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Calcium-independent phospholipase A_2-mediated formation of 1,2-diarachidonoyl-glycerophosphoinositol in monocytes

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Phagocytic cells exposed to exogenous arachidonic acid (AA) incorporate large quantities of this fatty acid into choline and ethanolamine glycerophospholipids, and into phosphatidylinositol (PtdIns). Utilizing liquid chromatography coupled to MS, we have characterized the incorporation of exogenous deuterated AA ([^{2}H]AA) into specific PtdIns molecular species in human monocyte cells. A PtdIns species containing two exogenous [^{2}H]AA molecules (1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol) was readily detected when human U937 monocyte-like cells and peripheral blood monocytes were exposed to [^{2}H]AA concentrations as low as 160 nM to 1 μM. Bromoenol lactone, an inhibitor of Ca^{2+}-independent phospholipase A_2 (iPLA$_2$), diminished lyso-PtdIns levels, and almost completely inhibited the appearance of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol, suggesting the involvement of deacylation reactions in the synthesis of this phospholipid. *De novo* synthesis did not appear to be involved, as no other diarachidonoyl phospholipid or neutral lipid was detected under these conditions. Measurement of the metabolic fate of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol after pulse-labeling of the cells with [^{2}H]AA showed a time-dependent, exponential decrease in the level of this phospholipid. These results identify 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol as a novel, short-lived species for the initial incorporation of AA into the PtdIns class of cellular phospholipids in human monocytes.

Arachidonic acid (AA) is the precursor of a family of compounds, collectively called the eicosanoids, with key roles in inflammation [1]. AA is an intermediate of a deacylation–reacylation cycle of membrane phospholipids, the Lands pathway, in which the fatty acid is cleaved by phospholipase A_2 (PLA$_2$) enzymes, and reincorporated by CoA-dependent acyltransferases [2–4]. In resting cells, reacylation dominates, and hence the bulk of cellular AA is found in esterified form. In stimulated cells, the dominant reaction is the PLA$_2$-mediated deacylation, which results in dramatic releases of free AA that is then available for eicosanoid synthesis [5–9]. However, under activation conditions, AA reacylation is still very significant, as manifested by the fact that only a minor fraction of the AA released by PLA$_2$ is available for eicosanoid synthesis, and the remainder is effectively incorporated back into phospholipids by acyltransferases.

The pathways for AA incorporation into and remodeling between various classes of glycerophospholipids

Abbreviations
AA, arachidonic acid; BEL, bromoenol lactone; cPLA$_2$, calcium-dependent cytosolic phospholipase A_2 (group IV); iPLA$_2$, calcium-independent phospholipase A_2 (group VI); LC, liquid chromatography; PC, choline glycerophospholipid; PE, ethanolamine glycerophospholipid; PLA$_2$, phospholipase A_2; PtdIns, phosphatidylinositol.

have been described in detail in inflammatory cells [3]. Two distinct pathways exist for the initial incorporation of AA. The first one is a high-affinity pathway that incorporates low concentrations of AA into phospholipids via direct acylation reactions catalyzed by CoA-dependent acyltransferases. This is thought to be the major pathway for AA incorporation into phospholipids under physiological conditions [3]; thus, the PLA_2-dependent availability of lysophospholipid acceptors may constitute a critical regulatory factor [4,10–12]. The second pathway operates at high levels of free AA, and leads to the incorporation of the fatty acid primarily via the *de novo* route for phospholipid biosynthesis, resulting ultimately in the accumulation of AA into triacylglycerols and diarachidonoyl phospholipids [3]. This 'high-capacity, low-affinity' pathway is thought to primarily operate after the high-affinity deacylation–reacylation pathway has been saturated due to the high AA concentrations [3].

Once the AA has been incorporated into phospholipids, a remodeling process carried out by CoA-independent transacylase transfers AA from choline glycerophospholipids (PCs) to ethanolamine glycerophospholipids (PEs). In inflammatory cells, a major consequence of these CoA-independent transacylase-driven remodeling reactions is that, despite PCs being the preferred acceptors for exogenous AA, under equilibrium conditions AA is more abundant in PEs than in PCs [3].

Whereas the AA incorporation and remodeling reactions involving PCs and PEs have been the subject of numerous studies, much less attention has been paid to the incorporation of AA into phosphatidylinositol (PtdIns). PtdIns generally incorporates less AA from exogenous sources than PCs or PEs, and, compared to AA-containing PCs or PEs, the levels of AA-containing PtdIns species vary little after the initial AA incorporation step has been completed [13–17].

