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Group V Phospholipase A_2-dependent Induction of Cyclooxygenase-2 in Macrophages*

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When exposed for prolonged periods of time (up to 20 h) to bacterial lipopolysaccharide (LPS) murine $P388D_1$ macrophages exhibit a delayed prostaglandin biosynthetic response that is entirely mediated by cyclooxygenase-2 (COX-2). Both the constitutive Group IV cytosolic phospholipase A_2 ($cPLA_2$) and the inducible Group V secretory phospholipase A_2 ($sPLA_2$) are involved in the cyclooxygenase-2-dependent generation of prostaglandins in response to LPS. Using the selective $sPLA_2$ inhibitor 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propane sulfonic acid (LY311727) and an antisense oligonucleotide specific for Group V $sPLA_2$, we found that induction of COX-2 expression is strikingly dependent on Group V $sPLA_2$, which was further confirmed by experiments in which exogenous Group V $sPLA_2$ was added to the cells. Exogenous Group V $sPLA_2$ was able to induce significant arachidonate mobilization on its own and to induce expression of the COX-2. None of these effects was observed if inactive Group V $sPLA_2$ was utilized, implying that enzyme activity is crucial for these effects to take place. Therefore, not only delayed prostaglandin production but also *COX-2* gene induction are dependent on a catalytically active Group V $sPLA_2$. COX-2 expression was also found to be blunted by the Group IV $cPLA_2$ inhibitor methyl arachidonyl fluorophosphonate, which we have previously found to block Group V $sPLA_2$ induction as well. Collectively, the results support a model whereby Group IV $cPLA_2$ activation regulates the expression of Group V $sPLA_2$, which in turn is responsible for delayed prostaglandin production by regulating COX-2 expression.

Phospholipase A_2 (PLA_2)[1] plays a key role in numerous cellular processes by regulating the release of arachidonic acid (AA) from membrane phospholipids. In turn, free AA can be converted into the prostaglandin (PG) precursor PGH_2 by the action of cyclooxygenases (COX). These two reactions constitute the regulatory checkpoints of the pathway leading to PG biosynthesis in mammalian cells.

A considerable body of evidence suggests that specific coupling between certain PLA_2 and COX forms accounts for the differential regulation of the immediate and delayed responses (1–11). We have shown that the immediate platelet-activating factor-receptor-mediated phase of PGE_2 production in LPS-primed $P388D_1$ macrophages involves Group V $sPLA_2$ coupling to COX-2 (3). More recently, we have discovered that the exact same coupling of Group V $sPLA_2$ to COX-2 also regulates the delayed PGE_2 response of $P388D_1$ macrophages to LPS alone (4). Under the latter conditions, expression of both Group V $sPLA_2$ and COX-2 was markedly induced and correlated with ongoing AA release and PG biosynthesis, respectively (4), suggesting that the AA produced by Group V $sPLA_2$ was used by COX-2 to produce PGE_2. Importantly, $sPLA_2$ expression could be abolished by pretreating the cells with the $cPLA_2$ inhibitor methyl arachidonyl fluorophosphonate, implying that a functionally active $cPLA_2$ is required for the delayed PGE_2 response to occur (4).

In the current study we extend our previous observations on the delayed phase of PGE_2 in $P388D_1$ macrophages to further investigate the regulatory relationships between the three effectors of the response (*i.e.* $cPLA_2$, $sPLA_2$, and COX-2). We now demonstrate tight coupling between $sPLA_2$ and COX-2 for the delayed phase of PGE_2 generation by showing that COX-2 induction by LPS is dependent upon a catalytically active Group V $sPLA_2$.

