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Antisense Inhibition of Group VI Ca^{2+}-independent Phospholipase A_2 Blocks Phospholipid Fatty Acid Remodeling in Murine $P388D_1$ Macrophages*

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A major issue in lipid signaling relates to the role of particular phospholipase A_2 isoforms in mediating receptor-triggered responses. This has been difficult to study because of the lack of isoform-specific inhibitors. Based on the use of the Group VI Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) inhibitor bromoenol lactone (BEL), we previously suggested a role for the $iPLA_2$ in mediating phospholipid fatty acid turnover (Balsinde, J., Bianco, I. D., Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92: 8527–8531). We have now further evaluated the role of the $iPLA_2$ in phospholipid remodeling by using antisense RNA technology. We show herein that inhibition of $iPLA_2$ expression by a specific antisense oligonucleotide decreases both the steady-state levels of lysophosphatidylcholine and the capacity of the cell to incorporate arachidonic acid into membrane phospholipids. These effects correlate with a decrease in both $iPLA_2$ activity and protein in the antisense-treated cells. Collectively these data provide further evidence that the $iPLA_2$ plays a major role in regulating phospholipid fatty acyl turnover in $P388D_1$ macrophages. In stark contrast, experiments with activated cells confirmed that the $iPLA_2$ does not play a significant role in receptor-coupled arachidonate mobilization in these cells, as manifested by the lack of an effect of the $iPLA_2$ antisense oligonucleotide on PAF-stimulated arachidonate release.

The phospholipase A_2 (PLA_2)[1] superfamily of enzymes includes a heterogeneous collection of proteins with diverse roles in cell function (1). The PLA_2s catalyze the hydrolysis of the *sn-2* fatty acyl moiety of phospholipids, generating a free fatty acid and a 2-lysophospholipid, both of which may serve significant biological roles. The latter reaction is particularly relevant when the free fatty acid generated is arachidonic acid (AA), as this is the common precursor of the biologically active eicosanoids, *i.e.* the prostaglandins, leukotrienes, thromboxane, and lipoxins (2).

Based on sequence data, nine different PLA_2 Groups have been identified to date (1). However, based on biochemical properties and structural features, the PLA_2 superfamily can be subdivided into three main types, *i.e.* the Ca^{2+}-dependent secretory enzymes ($sPLA_2$), the Ca^{2+}-dependent cytosolic enzymes ($cPLA_2$) and the Ca^{2+}-independent cytosolic enzymes ($iPLA_2$).

It is difficult to demonstrate specificity of function for a single PLA_2 isoform *in vivo* because most of the PLA_2 inhibitors currently available are not isoform-specific. However, bromoenol lactone (BEL) had been regarded as a specific $iPLA_2$ inhibitor since it manifests greater than 1000-fold selectivity for the $iPLA_2$ *versus* the $sPLA_2$ and $cPLA_2$ (3, 4). We recently found that BEL inhibits AA esterification into $P388D_1$ cell phospholipids in a dose-dependent and saturatable manner, with the decrease being directly related to inhibition of both cellular $iPLA_2$ activity and steady-state lysophospholipid levels (5). These data, along with the finding that the process takes place in a Ca^{2+}-independent manner, led us to implicate the $iPLA_2$ as the enzyme providing the lysophospholipid acceptors employed in the reaction (5, 6). Recently however, BEL has been found to inhibit another cellular phospholipase, the Mg^{2+}-dependent phosphatidate phosphohydrolase, with similar potency to that shown for the $iPLA_2$ (7).

The nucleotide sequence of the macrophage $iPLA_2$ has recently been elucidated (8). This has now allowed us to achieve the specific inhibition of the $iPLA_2$ by using antisense RNA technology. In this manner, the inherent lack of specificity associated with the use of chemical inhibitors such as BEL is circumvented. We have previously taken advantage of this technique to unravel the very important role that $sPLA_2$ plays in AA metabolism in $P388D_1$ cells (9–11). We report herein our results on the antisense inhibition of the macrophage $iPLA_2$, which provide further evidence for the involvement of this enzyme in regulating fatty acid remodeling reactions among membrane phospholipids.