Utilizing HPLC coupled to ion-trap ESI-MS, we have characterized the incorporation of AA into the various molecular species of PtdIns in human U937 monocyte-like cells and peripheral blood monocytes. Unexpectedly, we have found that the unusual species 1,2-diarachidonoyl-*sn*-glycero-3-phosphoinositol behaves as a significant acceptor of exogenous AA under physiologically relevant conditions (nanomolar levels of free fatty acid). Our studies describe a novel route for phospholipid AA incorporation at low AA concentrations that involves the direct acylation of both the *sn*-1 and *sn*-2 positions of PtdIns.

Results

Initial incorporation of [^{2}H]AA into PtdIns

When monocyte cells are exposed to exogenous AA (1 μM), approximately 20% of the incorporated fatty acid is found in PtdIns [4,17]. To unequivocally identify [^{2}H]AA-containing phospholipid species, two necessary criteria were taken into account. The first criterion was the different *m/z* signal shape produced by a deuterated species versus the one elicited by its nondeuterated counterpart. When free [^{2}H]AA was directly analyzed by MS, a bell-shaped set of peaks with a maximum at *m/z* 311 was observed, due to the presence of various isotopomers (Fig. 1A). The signal produced by native AA was very different, showing a decay from a maximum at *m/z* 303 (Fig. 1B). Thus, [^{2}H]AA-containing phospholipids must show a bell-shaped isotopic distribution with a maximum at $+8$ *m/z* apart from their native counterparts, due to the [^{2}H]AA isotopomers. The second criterion was the formation of characteristic daughter ions in MS/MS experiments, which were carried out in negative ion mode. When the most abundant isotopomer of a given species was fragmented, both the detection of *m/z* 311 ions from released [^{2}H]AA and the presence of the inositol ring in the daughter ions were considered to be evidence of the presence of an [^{2}H]AA-containing PtdIns in the sample.

With regard to C18 chromatography, we found that both the sum of acyl chain length and decreasing number of double bonds augmented the retention time of phospholipids. In addition, we found that when native and exogenous phospholipids were present, the retention time of the [^{2}H]AA-containing species was slightly shortened as compared to the retention time of the endogenous compound. This behavior has also been documented for [^{2}H]AA-labeled prostaglandins in C18 column chromatography [18].

Five PtdIns molecular species were found to initially incorporate [^{2}H]AA when U937 cells were exposed to low AA concentrations (1 μM). Three of these were identified, as 1-palmitoyl-2-[^{2}H]AA-glycero-3-phosphoinositol, 1-oleoyl-2-[^{2}H]AA-glycero-3-phosphoinositol, and 1-stearoyl-2-[^{2}H]AA-glycero-3-phosphoinositol (Fig. 2). Two unexpected species that coeluted at 5.0 min were detected as two groups of isotopomers at *m/z* 913.5 and *m/z* 920.6 (Fig. 3A). Fragmentation of *m/z* 913.5 (Fig. 3B) gave characteristic phosphoinositol ions at *m/z* 223, *m/z* 241 and *m/z* 297. Acyl chain fragments at *m/z* 303 and *m/z* 311 were attributed to endogenous AA and exogenous [^{2}H]AA, in accordance with the isotopic distribution of the mass spectra.