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 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. LPS (*Escherichia coli* 0111:B4) was from Sigma. Methyl arachidonyl fluorophosphonate (MAFP), *p*-bromophenacyl bromide, and manoalide were from Biomol (Plymouth Meeting, PA). Antibodies against murine COX isoforms were generously provided by Dr. W. L. Smith (Dept. of Biochemistry, Michigan State University, East Lansing, MI). The antibody against Group IV $cPLA_2$ was generously provided by Dr. Ruth Kramer (Lilly Research Laboratories, Indianapolis, IN). The $sPLA_2$ inhibitor 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propane sulfonic acid (LY311727) was generously provided by Dr. Edward Mihelich (Lilly). cDNA probes for murine COX-2 were from Cayman (Ann Arbor, MI). cDNA probes for murine glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were from Ambion (Austin, TX). *Pichia pastoris* strains and vectors were obtained from Invitrogen (Carlsbad, CA)

Cell Culture and Labeling Conditions—$P388D_1$ cells (MAB clone) (4) were maintained at 37 °C in a humidified atmosphere at 90% air and

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[1] The abbreviations used are: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $sPLA_2$, secretory PLA_2; COX, cyclooxygenase; AA, arachidonic acid; PG, protaglandin; LPS, lipopolysaccharide; MAFP, methyl arachidonyl fluorophosphonate; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; LY311727, 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propane sulfonic acid.

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. Radiolabeling of the P388D_1 cells with [^{3}H]AA was achieved by including 0.5 μCi/ml of [^{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 Extracellular [^{3}H]AA Release—The cells were placed in serum-free medium for 30 min before the addition of LPS or exogenous sPLA_2 for different periods of time 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.

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 elsewhere (4, 12). Briefly, P388D_1 cells were transfected with oligonucleotide (125 nM) in the presence of 5 μg/ml LipofectAMINE (Life Technologies) under serum-free conditions for 8 h before treating the cells with or without 100 ng/ml LPS for 10 h after transfection.

Group V sPLA_2 Expression in P. pastoris and Purification—A pCH10 vector containing the gene encoding human Group V sPLA_2 (13) was a generous gift of Dr. Jay A. Tischfield (Dept. of Genetics, Rutgers University, Piscataway, NJ). The gene was transferred from the pCH10 vector to the *P. pastoris* expression vector pPIC9K. Because of difficulties in purifying the expressed enzyme, we opted to incorporate an N-terminal HisTag that could be removed by enterokinase. The construct was then transferred into the pPIC9K *Pichia* expression vector using the pPIC9 vector as a shuttle vector (14). The protease-deficient *P. pastoris* strain SMD1168 was transformed using 10 μg of the *Bgl*II fragment of pPIC9KG5HT that had been linearized with *Bgl*II using a spheroplasting procedure described previously (14). Briefly, colonies were picked and used to inoculate 10 ml of glycerol-containing BMGY medium and allowed to grow. For induction, cells were switched to BMMY medium containing 1% methanol as a carbon source. After 2 days, the crude medium was assayed for PLA_2 activity as described below. The colony displaying the highest level of PLA_2 production was chosen for high level expression using a 5.0 l BioFlow 3000 fermentor (New Brunswick Scientific, Edison, NJ).

Following fermentation, cells were removed by centrifugation at 4,500 × *g* for 10 min, and crude medium was stored at −20 °C. For purification, the medium was thawed and centrifuged again at 16,000 × *g* for 20 min to discard any remaining cells and precipitated proteins. The crude medium was then adjusted to 500 mM NaCl, 500 mM guanidine-HCl, and 50 mM Tris-Cl, pH 8.0, and loaded onto a 1.0-cm column containing 10 ml of Chelating Sepharose Fast Flow resin (Amersham Pharmacia Biotech). After loading, the column was washed with 500 mM NaCl in 50 mM Tris-Cl, pH 8.0, and the Group V enzyme was eluted using a linear gradient from 0 to 500 mM imidazole in 500 mM NaCl, 50 mM Tris-Cl, pH 8.0. Fractions containing PLA_2 activity were pooled and dialyzed exhaustively against 25 mM Tris, pH 6.5. This material was then loaded on a DEAE column equilibrated with 25 mM Tris, pH 6.5, and eluted using a linear gradient from 0 to 1.0 M NaCl in 25 mM Tris, pH 6.5. The HisTag from the purified enzyme was removed with enterokinase according to the manufacturer's recommmendations.

Synthetic Group V sPLA_2—Group V sPLA_2 protein was synthesized from its constituent amino acids by a procedure previously employed for the Group IIA sPLA_2 (15), and the resultant protein was folded using a procedure developed for the protein expressed in *E. coli* (16).[2]

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 (100 μg) were separated by SDS-polyacrylamide gel electrophoresis (10% acrylamide gel) and transferred to Immobilon-P (Millipore). Hybridizations were performed exactly as described previously (4).

