

## Phospholipid sources for adrenic acid mobilization in RAW 264.7 macrophages. Comparison with arachidonic acid

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### ABSTRACT

Cells metabolize arachidonic acid (AA) to adrenic acid (AdA) via 2-carbon elongation reactions. Like AA, AdA can be converted into multiple oxygenated metabolites, with important roles in various physiological and pathophysiological processes. However, in contrast to AA, there is virtually no information on how the cells regulate the availability of free AdA for conversion into bioactive products. We have used a comparative lipidomic approach with both gas chromatography and liquid chromatography coupled to mass spectrometry to characterize changes in the levels of AA- and AdA-containing phospholipid species in RAW 264.7 macrophage-like cells. Incubation of the cells with AA results in an extensive conversion to AdA but both fatty acids do not compete with each other for esterification into phospholipids. AdA but not AA, shows preference for incorporation into phospholipids containing stearic acid at the *sn*-1 position. After stimulation of the cells with zymosan, both AA and AdA are released in large quantities, albeit AA is released to a greater extent. Finally, a variety of phosphatidylcholine and phosphatidylinositol molecular species contribute to AA; however, AdA is liberated exclusively from phosphatidylcholine species. Collectively, these results identify significant differences in the cellular utilization of AA and AdA by the macrophages, suggesting non-redundant biological actions for these two fatty acids.

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### 1. Introduction

In addition to the expression of arachidonic acid (AA)-metabolizing enzymes, availability of the fatty acid in free form is well established to constitute a limiting step for the biosynthesis of eicosanoids, a family of bioactive compounds with key roles in inflammation [1–3]. Cellular levels of free AA are controlled by two opposing reactions, i.e. phospholipid hydrolysis by one or several phospholipase  $A_2$  enzymes (PLA $_2$ ), and reacylation of the free fatty acid back into phospholipids by CoA-dependent acyltransferases [4]. In resting cells the reacylation reactions dominate, but in stimulated cells the dominant reaction is the PLA $_2$ -mediated deacylation step, which results in a dramatic increase in the levels of free AA, which is now available for eicosanoid synthesis [3–6]. It is now clear that calcium-dependent cytosolic group IVA PLA $_2\alpha$  (cPLA $_2\alpha$ ) is the critical enzyme in effecting the AA release [7–10] and that, depending on cell type and stimulation conditions, a secreted PLA $_2$  may also participate by amplifying the cPLA $_2\alpha$ -regulated response [11–17].

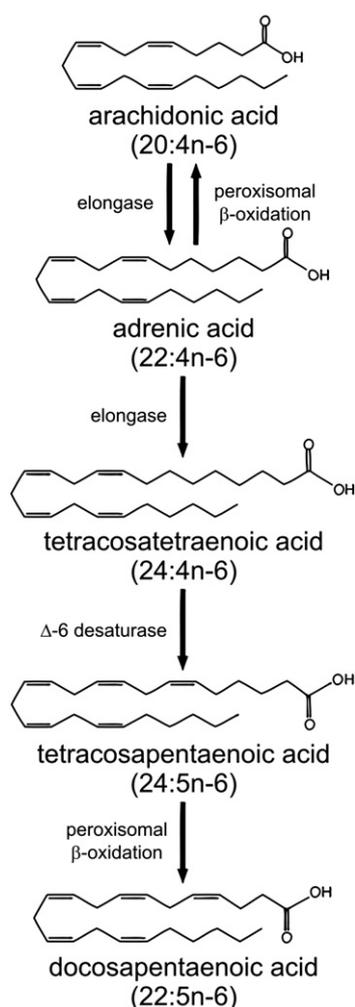
**Abbreviations:** AA, arachidonic acid; AdA, adrenic acid; PLA $_2$ , phospholipase  $A_2$ ; cPLA $_2\alpha$ , group IVA cytosolic phospholipase  $A_2$ ; PC, choline glycerophospholipid; PE, ethanolamine glycerophospholipid; PI, phosphatidylinositol

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Free AA can also be metabolized to adrenic acid (AdA, 22:4 $n$ –6) via 2-carbon elongation reactions catalyzed by specific elongases residing in the endoplasmic reticulum [18–20]. AdA in turn can be metabolized to other long-chain  $n$ –6 fatty acids [21] (Fig. 1). Similar to AA, AdA can also be oxygenated to form a variety of compounds collectively called dihomo-eicosanoids or docosanoids [22]. The initial assumption was that these oxygenated metabolites of AdA would function in a similar manner as their AA-derived counterparts but with somewhat lesser potency [22–25]. Thus AdA and its metabolites were envisioned as endogenous competitors of AA and the eicosanoids [22–26]. With the recent discovery of many more oxygenated metabolites of AdA it has become clear that, in some cases the AdA-derived docosanoids display the same or even higher potency as the AA-derived eicosanoids, or are produced at significantly higher quantities, suggesting that they may play specific biological roles on their own.

