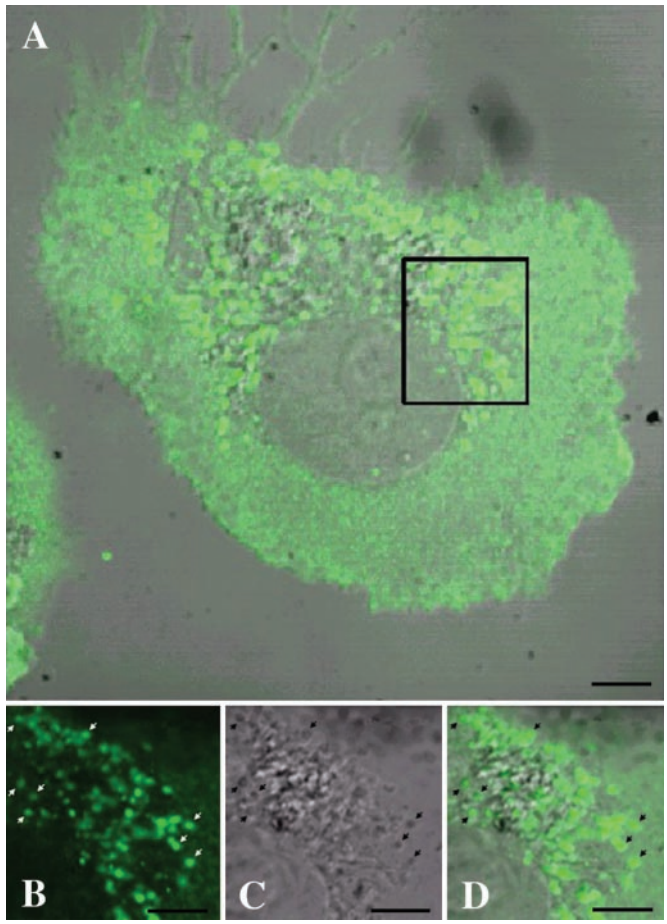

FIG. 3. **Magnification of Group V PLA_2-stained granules.** The cells were treated with 100 ng/ml LPS and stained with Group V PLA_2 antibody as described under "Experimental Procedures." *A*, single cell; *B–D*, magnification of the perinuclear granules (*B*, Group V PLA_2 fluorescence; *C*, Nomarski image; *A* and *D*, merged images). *Arrows* point to some of these granules. *Bar* in *panel A* = 2 μM; *bars* in *panels*, *B*, *C*, and *D* = 0.5 μM.

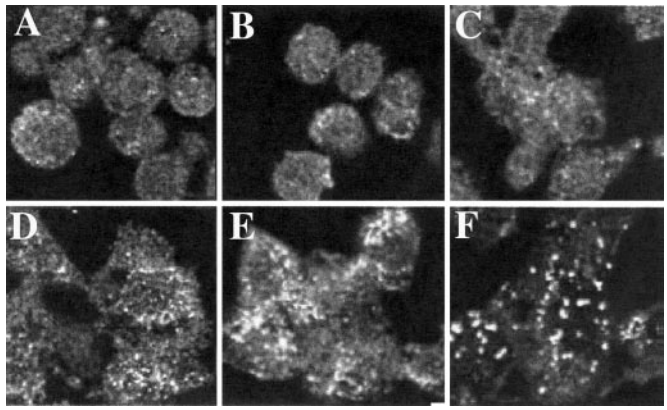

FIG. 4. **Time-course of changes in Group V $sPLA_2$ localization in response to LPS.** The cells were treated with 100 ng/ml LPS for the indicated time periods. After fixation and permeabilization, the cells were treated with rabbit anti-mouse Group V $sPLA_2$ followed by FITC-conjugated anti-rabbit IgG antibody. *A*, untreated cells; *B*, cells treated with LPS for 3 h; *C*, 6 h; *D*, 9 h; *E*, 12 h; and *F*, 18 h.

