Cytosolic Group IVA and Calcium-Independent Group VIA Phospholipase A_2s Act on Distinct Phospholipid Pools in Zymosan-Stimulated Mouse Peritoneal Macrophages

Luis Gil-de-Gómez,*,† Alma M. Astudillo,*,† Carlos Guijas,*,† Victoria Magrioti,‡ George Kokotos,‡ María A. Balboa,*,† and Jesús Balsinde*,†

Phospholipase A_2s generate lipid mediators that constitute an important component of the integrated response of macrophages to stimuli of the innate immune response. Because these cells contain multiple phospholipase A_2 forms, the challenge is to elucidate the roles that each of these forms plays in regulating normal cellular processes and in disease pathogenesis. A major issue is to precisely determine the phospholipid substrates that these enzymes use for generating lipid mediators. There is compelling evidence that group IVA cytosolic phospholipase A_2 ($cPLA_2\alpha$) targets arachidonic acid–containing phospholipids but the role of the other cytosolic enzyme present in macrophages, the Ca^{2+}-independent group VIA phospholipase A_2 ($iPLA_2\beta$) has not been clearly defined. We applied mass spectrometry–based lipid profiling to study the substrate specificities of these two enzymes during inflammatory activation of macrophages with zymosan. Using selective inhibitors, we find that, contrary to $cPLA_2\alpha$, $iPLA_2\beta$ spares arachidonate-containing phospholipids and hydrolyzes only those that do not contain arachidonate. Analyses of the lysophospholipids generated during activation reveal that one of the major species produced, palmitoyl-glycerophosphocholine, is generated by $iPLA_2\beta$, with minimal or no involvement of $cPLA_2\alpha$. The other major species produced, stearoyl-glycerophosphocholine, is generated primarily by $cPLA_2\alpha$. Collectively, these findings suggest that $cPLA_2\alpha$ and $iPLA_2\beta$ act on different phospholipids during zymosan stimulation of macrophages and that $iPLA_2\beta$ shows a hitherto unrecognized preference for choline phospholipids containing palmitic acid at the *sn*-1 position that could be exploited for the design of selective inhibitors of this enzyme with therapeutic potential. ***The Journal of Immunology*****, 2014, 192: 752–762.**

Macrophages contain large amounts of arachidonic acid (AA) esterified in membrane glycerophospholipids (1–3), which makes them a major source of eicosanoids during innate immune reactions. The eicosanoids affect immune regulation by modulating phagocyte cell activation at different points, including differentiation and migration, phagocytic capacity, and cytokine production (4–7).

The first committed step in the synthesis of eicosanoids is the phospholipase A_2 (PLA_2)-mediated cleavage of phospholipids to release free AA (8). There are multiple PLA_2 enzymes in cells, and many of them may be capable of catalyzing the release of AA from membrane phospholipids (8–11). Based on biochemical commonalities, mammalian PLA_2 enzymes can be classified into five major families: the Ca^{2+}-dependent secreted PLA_2s, the Ca^{2+}-dependent cytosolic PLA_2s, the Ca^{2+}-independent cytosolic PLA_2s, the platelet-activating factor acetyl hydrolases, and the lysosomal PLA_2s (11). Of these, the first two have been repeatedly implicated in AA mobilization in response to a variety of immunoinflammatory stimuli (8–11). There is general agreement that Ca^{2+}-dependent cytosolic group IVA PLA_2 ($cPLA_2\alpha$) is the critical enzyme in AA release and that, depending on the agonist, a secreted PLA_2, in particular that belonging to groups IIA, V, and X, may also participate by amplifying the $cPLA_2\alpha$-regulated response (8–11).

With regard to the other cytosolic PLA_2 form present in macrophages, the Ca^{2+}-independent cytosolic group VIA PLA_2 ($iPLA_2\beta$) (11), no evidence has been provided for its involvement in eicosanoid production under innate immunity and inflammation conditions, as manifested by studies in murine mesangial cells (12), murine peritoneal macrophages (13, 14), murine $P388D_1$ and RAW264.7 macrophage-like cells (15–18), human neutrophils (19, 20), human monocytes (21), and human U937 promonocytic cells (22, 23), in which selective inhibition of $iPLA_2\beta$ did not modify the stimulus-induced AA release. However $iPLA_2\beta$ is emerging as an important enzyme during stimulus-response coupling, and a variety of roles have been ascribed to this enzyme in various cells and tissues (24–27). In this regard, $iPLA_2\beta$ may play major roles in intracellular signaling cascades, in particular those regulating phospholipid hydrolysis during apoptosis (26–31).

Recently, we have extensively investigated the mechanisms of PLA_2-mediated AA mobilization in monocytes and macrophages in response to a variety of innate immune stimuli. In those studies

*Instituto de Biología y Genética Molecular, Consejo Superior de Investigaciones Científicas, Universidad de Valladolid, 47003 Valladolid, Spain; †Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, 08036 Barcelona, Spain; and ‡Laboratory of Organic Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis, Athens 15771, Greece

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Address correspondence and reprint requests to Prof. Jesús Balsinde, University of Valladolid School of Medicine, Calle Sanz y Forés 3, 47010 Valladolid, Spain. E-mail address: jbalsinde@ibgm.uva.es

Abbreviations used in this article: AA, arachidonic acid; $cPLA_2\alpha$, Ca^{2+}-dependent cytosolic group IVA phospholipase A_2; $iPLA_2\beta$, Ca^{2+}-independent cytosolic group VIA phospholipase A_2; LPC, lysoPC; LPE, lysoPE; LPI, lysoPI; lysoPC, choline lysoglycerophospholipid; lysoPE, ethanolamine lysoglycerophospholipid; lysoPI, lysophosphatidylinositol; MS, mass spectrometry; PC, choline glycerophospholipid; PE, ethanolamine glycerophospholipid; PI, phosphatidylinositol; PLA_2, phospholipase A_2.

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(32–38), we have taken advantage of mass spectrometry (MS)-based lipidomic approaches to define, at a molecular species level, the sources of AA liberation and incorporation. In this study, we have applied a similar approach to relate changes in phospholipid turnover with the activation of particular PLA_2 forms: $cPLA_2\alpha$ and $iPLA_2\beta$. For this purpose, we have used selective inhibitors of each of these forms. The use of inhibitors to address the role of intracellular PLA_2s during cell activation has a number of advantages over other widely used methods, such as small interfering RNA or cells from knockout mice, in that inhibition develops rapidly, which reduces the impact of unspecific effects that could occur over time, and no compensatory mechanisms take place that might obscure the interpretation of results (39). Our data show that cPLA2α and iPLA2β act on different phospholipid pools, thereby regulating separate pathways, one leading to AA mobilization and the other to choline lysoglycerophospholipid (lysoPC [LPC]) molecules containing palmitic acid at the *sn*-1 position [LPC(16:0)], respectively. These studies suggest a critical role not only for cPLA2α, but also for iPLA2β in the intracellular-signaling cascades initiated by innate immune receptors leading to generation of inflammatory lipid mediators.

Materials and Methods

Reagents

Cell culture medium was from Molecular Probes-Invitrogen (Carlsbad, CA). Chloroform and methanol (HPLC grade) were from Fisher Scientific (Hampton, NH). Lipid standards were from Avanti Polar Lipids (Alabaster, AL) or Larodan Fine Chemicals (Malmö, Sweden). The $cPLA_2\alpha$ inhibitor pyrrophenone (40) was synthesized and provided by Dr. A. Llebaria (Institute for Chemical and Environmental Research, Barcelona, Spain). The $iPLA_2\beta$ inhibitor FKGK18 (1,1,1-trifluoro-6-(naphthalen-2-yl)hexan-2-1) was synthesized as previously described (41). The CoA-independent transacylase inhibitor SK&F98625 (diethyl 7-(3,4,5-triphenyl-2-oxo-2,3-dihydroimidazol-1-yl)heptane phosphonate) (42–44) was synthesized and provided by Dr. A. Pérez (Department of Organic Chemistry, University of Valladolid). All other reagents were from Sigma-Aldrich.

Cell culture

Resident peritoneal macrophages from Swiss male mice (University of Valladolid Animal House, 10–12 wk old) were obtained by peritoneal lavage using 5 ml cold PBS, as described elsewhere (45, 46). The cells were plated at 2×10^6/well (six-well plates) in 2 ml RPMI 1640 medium with 10% heat-inactivated serum, 100 U/ml penicillin, and 100 mg/ml streptomycin and allowed to adhere for 20 h in a humidified atmosphere of 5% CO_2 at 37°C. Wells were washed extensively with PBS to remove non-adherent cells. Adherent macrophages were used for experimentation. When inhibitors were used, they were added to the incubation media 30 min before stimulating the cells with zymosan. All procedures involving animals were undertaken in accordance with the Spanish National Committee on Biosafety and Animal Care, under the guidelines established by the Spanish Ministry of Agriculture, Food, and Environment and the European Union.

Preparation of zymosan

Zymosan particles were suspended in PBS, boiled for 60 min, and washed three times. The final pellet was resuspended in PBS at 20 mg/ml and stored frozen. Zymosan aliquots were diluted in serum-free medium and sonicated before addition to the cells. No endogenous PLA_2 activity was detected in the zymosan batches used in this study, as assessed by in vitro assay under a variety of conditions (47, 48).

Liquid chromatography/MS analyses of macrophage glycerophospholipids

A cell extract corresponding to 10^7 cells was used for these analyses. The following internal standards were added: 600 pmol each 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine, 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoinositol, before lipid extraction, according to the method of Bligh and Dyer (49). After evaporation of organic solvent under vacuum, the lipids were redissolved in 100 μl methanol/water (9:1, v/v) and injected into a high-performance liquid chromatograph equipped with a binary pump Hitachi LaChrom Elite L-2130 and a Hitachi Autosampler L-2200 (Merck). The column was a SUPELCOSIL LC-18 (5-μm particle size, 250 × 2.1 mm) protected with a Supelguard LC-18 (20 × 2.1 mm) guard cartridge (both from Sigma-Aldrich). Mobile phase was a gradient of solvent A (methanol/water/*n*-hexane/32% ammonium hydroxide, 87.5:10.5:1.5:0.5, by volume) and solvent B (methanol/*n*-hexane/32% ammonium hydroxide, 87.5:12:0.5, by vol.). The gradient was started at 100% solvent A; it was decreased linearly to 65% solvent A, 35% solvent B in 20 min, to 10% solvent A, 90% solvent B in 5 min, and to 0% solvent A, 100% solvent B in an additional 5 min. Flow rate was 0.5 ml/min, and 80 μl the lipid extract was injected. The liquid chromatography system was coupled online to a Bruker esquire6000 ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). The total flow rate into the column was split, and 0.2 ml/min entered into the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 pounds per square inch, dry gas was set to 8 l/min, and dry temperature was set to 365°C. Ethanolamine glycerophospholipids (PE) species and phosphatidylinositol (PI) were detected in negative ion mode with the capillary current set at +3500 V over the initial 25 min as $[M-H]^-$ ions. Choline glycerophospholipid (PC) species were detected over the elution interval from 25 to 35 min in positive ion mode, as $[M+H]^+$ ions, with the capillary current set at −3500 V.