Abnormalities in AdA content have been described in several diseases such as Zellweger syndrome [27], schizophrenia [28] or Alzheimer disease [29], suggesting the involvement of this fatty acid and/or its metabolites in the development of these pathologies. It has also been reported that AdA (or, more precisely, AdA-containing phosphatidylethanolamine species) plays a role in mediating steroidogenesis in adrenal glands [30]. Like other polyunsaturated fatty acids, AdA is susceptible to free radical attack, and AdA-derived dihomo-isoprostanes have been suggested as possible selective markers of oxidative stress in the brain in Rett syndrome and Alzheimer disease [31,32]. Finally, studies with



**Fig. 1.** Biosynthetic pathway for AA-derived  $n-6$  fatty acids. The enzymes catalyzing each conversion are also given.

macrophage cell cultures have demonstrated that stimulation with bacterial endotoxin leads to the production of dihomoprostaglandin  $D_2$  at levels that are roughly similar to those of prostaglandin  $D_2$ , despite AdA being 5-fold less abundant than AA in these cells [33]. Collectively, these results have raised the possibility that cells and tissues may exhibit modes of regulating AdA-derived docosanoid production which differ from those regulating eicosanoid production.

While the processes of phospholipid fatty acid incorporation, remodeling and mobilization involving AA have been studied extensively [3–6,34–38], virtually no information exists on the cellular distribution and mobilization of phospholipid-bound AdA. In this work we have used a lipidomics approach to characterize AdA utilization in macrophages at the level of incorporation into and release from phospholipids. We have set up a cellular system that allows direct comparison with AA. Our data demonstrate that significant differences exist between the mechanism regulating AA and AdA availability in resting and receptor-stimulated macrophages. These differences suggest non-redundant biological actions for these two fatty acids that could be exploited to design strategies to control the production of AdA-derived products at the level of their precursor fatty acid.

## 2. Materials and methods

### 2.1. Reagents

Cell culture medium was from Lonza (Basel, Switzerland). Chloroform and methanol (HPLC grade) were from Fisher Scientific (Hampton,

NH). Phospholipid standards for mass spectrometry were purchased from Avanti (Alabaster, AL) or Cayman (Ann Arbor, MI). Silicagel thin-layer chromatography plates were from Macherey-Nagel (Düren, Germany). Pyrrophenone was synthesized and generously provided by Dr. Amadeu Llebaria (Institute for Chemical and Environmental Research, Barcelona, Spain). All other reagents were from Sigma-Aldrich.

### 2.2. Cell culture conditions

RAW 264.7 macrophage-like cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine at 37 °C in a humidified atmosphere of  $CO_2$ /air (1:19). For experiments, subconfluent cell monolayers were incubated with serum-free medium for 1 h before placing them in serum-free medium supplemented with AA for the indicated time. AA was added to the media in the form of complexes with fatty acid free-bovine serum albumin (Sigma-Aldrich) (2:1 molar ratio). The AA/albumin complexes were prepared as described elsewhere [39,40]. Afterward, the cells were washed three times with serum-free medium, and placed in serum-free medium for 1 h before addition of zymosan (1 mg/ml). When the cPLA $_2\alpha$  inhibitor pyrrophenone (1  $\mu$ M) was used [41,42], it was added to the incubation media 30 min before the addition of zymosan. After cellular stimulation, the cells were washed twice with PBS, scraped in ice-cold water and sonicated in a tip homogenizer twice for 15 s and prepared for their further analysis by mass spectrometry as described below.

Zymosan was prepared as described elsewhere [43]. Briefly, 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 at –20 °C. Zymosan aliquots were diluted in serum-free medium and sonicated before addition to the cells. No PLA $_2$  activity was detected in the zymosan batches used in this study, as assessed by *in vitro* activity assay [44–46].

Total cellular protein level was measured by the Bradford procedure [47] utilizing a commercial kit (BioRad Protein Assay). Protein levels did not significantly change over the course of the 16-h period of loading the cells with AA and subsequent zymosan exposure.

### 2.3. Gas chromatography/mass spectrometry analysis of fatty acid methyl esters

A cell extract corresponding to  $20 \times 10^6$  cells was used and, before the extraction and separation of lipid classes, internal standards were added. For choline glycerophospholipids (PC), 10 nmol of 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine was added; for ethanolamine glycerophospholipids (PE), 10 nmol of 1,2-diheptadecanoyl-glycero-3-phosphoethanolamine was added, and for phosphatidylinositol, 10 nmol of 1,2-dioctanoyl-glycero-3-phosphoinositol was added. Total lipids were extracted according to Bligh and Dyer [48], and the resulting lipid extract was separated into various fractions by thin-layer chromatography. Phospholipid classes (PC, PE, PI, phosphatidylserine) were separated with chloroform/methanol/acetic acid/acetone/water (45:25:7:4:2, by vol.), using plates previously impregnated with an aqueous solution of  $(NH_4)_2SO_4$  (0.4% w/v) [49]. Spots corresponding to each fraction were scraped and the lipids were extracted from the silica with 800  $\mu$ l methanol followed by 800  $\mu$ l chloroform/methanol (1:2, v/v), and 500  $\mu$ l chloroform/methanol (2:1, v/v). The extracts were transmethylated with 500  $\mu$ l of 0.5 M KOH in methanol for 30 min at 37 °C. 500  $\mu$ l of 0.5 M HCl was added to neutralize and fatty acid methyl esters were extracted twice with 1 ml of *n*-hexane.