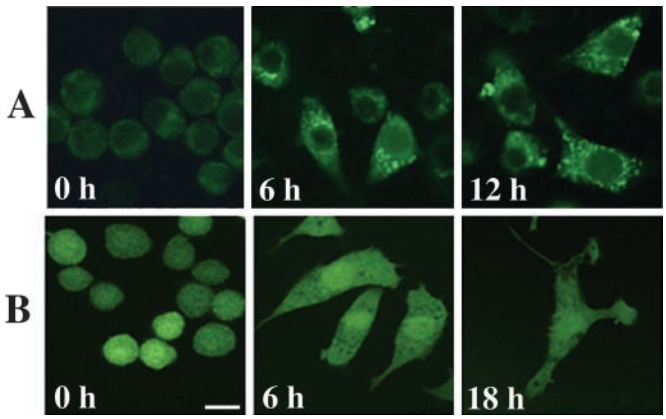

FIG. 5. **Subcellular distribution of GFP-GV $sPLA_2$ (GFP-GV) in response to LPS.** Stable transfectants of GFP-GV (*A*) or control GFP (*B*) were treated with 100 ng/ml LPS for the indicated periods of time, fixated with 4% paraformaldehyde, and fluorescence was observed by confocal microscopy. All images were taken with the same magnification. *Bar* = 20 μm.

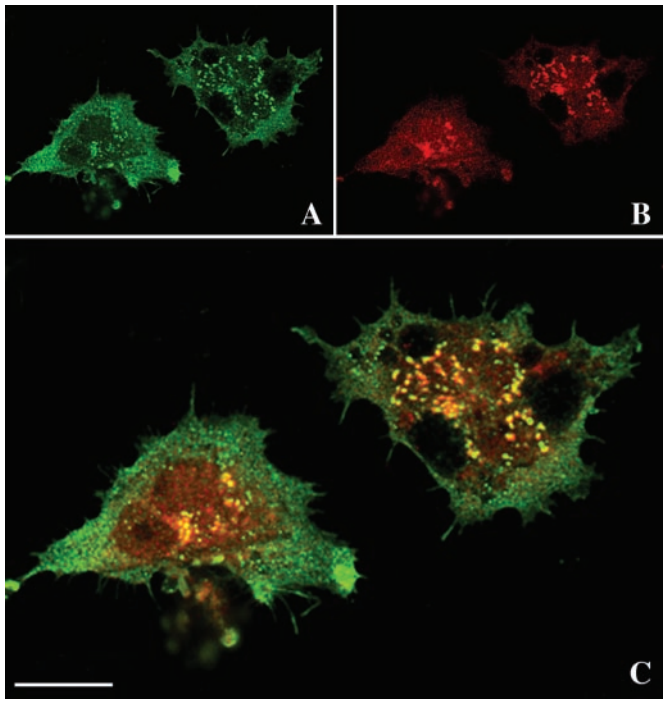

FIG. 6. **Co-localization of Group V $sPLA_2$ with caveolin-2 in intracellular granules of LPS-treated cells.** The macrophages were incubated with 100 ng/ml LPS for 18 h. After fixation and permeabilization, the cells were double-stained with anti-Group V $sPLA_2$ (*A*, *green*) and anti-caveolin-2 (*B*, *red*). *C* shows the merge. *Bar* = 10 μM.

cally reduced the appearance of Group V $sPLA_2$-containing perinuclear granules in LPS-activated cells (Fig. 7), suggesting an extracellular origin for those structures. Also, a side-by-side comparison of the effects of heparin on the co-localization of Group V $sPLA_2$ with caveolin-2 in LPS-treated cells is shown in Fig. 8. Because in the presence of heparin no perinuclear $sPLA_2$-containing granules are observed, no-colocalization between caveolin-2 and GV $sPLA_2$ was detected.

In macrophages, PGE_2 production in the delayed phase of LPS activation is due to the metabolism of AA by COX-2 (8, 9). It has been described that COX-2 localizes close to or by the nuclear envelope (23). We performed some experiments using antibodies against COX-2 to define the localization of this enzyme in LPS-treated cells. As shown in Fig. 9, COX-2 localizes in granules near the nucleus in close proximity to Group V PLA_2.