PE and PI molecular species were identified by multiple reaction monitoring experiments on chromatographic effluent by comparison with previously published data (32–38). Cutoff parameter was set at m/z 150, and fragmentation amplitude was set at 1 arbitrary unit. Because of the lability of vinyl ether linkages in acid media, plasmanyl (1-alkyl) and plasmenyl (1-alk′1′-enyl) glycerophospholipids were distinguished by acidifying the samples before lipid extraction. For the identification of acyl chains of PC species, ionization was carried out in negative mode with postcolumn addition of acetic acid at a flow rate of 100 μl/h as $[M+CH_3CO_2]^-$ adducts, and acyl chains were identified by MS^3 experiments. Stereospecific assignation of fatty acyl chains was carried out by comparing the relative intensities of the 1-lysophospholipid and 2-lysophospholipid compounds arising in the fragmentation experiments (the signal of the latter predominates over that of the former in ion-trap MS) (32–38).

Liquid chromatography/MS analyses of lysophospholipids

A cell extract corresponding to 10^7 cells was used for these analyses. The following internal standards were added: 200 pmol each 1-tridecanoyl-*sn*-glycero-3-phosphocholine and 1-miristoyl-*sn*-glycero-3-phosphoethanolamine, before lysophospholipid extraction with *n*-butanol. After evaporation of the organic solvent under vacuum, the lipids were redissolved in 100 μl chloroform and injected into a high-performance liquid chromatograph equipped with a binary pump Hitachi LaChrom Elite L-2130 and a Hitachi Autosampler L-2200 (Merck). The column was a SUPELCOSIL LC-Si protected with a Supelguard LC-Si guard cartridge (Sigma-Aldrich). Mobile phase was a gradient of solvent A (chloroform/methanol/water/32% ammonium hydroxide, 75:24:5:0.5, by volume) and solvent B (chloroform/methanol/water/32% ammonium hydroxide, 55:39:5.5:0.5, by volume). The gradient was started at 100% solvent A, it was decreased linearly to 50% solvent A in 2 min and maintained for 8 min, and finally it was decreased to 0% solvent A in 2 min. Flow rate was 0.5 ml/min, and 80 μl the lipid extract was injected. The liquid chromatography system was coupled online to a Bruker Esquire 6000 Ion Trap Mass Spectrometer (Bruker Daltonics). The total flow rate into the column was split, and 0.2 ml/min entered into the electrospray interface of the mass spectrometer. Nebulizer gas was set to 30 pounds per square inch, dry gas was set to 8 l/min, and dry temperature was set to 365°C. Ethanolamine and inositol lysophospholipids were detected in negative ion mode with the capillary current set at +3500 V as $[M-H]^-$ ions. Choline lysophospholipids were detected in positive ion mode as $[M+H]^+$ ions, with the capillary current set at −4000 V. Quantification of inositol lysophospholipid species was carried out by comparison with an external calibration curve made with 1-palmitoyl-*sn*-glycero-3-phosphoinositol.

Liquid chromatography/MS analyses of eicosanoids

A small amount of butylated hydroxytoluene in methanol (0.01%, w/v) was added to the supernatants to prevent eicosanoid degradation. Deuterated PGE_2 and LTB_4 (400 pmol each) were added as internal standards before extraction. Eicosanoids were extracted using Bond Elut Plexa solid-phase extraction columns (Agilent), following the manufacturer's instructions. Columns were pretreated with 3 ml methanol and 3 ml water. Supernatants were acidified with 0.5% acetic acid, and 10% methanol was also added before sample loading. Samples were washed with 3 ml 10% methanol, and lipid products were eluted with 1.5 ml 100% methanol twice. Lipid products were concentrated under vacuum and redissolved in 100 μl solvent A (see below).

A total of 90 μl of the extract was injected into an Agilent 1260 Infinity high-performance liquid chromatograph equipped with an Agilent G1311C quaternary pump and an Agilent G1329B Autosampler. The column was

a SUPELCOSIL LC-18 (250 × 2.1 mm, 5 μm particle size) protected with a Supelguard LC-18 (20 × 2.1 mm) guard cartridge (Sigma-Aldrich). Eicosanoids were separated according to the procedure described by Dumlao et al. (50), with minor modifications. Briefly, the mobile phase consisted of a gradient of solvent A (water/acetonitrile/acetic acid, 70:30:0.02, by volume) and solvent B (acetonitrile/isopropanol, 50:50, by volume). The gradient was started at 100% solvent A, which was decreased linearly to 75% at 3 min, 55% at 11 min, 40% at 13 min, 25% at 18 min, and 10% at 18.5 min. The last solvent mixture was held for an additional 1.5 min; finally, the column was re-equilibrated with 100% solvent A for 10 min before the next sample injection.

The flow rate through the column was fixed at 0.6 ml/min, and this flow entered into the electrospray interface of an API2000 triple quadrupole mass spectrometer (Applied Biosystems). The parameters of the source were set as follows: ion spray voltage, −4500 V; curtain gas, 25 pounds per square inch; nebulizer gas, 40 pounds per square inch; desolvation gas, 80 pounds per square inch; temperature, 550°C. The analyzer mode was set to scheduled multiple-reaction monitoring with negative ionization, defining for each analyte the m/z of the parent ion as Q1 mass and the m/z of its daughter ion fragment (transition) as Q3 mass, and associating them to the chromatographic retention time to improve the number of analytes collected in a single chromatographic run. The retention time window was set to 120 s. The declustering potential and collision energy for each analyte were optimized by the use of analytical standards. Other parameters were fixed for all analytes: entrance potential, −10 V; focusing potential, −350 V; and collision cell exit potential, −10 V. Quantification was carried out by integrating the chromatographic peaks of each species and comparing with an external calibration curve made with analytical standards.

Results

AA-containing species in macrophages

The profile of AA-containing glycerophospholipid species of peritoneal macrophages was measured by liquid chromatography/ion-trap MS (Fig. 1). The lipids were unequivocally identified by determining the production of an m/z 303 fragment in MS^n experiments, which corresponds to AA. Structural identification of the glycerophospholipids was achieved by looking at the fragments and/or neutral losses obtained in MS^2 experiments (PE, PI) or MS^3 experiments (PC) (32–38). Fatty chains within the different phospholipid species are designated by their number of carbons:double bonds. A designation of *O-* before the first fatty chain indicates that the *sn*-1 position is ether linked, whereas a *P-* designation indicates a plasmalogen form (*sn*-1 vinyl ether linkage) (51). In agreement with previous observations by us (38) and other investigators (2, 52), we found that most of the AA was found in PE species (32.8 nmol/mg protein), followed by PC (25.9 nmol/mg protein) and PI (10.3 nmol/mg protein). With regard to molecular species, the major AA-containing ones were: PE(P-16:0/20:4), PC(16:0/20:4), PE(P-18:0/20:4), PI(18:0/20:4), PE (18:0/20:4), and PC(18:0/20:4), again in agreement with previous estimates (38). Stimulation of the cells with the phagocytosable particle zymosan promoted marked changes in the content of cellular AA-containing phospholipids (Fig. 1). All major AA-containing PC species decreased, and so did PI(18:0/20:4). However, the PE species remained unchanged (Fig. 1). These changes were fully prevented when the incubations were carried out in the presence of the well-established cPLA$_2\alpha$ inhibitor pyrrophenone (Fig. 1).

In parallel studies, we also determined the effect of inhibiting iPLA$_2\beta$ with the novel selective inhibitor FKGK18 (41). In stark contrast with the experiments using pyrrophenone, the zymosan-stimulated decreases in PC and PI molecular species were the same whether or not FKGK18 was present in the incubations (Fig. 1). Thus, the data suggest that AA-containing phospholipids are hydrolyzed by cPLA$_2\alpha$, but not by iPLA$_2\beta$, in zymosan-stimulated macrophages.

The above results were obtained after a 1-h stimulation with zymosan, because AA release in response to this agonist is maximal at that time (15, 53). However, because at later times cyclooxygenase-2 is upregulated and PGs are most robustly produced, we also examined the distribution of AA-containing phospholipid species at these later time points (8 h). The results indicated no further differences in phospholipid turnover beyond those already observed at 1 h (Fig. 1), thus confirming that the bulk of AA mobilization in response to zymosan occurs at early stimulation times (data not shown).

Role of AA-containing PE species

The finding that the levels of all AA-containing PE species do not change appreciably during zymosan stimulation, despite them constituting the major cellular reservoirs of AA, is striking and deserved further investigation. AA is known to be incorporated into PE species by the Lands cycle, as well as via a transacylation reaction that uses AA-containing PC as donor and occurs in a CoA-independent manner (54). This reaction proceeds at a very slow rate in resting cells, but it is activated in receptor-stimulated cells as a part of a phospholipid-remodeling pathway that seems to be necessary for a full AA mobilization response (54). Thus, we hypothesized that AA-containing PE formation via CoA-independent transacylation could be taking place in zymosan-stimulated macrophages, which would account for the apparent lack of AA-containing PE hydrolysis under these conditions. To study this possibility, we conducted experiments using the well-established CoA-independent transacylase inhibitor SK&F98625 (42, 43). This inhibitor was designed on the basis of analogy with compounds that inhibit acyl-CoA:cholesterol acyltransferase and was found to potently block the movement of AA to PE in a variety of cells (42–44). Preliminary determinations confirmed that, at 10 μM, SK&F98625 quantitatively blocked the transfer of AA from PC to PE in RAW264.7 macrophage-like cells and peritoneal macrophages, and it exerted only little inhibitory effect (<15%) on the Ca^{2+}-dependent PLA$_2$ activity of a macrophage homogenate, as measured in vitro under a variety of conditions (data not shown). Fig. 2 shows that, in the presence of SK&F98625, the zymosan-stimulated cells showed significant decreases in the content of all major AA-containing PE species. Correspondingly, the levels of AA-containing PC species were preserved in the SK&F98625-treated cells (Fig. 2). Collectively, these results demonstrate that AA-containing PE species are indeed hydrolyzed during zymosan stimulation of the macrophages. In turn, the data also suggest that a major portion of the AA lost from PC (Fig. 1) is not released as free fatty acid but rather is channeled to PE (Fig. 2).