Analysis of fatty acid methyl esters was carried out in a Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass-selective detector operated in electron impact mode (EI, 70 eV) equipped with an Agilent

7693 autosampler and an Agilent DB23 column (60 m length  $\times$  250  $\mu$ m internal diameter  $\times$  0.15  $\mu$ m film thickness) under the conditions described previously [50,51] with a slight modification of the procedure to improve separation of fatty acid methyl esters. Briefly, oven temperature was held at 50 °C for 1 min, then increased to 175 °C at a rate of 25 °C/min, then increased to 215 °C at a rate of 1.5 °C/min, and the final ramp being reached at 235 °C at a rate of 10 °C/min. The final temperature was maintained for 5 min, and the run time was 39.67 min. Data analysis was carried out with the Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00.

#### 2.4. High performance liquid chromatography/mass spectrometry analysis of AA- and AdA-containing phospholipid species

A cell extract corresponding to  $10^7$  cells was used for these analyses. The following internal standards were added: 600 pmol each of 1,2-dipentadecanoyl-*sn*-glycero-3-phosphocholine, 1,2-dilauroyl-*sn*-glycero-3-phospho-ethanolamine and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoinositol, before lipid extraction according to the method of Bligh and Dyer [48]. After evaporation of organic solvent under vacuum, the lipids were redissolved in 100  $\mu$ l of 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  $\mu$ m particle size, 250  $\times$  2.1 mm) (Sigma-Aldrich) protected with a Supelguard LC-18 (20  $\times$  2.1 mm) guard cartridge (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, v/v/v/v) and solvent B (methanol/*n*-hexane/32% ammonium hydroxide, 87.5:12:0.5, v/v/v). 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  $\mu$ l of 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 psi, dry gas to 7 l/min and dry temperature to 325 °C. PE and PI were detected in negative ion mode with the capillary current set at +3500 V over the initial 25 min as  $[M - H]^-$  ions. 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.

AA- and AdA-containing PI and PE species were identified by multiple reaction monitoring MS/MS experiments on chromatographic effluent by comparison with previously published data [52–55]. Cutoff parameter was set at  $m/z$  150 and fragmentation amplitude 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 [56]. For the identification of acyl chains of AA-containing PC species, ionization was carried out in negative mode with post-column addition of acetic acid at a flow rate of 100  $\mu$ l/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 mass spectrometry) [53,54,57].

#### 2.5. Statistical analysis

Assays were carried out in triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data shown are from representative experiments and are expressed as means  $\pm$  SE.

### 3. Results

#### 3.1. Conversion of AA to AdA in RAW 264.7 macrophages

Incubation of RAW 264.7 cells in serum-free medium with varying concentrations of AA resulted in the fatty acid being readily incorporated into cellular phospholipids. In parallel with AA incorporation, also observed was an increase in the phospholipid content of AdA (22:4n-6), the 2-carbon elongation product of AA (Fig. 2A). Interestingly, addition of fatty acid concentrations leading to saturation of phospholipid AA incorporation also ultimately led to saturation of phospholipid AdA incorporation, in a manner that the amount of both AA and AdA in phospholipids was nearly the same after 16 h (Fig. 2A). This circumstance provides us with a unique system to comparatively study the dynamics of mobilization of the two fatty acids under identical conditions.

Further elongation/desaturation of AdA by cells yields tetracosatetraenoic acid (24:4n-6), tetracosapentaenoic acid (24:5n-6), and docosapentaenoic acid (22:5n-6) (Fig. 1), and these three fatty acids were also detected in phospholipids at practically all concentrations of added exogenous AA, albeit at comparatively much lower levels than those of AA or AdA (Fig. 2B).

To ensure conditions under which the amount of AA and AdA in cellular phospholipids is similar, and thus allow for direct comparison of results, a concentration of 200  $\mu$ M exogenous AA was used for the subsequent experiments described here. It should be noted that this relatively large AA concentration was presented to the cells as a complex with BSA (ratio 2:1), which preserved cell viability and allowed fatty acid incorporation to proceed in the physiological manner. Under these conditions, 1% of the added AA was incorporated in phospholipids at the end of the incubations. The amount of AA present in phospholipids