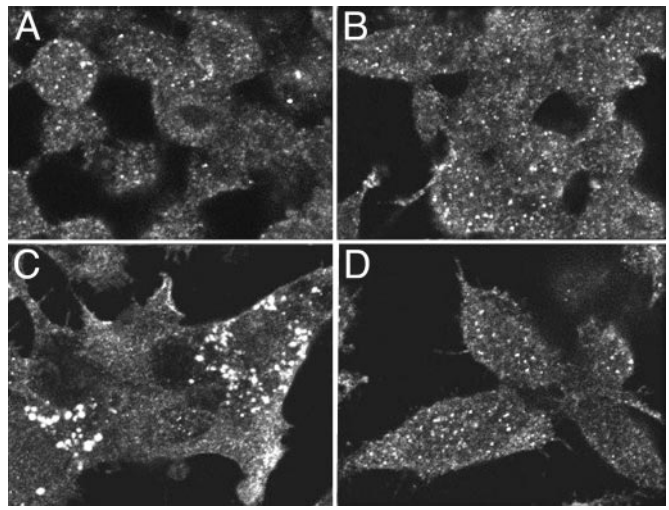

FIG. 7. **Effect of heparin on the subcellular distribution of Group V $sPLA_2$.** The cells were either untreated (*A* and *B*) or treated with 100 ng/ml LPS (*C* and *D*) for 18 h in the absence (*A* and *C*) or presence (*B* and *D*) of 1 mg/ml heparin. After fixation and permeabilization, the cells were treated with rabbit anti-mouse Group V $sPLA_2$ followed by FITC-conjugated anti-rabbit IgG antibody.

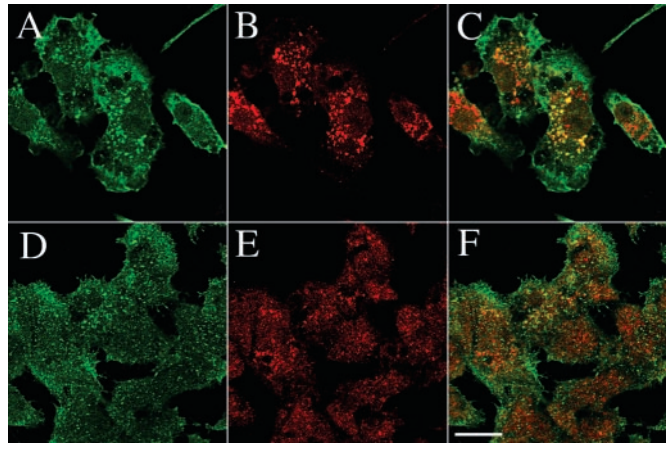

FIG. 8. **Effect of heparin on the colocalization of Group V $sPLA_2$ with caveolin-2 in LPS-treated cells.** The macrophages were treated with 100 ng/ml LPS for 18 h in the absence (*A–C*) or presence (*D–F*) of 1 mg/ml heparin. After fixation and permeabilization, the cells were double-stained with anti-Group V $sPLA_2$ (*green*; *A* and *D*) and anti-caveolin-2 (*red*; *B* and *E*). *C* and *F* are merged images. *Bar* = 10 μM.

As indicated above, long term stimulation by LPS promotes activation of $cPLA_2$, which leads to an early increase of free AA. Blocking $cPLA_2$ activity with MAFP prevents PGE_2 synthesis in LPS-treated cells, due to inhibition of the induction of Group V PLA_2. Confocal microscopy experiments were performed in the LPS-treated cells in the presence of MAFP. As shown in Fig. 10, no perinuclear Group V PLA_2-enriched granules were found under those conditions (Fig. 10, *C* and *D*).

DISCUSSION

Ongoing studies in our laboratory for several years have delineated two pathways for AA release and metabolism in LPS-treated macrophages. The first one, referred to as the "primed immediate pathway" takes place in minutes and is elicited by the Ca^{2+}-mobilizing agonist platelet-activating factor, but requires the cells to be exposed first to LPS for 1 h (4–7, 16). The second route, or "delayed pathway," is elicited by LPS for periods of time spanning several hours (8, 9). Interestingly, both pathways utilize the same effectors, namely Group IVA $cPLA_2$, Group V $sPLA_2$, and COX-2, although the molecular mechanisms involved dramatically differ. In both of these