The effect of SK&F98625 on the production of eicosanoids by the zymosan-stimulated cells is shown in Fig. 3. In agreement with previous observations (1), PGE$_2$ was the most abundant eicosanoid produced under these conditions, and the presence of SK&F98625 reduced its formation by ~30–40% (Fig. 3A). Similar decreases were also observed for the other cyclooxygenase metabolites measured: PGI$_2$ (measured as 6-keto PGF$_{1\alpha}$), PGF$_{2\alpha}$, TXA$_2$ (measured as TXB$_2$), and 11-hydroxyeicosatetraenoic acid (Fig. 3A). This general decrease in prostanoid production when AA transfer from PC to PE is blocked by SK&F98625 was not unexpected; this compound is known to reduce stimulus-induced AA mobilization in a variety of cells (54), and our preliminary studies confirmed that this is also the case in zymosan-stimulated macrophages, in which SK&F98625 reduced AA release by ~25–30%, as measured by gas chromatography/MS. Importantly, however, production of lipoxygenase metabolites was strongly increased in cells treated with SK&F98625 (Fig. 3B), suggesting that the transfer of AA from PC to PE also serves to modulate the relative amount of lipoxygenase products formed.

Profiling of phospholipid species not containing AA in macrophages

Liquid chromatography/MS analyses of major phospholipid species not containing AA were conducted next (Fig. 4). Because in

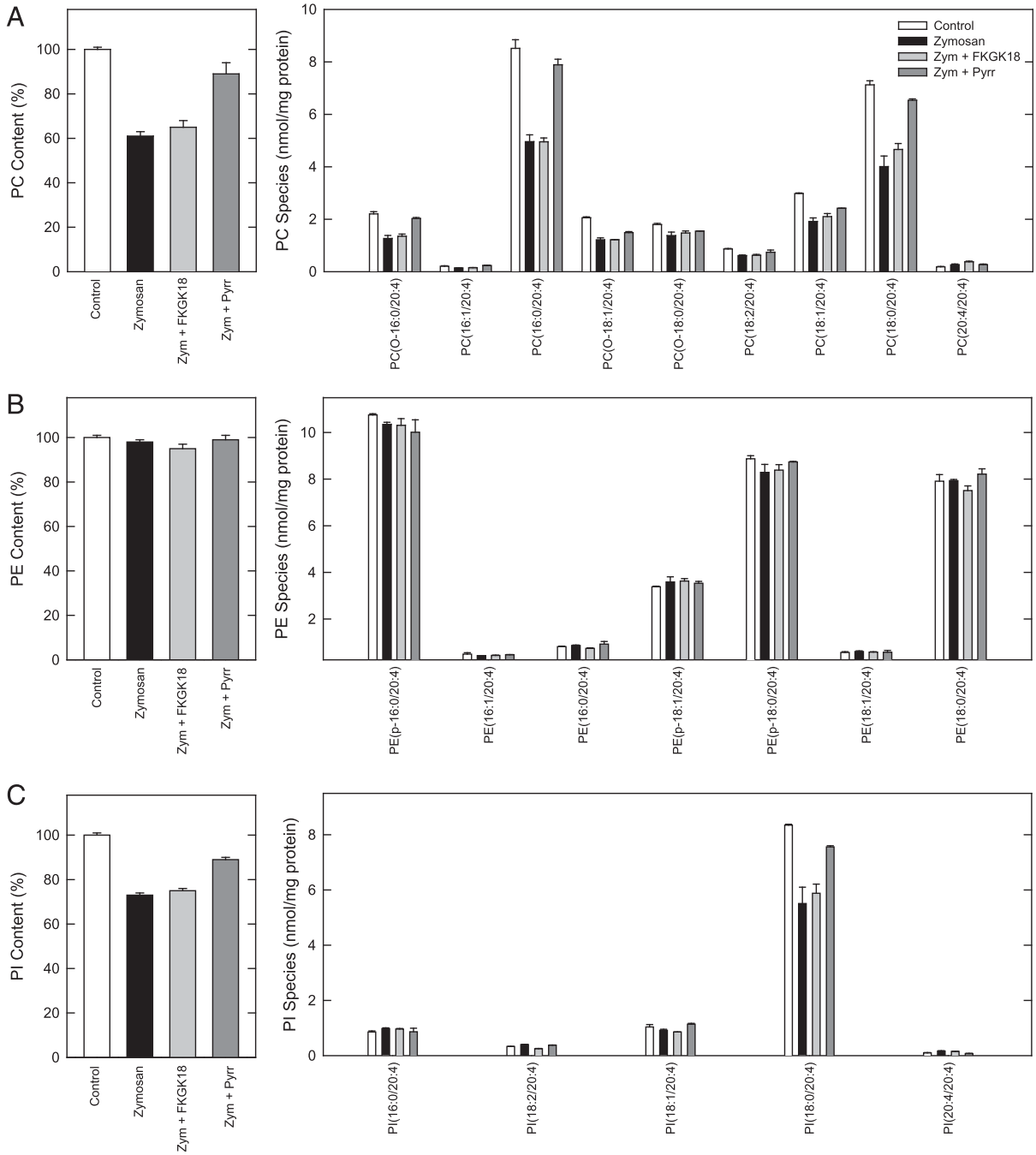

FIGURE 1. AA-containing phospholipid species in control and zymosan-stimulated macrophages. The cells were not stimulated (open bars) or stimulated by 1 mg/ml zymosan in the absence (black bars) or presence of 1 μM FKGK18 (light gray bars) or 1 μM pyrrophenone (dark gray bars). After 1 h, the cellular content of PC (**A**), PE (**B**), and PI (**C**) molecular species was determined by liquid chromatography/MS. FKGK18 and pyrrophenone exerted no effect on the unstimulated levels of any phospholipid species, and these data have been omitted to make the figure clearer. Data are mean ± SEM of three independent determinations.

many cases fragmentation of the m/z peaks detected in MS analyses yielded fragments corresponding to various species, it was not always possible to unequivocally assign structures to these m/z peaks. Thus, the data are given in abbreviated form, indicating phospholipid class and number of carbon atoms and double bonds of the two lateral chains together.

A number of PC species were significantly decreased after zymosan activation of the cells, indicating that the stimulus also activates phospholipid hydrolytic pathways unrelated to eicosanoid signaling (Fig. 3A). The species that decreased most prominently were PC(32:0), PC(34:2), PC(34:1), PC(36:3), PC(36:2), PC(36:1), and PC(38:3). When the zymosan stimulations were conducted in the presence of pyrrophenone, the levels of all PC species remained essentially the same as those found in the absence of the inhibitor. Thus, under zymosan-activation conditions in intact cells, cPLA$_2$α does not hydrolyze phospholipid substrates that do not contain AA. This is an important observation, because although cPLA$_2$α shows marked preference for AA-containing phospholipids in in vitro assays, such a preference is not absolute, and the enzyme also hydrolyzes phospholipid substrates that contain other fatty acids instead of AA in the *sn*-2 position in vitro (11).

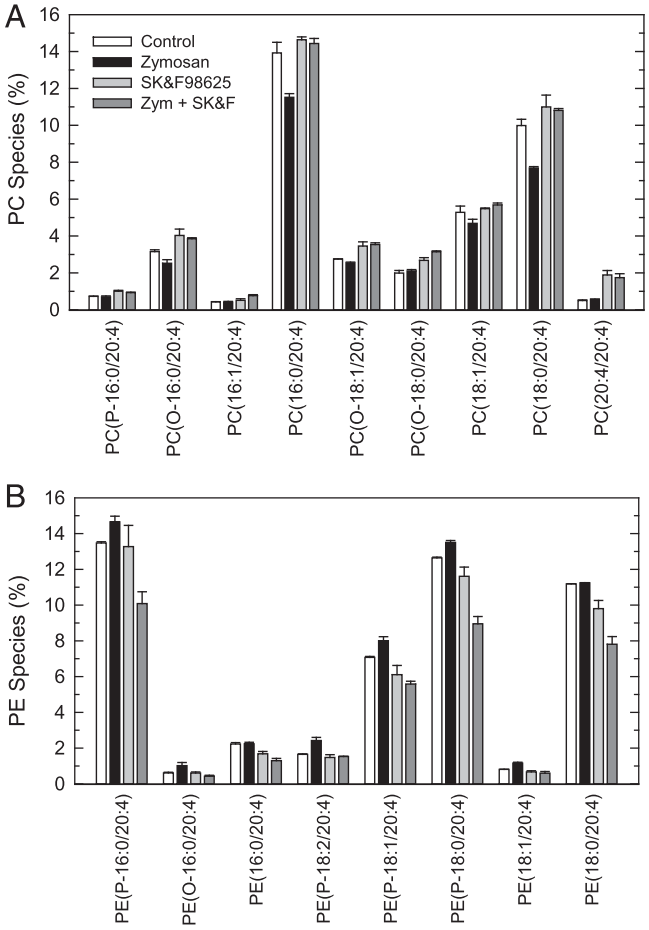

FIGURE 2. Effect of SK&F98625 on the profile of AA-containing species in zymosan-stimulated macrophages. The cells were not stimulated (open bars) or were stimulated by 1 mg/ml zymosan in the absence (black bars) or presence (dark gray bars) of 10 μM SK&F98625. After 1 h, the cellular content of PC (**A**) and PE (**B**) molecular species was determined by liquid chromatography/MS. Incubations that received SK&F98625 but not zymosan are shown in light gray. Data are mean ± SEM of three independent determinations.