EXPERIMENTAL PROCEDURES

Materials—Mouse $P388D_1$ macrophage-like cells were obtained from the American Type Culture Collection (Rockville, MD). Iscove's modified Dulbecco's medium (endotoxin <0.05 ng/ml) was from Whittaker Bioproducts (Walkersville, MD). Fetal bovine serum was from Hyclone 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) and [methyl-^{3}H]choline chloride (specific activity 79 Ci/mmol) were obtained from New England Nuclear (Boston, MA). 1-Palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (specific activity 59 mCi/mmol) and [2-^{14}C]ethanolamine (specific activity 57 mCi/mmol) were from Amersham Corp. BEL was from Biomol (Plymouth Meeting, PA). Bacterial lipopolysaccharide (LPS) and platelet-activating factor (PAF) were from Sigma. Group VI $iPLA_2$ antiserum was generously provided by Dr. S. Jones (Genetics Institute, Cambridge, MA) (8, 12)

Oligonucleotide Treatment and Cell Culture Conditions—A 20-base-

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[1] The abbreviations used are: PLA_2, phospholipase A_2: $cPLA_2$, Group IV Ca^{2+}-dependent cytosolic phospholipase A_2; $iPLA_2$, Group VI Ca^{2+}-independent cytosolic phospholipase A_2; $sPLA_2$, Ca^{2+}-dependent secretory phospholipase A_2; AA, arachidonic acid; BEL, bromoenol lactone; LPS, bacterial lipopolysaccharide, PAF, platelet-activating factor; lyso-PC, lysophosphatidylcholine; lyso-PE, lysophosphatidylethanolamine.

long antisense corresponding to nucleotides 59–78 in the murine Group VI iPLA$_2$ sequence (8) was utilized (ASGVI-18; 5′-CUC CUU CAC CCG GAA UGG GU). As a control, the sense complement of ASGVI-18 was used (SGV-18; 5′-ACC CAU UCC GGG UGA AGG AG). Both ASGVI-18 and SGVI-18 contained phosphorothioate linkages to limit degradation. The transfection procedure was adapted from that described by Locati *et al.* (13) for inhibition of the cPLA$_2$. Briefly, 2.5×10^5 cells were cultured in the presence of different oligonucleotide concentrations in Iscove's modified Dulbecco's medium for 4 h at 37 °C in a humidified atmosphere at 90% air and 10% CO_2. A final concentration of 10% fetal bovine serum was then added, and the cells were kept in culture for an additional 48-h time period. The culture medium was supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids. 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.

Measurement of [^{3}H]AA Incorporation into Cellular Phospholipids—After the treatment described above, the cells were placed in phosphate-buffered saline containing 1 mM EGTA for 60 min, washed, and then exposed to exogenous [^{3}H]AA (5 nM, 0.5 μCi/ml). After 10 min, supernatants were removed, and the cell monolayers were gently washed with buffer containing 5 mg/ml albumin to remove the labeled AA that had not been incorporated into cellular lipids. The cell monolayers were scraped twice with 0.5% Triton X-100, and total lipids were extracted according to Bligh and Dyer (14). Phospholipids were separated from the rest of cellular lipids by thin-layer chromatography with *n*-hexane/ethyl ether/acetic acid (70:30:1). In this system, phospholipids remain at the origin of the plate. Radioactivity content in phospholipids was quantitated by liquid scintillation counting. When BEL was used (25 μM), it was added to the cells 30 min before addition of [^{3}H]AA.