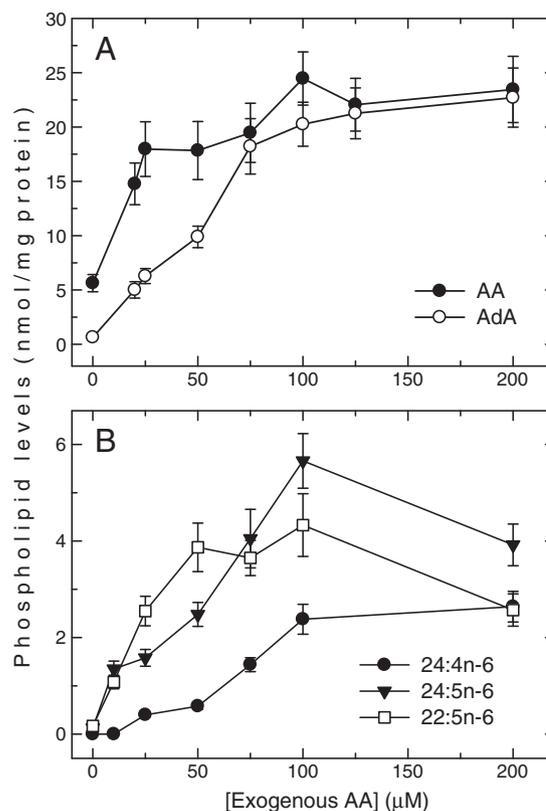


Fig. 2. Incorporation of AA and related *n*-6 fatty acids into phospholipids. The cells were incubated for 16 h with the indicated concentrations of AA (complexed with albumin at a 2:1 ratio) and the levels of phospholipids containing AA, AdA (A), 24:4n-6, 24:5n-6, and 22:5n-6 (B) were determined by gas chromatography/mass spectrometry.

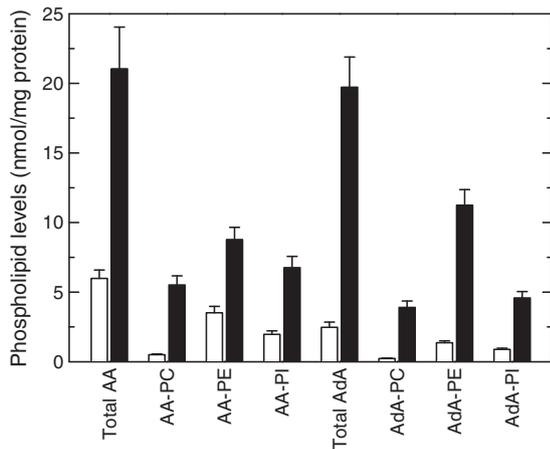
was  $2.9 \pm 0.2$  nmol per million cells ( $n = 4$ , mean  $\pm$  SEM), which is very similar to the physiological level of AA in the phospholipids of murine peritoneal macrophages ( $3.5 \pm 0.6$  nmol per million cells) [55].

Fig. 3 shows the distribution of AA and AdA among the various phospholipid classes in the exogenous AA-fed cells versus otherwise-untreated cells. It is remarkable that, in qualitative terms, the relative distribution of AA and/or AdA between phospholipid classes was very similar in the AA-fed cells versus the untreated cells, i.e. the rank order of AA and/or AdA content being PE > PI > PC, with PE comprising roughly 50% of all the AA or AdA present in phospholipids under the various different conditions.