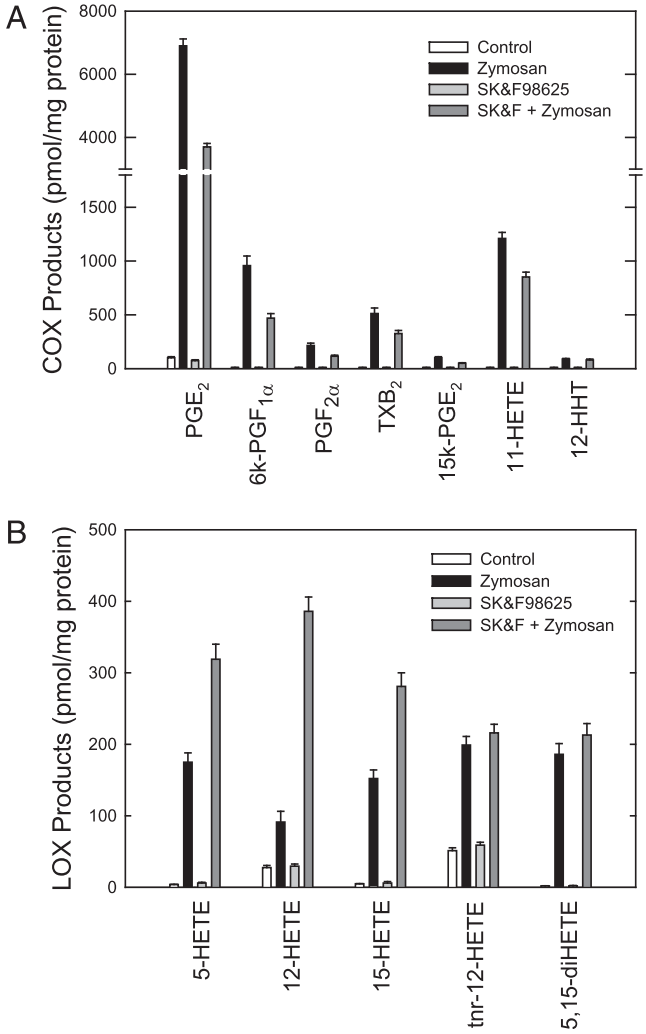

FIGURE 3. Effect of SK&F98625 on the profile of eicosanoids produced by zymosan-stimulated macrophages. The cells were not stimulated (open bars) or were stimulated with 1 mg/ml zymosan in the absence (black bars) or presence (dark gray bars) of 10 μM SK&F98625. After 8 h, supernatants were collected, and the production of cyclooxygenase (COX) (**A**) or lipoxygenase (LOX) (**B**) metabolites was determined by liquid chromatography/MS. Incubations that received SK&F98625 but not zymosan are shown in light gray. Data are mean ± SEM of three independent determinations. tnr, Tetranor.

Conversely, the use of the iPLA$_2\beta$ inhibitor FKGK18 restored the levels of some of the PC species to those found in unstimulated cells (Fig. 4A). The most prominent effects were found in the two major species measured—PC(32:0) and PC(34:1)—the levels of which were completely restored in the presence of FKGK18. These two species could be identified as PC(16:0/16:0) and PC (16:0/18:1) in fragmentation experiments. Minor species whose hydrolysis was completely prevented by FKGK18 were PC(32:1) and PC(34:2), and these also could be identified by fragmentation experiments as PC(16:0/16:1) and PC(16:0/18:2). Therefore, it is striking that four phospholipids unequivocally identified as iPLA$_2\beta$ substrates during zymosan activation all contain palmitic acid (16:0) esterified at the *sn*-1 position. This finding strongly suggests that, in activated cells, iPLA$_2\beta$ displays some sort of specificity for PC molecules containing palmitic at the *sn*-1 position and, by inference, that lysoPC molecules containing *sn*-1 palmitic acid could serve a biological role.

Fig. 4B shows that zymosan induced no significant changes in the levels of PE species that do not contain AA and that the presence of either pyrrophenone or FKGK18 along with zymosan did not produce any effect. With regard to PI species not containing AA, quite significant increases in the mass of some of them were detected, particularly PI(34:1) and PI(36:0) (Fig. 4C). Neither pyrrophenone nor FKGK18 exerted significant effects on the levels of these PI species. We speculate that these increases may be consequent on the activation of the PI cycle leading to sustained supply of phosphatidylinositol 4,5-bisphosphate to generate calcium signals during zymosan stimulation of the macrophages (55, 56). Fragmentation experiments indicated that PI(34:1) was a mix of PI(18:0/16:1) plus PI(16:0/18:1) and that PI(36:0) was PI(18:0/18:0).

Profiling of lysophospholipid species in macrophages

To obtain a more complete picture of the changes occurring via PLA$_2$-mediated deacylation reactions in zymosan-stimulated macrophages, measurement of lysophospholipid species were also carried out, and the effects of pyrrophenone and FKGK18 were studied as well. The profile of lysoPC species generated after zymosan stimulation of the macrophages is shown in Fig. 5A. In general, there was a good correlation between the appearance of lysoPC species and the disappearance of phospholipids shown in Figs. 1 and 4. Major lysoPC species detected were LPC(16:0) and

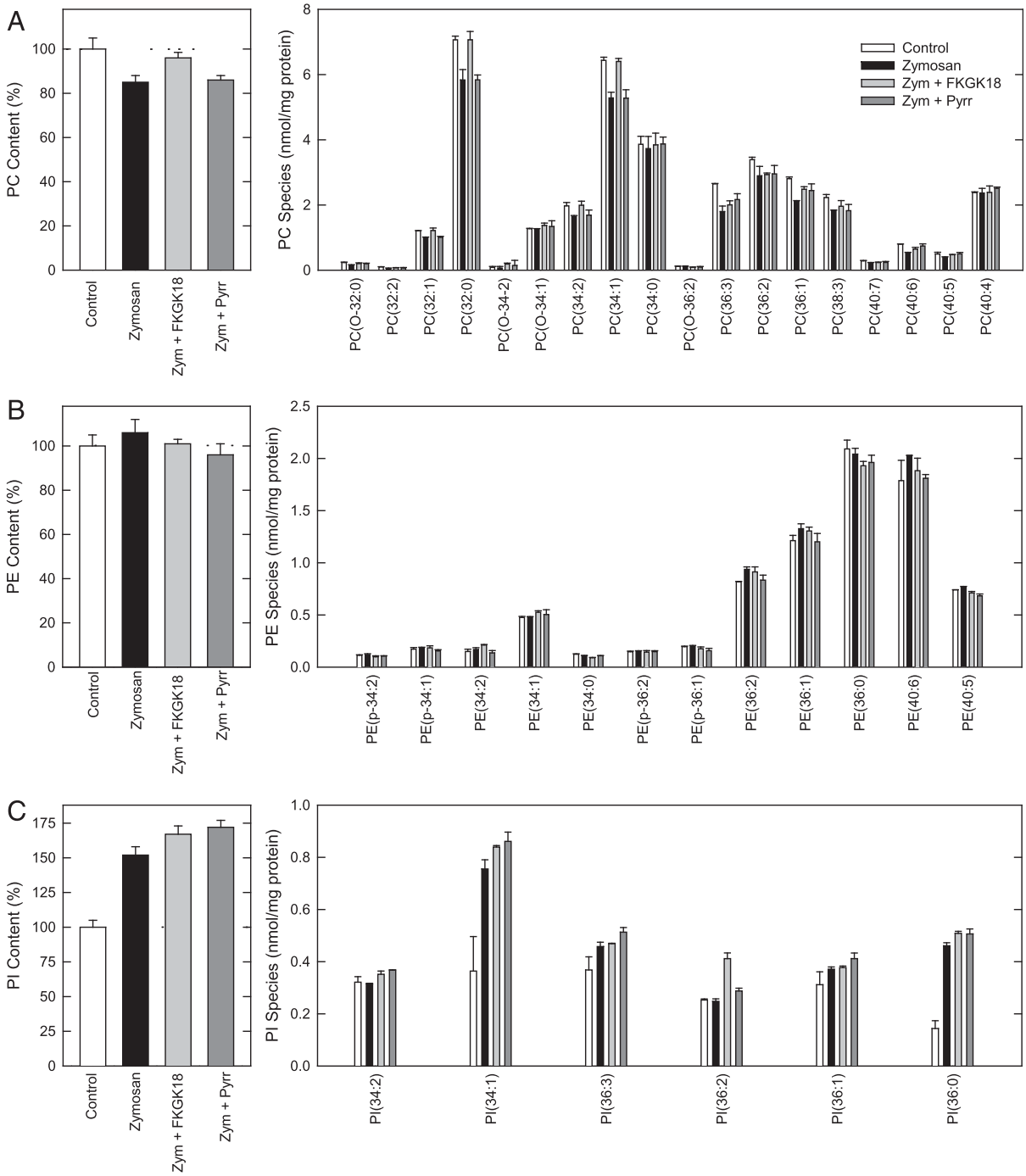

FIGURE 4. Phospholipid species not containing AA in control and zymosan-stimulated macrophages. The cells were not stimulated (open bars) or were stimulated by 1 mg/ml zymosan in the absence (black bars) or presence of 1 μM FKGK18 (light gray bars) or 1 μM pyrrophenone (dark gray bars). After 1 h, the cellular content of PC (**A**), PE (**B**), and PI (**C**) molecular species was determined by liquid chromatography/MS. FKGK18 and pyrrophenone had no significant effect on the unstimulated levels of any phospholipid species, and these data have been omitted to make the figure clearer. Data are mean ± SEM of three independent determinations.

LPC(18:0). When the effects of pyrrophenone and FKGK18 were examined, a few interesting findings could be appreciated. Pyrrophenone inhibited formation of both LPC(16:0) and LPC(18:0) in the zymosan-stimulated cells, albeit inhibition of the latter was stronger than that of the former. These results correspond well with the data shown in Fig. 1A, indicating that palmitic acid– and stearic acid–containing PC species are the major contributors to AA release in the activated cells. Conversely, FKGK18 strongly inhibited LPC(16:0) formation but exerted little effect on LPC (18:0) formation (Fig. 5A). Again, these data correspond well with the data in Fig. 4A, suggesting that iPLA$_2$β shows selectivity for PC species containing palmitic acid at the *sn*-1 position. Collectively, these results suggest that, although both cPLA$_2$α and iPLA$_2$β contribute to lysoPC accumulation during stimulation of the cells with zymosan, LPC(18:0) appears to be produced primarily by cPLA$_2$α, whereas LPC(16:0) appears to be produced primarily by iPLA$_2$β.