Measurement of Lysophospholipid Levels—The cells were labeled with 0.5 μCi/ml [^{3}H]choline or 0.5 μCi/ml [^{14}C]ethanolamine for 2 days. When oligonucleotides were used, they were added to the cells at the same time as the radioactive compounds. Cellular uptake of the radioactive compounds was not affected by the oligonucleotides. Afterward, the cell monolayers were scraped in 0.5 ml of 0.5% Triton X-100. For separation of lyso-PC and lyso-PE, the lipids were extracted with ice-cold *n*-butyl alcohol and separated by thin-layer chromatography with chloroform/methanol/acetic acid/water (50:40:6:0.6) as a solvent system. Spots corresponding to lyso-PC or lyso-PE were scraped into scintillation vials, and the amount of radioactivity was estimated by liquid scintillation counting.

Immunoblot Analysis of iPLA$_2$—The cells were washed twice with serum-free medium and homogenized by 25 strokes in a Dounce homogenizer in a buffer consisting of 20 mM Tris-HCl, 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 20 μM aprotinin, 0.1% 2-mercaptoethanol, pH 7.5. The homogenates were centrifuged at $500 \times g$ for 5 min at 4 °C to separate nuclei. Samples (50 μg) were separated by SDS-PAGE (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 1:200 dilution for 30 min and then treated with horseradish peroxidase-conjugated protein A (Amersham Life Science, Inc.). Bands were detected by enhanced chemiluminescence (ECL, Amersham Life Science, Inc.).

iPLA$_2$ Assay—Homogenates from P388D$_1$ cells were prepared by sonication as described previously (5). iPLA$_2$ activity in the homogenates was assayed in a buffer consisting of 100 mM Hepes, 400 μM Triton X-100, 100 μM phospholipid substrate (1-palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine; 300,000 cpm), 5 mM EDTA, and the indicated amounts of ATP in a final volume of 0.2 ml (pH 7.5) (15, 16). The mixture was incubated at 40 °C for 1 h with shaking. The reaction was stopped by adding 0.75 ml of choloroform/methanol (1:2). The products were extracted according to Bligh and Dyer (14) and separated by thin-layer chromatography with *n*-hexane/ethyl ether/acetic acid (70:30:1) as a mobile phase.

[^{3}H]AA Release Measurement—The standard procedure for activating the cells with LPS and PAF has been previously described (4). Briefly, the cells (10^6 cells/ml), labeled for 20 h with 0.5 μCi/ml of [^{3}H]AA, were placed in serum-free medium for 1 h before the addition of LPS (200 ng/ml) for 1 h. Subsequently, 10 μl of a 10 mg/ml albumin solution was added per well (final concentration of albumin in each well was 0.1 mg/ml), and after 5 min, PAF (100 nM) was added. After a 15-min incubation period, supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