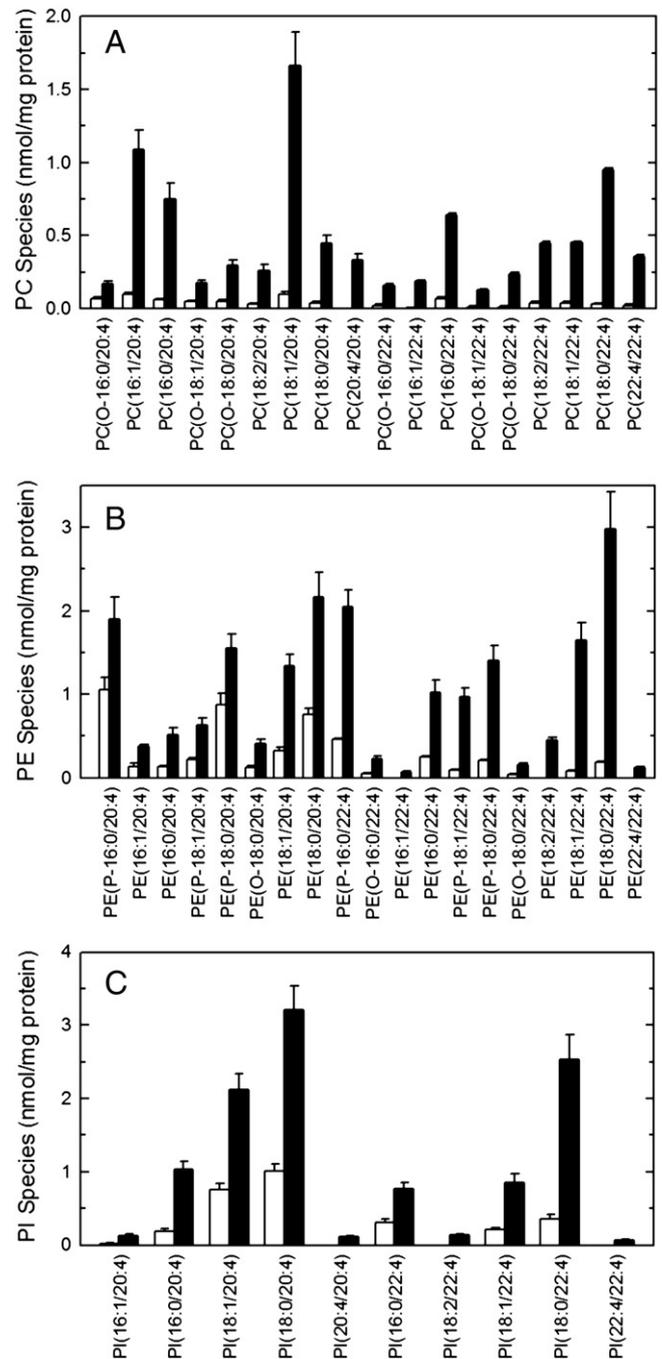
To further substantiate the above finding, a full lipidomic analysis of molecular species containing either AA or AdA was conducted. Fig. 4 shows the profile of AA- and AdA-containing phospholipids of RAW 264.7 cells treated or not with exogenous AA. In keeping with the results of Fig. 3, the profile of AA-containing phospholipids in control versus AA-fed cells was similar from a qualitative point of view, i.e. the major AA-containing species in untreated cells were also the major ones in the AA-fed cells. These were, in order of abundance: PI(18:0/20:4), PE(18:0/20:4), PI(18:1/20:4), PE(P-16:0/20:4), PE(P-18:0/20:4), PC(18:1/20:4), PE(18:1/20:4), and PC(16:1/20:4) (Fig. 4). With regard to the distribution of AdA in the AA-fed cells, a preference was found for the incorporation of AdA into phospholipids containing stearic acid (18:0) in the *sn*-1 position of the glycerol backbone, regardless of the headgroup. PE(18:0/22:4) and PI(18:0/22:4) were, by far, the richest AdA-containing phospholipids in the AA-fed cells, and high amounts of PC(18:0/22:4) were detected as well (Fig. 4). Collectively, these results highlight a significant difference between AA and AdA with respect to their features of incorporation into cellular phospholipids. While the profile of distribution of AA among phospholipid classes and molecular species in AA-fed cells versus untreated cells is preserved, there is a tendency for AdA to accumulate in species containing stearic acid in the *sn*-1 position in the AA-fed cells. This preference was not manifested in the untreated cells, possibly due to their low AdA content.

### 3.2. Stimulus-induced mobilization of AA and AdA

RAW 264.7 cells, containing similar amounts of AA and AdA esterified in phospholipids by prolonged incubation with exogenous AA (Fig. 2A), were challenged with yeast-derived zymosan, a potent inducer of the AA mobilization response in murine macrophages [58,59], and total AA mobilized from cellular phospholipids was analyzed by gas chromatography/mass spectrometry. Despite the cells containing similar amounts of AA and AdA esterified in phospholipids, the liberation of AA was always



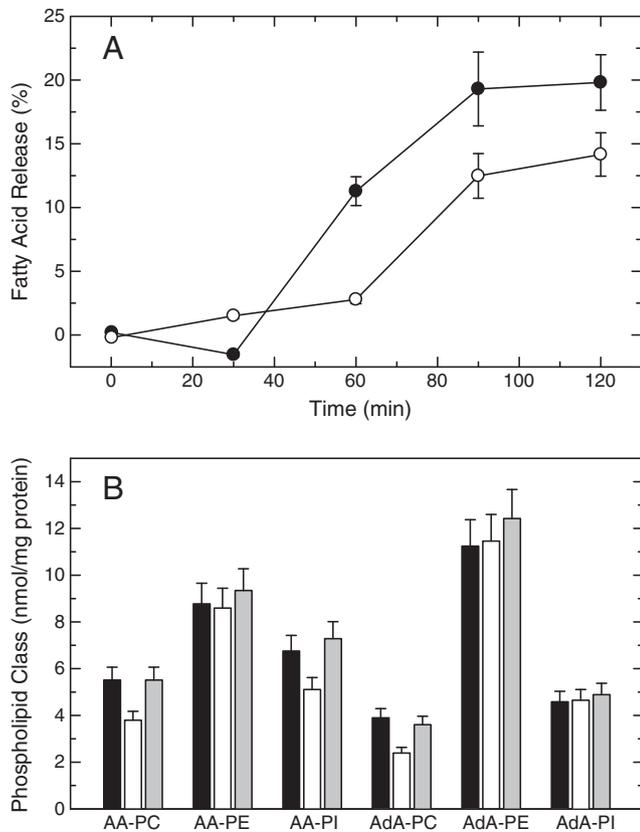
**Fig. 3.** Distribution of AA and AdA among phospholipid classes. The distribution of AA and AdA among PC, PE and PI in untreated cells (open bars) or cells preincubated for 16 h with 200  $\mu$ M exogenous AA (complexed with albumin at a 2:1 ratio) (black bars) was determined by gas chromatography/mass spectrometry.