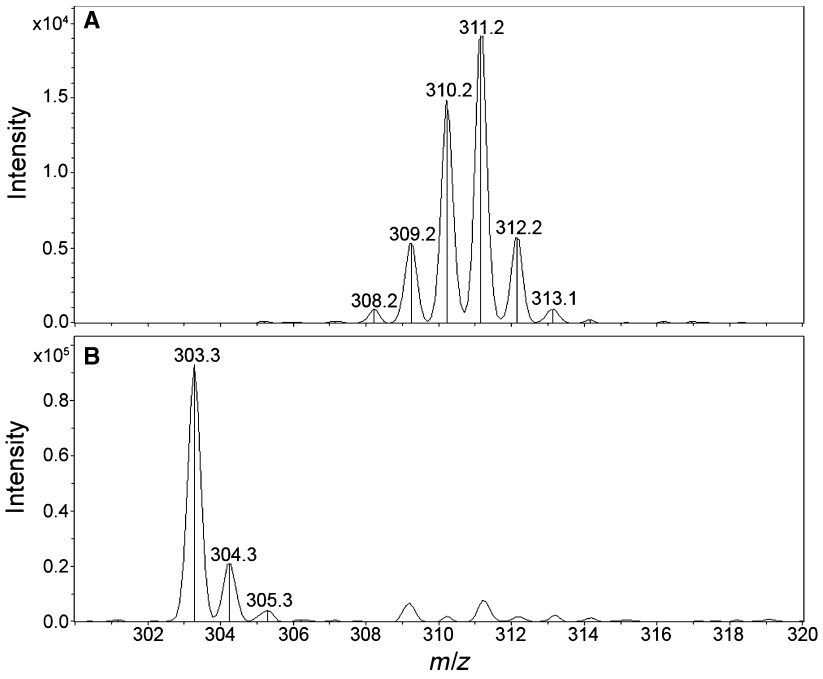

Fig. 1. Detection of AA by MS. [^{2}H]AA (A) or naturally occurring AA (B) were injected directly into the mass spectrometer.

Moreover, due to the increased intensity of the fragment corresponding to the neutral loss of the *sn*-2 acyl chain [19], we identified the species containing the exogenous [^{2}H]AA in the *sn*-1 position (the ion intensity of the fragment at *m/z* 609 was greater than the intensity of the fragment at *m/z* 601). Thus, the group of isotopomers at *m/z* 913.5 was identified as 1-[^{2}H]AA-2-AA-glycero-3-phosphoinositol (Fig. 3B).

Fragmentation of *m/z* 920.6 also yielded the characteristic phosphoinositol fragments at *m/z* 223, *m/z* 241, and *m/z* 297, along with a fragment at *m/z* 311 corresponding to the acyl chains (Fig. 3C). As this *m/z* could derive from [^{2}H]AA but also from arachidic acid, the observed isotopic distribution was compared with the calculated isotopic distribution of a PtdIns containing either acyl chain, namely di[^{2}H]arachidonoyl or arachidyl-[^{2}H]arachidonoyl. As shown in Fig. 3D, the observed isotopic distribution closely matches the one calculated for 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol. Theoretical isotopic distributions were calculated by computing the isotopic distribution of the glycerophosphoinositol moiety, and calculating afterwards how this isotopic distribution would be modified by the presence of either one or two arachidonoyl substituents. The SIMULATED PATTERN tool of the DATA ANALYSIS software from Bruker Daltonics S.A. was used for these calculations.

To confirm that the production of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol by U937 cells was physiologically meaningful, studies were also carried out with human peripheral blood monocytes exposed to 1 μM [^{2}H]AA. The results, shown in Fig. 4, indicated that monocytes indeed produce significant quantities of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol under these conditions (set of peaks with a maximun at *m/z* 920.6). The PtdIns species containing both a [^{2}H]AA and a natural AA was also readily detected in blood monocytes (set of peaks with a maximum at *m/z* 913.6) (Fig. 4).

Interestingly, 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol was also readily detected when the analyses of AA incorporation into PtdIns were carried out in cells exposed to very low levels of exogenous ^{2}H-labeled fatty acid, i.e. 160 nM (data not shown). These data strongly suggest that synthesis of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol proceeds via the high-affinity pathway of direct reacylation of phospholipids, not via *de novo* synthesis.

Effect of PLA_2 inhibitors on the incorporation of exogenous [^{2}H]AA into PtdIns

To directly study the role of deacylation–reacylation reactions in the incorporation of AA into PtdIns, we conducted experiments in the presence of the well-established PLA_2 inhibitors pyrrophenone (1 μM), an