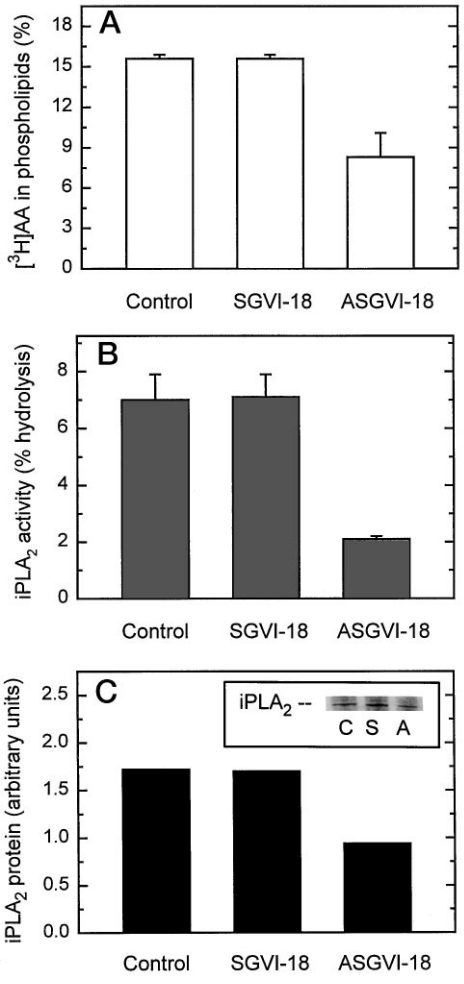

FIG. 1. **iPLA$_2$ antisense oligonucleotides inhibit iPLA$_2$ protein expression and activity and AA incorporation into phospholipids in P388D$_1$ macrophages.** The cells were either untreated (*Control*) or treated with sense (*SGVI-18*) or antisense (*ASGVI-18*) oligonucleotides for 40 h. *A*, effect on AA incorporation into phospholipids; *B*, effect on iPLA$_2$ activity; and *C*, effect on iPLA$_2$ protein. The *inset* in *panel C* shows the Western blot from which the densitometry data were obtained. *Lane 1*, control (*C*); *lane 2*, SGVI-18 (*S*); *lane 3,* ASGVI-18 (*A*).

RESULTS

Effect of an iPLA$_2$ Antisense Oligonucleotide on AA Incorporation into Phospholipids—The iPLA$_2$ of P388D$_1$ cells, now designated as a Group VI PLA$_2$ (1), is an 80–85 kDa protein that shows no specificity for the fatty acid present at the *sn-2* position of the phospholipid (16). This enzyme appears to be implicated in regulating basal phospholipid remodeling in P388D$_1$ cells (4, 5). Fig. 1*A* shows that treatment of the P388D$_1$ cells with an iPLA$_2$ antisense oligonucleotide (referred to as ASGVI-18) led to a marked decrease in their capacity to incorporate exogenous [^{3}H]AA into membrane phospholipids. This inhibition was not due to loss of cell viability, as judged by trypan blue exclusion and by quantitation of adherent cell protein. Cell viability was further assessed by monitoring [^{3}H]thymidine incorporation, which was the same in control or oligonucleotide-treated cells (not shown). AA incorporation experiments carried out in the presence of 1 mM $CaCl_2$ in the incubation medium instead of 1 mM EGTA gave the same results, in agreement with previous data (5). More importantly, ASGVI-18 reduced the iPLA$_2$ activity of cell homogenates by 75–80% (Fig. 1*B*).

A polyclonal antibody against the Group VI iPLA$_2$ has recently become available (12). This antibody was generated to a glutathione *S*-transferase fusion of the C-terminal half of the

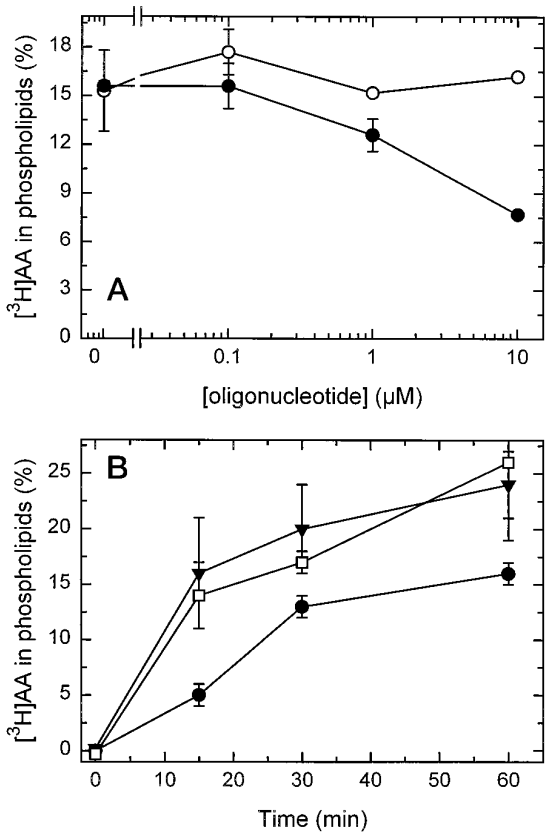

FIG. 2. **AA incorporation into phospholipids is inhibited by ASGVI-18 in a concentration- and time-dependent manner.** *A*, the extent of AA incorporation into phospholipids was determined in cells that had been treated with the indicated amounts of ASGVI-18 (●) or SGVI-18 (○) for 40 h. *B*, AA incorporation was measured at the indicated times in cells treated with ASGVI-18 (●), SGVI-18 (□), or neither (▼).

hamster Group VI iPLA$_2$ (12). Using this antibody, we observed a 55–60% decrease of the immunoreactive band, detected by Western blot, in homogenates from ASGVI-18-treated cells compared with control or sense-treated cells (Fig. 1*C*).