**Fig. 4.** AA-containing phospholipid species in RAW 264.7 cells. The profile of AA- or AdA-containing PC (A), PE (B), or PI (C) species in untreated cells (open bars) or cells preincubated for 16 h with 200  $\mu$ M exogenous AA (black bars) was determined by liquid chromatography/mass spectrometry.

higher than that of AdA, albeit both processes showed similar dependence with time (Fig. 5A). The zymosan-stimulated mobilization of the two fatty acids was completely abrogated by the selective cPLA<sub>2</sub> $\alpha$  inhibitor pyrrophenone at concentrations as low as 1  $\mu$ M (Fig. 5B), indicating that cPLA<sub>2</sub> $\alpha$  is responsible not only for the mobilization of AA, but also of AdA during cellular activation. No significant mobilization of any of the AdA elongation/desaturation products tetracosatetraenoic acid (24:4n-6), tetracosapentaenoic acid (24:5n-6), and docosapentaenoic acid (22:5n-6) could be detected (data not shown).

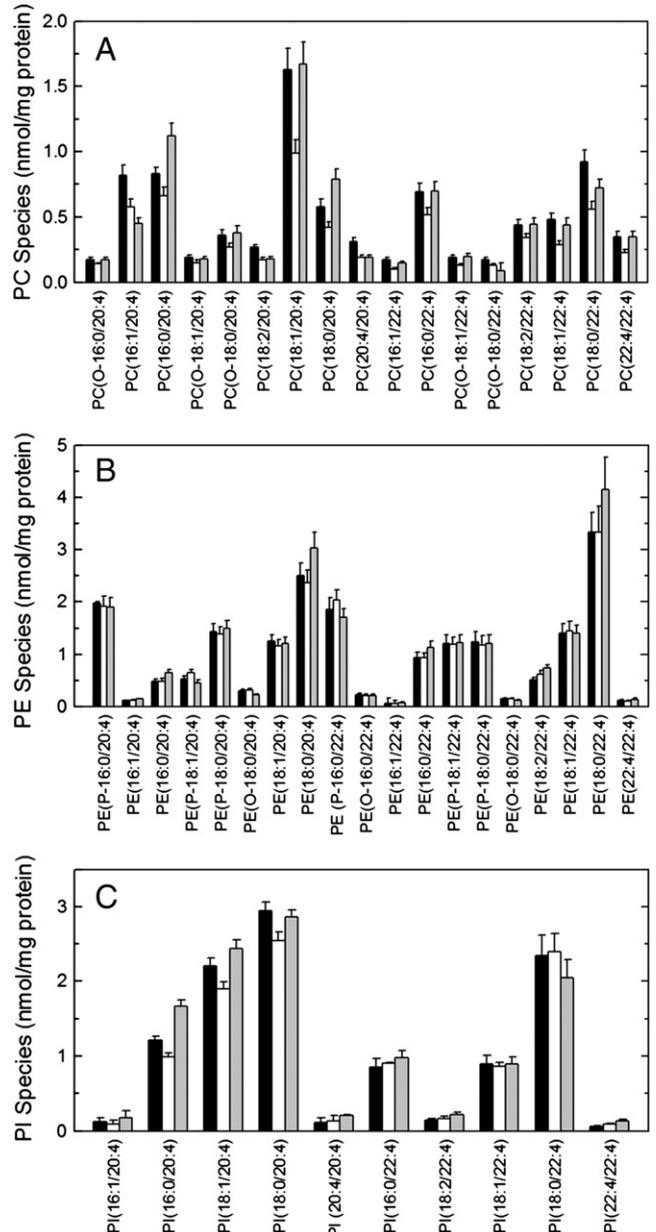
Fig. 5B also shows the phospholipid classes that originated the free AA and AdA that were liberated after zymosan stimulation. In keeping with previous reports using zymosan-stimulated human monocytes,



**Fig. 5.** AA and AdA mobilization in zymosan-stimulated RAW 264.7 macrophages. (A) The cells, preincubated with exogenous AA for 16 h, were stimulated with 1 mg/ml zymosan for the indicated times. Afterward, total cellular content of AA (closed symbols) or AdA (open symbols) in phospholipids was measured by gas chromatography/mass spectrometry. The fatty acid release was calculated by subtracting the amount of phospholipid-bound AA or AdA in stimulated cells at each time from that in unstimulated cells (zero time). (B) The amount of AA or AdA in PC, PE and PI classes in unstimulated cells (black bars), and zymosan-stimulated cells for 1 h in the absence (open bars) or presence (gray bars) of 1  $\mu$ M pyrrophenone was determined by gas chromatography/mass spectrometry. When pyrrophenone was used, it was added 30 min before the zymosan stimulation period.

murine macrophages and RAW 264.7 macrophage-like cells [54,55,60], PC and PI, but not PE, behaved as sources of released AA during zymosan stimulation. Strikingly, AdA release appears to have arisen only from PC, since this was the only phospholipid class that showed a significant decrease after the zymosan challenge (Fig. 5B).