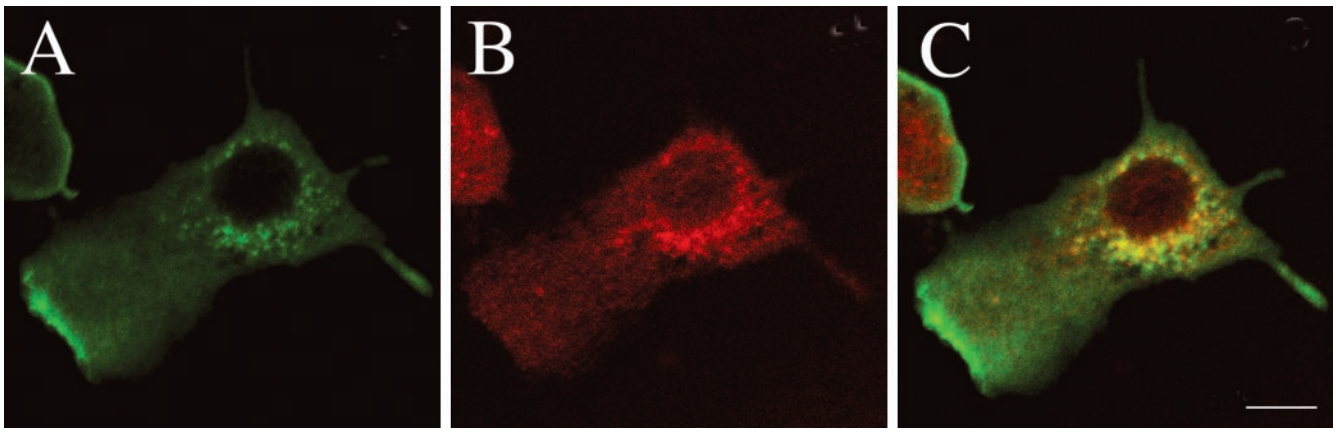

FIG. 9. **Localization of COX-2 and Group V PLA_2 in LPS-treated cells.** The cells were treated with 100 ng/ml LPS for 20 h. After fixation and permeabilization, the cells were stained with anti-Group V PLA_2 (*A*, *green*) and COX-2 (*B*, *red*) antibodies. *C* shows the merged image. *Bar* = 10 μm.

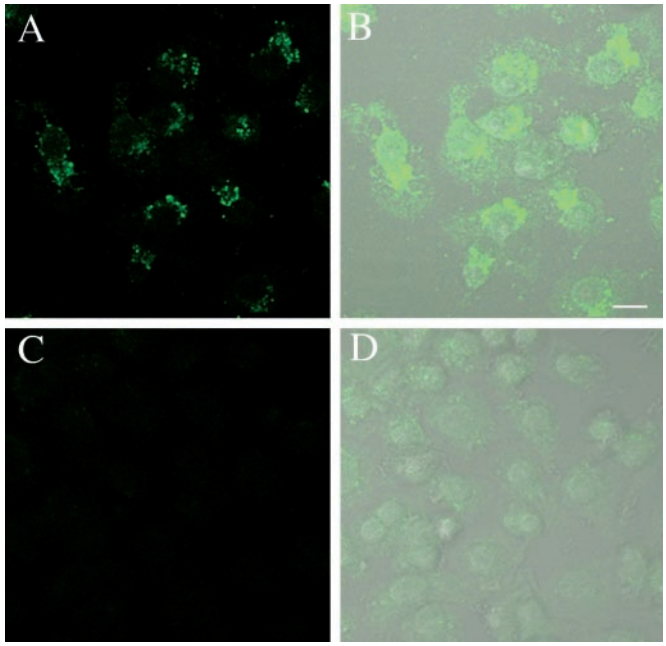

FIG. 10. **Effect of $cPLA_2$ inhibition on Group V PLA_2 subcellular location on LPS-stimulated $P388D_1$ macrophages.** The cells were treated with 100 ng/ml LPS for 20 h in the absence (*A* and *B*) or presence (*C* and *D*) of 50 μM MAFP. After fixation and permeabilization, the cells were stained with anti-Group V PLA_2 antibody. *B* and *D* show the fluorescence with Nomarski image. *Bar* = 10 μm.

routes the $cPLA_2$ appears to behave primarily as an initiator of the response, whereas Group V $sPLA_2$ plays an augmentative role by providing most of the AA to be converted to prostaglandins via COX-2 (24). Although in the immediate pathway all the enzymes implicated in PGE_2 production are already present in the cell, in the long term pathway both Group V PLA_2 and COX-2 are up-regulated, and these events are triggered after the $cPLA_2$ has become activated. Expression of COX-2 is also dependent on the activation of Group V PLA_2 (8, 9).

The importance of Group V $sPLA_2$ in AA mobilization and prostaglandin production by major immunoinflammatory cells has been clearly recognized (24, 25). Group V $sPLA_2$ is rapidly secreted by the activated cells to the extracellular medium. The enzyme has traditionally been thought to re-associate with the outer leaflet of the plasma membrane to hydrolyze phospholipids and release AA and, in this manner, amplify the response already initiated by Group IVA PLA_2 in the interior of the cell. According to this view, it was assumed that part of the AA released by Group V $sPLA_2$ at the plasma membrane would travel to the nuclear envelope and endoplasmic reticulum, either by passive diffusion or active transport mechanisms, for metabolism by 5-lipoxygenase or COX isoforms (5–7, 21, 26, 27).