With regard to other lysophospholipid species, no increases in ethanolamine lysoglycerophospholipid (lysoPE) were detected under any condition (Fig. 5B). Definite increases in lysophosphatidylinositol (lysoPI) species were detected, which could be abrogated

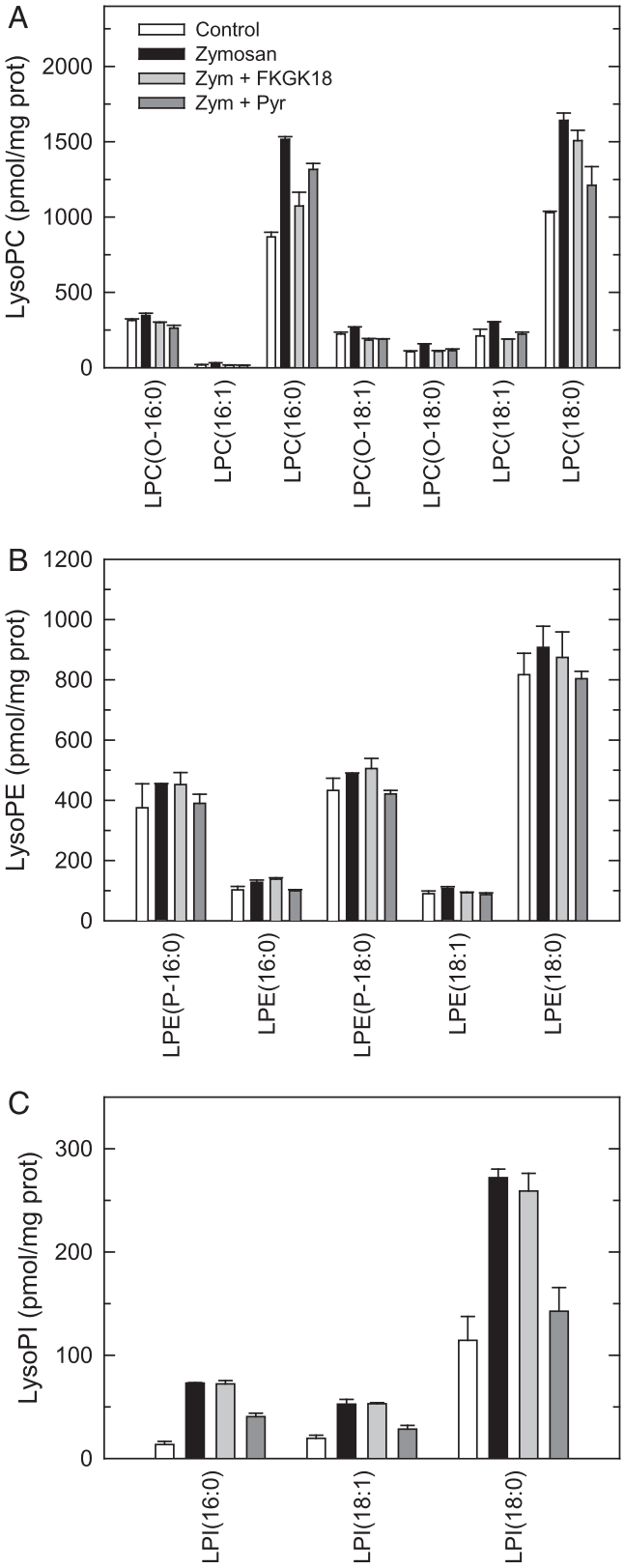

FIGURE 5. Lysophospholipid molecular species generated by zymosan-stimulated macrophages. The cells were not stimulated (open bars) or were stimulated by 1 mg/ml zymosan in the absence (black bars) or presence of 1 μM FKGK18 (light gray bars) or 1 μM pyrrophenone (dark gray bars). After 1 h, the cellular content of lysoPC (**A**), lysoPE (**B**), and lysoPI (**C**) molecular species was determined by liquid chromatography/MS. FKGK18 and pyrrophenone exerted no effect on the unstimulated levels of any lysophospholipid species, and these data have been omitted to make the figure clearer. Data are mean ± SEM of three independent determinations.

by pyrrophenone but not by FKGK18, suggesting that lysoPI production in activated cells is linked primarily to AA mobilization (Figs. 1C, 5C).

Role of lysoPC species produced by zymosan-stimulated macrophages

It is well established that not all of the AA liberated following cellular stimulation is converted into biologically active eicosanoids; a significant portion of the free AA is reincorporated back into phospholipids by the action of CoA-dependent acyltransferases (8, 54). Although it was described that this free AA that returns to phospholipids is incorporated primarily into PC (57), the exact molecular species involved have not been identified. To study the phospholipid species that reincorporate AA during zymosan activation of the macrophages, a liquid chromatography/MS metabolipidomic approach using [^{2}H]AA to label the acceptors involved was used. This procedure consists of incubating the cells with [^{2}H]AA at the time they are stimulated with zymosan. We recently used this technique to identify novel AA-containing inositol phospholipids in human monocytes and murine macrophages (32, 38). Newly synthesized [^{2}H]AA-containing phospholipids were unequivocally identified by their bell-shaped isotopic distribution, with a maximum at +8 m/z apart from their native counterparts, due to the [^{2}H]AA isotopomers. Fig. 6 shows the phospholipid species that incorporated [^{2}H]AA after a 30-min incubation of the cells with zymosan in the presence of the fatty acid. The most abundant species, accounting for ~85–90% of total [^{2}H]AA incorporated, were PC (16:0/[^{2}H]AA) and PC(18:0/[^{2}H]AA). This finding was not unexpected given the previous data showing that LPC(16:0) and LPC (18:0) were the major lysoPC species in zymosan-treated cells (Fig. 5A). Small amounts of other species were also produced: PC(18:1/[^{2}H]AA) and three PI molecular species. No incorporation of [^{2}H]AA into PE species was observed (Fig. 6).

Discussion

Macrophages, like most mammalian cells, express the two major cytosolic PLA$_2$ forms: cPLA$_2\alpha$ and iPLA$_2\beta$. Although the role of

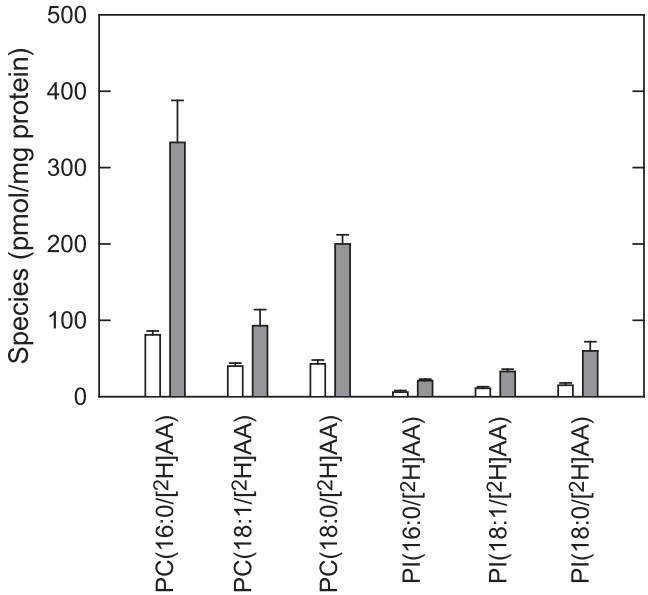

FIGURE 6. Phospholipid molecular species that initially incorporate [^{2}H]AA. The cells were exposed to 1 μM [^{2}H]AA at the time that they were stimulated with 1 mg/ml zymosan (filled bars) or not (open bars) for 30 min. The incorporation of [^{2}H]AA was determined by liquid chromatography/MS. Data are mean ± SEM of three independent determinations.

cPLA$_2$α as the rate-limiting provider of free AA release for eicosanoid synthesis in macrophages and other cells is well established (8–11), the cellular role of iPLA$_2$β has not been clearly defined. Initially described as a homeostatic enzyme involved in phospholipid fatty acid remodeling (58–60), more recent data suggested that the enzyme also plays multiple roles in activated cells, highlighting its potential as a therapeutic candidate for a number of pathophysiological conditions (24–27, 61). In this work, we aimed at both defining and differentiating the contribution of these two PLA$_2$s to zymosan-stimulated phospholipid turnover in macrophages. We find that both enzymes act on different phospholipid substrates during zymosan stimulation. Our key findings can be summarized as follows: cPLA$_2$α targets AA-containing phospholipids and appears not to hydrolyze phospholipids that do not contain AA; iPLA$_2$β does not hydrolyze AA-containing phospholipids during zymosan activation; iPLA$_2$β appears to specifically target phospholipids carrying palmitic acid in the *sn*-1 position to generate LPC(16:0), which is a major acceptor for AA incorporation back into phospholipids; AA is released not only from PC and PI, but also from PE species; the amount of AA in PE is restored by the action of transacylation mechanisms using various PC species as donors; and the loss of AA from PC as a consequence of zymosan-activation is a composite of the direct hydrolytic attack of cPLA$_2$α and the transfer of AA to PE molecules. Taken together, the data presented in this article demonstrate the diversity of phospholipid pathways that operate in macrophages, as well as their independent, but converging, regulation, which allows the cells to orchestrate tightly integrated responses during stimulation. On the one hand, there is a pathway that culminates in AA mobilization that involves the participation of cPLA$_2$α and CoA-independent transacylation reactions that remodel the various AA pools. On the other hand, there is a phospholipid hydrolytic pathway that operates on phospholipid pools that do not contain AA but serves to generate high amounts of palmitate-containing lysoPC, which, in turn, can be used to reincorporate part of the free AA that was liberated via cPLA$_2$α-mediated reactions. This can be envisioned as a way to protect against excessive eicosanoid production during inflammation. In addition, iPLA$_2$β-derived lysoPC may also serve other biological roles. For example, this molecule also has been described to be antigenic for natural IgM Abs, to constitute a ligand for C-reactive protein, and to act as a "find me" signal to attract phagocytes to migrate toward phagocytic targets (29–31). The novel finding that iPLA$_2$β shows specificity for palmitic acid–containing choline phospholipids and does not participate in AA release may have implications for the development of strategies for therapeutic intervention against different pathophysiological responses of the macrophages.

The use of cell-permeable inhibitors to comparatively study the functioning of different PLA$_2$ forms in cells has the key advantage that inhibition is achieved very rapidly, within minutes of addition to the cells. Thus, effects on transcription, translation, or other long-term alterations of gene expression, which often occur with genetic strategies, such as small interfering RNA knockdown or gene knockout, are avoided. Another potential problem with genetic strategies, which the use of chemical inhibitors also avoids, is the existence of compensatory mechanisms arising from the expression of multiple related PLA$_2$ isoforms in a cell or tissue. This can be exemplified by the strong body of evidence suggesting a role for cPLA$_2$α in regulating tubulation and intra-Golgi transport, yet these processes function normally in fibroblasts from cPLA$_2$α-knockout mice (39, 62). Also, we have repeatedly noted that inhibition of iPLA$_2$β in phagocytic cells of various origins results in slowed cellular use of AA, presumably due to acute reduction in lysoPC levels (58–60, 63, 64); however, this effect is not observed in macrophages from iPLA$_2$β-knockout mice, which show normal lysoPC levels (14). Finally, it has been appreciated that skeletal muscle from mice lacking group VIB phospholipase A$_2$, a Ca^{2+}-independent PLA$_2$ form with similarities to iPLA$_2$β, by genetic ablation shows increased levels of both cPLA$_2$α and iPLA$_2$β (65).