The dose dependence and time course of the effect of the iPLA$_2$ antisense oligonucleotide was investigated, and the results are shown in Fig. 2. Concentrations of ASGVI-18 below 1 μM exerted little effect; whereas, maximal effects were observed at an oligonucleotide concentration of 10 μM (Fig. 2*A*). Oligonucleotide concentrations higher than 10 μM induced excessive detachment of the cells from the plastic wells and, therefore, could not be used. Consistent with an antisense-type inhibition, ASGVI-18 decreases cellular AA esterification at all times measured (Fig. 2*B*).

The above experiments determined incorporation of AA at short times in cells that were already deficient in iPLA$_2$. Thus it was important to determine whether iPLA$_2$ depletion changes the endogenous AA pools. To that end, experiments were conducted wherein the radioactive AA was added at the time the cells were exposed to the oligonucleotides. As AA incorporation into phospholipids in P388D$_1$ takes place very rapidly (10), it will be completed long before the cellular iPLA$_2$ levels begin to drop as a result of the antisense treatment. Thus, the relative distribution of AA among phospholipids in iPLA$_2$-depleted cells can be determined and compared with that in control untreated cells. As shown in Table I, the relative distribution of AA among phospholipids did not change after ASGVI-18 treatment, indicating that ASGVI-18 treatment does not change the endogenous AA pools.

Fig. 3 shows that ASGVI-18 treatment of P388D$_1$ cells resulted in a 50–55% decrease of the steady-state levels of lyso-PC, which corresponds well with the decrease in AA incorporation into phospholipids shown in Fig. 1*A*. Lyso-PC levels in SGVI-18-transfected cells were the same as those found in control untreated cells (Fig. 3). Within experimental error, no effect of ASGVI-18 on lyso-PE levels could be detected (data not shown).

TABLE I

Effect of ASGVI-18 on the distribution of AA among phospholipids

P388D$_1$ cells were labeled with [^{3}H]AA (0.5 μCi/ml) at the time they were treated with SGVI-18, ASGVI-18, or neither (control) as indicated. After 2 days, the amount of AA in the different lipid classes was determined after separation by thin-layer chromatography. Data are given as a percentage of the total radioactivity found in all phospholipid classes and are expressed as means ± S.E. of three independent determinations.

Phospholipid	Control	SGVI-18	ASGVI-18
		%	
PC	33 ± 10	28 ± 3	28 ± 5
PE	55 ± 5	60 ± 3	57 ± 5
PI	15 ± 3	14 ± 1	20 ± 6

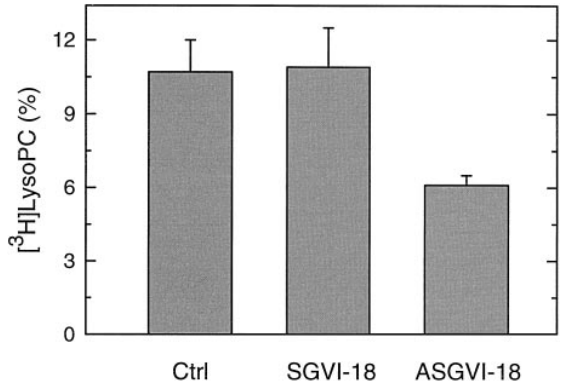

FIG. 3. **Effect of iPLA$_2$ antisense oligonucleotide on lyso-PC levels.** The cells were labeled with [^{3}H]choline at the time they were treated with oligonucleotides, as described under "Experimental Procedures." The cellular amount of lyso-PC in control (*Ctrl*), sense-treated (*SGVI-18*), or antisense-treated (*ASGVI-18*) cells was determined by thin-layer chromatography. Radioactivity in lyso-PC is expressed as a percentage of total [^{3}H]choline incorporated into glycerophospholipids.