Structurally, Group V $sPLA_2$ is remarkably similar to other $sPLA_2$ family members present in mammalian cells (1, 3), most of which seem to have limited or no role in AA mobilization and attendant eicosanoid production (2, 28). This has made it difficult to establish the exact site(s) of action of this enzyme within the cell. In fact, most of the anti-$sPLA_2$ antibodies that have been used in subcellular localization studies have later been found not to distinguish among the different $sPLA_2$ forms. Recently however, Arm and co-workers (see Ref. 16 and "Experimental Procedures") have described a polyclonal antibody directed against a unique 19-mer peptide sequence in the N-terminal end of the molecule. Utilizing this antibody, Bingham *et al.* (16) have studied the subcellular localization of Group V $sPLA_2$ in resting mast cells and compared it with the distribution of Group IIA $sPLA_2$ in these cells. Both $sPLA_2$ types were found to localize in a distinct manner. Although Group IIA $sPLA_2$ was found in secretory granules, Group V $sPLA_2$ was found on the plasma membrane but also on cytoplasmic membranes, particularly those of the Golgi and the nuclear envelope (16). Unfortunately, Bingham *et al.* (16) did not extend their studies to stimulated cells and thus the significance of intracellularly located Group V $sPLA_2$ was not ascertained.

In the present study we have carried out studies with the same antibody employed in the studies of Bingham *et al.* (16) to determine the localization of Group V PLA_2 in stimulated cells. In our cellular model, the $P388D_1$ macrophage cell line Group V $sPLA_2$ has a diffuse distribution across the cell during resting conditions (Fig. 1*A*). After treatment with LPS for periods of time longer than 6 h, cell-associated Group V $sPLA_2$ is found to be present in cytoplasmic granules. These large size granules are located in the perinuclear region and contain caveolin-2 together with Group V $sPLA_2$, although some of the Group V $sPLA_2$ is not associated with these large granules. Given the presence of caveolin-2 in those granules, and the fact that treatment with heparin blocks the Group V $sPLA_2$ present therein (Figs. 7 and 8), it seems logical to suggest that these granules are formed by internalization of the Group V $sPLA_2$ that has associated with the cellular surface after being secreted to the incubation medium, via a caveolae-mediated endocytotic event (Fig. 11). A similar process was suggested by Murakami *et al.* (11) utilizing transfected enzyme.

Caveolae are known to form a unique endocytic and exocytic compartment at the surface of most cells, capable of importing

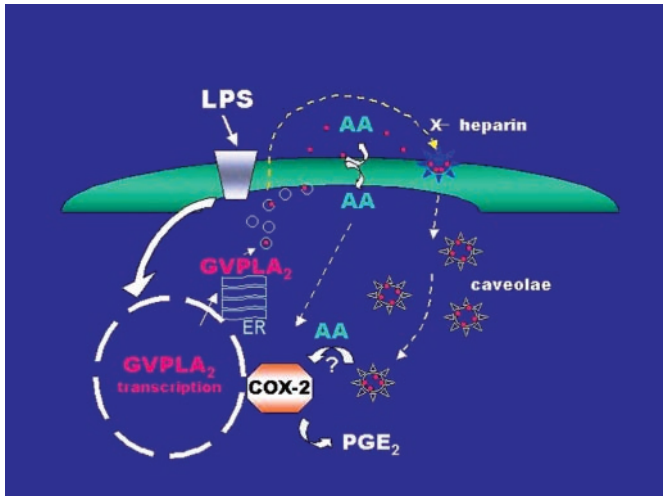

FIG. 11. **Release and uptake of Group V sPLA$_2$ into caveolin-rich granules.** When macrophages are stimulated by LPS, Group V PLA$_2$ is produced. The enzyme is released to the extracellular medium, from which it re-associates with discrete sites on the cell surface, such as caveolae. The caveolae can be internalized back into the cell. In this manner, Group V PLA$_2$ comes closer to other enzymes involved in AA metabolism, like COX-2. The caveola interior contains the appropriate millimolar Ca^{2+} levels that could allow the enzyme to be active and, in addition, preserves the enzyme from the reducing environment of the cytosol.