Another advantage of the use of inhibitors stems from the recent discovery that some phospholipases, in addition to acting as enzymes, may exert other important noncatalytic functions in cells. These functions would be lost if inhibition were accomplished by genetic approaches, but not if chemical inhibitors were used. In this regard, recent data have shown that, during phagocytosis, cPLA$_2$α translocates to the site of nascent phagosomes to modulate the phagocytic process in a manner that is independent of its enzymatic activity (66). Of course, the use of chemical inhibitors has its own issues, such as the lack of specificity, and we acknowledge this as a limitation of the current study. It should be noted in this regard that the two inhibitors used in this work, pyrrophenone and FKGK18, are the most potent and selective inhibitors available to block cPLA$_2$α and iPLA$_2$β in cells. Pyrrophenone potently and selectively inhibits cPLA$_2$α activity using a number of in vitro lipolysis assays without detectable effects on other PLA$_2$ activities, blocks AA release in mammalian cells in the 0.01–1 μM range (40, 67, 68), and has been shown to be effective in experimental models of disease involving cPLA$_2$α, such as collagen-induced arthritis and experimental autoimmune encephalomyelitis (11, 39). We have also shown that, at the concentration used in this study (1 μM), the inhibitor blocks essentially all measurable Ca^{2+}-dependent PLA$_2$ activity of cell homogenates, leaving the Ca^{2+}-independent activity unchanged (47, 67, 69). FKGK18, a member of a family of polyfluoroketone-containing compounds, is the most potent iPLA$_2$β inhibitor reported, completely blocking iPLA$_2$β activity at concentrations ≥200 times lower than those required for inhibition of other PLA$_2$ activities, which makes it a valuable tool to explore the role of iPLA$_2$β in cells and in in vivo models (11, 41). In keeping with this observation, very recent data in pancreatic β cells show that FKGK18 blocks iPLA$_2$β-mediated responses in these cells while leaving the cPLA$_2$α-dependent ones unchanged (70). Moreover, at the concentrations used in this study (1 μM), FKGK18 completely inhibited the Ca^{2+}-independent PLA$_2$ activity of macrophage homogenates while exerting no effect on the Ca^{2+}-dependent activity, as measured under a variety of assay conditions. Together, all of these control measurements provide confidence on the specificity of the inhibitors used in this study, yet the existence of unknown targets that could obscure interpretation of the results is a possibility that cannot be ruled out.

Liquid chromatography/MS–based approaches have been used in this study to track the changes in phospholipid metabolism that occur after zymosan addition to the macrophages. This technology offers great advantages, both at qualitative and quantitative levels, over traditional methods that relied primarily on the use of radioactive precursors (71). Although the latter kind of studies has been immensely useful to delineate pathways, intermediates, and enzymes involved, radioactive tracers do not always distribute homogeneously among the various phospholipid pools, which has often led to inaccurate estimation of turnover rates (72, 73). Actually, previous studies using macrophages prelabeled with [^{3}H]AA have shown that PE is a source of free AA when exposed to a variety of stimuli, including zymosan (57, 74–76). These results contrast with our current mass measurements showing no changes in the AA content of all PE molecular species under activation conditions. The lack of an apparent contribution of PE species to zymosan-stimulated AA mobilization in macrophages, as studied by

MS, also was reported by other laboratories (2), and we have obtained the same result in zymosan-stimulated human monocytes (33). This discrepancy provides a germane example of the differences between using label or mass to determine sources of AA release in stimulated cells. However, a scenario that reconciles all of these observations would be one in which PE species are indeed substrates for cPLA$_2$α-mediated AA mobilization but, once hydrolyzed by cPLA$_2$α, they are rapidly reformed with AA coming from other sources: PC via CoA-independent transacylation reactions. We tested this possibility using SK&F98625, a well-established CoA-independent transacylase inhibitor (42, 43). We found that, in the presence of the inhibitor, marked decreases in the cellular levels of AA-containing PE species were measured that were accompanied by the corresponding increases in AA-containing PC species. Thus, these results provide direct evidence that, in zymosan-stimulated cells, the levels of AA-containing PE species are tightly controlled by the opposing actions of cPLA$_2$α and CoA-independent transacylase.

A striking feature of this work is that inhibition of the transfer of AA from PC to PE by SK&F98625 leads to a strong increase in lipoxygenase metabolite formation in response to zymosan. From a biochemical point of view, the data are consistent with AA-containing PC molecules being the major precursors of lipoxygenase metabolites under zymosan stimulation, because blockade of CoA-independent transacylation by SKF98625 would favor the cPLA$_2$α-mediated cleavage of AA-containing PC over the AA transfer from PC to PE. This view is reminiscent of early work by Chilton (77), suggesting that ether-linked PC species, which are major donors of AA in the CoA-independent transacylation reaction (54), are also important sources of AA used for lipoxygenase metabolite production in neutrophils. More interestingly, these data are immediately relevant to innate immune signaling in that they unveil a previously unrecognized mechanism to protect against excessive production of certain eicosanoids during inflammation. Thus, by channeling the AA present in PC toward PE, the CoA-independent transacylation reactions may limit lipoxygenase product formation under zymosan stimulation. Although much data exist on the role of cyclooxygenase products in regulating innate immune responses, the contribution of lipoxygenase products is less well appreciated. Relevant to the results of this study, increased amounts of 5-, 12-, and 15-hydroxyeicosatetraenoic acid are synthesized when CoA-independent transacylation is inhibited. These metabolites are known to interact with specific receptors on the surface of immune cells, thereby impacting on intracellular signaling and ultimately modulating various innate immune responses, such as leukocyte migration or generation of other proinflammatory mediators (78–80).

In addition to cPLA$_2$α and iPLA$_2$β, murine peritoneal macrophages are known to express another PLA$_2$ form, the secreted group V enzyme, and this form was shown to participate in zymosan-stimulated phospholipid turnover in these cells (81). It is assumed that group V PLA$_2$ participates in zymosan-induced signaling by directly hydrolyzing membrane phospholipids via its intrinsic enzymatic activity; however, it has not been ruled out that the enzyme may also act as a surface receptor ligand in an activity-independent manner (81). In any case, it has become clear over the years that secreted PLA$_2$ enzymes act after cPLA$_2$α by amplifying the response that the latter enzyme initiates (8–11). Therefore, it is conceivable that the contribution of group V PLA$_2$ to phospholipid hydrolysis will be blocked if cPLA$_2$α is inhibited. Thus, our pyrrophenone data would include the contribution of group V PLA$_2$ to overall phospholipid hydrolysis. We have not been able to locate a reliable, reasonably selective, and cell-permeable group V PLA$_2$ inhibitor. Thus, we cannot directly examine the effect of group V PLA$_2$ during zymosan stimulation of the macrophages. In the absence of such an inhibitor, the use of macrophages isolated from group V PLA$_2$–knockout animals could be envisioned as a useful alternative, especially because these cells show reduced responses to zymosan under certain conditions (82, 83). However, aside from the potential pitfalls associated with the use of cells from knockout animals discussed above, a significant problem with the group V–knockout mouse is that the effects observed depend on the genetic background of the animal; group V PLA$_2$–null cells on a C57BL/6 genetic background show altered responses to stimuli, but group V PLA$_2$–null cells on a BALB/c background do not (84). This argues again for the importance of developing potent and selective inhibitors for the various PLA$_2$ forms, including group V PLA$_2$, to more accurately assess the involvement of these key enzymes in regulating both physiological and pathophysiological processes. In this regard, the inhibitor studies reported in this article provide clues to understand the independent, but coordinate, regulation of phospholipid turnover in activated macrophages by two distinct PLA$_2$s.

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Disclosures

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References

1. Scott, W. A., J. M. Zrike, A. L. Hamill, J. Kempe, and Z. A. Cohn. 1980. Regulation of arachidonic acid metabolites in macrophages. *J. Exp. Med.* 152: 324–335.
2. Rouzer, C. A., P. T. Ivanova, M. O. Byrne, H. A. Brown, and L. J. Marnett. 2007. Lipid profiling reveals glycerophospholipid remodeling in zymosan-stimulated macrophages. *Biochemistry* 46: 6026–6042.
3. Astudillo, A. M., D. Balgoma, M. A. Balboa, and J. Balsinde. 2012. Dynamics of arachidonic acid mobilization by inflammatory cells. *Biochim. Biophys. Acta* 1821: 249–256.
4. Peters-Golden, M., C. Canetti, P. Mancuso, and M. J. Coffey. 2005. Leukotrienes: underappreciated mediators of innate immune responses. *J. Immunol.* 174: 589–594.
5. Serhan, C. N. 2007. Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. *Annu. Rev. Immunol.* 25: 101–137.
6. Ricciotti, E., and G. A. FitzGerald. 2011. Prostaglandins and inflammation. *Arterioscler. Thromb. Vasc. Biol.* 31: 986–1000.
7. Kalinski, P. 2012. Regulation of immune responses by prostaglandin E$_2$. *J. Immunol.* 188: 21–28.
8. Pérez-Chacón, G., A. M. Astudillo, D. Balgoma, M. A. Balboa, and J. Balsinde. 2009. Control of free arachidonic acid levels by phospholipases A$_2$ and lysophospholipid acyltransferases. *Biochim. Biophys. Acta* 1791: 1103–1113.
9. Balsinde, J., M. V. Winstead, and E. A. Dennis. 2002. Phospholipase A$_2$ regulation of arachidonic acid mobilization. *FEBS Lett.* 531: 2–6.
10. Leslie, C. C. 2004. Regulation of arachidonic acid availability for eicosanoid production. *Biochem. Cell Biol.* 82: 1–17.
11. Dennis, E. A., J. Cao, Y. H. Hsu, V. Magrioti, and G. Kokotos. 2011. Phospholipase A$_2$ enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. *Chem. Rev.* 111: 6130–6185.
12. Han, W. K., A. Sapirstein, C. C. Hung, A. Alessandrini, and J. V. Bonventre. 2003. Cross-talk between cytosolic phospholipase A$_2$α (cPLA$_2$α) and secretory phospholipase A$_2$ (sPLA$_2$) in hydrogen peroxide-induced arachidonic acid release in murine mesangial cells: sPLA2 regulates cPLA$_2$α activity that is responsible for arachidonic acid release. *J. Biol. Chem.* 278: 24153–24163.
13. Panini, S. R., L. Yang, A. E. Rusiñol, M. S. Sinensky, J. V. Bonventre, and C. C. Leslie. 2001. Arachidonate metabolism and the signaling pathway of induction of apoptosis by oxidized LDL/oxysterol. *J. Lipid Res.* 42: 1678–1686.
14. Bao, S., Y. Li, X. Lei, M. Wohltmann, W. Jin, A. Bohrer, C. F. Semenkovich, S. Ramanadham, I. Tabas, and J. Turk. 2007. Attenuated free cholesterol loading-induced apoptosis but preserved phospholipid composition of peritoneal macrophages from mice that do not express group VIA phospholipase A$_2$. *J. Biol. Chem.* 282: 27100–27114.
15. Balsinde, J., M. A. Balboa, and E. A. Dennis. 2000. Identification of a third pathway for arachidonic acid mobilization and prostaglandin production in activated P388D$_1$ macrophage-like cells. *J. Biol. Chem.* 275: 22544–22549.