To characterize further the phospholipid sources involved in AA and AdA release in response to zymosan, we conducted a full lipidomic analysis of molecular species to determine the changes in the levels of those containing either AA or AdA (Fig. 6). Essentially all AA-containing PC and PI species experienced dramatic decreases after zymosan stimulation, which were prevented by the presence of the selective cPLA<sub>2</sub> $\alpha$  inhibitor pyrrophenone. This provides unambiguous evidence that such losses followed from cPLA<sub>2</sub> $\alpha$  activation. One notable major exception to the latter was PC(16:1/20:4), the levels of which were not recovered in the presence of pyrrophenone (Fig. 6A). Also, the zymosan-stimulated hydrolysis of two other minor species, namely PC(20:4/20:4) and PC(18:2/20:4) was not prevented by pyrrophenone. It is possible that these particular species may break down via mechanisms not involving cPLA<sub>2</sub> $\alpha$  and hence, in a manner not inhibitable by pyrrophenone. Another possibility is that these particular phospholipid species are hydrolyzed primarily by a PLA<sub>2</sub> distinct from cPLA<sub>2</sub> $\alpha$ , e.g. group V sPLA<sub>2</sub>, which is known to be expressed in macrophages and to participate in AA release during cell stimulation [14,17,61,62]. On the other hand, no AA-containing PE species decreased significantly as a consequence of treating the cells with zymosan (Fig. 6B).



**Fig. 6.** Changes in AA- and AdA-containing species after zymosan-stimulation. RAW 264.7 cells, preincubated with exogenous AA for 16 h, were either untreated (black bars) or treated with 1 mg/ml zymosan for 1 h in the absence (open bars) or presence (gray bars) of 1  $\mu$ M pyrrophenone. Phospholipid molecular species containing either AA or AdA were determined by liquid chromatography/mass spectrometry. When pyrrophenone was used, it was added 30 min before the zymosan stimulation. (A) PC species; (B) PE species; (C) PI species.

Regarding AdA-containing phospholipids, all PC molecular species experienced significant losses of this fatty acid which, with the exception of PC(O-18:0/22:4), were fully prevented by the pyrrophenone treatment (Fig. 6). In contrast, no changes were detected in any of the AdA-containing PE or PI molecular species.

#### 4. Discussion

The cellular mechanisms that underlie the formation of phospholipid pools of AA, i.e. routes of phospholipid fatty acid incorporation and remodeling, as well as their differential mobilization during stimulus-response coupling have been extensively studied over the years [3–6,34–38]. In contrast, practically nothing is known on the mechanisms regulating AdA availability in cells, despite this fatty acid being

also established as the precursor of a family of biologically-active compounds as diverse as that of the AA-derived eicosanoids. Conversion of AA into AdA has been recently described in RAW 264.7 macrophages [33]; however, the lipid pools to which the fatty acid incorporates and from which the fatty acid is mobilized following cell activation, and the molecular species involved have not been documented. In this work we present a full lipidomic analysis of AdA-containing phospholipids which defines both the phospholipid acceptors for fatty acid incorporation and the phospholipid molecular sources for fatty acid mobilization. Moreover, we have compared the cellular utilization of AdA-containing phospholipids with that of AA-containing phospholipids and found significant differences that suggest that separate and selective mechanisms may exist for regulating AA and AdA availability in macrophages.

As cells in culture, RAW 264.7 macrophages are typically deficient in essential polyunsaturated fatty acids, which results in an enrichment of their membrane phospholipids with mead acid (20:3n-9), produced by elongation/desaturation of oleic acid (18:1n-9) [63]. In keeping with previous estimates, these cells avidly incorporate AA from exogenous sources and incorporate it into various phospholipid molecular species [64,65]. It is notable that supplementation of these cells with AA typically results in the almost complete displacement of mead acid – and also of a large portion of oleic acid – from phospholipids, which is diverted to triacylglycerol formation (C. Guijas and J. Balsinde, unpublished observations).

Importantly, the profile of distribution of the exogenous AA among phospholipid classes in the RAW 264.7 cells (i.e. PC, PE and PI) is essentially the same as that found in the otherwise untreated cells. This is a striking finding, since it shows that the affinity of the various phospholipid classes for AA does not depend on the relative concentration of available fatty acid. Rather, the pools of AA-containing phospholipids may be expanded to accommodate the fatty acid incorporated from exogenous sources in a manner that, under equilibrium conditions, the relative proportions of AA among the various phospholipid classes are kept relatively constant.

Also striking is the finding that a very significant part of the AA incorporated by the cells is converted to AdA, which is in turn efficiently incorporated into phospholipids. We have measured a concentration-dependent linear increase of AdA incorporation into phospholipids within the 0–100  $\mu\text{M}$  range of added exogenous AA. At higher concentrations of exogenous AA (100–200  $\mu\text{M}$ ), AdA incorporation into phospholipids proceeds at a much slower rate, indicating saturation of AdA esterification. Note that saturation of AA incorporation into RAW 264.7 cell phospholipids is reached much earlier, i.e. at an exogenous AA concentration of around 50  $\mu\text{M}$ . The finding that AdA incorporation into phospholipids takes place at a high rate even after saturation of AA incorporation was reached, clearly suggests that the metabolic fates of the two fatty acids are different. Thus, these data do not support a scenario whereby the increased incorporation of AdA into the *sn*-2 position of phospholipids merely serves to compete with that of AA, thereby reducing the amount of ‘mobilizable’ AA and hence limiting eicosanoid synthesis [23,29]. If that was the case, we would have expected a reduction in the levels of phospholipid-bound AA with increased AdA incorporation, which does not occur.