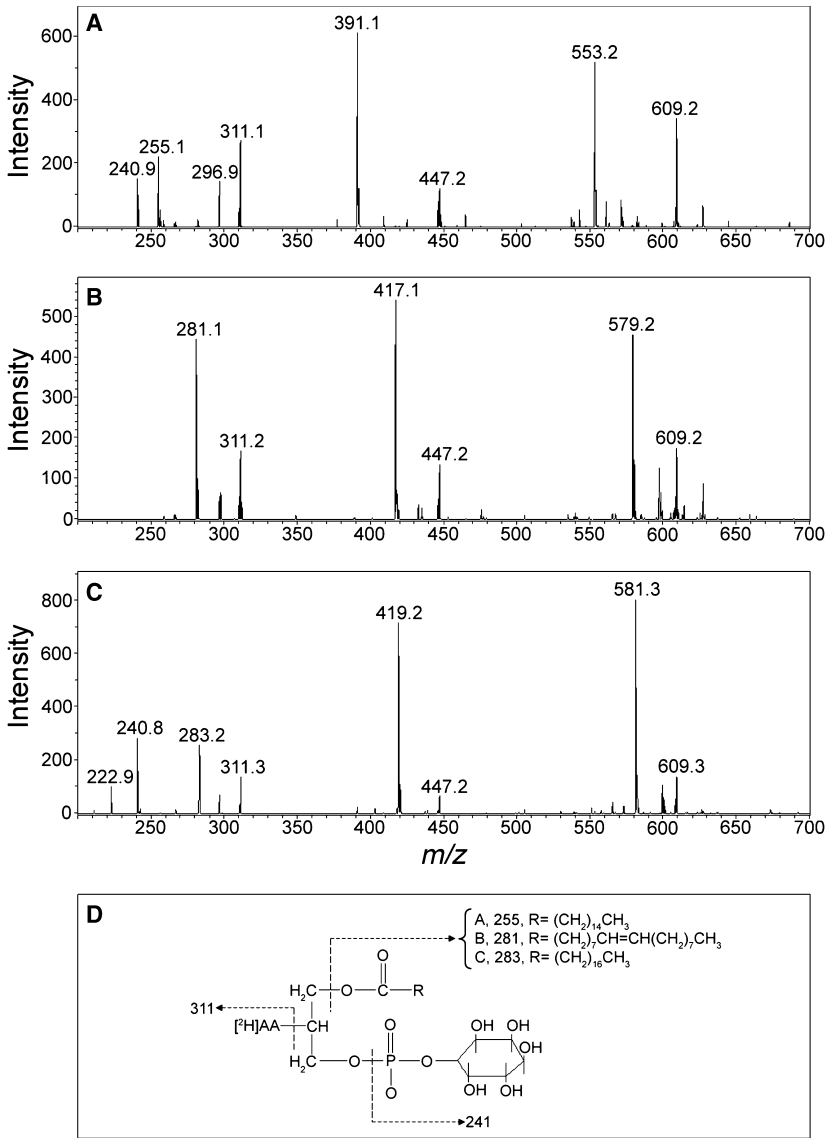

Fig. 2. Identification of common [^{2}H]AA-containing PtdIns species in U937 cells. The cells were exposed to 1 μM [^{2}H]AA for 30 min. [^{2}H]AA-containing PtdIns species were then analyzed by LC/MS. (A) 1-Palmitoyl-2-[^{2}H]AA-glycero-3-phosphoinositol. (B) 1-Oleoyl-2-[^{2}H]AA-glycero-3-phosphoinositol. (C) 1-Stearoyl-2-[^{2}H]AA-glycero-3-phosphoinositol. (D) Chemical structures and MS/MS ion fragmentation of the identified PtdIns species.

inhibitor of group IV calcium-dependent cytosolic PLA_2 ($cPLA_2$) [20,21], and bromoenol lactone (BEL, 10 μM), an inhibitor of group VI calcium-independent PLA_2 ($iPLA_2$) [22,23]. We have previously shown that, at the concentrations utilized in this study, both pyrrophenone and BEL quantitatively inhibit cellular $cPLA_2$ and $iPLA_2$ activities, respectively [24–29]. Figure 5 shows that, whereas pyrrophenone had no inhibitory effect on any of the five PtdIns species incorporating [^{2}H]AA, BEL exerted dramatic inhibitory effects on most of them, particularly on 1-[^{2}H]AA-2-AA-glycero-3-phosphoinositol and 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol, which almost completely disappeared in the presence of BEL. Collectively, these data suggest the involvement of $iPLA_2$ but not $cPLA_2$ in [^{2}H]AA incorporation into PtdIns molecular species.

Analysis of lyso-PtdIns levels

In previous studies, we have shown that BEL is capable of decreasing the steady-state levels of lyso-PC in $P388D_1$ macrophage-like cells, an event that paralleled the inhibition of AA incorporation into phospholipids [10,11,30–32]. Given the above data showing that BEL blocks [^{2}H]AA incorporation into PtdIns species, we reasoned that BEL, if acting via $iPLA_2$ inhibition, would also reduce cellular lyso-PtdIns levels. Accord-