16. Balsinde, J., M. A. Balboa, S. Yedgar, and E. A. Dennis. 2000. Group V phospholipase A_2-mediated oleic acid mobilization in lipopolysaccharide-stimulated $P388D_1$ macrophages. *J. Biol. Chem.* 275: 4783–4786.
17. Pindado, J., J. Balsinde, and M. A. Balboa. 2007. TLR3-dependent induction of nitric oxide synthase in RAW 264.7 macrophage-like cells via a cytosolic phospholipase A_2/cyclooxygenase-2 pathway. *J. Immunol.* 179: 4821–4828.
18. Ruipérez, V., A. M. Astudillo, M. A. Balboa, and J. Balsinde. 2009. Coordinate regulation of TLR-mediated arachidonic acid mobilization in macrophages by group IVA and group V phospholipase A_2s. *J. Immunol.* 182: 3877–3883.
19. Marshall, J., E. Krump, T. Lindsay, G. Downey, D. A. Ford, P. Zhu, P. Walker, and B. Rubin. 2000. Involvement of cytosolic phospholipase A_2 and secretory phospholipase A_2 in arachidonic acid release from human neutrophils. *J. Immunol.* 164: 2084–2091.
20. Degousee, N., F. Ghomashchi, E. Stefanski, A. G. Singer, B. P. Smart, N. Borregaard, R. Reithmeier, T. F. Lindsay, C. Lichtenberger, W. Reinisch, et al. 2002. Groups IV, V, and X phospholipases A_2s in human neutrophils: role in eicosanoid production and gram-negative bacterial phospholipid hydrolysis. *J. Biol. Chem.* 277: 5061–5073.
21. Pérez-Chacón, G., A. M. Astudillo, V. Ruipérez, M. A. Balboa, and J. Balsinde. 2010. Signaling role for lysophosphatidylcholine acyltransferase 3 in receptor-regulated arachidonic acid reacylation reactions in human monocytes. *J. Immunol.* 184: 1071–1078.
22. Balsinde, J. 2002. Roles of various phospholipases A_2 in providing lysophospholipid acceptors for fatty acid phospholipid incorporation and remodelling. *Biochem. J.* 364: 695–702.
23. Hsu, F. F., Z. Ma, M. Wohltmann, A. Bohrer, W. Nowatzke, S. Ramanadham, and J. Turk. 2000. Electrospray ionization/mass spectrometric analyses of human promonocytic U937 cell glycerolipids and evidence that differentiation is associated with membrane lipid composition changes that facilitate phospholipase A_2 activation. *J. Biol. Chem.* 275: 16579–16589.
24. Balsinde, J., and M. A. Balboa. 2005. Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A_2 in activated cells. *Cell. Signal.* 17: 1052–1062.
25. Hooks, S. B., and B. S. Cummings. 2008. Role of Ca^{2+}-independent phospholipase A_2 in cell growth and signaling. *Biochem. Pharmacol.* 76: 1059–1067.
26. Wilkins, W. P., III, and S. E. Barbour. 2008. Group VI phospholipases A_2: homeostatic phospholipases with significant potential as targets for novel therapeutics. *Curr. Drug Targets* 9: 683–697.
27. Lei, X., S. E. Barbour, and S. Ramanadham. 2010. Group VIA Ca^{2+}-independent phospholipase A_2 ($iPLA_2\beta$) and its role in beta-cell programmed cell death. *Biochimie* 92: 627–637.
28. Lei, X., S. Zhang, A. Bohrer, S. E. Barbour, and S. Ramanadham. 2012. Role of calcium-independent phospholipase $A_2\beta$ in human pancreatic islet β-cell apoptosis. *Am. J. Physiol. Endocrinol. Metab.* 303: E1386–E1395.
29. Pérez, R., M. A. Balboa, and J. Balsinde. 2006. Involvement of group VIA calcium-independent phospholipase A_2 in macrophage engulfment of hydrogen peroxide-treated U937 cells. *J. Immunol.* 176: 2555–2561.
30. Kim, S. J., D. Gershov, X. Ma, N. Brot, and K. B. Elkon. 2002. I-PLA_2 activation during apoptosis promotes the exposure of membrane lysophosphatidylcholine leading to binding by natural immunoglobulin M antibodies and complement activation. *J. Exp. Med.* 196: 655–665.
31. Lauber, K., E. Bohn, S. M. Kröber, Y. J. Xiao, S. G. Blumenthal, R. K. Lindemann, P. Marini, C. Wiedig, A. Zobywalski, S. Baksh, et al. 2003. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. *Cell* 113: 717–730.
32. Balgoma, D., O. Montero, M. A. Balboa, and J. Balsinde. 2008. Calcium-independent phospholipase A_2-mediated formation of 1,2-diarachidonoylglycerophosphoinositol in monocytes. *FEBS J.* 275: 6180–6191.
33. Balgoma, D., A. M. Astudillo, G. Pérez-Chacón, O. Montero, M. A. Balboa, and J. Balsinde. 2010. Markers of monocyte activation revealed by lipidomic profiling of arachidonic acid-containing phospholipids. *J. Immunol.* 184: 3857–3865.
34. Astudillo, A. M., G. Pérez-Chacón, D. Balgoma, L. Gil-de-Gómez, V. Ruipérez, C. Guijas, M. A. Balboa, and J. Balsinde. 2011. Influence of cellular arachidonic acid levels on phospholipid remodeling and CoA-independent transacylase activity in human monocytes and U937 cells. *Biochim. Biophys. Acta* 1811: 97–103.
35. Valdearcos, M., E. Esquinas, C. Meana, L. Gil-de-Gómez, C. Guijas, J. Balsinde, and M. A. Balboa. 2011. Subcellular localization and role of lipin-1 in human macrophages. *J. Immunol.* 186: 6004–6013.
36. Astudillo, A. M., G. Pérez-Chacón, C. Meana, D. Balgoma, A. Pol, M. A. Del Pozo, M. A. Balboa, and J. Balsinde. 2011. Altered arachidonate distribution in macrophages from caveolin-1 null mice leading to reduced eicosanoid synthesis. *J. Biol. Chem.* 286: 35299–35307.
37. Guijas, C., A. M. Astudillo, L. Gil-de-Gómez, J. M. Rubio, M. A. Balboa, and J. Balsinde. 2012. Phospholipid sources for adrenic acid mobilization in RAW 264.7 macrophages. Comparison with arachidonic acid. *Biochim. Biophys. Acta* 1821: 1386–1393.
38. Gil-de-Gómez, L., A. M. Astudillo, C. Meana, J. M. Rubio, C. Guijas, M. A. Balboa, and J. Balsinde. 2013. A phosphatidylinositol species acutely generated by activated macrophages regulates innate immune responses. *J. Immunol.* 190: 5169–5177.
39. Leslie, C. C., T. A. Gangelhoff, and M. H. Gelb. 2010. Localization and function of cytosolic phospholipase $A_2\alpha$ at the Golgi. *Biochimie* 92: 620–626.
40. Ono, T., K. Yamada, Y. Chikazawa, M. Ueno, S. Nakamoto, T. Okuno, and K. Seno. 2002. Characterization of a novel inhibitor of cytosolic phospholipase $A_2\alpha$, pyrrophenone. *Biochem. J.* 363: 727–735.
41. Kokotos, G., Y. H. Hsu, J. E. Burke, C. Baskakis, C. G. Kokotos, V. Magrioti, and E. A. Dennis. 2010. Potent and selective fluoroketone inhibitors of group VIA calcium-independent phospholipase A_2. *J. Med. Chem.* 53: 3602–3610.
42. Chilton, F. H., A. N. Fonteh, C. M. Sung, D. M. Hickey, T. J. Torphy, R. J. Mayer, L. A. Marshall, J. D. Heravi, and J. D. Winkler. 1995. Inhibitors of CoA-independent transacylase block the movement of arachidonate into 1-ether-linked phospholipids of human neutrophils. *Biochemistry* 34: 5403–5410.
43. Winkler, J. D., A. N. Fonteh, C. M. Sung, J. D. Heravi, A. B. Nixon, M. Chabot-Fletcher, D. Griswold, L. A. Marshall, and F. H. Chilton. 1995. Effects of CoA-independent transacylase inhibitors on the production of lipid inflammatory mediators. *J. Pharmacol. Exp. Ther.* 274: 1338–1347.
44. Pérez, R., X. Matabosch, A. Llebaria, M. A. Balboa, and J. Balsinde. 2006. Blockade of arachidonic acid incorporation into phospholipids induces apoptosis in U937 promonocytic cells. *J. Lipid Res.* 47: 484–491.
45. Balsinde, J., B. Fernández, and E. Diez. 1990. Regulation of arachidonic acid release in mouse peritoneal macrophages. The role of extracellular calcium and protein kinase C. *J. Immunol.* 144: 4298–4304.
46. Balsinde, J., B. Fernández, J. A. Solís-Herruzo, and E. Diez. 1992. Pathways for arachidonic acid mobilization in zymosan-stimulated mouse peritoneal macrophages. *Biochim. Biophys. Acta* 1136: 75–82.
47. Balboa, M. A., R. Pérez, and J. Balsinde. 2008. Calcium-independent phospholipase A_2 mediates proliferation of human promonocytic U937 cells. *FEBS J.* 275: 1915–1924.
48. Guijas, C., G. Pérez-Chacón, A. M. Astudillo, J. M. Rubio, L. Gil-de-Gómez, M. A. Balboa, and J. Balsinde. 2012. Simultaneous activation of p38 and JNK by arachidonic acid stimulates the cytosolic phospholipase A_2-dependent synthesis of lipid droplets in human monocytes. *J. Lipid Res.* 53: 2343–2354.
49. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37: 911–917.
50. Dumlao, D. S., M. W. Buczynski, P. C. Norris, R. Harkewicz, and E. A. Dennis. 2011. High-throughput lipidomic analysis of fatty acid derived eicosanoids and N-acylethanolamines. *Biochim. Biophys. Acta* 1811: 724–736.
51. Fahy, E., S. Subramaniam, H. A. Brown, C. K. Glass, A. H. Merrill, Jr., R. C. Murphy, C. R. Raetz, D. W. Russell, Y. Seyama, W. Shaw, et al. 2005. A comprehensive classification system for lipids. *J. Lipid Res.* 46: 839–861.
52. Rouzer, C. A., P. T. Ivanova, M. O. Byrne, S. B. Milne, L. J. Marnett, and H. A. Brown. 2006. Lipid profiling reveals arachidonate deficiency in RAW264.7 cells: Structural and functional implications. *Biochemistry* 45: 14795–14808.
53. Gijón, M. A., D. M. Spencer, A. R. Siddiqi, J. V. Bonventre, and C. C. Leslie. 2000. Cytosolic phospholipase A_2 is required for macrophage arachidonic acid release by agonists that do and do not mobilize calcium. Novel role of mitogen-activated protein kinase pathways in cytosolic phospholipase A_2 regulation. *J. Biol. Chem.* 275: 20146–20156.
54. Chilton, F. H., A. N. Fonteh, M. E. Surette, M. Triggiani, and J. D. Winkler. 1996. Control of arachidonate levels within inflammatory cells. *Biochim. Biophys. Acta* 1299: 1–15.
55. Emilsson, A., and R. Sundler. 1984. Differential activation of phosphatidylinositol deacylation and a pathway via diphosphoinositide in macrophages responding to zymosan and ionophore A23187. *J. Biol. Chem.* 259: 3111–3116.
56. Majerus, P. W., E. J. Neufeld, and D. B. Wilson. 1984. Production of phosphoinositide-derived messengers. *Cell* 37: 701–703.
57. Balsinde, J., B. Fernández, and J. A. Solís-Herruzo. 1994. Increased incorporation of arachidonic acid into phospholipids in zymosan-stimulated mouse peritoneal macrophages. *Eur. J. Biochem.* 221: 1013–1018.
58. Balsinde, J., I. D. Bianco, E. J. Ackermann, K. Conde-Frieboes, and E. A. Dennis. 1995. Inhibition of calcium-independent phospholipase A_2 prevents arachidonic acid incorporation and phospholipid remodeling in $P388D_1$ macrophages. *Proc. Natl. Acad. Sci. USA* 92: 8527–8531.
59. Balsinde, J., M. A. Balboa, and E. A. Dennis. 1997. Antisense inhibition of group VI Ca^{2+}-independent phospholipase A_2 blocks phospholipid fatty acid remodeling in murine $P388D_1$ macrophages. *J. Biol. Chem.* 272: 29317–29321.
60. Balsinde, J., and E. A. Dennis. 1997. Function and inhibition of intracellular calcium-independent phospholipase A_2. *J. Biol. Chem.* 272: 16069–16072.
61. Kalyvas, A., C. Baskakis, V. Magrioti, V. Constantinou-Kokotou, D. Stephens, R. López-Vales, J. Q. Lu, V. W. Yong, E. A. Dennis, G. Kokotos, and S. David. 2009. Differing roles for members of the phospholipase A_2 superfamily in experimental autoimmune encephalomyelitis. *Brain* 132: 1221–1235.
62. San Pietro, E., M. G. Capestrano, E. V. Polishchuk, A. DiPentima, A. Trucco, P. Zizza, S. Mariggiò, T. Pulvirenti, M. Sallese, S. Tete, et al. 2009. Group IV phospholipase $A_2\alpha$ controls the formation of inter-cisternal continuities involved in intra-Golgi transport. *PLoS Biol.* 7: e1000194.
63. Balboa, M. A., Y. Sáez, and J. Balsinde. 2003. Calcium-independent phospholipase A_2 is required for lysozyme secretion in U937 promonocytes. *J. Immunol.* 170: 5276–5280.
64. Pérez, R., R. Melero, M. A. Balboa, and J. Balsinde. 2004. Role of group VIA calcium-independent phospholipase A_2 in arachidonic acid release, phospholipid fatty acid incorporation, and apoptosis in U937 cells responding to hydrogen peroxide. *J. Biol. Chem.* 279: 40385–40391.
65. Yoda, E., K. Hachisu, Y. Taketomi, K. Yoshida, M. Nakamura, K. Ikeda, R. Taguchi, Y. Nakatani, H. Kuwata, M. Murakami, et al. 2010. Mitochondrial dysfunction and reduced prostaglandin synthesis in skeletal muscle of Group VIB Ca^{2+}-independent phospholipase $A_2\gamma$-deficient mice. *J. Lipid Res.* 51: 3003–3015.
66. Zizza, P., C. Iurisci, M. Bonazzi, P. Cossart, C. C. Leslie, D. Corda, and S. Mariggiò. 2012. Phospholipase A_2 IVα regulates phagocytosis independent of its enzymatic activity. *J. Biol. Chem.* 287: 16849–16859.
67. Balboa, M. A., and J. Balsinde. 2002. Involvement of calcium-independent phospholipase A_2 in hydrogen peroxide-induced accumulation of free fatty acids in human U937 cells. *J. Biol. Chem.* 277: 40384–40389.