Northern Blot Analyses—Total RNA was isolated from unstimulated or LPS-stimulated cells by the TRIZOL reagent method (Life Technologies), exactly as indicated by the manufacturer. Fifteen μg of RNA was electrophoresed in an 1% formaldehyde-agarose gel and transferred to nylon filters (Hybond, Amersham Pharmacia Biotech) in 10× SSC buffer. Hybridizations were performed exactly as described previously (4). Bands were visualized by autoradiography.

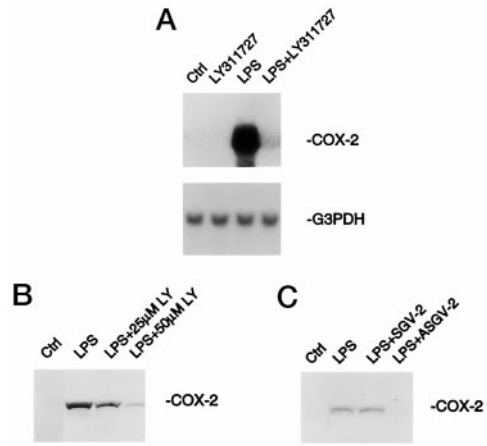

FIG. 1. **Effect of inhibition of endogenous Group V sPLA_2 on COX-2 expression.** *A*, effect of LY311727 on COX-2 mRNA levels as determined by Northern blot. Total mRNA from cells incubated with or without LPS (100 ng/ml, 18 h) and with or without LY311727 (50 μM), as indicated, was isolated and analyzed by Northern blot with probes specific for COX-2 or G3PDH. *Ctrl*, control. *B*, effect of LY311727 on COX-2 protein levels as determined by immunoblot. The effect of LY31727 (25 or 50 μM) on LPS-induced COX-2 protein is shown (100 ng/ml LPS, 18 h). The inhibitor was present throughout the entire 18-h incubation period. *C*, effect of a specific antisense oligonucleotide (*ASGV-2*) or its sense control (*SGV-2*) on COX-2 protein induction by LPS (100 ng/ml, 18 h).

Phospholipase A_2 Assay—The assay mix (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 were allowed to proceed at 40 °C for 30 min, after which [^{14}C]palmitate release was determined by a modified Dole procedure (17).

RESULTS

Inhibition of Endogenous Group V sPLA_2 Blocks COX-2 Expression in Activated P388D_1 Macrophages—We have recently shown that the selective sPLA_2 inhibitor LY311727 (18) is able to suppress long-term PGE_2 production in LPS-treated P388D_1 macrophages (4). We have taken these results as evidence that a catalytically active Group V sPLA_2 is needed for the cells to make PGE_2 at long stimulation times with LPS (4). We have found as well that the enzyme responsible for PGE_2 production in activated P388D_1 macrophages is exclusively COX-2. Albeit present in the cells, COX-1 is spared from the process (4). Thus a tight coupling between Group V sPLA_2 and COX-2 appears to exist for the regulation of PGE_2 production in activated P388D_1 macrophages. To further explore this coupling, we studied the effect of LY311727 on *COX-2* gene expression. Unexpectedly, LY311727 was found to suppress *COX-2* expression at both the mRNA (Fig. 1*A*) and the protein (Fig. 1*B*) levels.

Another method of inhibiting endogenous Group V sPLA_2 is to use antisense oligonucleotide technology (4, 12). An antisense oligonucleotide specific for Group V sPLA_2 (ASGV-2), which is identical to the one we have previously employed (4, 12), strongly inhibited COX-2 protein expression in LPS-treated cells, whereas the sense control (SGV-2) had no effect (Fig. 1*C*). The above results indicate that Group V sPLA_2 is required for COX-2 expression in activated P388D_1 macrophages.