In addition, our findings applying a lipidomics approach highlight significant differences between the molecular species distribution and mobilization of AA and AdA-containing phospholipids, which are again more consistent with non-redundant roles for these two fatty acids. A conspicuous difference between the profiles of AA and AdA incorporation is the preference of the latter to incorporate into phospholipids containing stearic acid (18:0) at the *sn*-1 position. This suggests that cells contain acyltransferases utilizing stearic acid-containing lysophospholipid acceptors that recognize and incorporate AdA with high affinity.

Previous studies have identified biochemical pathways in phagocytic cells for the formation and degradation of two diarachidonoylated

phospholipids, namely PC(20:4/20:4) and PI(20:4/20:4), under various experimental conditions [53,54,66]. These two phospholipids and their two diarachidonoylated analogs have been identified as well in this study in the AA-enriched RAW 264.7 cells. However, to our knowledge the analogous species PE(20:4/20:4) has never been identified; thus it seems most remarkable that the diarachidonoylated species PE(22:4/22:4) is also readily detected in the AA-enriched RAW 264.7 macrophages. Whether the appearance of PE(22:4/22:4) bears any biological significance will be the subject of future studies.

Experiments with zymosan-activated cells have demonstrated that both AA- and AdA-containing phospholipids are hydrolyzed via a cPLA<sub>2</sub> $\alpha$ -mediated mechanism to generate free AA and AdA with similar time-courses. This is a significant finding, since a previous study that examined the release of the two fatty acids in thrombin-stimulated endothelial cells failed to detect significant mobilization of AdA under conditions in which AA was abundantly liberated [26]. Despite the fact that the cells used in our experiments contained nearly the same amounts of AA and AdA esterified in phospholipids, AA release in response of zymosan was always higher than that of AdA (20% versus 14% of total phospholipid fatty acid content at 120 min). This circumstance may just reflect the higher affinity of cPLA<sub>2</sub> $\alpha$  for AA-containing phospholipids. However, it is also possible that the AA pools within the cell are more accessible to the hydrolytic action of cPLA<sub>2</sub> $\alpha$  than the AdA pools. In this regard, our lipidomic analyses have highlighted a very noticeable difference between the phospholipid pools that are used as sources for AA and AdA release after zymosan activation. In keeping with previous data utilizing zymosan-stimulated murine peritoneal macrophages and human monocytes [54,55], a number of PC and PI molecular species, but not of PE, acted as donors of the AA released after cell activation. These include PC(18:1/20:4), PC(16:1/20:4), PC(16:0/20:4), PC(18:0/20:4), PC(O-18:0/20:4), PC(18:0/20:4), PI(18:0/20:4), PI(18:1/20:4), and PI(16:0/20:4). As discussed elsewhere [54,55,60], the lack of AA mobilization from PE probably reflects the activation of CoA-independent transacylases, which rapidly restore the levels of AA in PE at the expense of other AA sources, i.e. PC. Regarding the sources for AdA release, only PC species were found to contribute. No molecular species of PE or PI were found to change their AdA content after cell activation. While the apparent lack of AdA release from PE could be explained by the involvement of CoA-independent transacylation reactions in an analogous manner to the situation described above for AA, to the best of our knowledge, no transacylation reactions utilizing lysoPI as acceptors have been described in intact cells. Thus the results suggest that AdA-containing PI is spared from cPLA<sub>2</sub> $\alpha$  hydrolysis. Since cPLA<sub>2</sub> $\alpha$  recognizes the fatty acid esterified in the *sn*-2 position with little or no regard for the chemical nature of the *sn*-3 substituent [67], and AdA-containing PC is indeed hydrolyzed, the most logical explanation for these findings is that, unlike AA-containing PI, the AdA-containing PI pools are not accessed by the enzyme. This in turn implies that differential compartmentalization of PI phospholipids depending on their fatty acid composition occurs in the macrophages. Thus incorporation of AdA into a stable pool may serve a role(s) in cell physiology distinct from serving as a cPLA<sub>2</sub> $\alpha$  substrate for the ulterior synthesis of bioactive lipid mediators.

Macrophages and macrophage-cell lines are known to respond to a wide variety of stimuli by releasing AA [3]. While the current studies have been carried out utilizing only zymosan as a cell stimulant, it appears likely that AdA mobilization may also be a common feature of a variety of stimuli acting on different receptors. In this regard, zymosan is recognized by cells via multiple different receptors, which suggests that the zymosan-triggered phospholipid hydrolysis is a complex response that may be specific for this kind of stimulus. Studies are currently in progress with defined stimuli that activate the cells by engaging only a specific set of receptors [17] to address whether specific and particular lipid profiles for AA and/or AdA mobilization exist that may help define specific activation states of the macrophage.

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