68. Ghomashchi, F., A. Stewart, Y. Hefner, S. Ramanadham, J. Turk, C. C. Leslie, and M. H. Gelb. 2001. A pyrrolidine-based specific inhibitor of cytosolic phospholipase A$_2\alpha$ blocks arachidonic acid release in a variety of mammalian cells. *Biochim. Biophys. Acta* 1513: 160–166.
69. Balboa, M. A., R. Pérez, and J. Balsinde. 2003. Amplification mechanisms of inflammation: paracrine stimulation of arachidonic acid mobilization by secreted phospholipase A$_2$ is regulated by cytosolic phospholipase A$_2$-derived hydroperoxyeicosatetraenoic acid. *J. Immunol.* 171: 989–994.
70. Ali, T., G. Kokotos, V. Magrioti, R. N. Bone, J. A. Mobley, W. Hancock, and S. Ramanadham. 2013. Characterization of FKGK18 as inhibitor of group VIA Ca^{2+}-independent phospholipase A$_2$ (iPLA$_2\beta$): candidate drug for preventing beta-cell apoptosis and diabetes. *PLoS ONE* 8: e71748.
71. Wenk, M. R. 2005. The emerging field of lipidomics. *Nat. Rev. Drug Discov.* 4: 594–610.
72. Chilton, F. H., and T. R. Connell. 1988. 1-ether-linked phosphoglycerides. Major endogenous sources of arachidonate in the human neutrophil. *J. Biol. Chem.* 263: 5260–5265.
73. Ralston, N. V., and M. S. Rohrbach. 1994. Mass determination of the fatty acids released from tannin-stimulated rabbit alveolar macrophages. *Lipids* 29: 103–109.
74. Emilsson, A., and R. Sundler. 1985. Studies on the enzymatic pathways of calcium ionophore-induced phospholipid degradation and arachidonic acid mobilization in peritoneal macrophages. *Biochim. Biophys. Acta* 846: 265–274.
75. Diez, E., J. Balsinde, M. Aracil, and A. Schüller. 1987. Ethanol induces release of arachidonic acid but not synthesis of eicosanoids in mouse peritoneal macrophages. *Biochim. Biophys. Acta* 921: 82–89.
76. Balsinde, J., and E. A. Dennis. 1996. Distinct roles in signal transduction for each of the phospholipase A$_2$ enzymes present in P388D$_1$ macrophages. *J. Biol. Chem.* 271: 6758–6765.
77. Chilton, F. H. 1989. Potential phospholipid source(s) of arachidonate used for the synthesis of leukotrienes by the human neutrophil. *Biochem. J.* 258: 327–333.
78. Bolick, D. T., A. W. Orr, A. Whetzel, S. Srinivasan, M. E. Hatley, M. A. Schwartz, and C. C. Hedrick. 2005. 12/15-lipoxygenase regulates intercellular adhesion molecule-1 expression and monocyte adhesion to endothelium through activation of RhoA and nuclear factor-kappaB. *Arterioscler. Thromb. Vasc. Biol.* 25: 2301–2307.
79. Wen, Y., J. Gu, S. K. Chakrabarti, K. Aylor, J. Marshall, Y. Takahashi, T. Yoshimoto, and J. L. Nadler. 2007. The role of 12/15-lipoxygenase in the expression of interleukin-6 and tumor necrosis factor-alpha in macrophages. *Endocrinology* 148: 1313–1322.
80. Blaho, V. A., M. W. Buczynski, C. R. Brown, and E. A. Dennis. 2009. Lipidomic analysis of dynamic eicosanoid responses during the induction and resolution of Lyme arthritis. *J. Biol. Chem.* 284: 21599–21612.
81. Balestrieri, B., and J. P. Arm. 2006. Group V sPLA$_2$: classical and novel functions. *Biochim. Biophys. Acta* 1761: 1280–1288.
82. Satake, Y., B. L. Diaz, B. Balestrieri, B. K. Lam, Y. Kanaoka, M. J. Grusby, and J. P. Arm. 2004. Role of group V phospholipase A$_2$ in zymosan-induced eicosanoid generation and vascular permeability revealed by targeted gene disruption. *J. Biol. Chem.* 279: 16488–16494.
83. Ohta, S., M. Imamura, W. Xing, J. A. Boyce, and B. Balestrieri. 2013. Group V secretory phospholipase A$_2$ is involved in macrophage activation and is sufficient for macrophage effector functions in allergic pulmonary inflammation. *J. Immunol.* 190: 5927–5938.
84. Diaz, B. L., Y. Satake, E. Kikawada, B. Balestrieri, and J. P. Arm. 2006. Group V secretory phospholipase A$_2$ amplifies the induction of cyclooxygenase 2 and delayed prostaglandin D$_2$ generation in mouse bone marrow culture-derived mast cells in a strain-dependent manner. *Biochim. Biophys. Acta* 1761: 1489–1497.