molecules from the exterior of the cells and delivering them to specific locations within the cell (29). Moreover, caveolae are sites of Ca^{2+} storage and entry into the cell (29). This is important because Group V sPLA$_2$ absolutely requires millimolar levels of calcium for activity (1). Moreover, encapsulation of the Group V sPLA$_2$ in the caveolin-2-containing granules would protect the enzyme from the reducing cytosolic environment that would rapidly denaturate the enzyme. Thus, Group V sPLA$_2$ inside caveolin-2 granules may well retain enzyme activity after internalization and translocation to the perinuclear membrane. Because the perinuclear membrane is precisely the site where upstream (cPLA$_2$) (10) and downstream (5-lipoxygenase and cyclooxygenase-2) (23) eicosanoid-metabolic enzymes reside, such a mechanism would result in an extremely efficient utilization of the free AA liberated by the Group V sPLA$_2$ at this location (Fig. 11). This is an important consideration because prostaglandin formation during the delayed phase of AA mobilization in macrophages normally occurs at levels of free AA that are much lower than those available during the immediate pathway of AA mobilization. For instance, analyses of the ^{3}H-radioactive material released during chronic exposure of the [^{3}H]AA-labeled cells to LPS reveals that free unmetabolized AA represents less than 2% of total. This is in stark contrast with the primed immediate pathway, where free AA constitutes more than 95% of the released ^{3}H-radioactive material.[3]

Recent data by Cho and co-workers (12) utilizing exogenous Group V sPLA$_2$ addition to human neutrophils showed the internalization of the enzyme only after all the AA release was completed. Such an internalization resulted in the enzyme being slowly degraded in the interior of the cell, which prompted the authors to suggest that Group V sPLA$_2$ internalization would serve as a mechanism to modulate the extent of membrane hydrolysis of cell surface bound Group V sPLA$_2$ (12). The results by Cho and co-workers (12) directly relate to signal termination and thus are both experimentally and phenomenologically unrelated to the internalization event herein described for Group V sPLA$_2$ during chronic exposure of the cells to LPS. We have failed to co-localize Group V sPLA$_2$ with the lysosomal markers LysoTracker and cathepsin D, which suggests that caveolin-2-granules containing Group V sPLA$_2$ do not fuse with lysosomes after internalization. Interestingly as well, we have found that even after 36 h following LPS addition to the cells, a time at which fatty acid release and prostaglandin release have long ceased, a large number of Group V sPLA$_2$-containing granules are still observable in the perinuclear area, as judged by both immunofluorescence and GFP fluorescence. Interestingly, other recent reports also from Cho's group described the internalization and apparent action of Group V sPLA$_2$ on perinuclear membranes of HEK cells, human neutrophils, and human eosinophils (13–15), a view that is consistent with the results of this study.

The studies by Kim *et al.* and Muñoz *et al.* (13–15) were conducted with exogenous, and in some cases, mutated enzymes and not with endogenous native enzyme, as performed in the current study. Nevertheless, as a whole, these studies clearly indicate that Group V PLA$_2$ may have different modes of action in different cells. This view is also highlighted by the recent studies of Murakami *et al.* (11). These investigators have proposed a "glypican-shuttling mechanism" of action on adherent cells, like fibroblasts or HEK cells, where secreted PLA$_2$s that bind heparin (Groups IIA, IID, and V) would directly bind to heparan sulfate chains of glypican inside secretory vesicles prior to being released to the extracellular space. Then, those enzymes would be redirected to caveolae-rich domains and reinternalized by potocytosis. They have proposed also a "glypican-independent" model that would be prevalent on mast cells and other hematopoietic cells poor in caveolae and/or glypican (11), where enzymes like Group V and Group X PLA$_2$s would act on the phosphatidylcholine present at the plasma membrane.

Our current work fits well with the glypican-shuttling mechanism proposed by Murakami *et al.* (11) on the basis of the following evidence: (i) P388D$_1$ cells do express caveolin and caveolae, (ii) Group V PLA$_2$ colocalizes with caveolin-2 in the same intracellular granules, and (iii) PGE$_2$ production depends on COX-2, in contrast to the COX-1-dependent PGE$_2$ production postulated for the glypican-independent model. Importantly, however, we have performed Group V PLA$_2$ staining experiments during the primed immediate phase of AA release in activated P388D$_1$ macrophages and found that under those conditions the enzyme is relocated in some patches in the plasma membrane of the cells, but it does not localize in intracellular granules.[4] These results would fit better with the glypican-independent mechanism proposed by Murakami *et al.* (11). Thus, it seems possible that the two mechanisms of action proposed by Murakami *et al.* (11) may occur within the same cell, and that the stimulation conditions dictate which mechanism takes place.

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Localization of Group V Phospholipase A_2 in Caveolin-enriched Granules in Activated P388D $_1$ Macrophage-like Cells

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