Effect of BEL on iPLA$_2$ Antisense-treated Cells—Collectively, the above data suggest that selective inhibition of iPLA$_2$ expression by antisense RNA technology reduces AA incorporation due to a decrease in the amount of cellular lyso-PC acceptors available for the esterification reaction. These data are consistent with our previous results using BEL to inhibit the iPLA$_2$ (5). However, it is now known that BEL also inhibits the Mg^{2+}-dependent phosphatidate phosphohydrolase, which is another key enzyme in phospholipid metabolism (7, 17). To verify the specificity of BEL in our previous studies (5), it seemed important to study the effects of BEL on AA esterification in antisense-treated cells. The results from these experiments are summarized in Fig. 4. In agreement with our previous data (8), AA incorporation into phospholipids was blocked up to 60% by BEL in control and SGVI-18-treated cells. In ASGVI-18-treated cells, which already showed a 60% decrease in AA incorporation, BEL was ineffective in further increasing this inhibition (Fig. 4). We have previously found that BEL has no effect on the relative distribution of AA among the different phospholipids of P388D$_1$ cells (5).

Effect of iPLA$_2$ Antisense on Receptor-coupled AA Release—The above data suggest that the Group VI iPLA$_2$ may function as a *housekeeping* enzyme involved in the regulation of basal deacylation/reacylation reactions among phospholipids. To further assess its role in cellular function, we sought to assess its

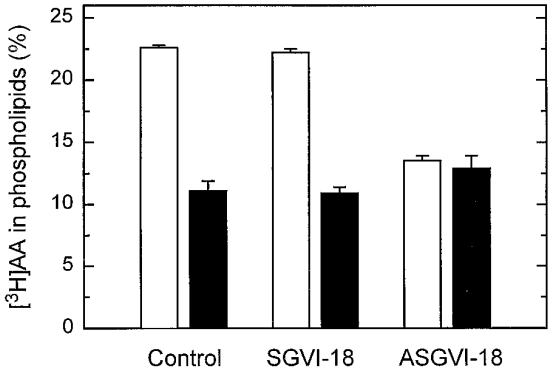

FIG. 4. **Effect of BEL on AA incorporation into phospholipids in cells treated with iPLA$_2$ antisense oligonucleotides.** Control, sense-treated (*SGVI-18*), or antisense-treated (*ASGVI-18*) cells were incubated without (*open bars*) or with (*black bars*) 25 μM BEL for 30 min. Afterward, the extent of AA incorporation into phospholipids was determined as described under "Experimental Procedures."

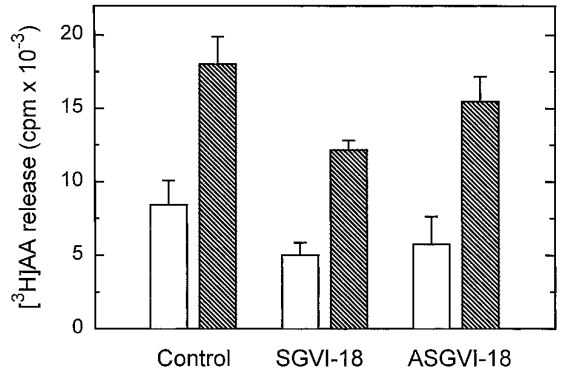

FIG. 5. **Effect of ASGVI-18 on PAF-stimulated [^{3}H]AA mobilization.** [^{3}H]AA-labeled, LPS-treated cells were treated with ASGVI-18, SGVI-18 or neither, as indicated. The cells were incubated without (*open bars*) or with (*hatched bars*) 100 nM PAF for 15 min. Extracellular [^{3}H]AA release was quantitated as described under "Experimental Procedures."