Effects of Exogenous Group V sPLA_2 on P388D_1 Cell Responses—We have recently succeeded in expressing Group V sPLA_2 in the *P. pastoris* yeast expression system. In this system, the enzyme is secreted to the extracellular medium in its catalytically active form. We have purified the enzyme from the supernatants to apparent homogeneity (see "Experimental Procedures") and have studied its effect when added to the P388D_1

[2] L. E. Cannie, P. Botti, R. J. Simon, Y. Chen, E. A. Dennis, and S. B. H. Kent, manuscript in preparation.

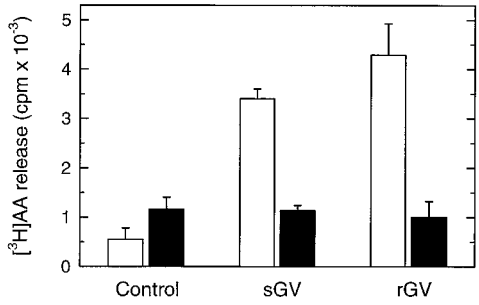

FIG. 2. **Effect of exogenous Group V $sPLA_2$ induces AA release.** *A*, synthetic (*sGV*) or recombinant (*rGV*) Group V $sPLA_2$ (200 ng/ml) was added to the cells in the absence (*open bars*) or presence (*closed bars*) of LY311727 (25 μM) for 1 h. Afterward, supernatants were assayed for [^{3}H]AA release. *Control* denotes incubations in the absence of added enzyme.

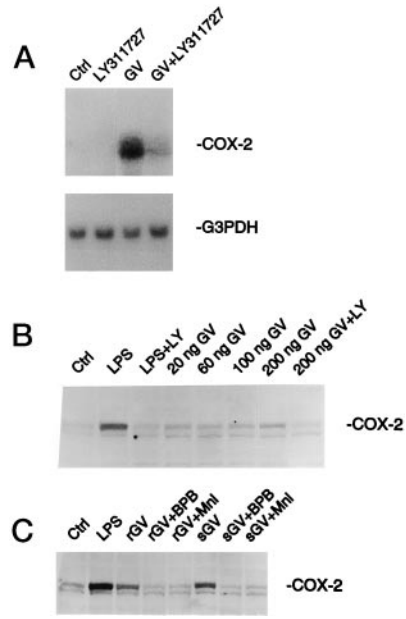

FIG. 3. **Effect of exogenous Group V $sPLA_2$ on COX-2 expression.** *A*, effect on COX-2 mRNA levels as determined by Northern blot. Total mRNA from cells incubated with or without exogenous Group V $sPLA_2$ (200 ng/ml, 18 h) and with or without LY311727 (50 μM), as indicated, was isolated and analyzed by Northern blot with probes specific for COX-2 or G3PDH. *Ctrl*, control. *B*, effect on COX-2 protein levels as determined by immunoblot. The effect of the indicated amounts of Group V $sPLA_2$ on COX-2 protein is shown (18-h incubation). The figure also shows the effect of LY31727 (50 μM) on both LPS- and Group V $sPLA_2$-induced COX-2 protein (100 ng/ml LPS, 18 h). The inhibitor was present throughout the entire 18-h incubation period. *C*, chemically inactivated $sPLA_2$ fails to induce COX-2 protein. Exogenous Group V $sPLA_2$ (200 ng) (both recombinant, rGV, and synthetic, sGV) was inactivated by treating it with either 10 μM *p*-bromophenacyl bromide (*BPB*) or 10 μM manoalide (Mnl) for 1 h at 37 °C.

macrophage cells. Within 1 h of addition to the cells, exogenous Group V $sPLA_2$ was already able to induce substantial AA release by itself (Fig. 2*A*). This finding is particularly interesting in view of the fact that exogenous Group IIA fails to elicit any AA release under similar experimental conditions (3). If the enzyme was preincubated with LY311727 before adding it to the cells, no AA release was observed, implying that the AA release response observed requires a catalytically active enzyme (Fig. 2).

Recently, we[2] have succeeded in chemically synthesizing Group V $sPLA_2$ utilizing the same procedure previously used for Group IIA $sPLA_2$ (15). Under our assay conditions, the synthetic enzyme displayed the same specific activity as the recombinant enzyme obtained from *P. pastoris*. When this synthetic Group V $sPLA_2$ was added to the $P388D_1$ macrophages, essentially the same results as found with the recombinant enzyme were observed, *i.e.* the synthetic enzyme itself induced an AA release response that was inhibited by LY311727 (Fig. 2). These results show that the synthetic enzyme is indistinguishable from the recombinant one in terms of biological activity, just as synthetic Group IIA $sPLA_2$ was found to be biologically indistinguishable from its recombinant counterpart (15). Therefore we have used both the recombinant and synthetic enzymes for the experiments reported in this study.

When measurements were conducted over longer incubation times (up to 20 h), exogenous Group V was found to increase the AA release well above unstimulated controls at every time point tested (not shown). Interestingly, when added along with LPS, exogenous Group V $sPLA_2$ increased the AA release in an additive manner (not shown), which provides additional support to our previous proposal that Group V $sPLA_2$ levels determine long-term AA release in the LPS-treated macrophages (4). Prolonged incubation with exogenous Group V $sPLA_2$ did not affect cell viability, as judged by the trypan blue assay.

Likewise, exogenous Group V $sPLA_2$ was found to induce the expression of COX-2 at long incubation times at both the mRNA (Fig. 3*A*) and the protein (Fig. 3*B*) level. If the enzyme was preincubated with LY311727 before adding it to the cells, no effects were observed (Fig. 3, *A* and *B*). These effects were also not observed if the cells were incubated with inactive $sPLA_2$, which was prepared by treating the enzyme with the irreversible inhibitors *p*-bromophenacyl bromide (19) and manoalide (19) (Fig. 3*C*). *In vitro* activity assays had demonstrated that under the conditions employed, both *p*-bromophenacyl bromide and manoalide totally inactivated Group V $sPLA_2$ activity (not shown). The inactivated enzyme failed to induce AA release from the $P388D_1$ cells (not shown). Thus, these data indicate that a functionally active $sPLA_2$ is required not only to elicit AA release from the cells but also to induce the expression of the *COX-2* gene.

$cPLA_2$ Inhibition Blocks COX-2 Expression—In a previous report we demonstrated that induction of Group V $sPLA_2$ was dependent upon a functionally active Group IV $sPLA_2$ (4). Because we have shown above that a functionally active Group V $sPLA_2$ is needed for expression of COX-2, it would be logical to anticipate that inhibition of the $cPLA_2$ also leads to a blockade in the induction of the *COX-2* gene. Utilizing the conditions described previously (4), we confirmed that inhibition of $cPLA_2$ by methyl arachidonyl fluorophosphonate completely blocked COX-2 expression in the LPS-treated cells (Fig. 4). This inhibition could not be reversed by supplementing the incubation medium with exogenous AA (up to 100 μM), suggesting that either free AA is not responsible for COX-2 induction or COX-2 induction may depend on a specific eicosanoid whose production is not adequately mimicked by exposing the cells to exogenous AA.

DISCUSSION

We have recently identified in $P388D_1$ macrophages a pathway for delayed prostaglandin synthesis in response to LPS that involves the participation of three major effectors, namely Group IV $cPLA_2$, Group V $sPLA_2$, and COX-2 (4). Because of the existence of cross-talk between $cPLA_2$ and $sPLA_2$ (2, 3), the exact contribution of each of these enzymes to the bulk of AA release that is metabolized to prostaglandins during the delayed phase has not been easy to quantify.

However, several lines of evidence based on inhibition of the response by selective chemical inhibitors or antisense oligonucleotide technology suggest that the $cPLA_2$ behaves primarily as an initiator of the response, whereas the $sPLA_2$ plays an augmentative role by providing the bulk of the free fatty acid

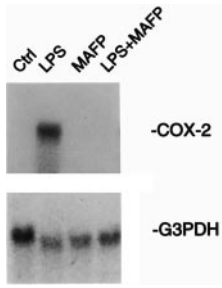

FIG. 4. **The effect of MAFP on LPS-induced COX-2 expression as determined by Northern blot.** Total mRNA from cells incubated with or without LPS (100 ng/ml, 18 h) and with or without MAFP (25 μM), as indicated, was isolated and analyzed by Northern blot with probes specific for COX-2 or G3PDH. The inhibitor was present throughout the entire 18-h incubation period. *Ctrl*, control.

used for prostaglandin production by COX-2. The strong correlation between induction of the Group V $sPLA_2$ gene and the time courses of both AA release and PG production provides further support for the central role of this enzyme in this process. An essential requirement for $sPLA_2$ in COX-2-dependent PG production has now been found in a variety of other cellular systems as well (6, 7, 20–22).