role during receptor activation conditions. The effect of ASGVI-18 on LPS/PAF-stimulated AA release from P388D$_1$ cells is shown in Fig. 5. Stimulation of murine P388D$_1$ macrophages with nanomolar amounts of the inflammatory mediator PAF results in negligible cellular responses unless the cells are first treated with LPS. LPS acts just as a primer, *i.e.* it does not stimulate the P388D$_1$ cells by itself but enables the cells to optimally respond to PAF (23). The sense and antisense oligonucleotides both slightly decreased the AA release response, which was detected both in resting and PAF-activated cells. However, the ratio of stimulated *versus* unstimulated release remained the same under all conditions (Fig. 5). This result indicates that the iPLA$_2$ does not play a significant role in mediating agonist-induced AA mobilization in these cells.

DISCUSSION

It is now well established that the availability of free AA limits the synthesis of the biologically active eicosanoids (2). As AA is mainly found esterified at the *sn-2* position of cellular phospholipids, PLA$_2$ has emerged as a key enzyme responsible for controlling the levels of free fatty acid (18). However, the amount of free AA available for eicosanoid synthesis represents a balance between what is being liberated by the activated PLA$_2$s, minus what is reincorporated back into phospholipids by the highly active acyltransferases (19). Thus, free fatty AA levels are also efficiently controlled by the AA reacylation pathway (19).

Even under resting conditions, the capacity of certain cells such as macrophages to incorporate AA into phospholipids via reacylation reactions is exceedingly high (6, 20, 21). Thus these cells should possess a basal PLA$_2$ activity high enough to account for their high AA reacylation capacity. Interestingly, AA incorporation into phospholipids in macrophages is Ca^{2+}-independent, *i.e.* it takes place normally at free Ca^{2+} concentrations lower than 10 nM (5). As under these conditions neither the cPLA$_2$ nor the sPLA$_2$ must be active, these results suggest that the enzyme providing lysophospholipid acceptors for the AA reacylation pathway is an iPLA$_2$. Further evidence for such a role was obtained by the use of BEL (5). Using this inhibitor, we found a direct correlation between endogenous iPLA$_2$ activity, steady-state lysophospholipid levels, and AA incorporation capacity of the cells (5). However, BEL, being selective for the iPLA$_2$ over the other PLA$_2$s (3, 4), is not devoid of other effects. For example, we have recently found that BEL also potently blocks the Mg^{2+}-dependent phosphatidate phosphohydrolase, a key enzyme in cellular phospholipid metabolism (7).

The inherent problems associated with the use of chemical inhibitors can be potentially circumvented by inhibiting expression of the iPLA$_2$ with antisense RNA oligonucleotides. Using this strategy, we have succesfully achieved the specific inhibition of the macrophage Group VI PLA$_2$ and confirmed our previous findings with BEL. Thus, the iPLA$_2$ antisense oligonucleotide ASGVI-18 reduces the cellular iPLA$_2$ activity by 75–80% (Fig. 1*B*) and iPLA$_2$ protein by at least 50–60% (Fig. 1*C*), which results in a decrease of the capacities of the cells to incorporate AA into phospholipids (Figs. 1*A* and 2) as well as the steady-state lyso-PC levels (Fig. 3). These latter effects correspond precisely, and are specific, as parallel experiments using the sense control SGVI-18 did not reproduce any of the effects induced by ASGVI-18. Specificity of ASGVI-18 is also stressed by the fact that sPLA$_2$ antisense oligonucleotides do not affect macrophage AA esterification (10). These results provide definitive evidence for the key role that the iPLA$_2$ plays in regulating basal phospholipid remodeling reactions in macrophages.