In this study we have provided further evidence to substantiate the notion that the $sPLA_2$ is tightly linked to COX-2 in the chain of events leading to delayed PG production. Quite unexpectedly, we discovered that $sPLA_2$ activation controls expression of the *COX-2* gene. Hence, COX-2-derived PG production is critically dependent on a functionally active Group V $sPLA_2$. Three different experimental approaches support this conclusion: (i) the specific $sPLA_2$ inhibitor LY311727 greatly diminishes the induction of COX-2 protein and mRNA by LPS; (ii) antisense inhibition of Group V $sPLA_2$ expression blocks COX-2 expression; (iii) exogenous Group V $sPLA_2$ is able by itself to both activate the cells to release AA and to induce COX-2 expression.

We have previously reported that Group V $sPLA_2$ expression is dependent on activation of the $cPLA_2$ (4). As expected, here we have shown that COX-2 expression is also inhibited if the $cPLA_2$ is inhibited. As a whole, these data demonstrate that the sequence of events leading to delayed PGE_2 involves activation of the $cPLA_2$ as the foremost event, followed by $cPLA_2$-dependent induction and of the Group V $sPLA_2$, which in turn controls the expression and activity of COX-2. Within this signaling cascade, two important points should be emphasized further. (*a*) Even though the $cPLA_2$ appears not to be the main provider of free AA for PG production, its upstream position in the cascade clearly shows that it is the key enzyme in AA signaling in macrophages. It follows from this observation that any circumstance that leads to $cPLA_2$ inhibition will prevent the cell from being able to generate PGs via COX-2 in response to stimuli. (*b*) The fact that COX-2 expression is dependent on both $cPLA_2$ activation and Group V $sPLA_2$ expression offers alternative avenues for the selective inhibition of COX-2 activity and conclusively supports our long-standing hypothesis (19, 23) that stimulated PG production can be efficiently controlled by controlling the upstream phospholipolytic step.

A striking feature of our study is that the effects of exogenous Group V $sPLA_2$ on $P388D_1$ cells reported here (AA release from cells and induction of COX-2 expression) appear to require the catalytic activity of the enzyme. In agreement with our data, Tada *et al.* (6) have reported very recently that exogenous Group IIA $sPLA_2$ induces AA release from rat mast cells and fibroblasts by a mechanism that is dependent on enzyme activity (6). Our results using the irreversible inhibitors *p*-bromophenacyl bromide and manoalide suggest that enzyme activity is needed as well for the Group V $sPLA_2$ to induce expression of COX-2. We cannot rule out at this time the possibility that at least part of the effect of $sPLA_2$ on COX-2 expression may be due to the $sPLA_2$ behaving as a ligand-like molecule if the inhibitors were preventing interaction of the enzyme with a putative binding site on the surface of the cell.

Whether there are true receptors (*i.e.* of proteinaceous nature) on cells that account for some of the effects of exogenous $sPLA_2$s reported in the literature remains to be clarified. To date, only one true protein receptor, the M-type receptor, which shows significant homology with the mannose scavenger receptor of phagocytes, has been cloned (24–26). This receptor binds certain $sPLA_2$ forms with very high affinity, which suggests that these enzymes may be natural ligands for the receptor (27). Some cellular responses to exogenous $sPLA_2$s, particularly Group IB, have been ascribed to interaction with the M-type receptor (28, 29). However, other putative ligand-like actions of Group IIA $sPLA_2$ have been shown not to involve the M-type receptor (6, 30).

In summary, our data demonstrate that Group V $sPLA_2$ regulates COX-2 expression in activated $P388D_1$ macrophages by a mechanism that is best explained by the hydrolytic action of the enzyme on the outer surface of the cells. These findings add to the growing concept that Group V $sPLA_2$ plays a fundamental role in AA release and PG synthesis in activated macrophage cells.

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