Strikingly, ASGVI-18 treatment was found to significantly decrease the steady state of lyso-PC levels but not of lyso-PE. It is likely that our inablity to detect any effect of ASGVI-18 on lyso-PE levels merely reflects some sort of experimental limitations. However, it could indicate as well that the Group VI iPLA$_2$ preferentially attacks PC over PE *in vivo* even though the enzyme has been found to lack headgroup specificity *in vitro* (12, 16). It is possible that PC pools within the cells are more accesible to iPLA$_2$ attack than the PE pools. Interestingly, in regards to AA metabolism, it is lyso-PC, not lyso-PE, that is the major acceptor for esterification of free AA (19). Over time, the AA accumulates into PE as a consequence of direct transfer from PC by CoA-independent transacylase, not by direct acylation of lyso-PE with AA (19). Therefore, selective inhibition of lyso-PC but not of lyso-PE would result in decreased labeling of phospholipids with AA, and the ratio AA-PC to AA-PE remaining unchanged. This is exactly what was found in the experiments determining the effect of ASGVI-18 treatment on AA distribution among phospholipids (Table I). Thus, the current data give additional support to the notion that the different phospholipid classes and subclasses serve different roles for AA incorporation and redistribution within different pools (19).

Under our experimental conditions, we were not able to achieve complete inhibition of the iPLA$_2$ but did achieve a 60% or 80% disappearance at best, as judged by protein content or activity, respectively. This reduces AA incorporation into phospholipids by about 60% in antisense-treated cells. It could thus be argued that achieving 100% inhibition of Group VI iPLA$_2$

expression would result in almost complete ablation of the capacity of the cell to incorporate AA into phospholipids. However, the fact that the decrease in iPLA$_2$ protein detected by Western blot does not correspond with the decrease in cellular iPLA$_2$ activity suggests the possibility that, in addition to the iPLA$_2$, the antibody used may be recognizing another 85 kDa-protein in P388D$_1$ macrophages. If this were the case, then the remainder of the 85-kDa protein band that is not blocked by ASGVI-18 could correspond to an antigenically related protein. Moreover, it is also possible that the 20% of iPLA$_2$ activity that is not eliminated by ASGVI-18, corresponds to another iPLA$_2$ distinct from the Group VI enzyme. The possibility that the P388D$_1$ cells contain more than one iPLA$_2$ form has previously been considered (16). Finally, BEL, either alone (5) or together with the iPLA$_2$ antisense (Fig. 4) fails to completely inhibit the response. Saturation of inhibition by BEL is reached at about 60–70% (5). Altogether, these facts seem to suggest that the cell possesses other mechanisms for regulating AA esterification besides the one provided by the Group VI iPLA$_2$. It should be remarked here that the current experiments were done in the absence of Ca^{2+}. Therefore, if a second PLA$_2$ distinct from the Group VI enzyme was involved in regulating AA esterification, it would have to be another Ca^{2+}-independent isoform.

As discussed elsewhere (5, 6), the contribution of the *de novo* biosynthetic pathway to AA incorporation into phospholipids is minimal in P388D$_1$ macrophages. This contrasts with results obtained with peritoneal macrophages (21) and neutrophils (22), wherein the *de novo* pathway has been demonstrated to provide a minor but significant route for the generation of highly polyunsaturated phospholipid species such as 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine. However the phospholipids of peritoneal macrophages and neutrophils are already very enriched in AA (21, 22). Such a circumstance likely explains why a significant portion of the free AA is shunted to the low-affinity biosynthetic route in these cells. In contrast, P388D$_1$ cells are very scarce in endogenous AA (23). Hence, most of the AA incorporation in these cells occurs through the high-affinity remodeling pathway (5, 6). Nonetheless, recent data have highlighted the fact that the *sn-1* position of phospholipids is also extensively remodeled in macrophages (21), which means that a phospholipase A$_1$ could also eventually participate in regulating AA esterification via fatty acid remodeling. Such a possibility, as well as the possible involvement of the recently described CoA-dependent transacylase reaction (24, 25), is currently under investigation in our laboratory.

In summary, in the present study we have utilized antisense RNA technology to obtain independent conclusive confirmation that the macrophage Group VI iPLA$_2$ does play an important role in modulating phospholipid fatty acid turnover by providing the 2-lysophospholipid acceptors required for the reaction. In addition, our results demonstrate that the Group VI iPLA$_2$ does not appear to play a significant role in the stimulation of AA release mediated via the PAF surface receptor.

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