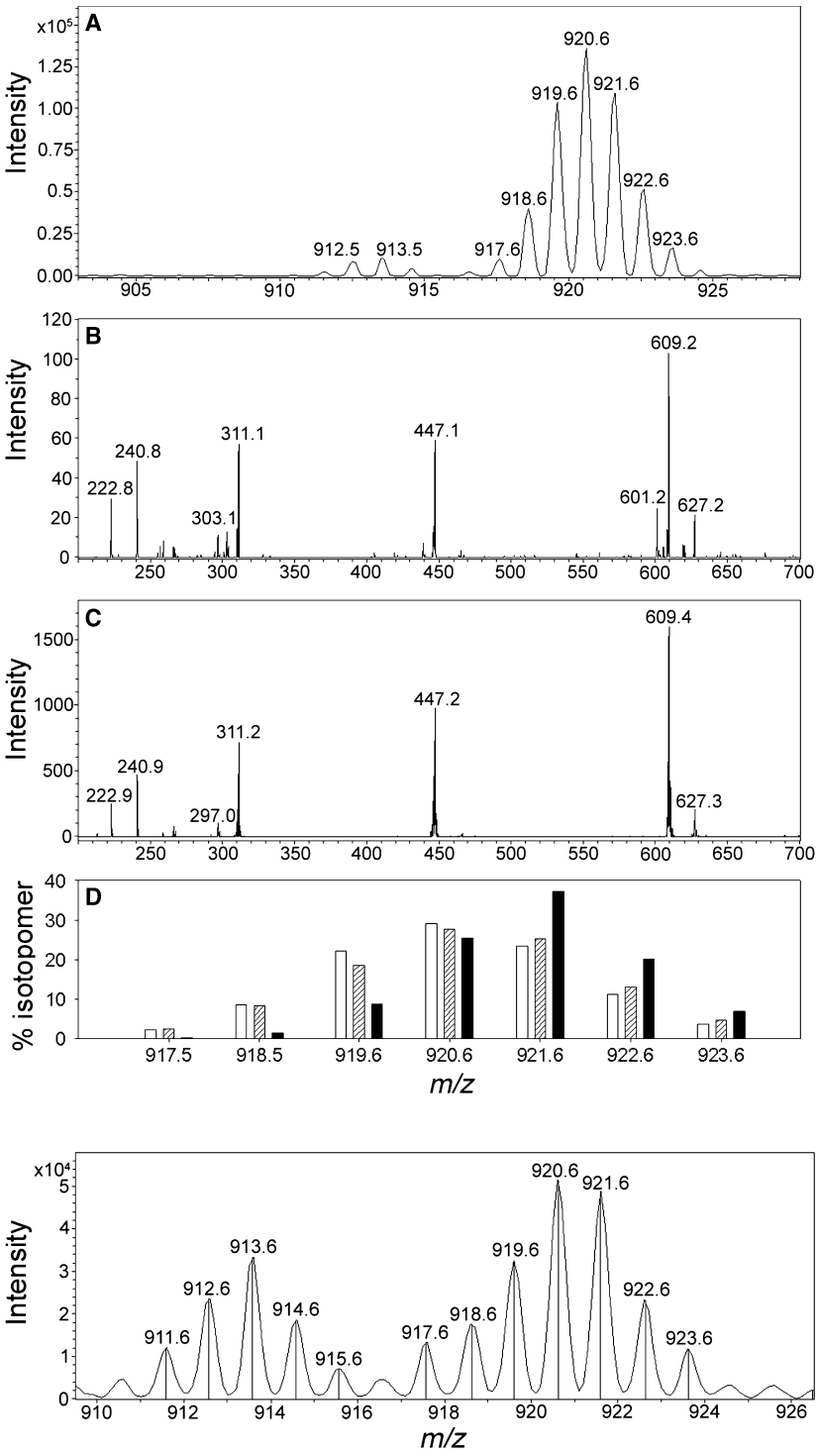

Fig. 3. Identification of unexpected [^{2}H]AA-containing PtdIns species in U937 cells. The cells were exposed to 1 μM [^{2}H]AA for 30 min. [^{2}H]AA-containing PtdIns species were then analyzed by LC/MS. (A) Isotopic distribution of two species that coeluted from the column. (B) Daughter ions produced after fragmentation of the peak at *m/z* 913.5. (C) Daughter ions produced after fragmentation of the peak at *m/z* 920.6. (D) Comparison between the experimental isotopomer distribution of the compound with maximum at *m/z* 920.6 (open bars) and the calculated distributions for di[^{2}H]AA-PtdIns (hatched bars) and arachidyl-[^{2}H]arachidonyl-PtdIns (black bars).

Fig. 4. Detection of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol in human monocytes. Human monocytes were exposed to 1 μM [^{2}H]AA for 30 min. 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol (set of peaks with a maximum at *m/z* 920.6) and 1-[^{2}H]AA-2-AA-glycero-3-phosphoinositol (set of peaks with a maximum at *m/z* 913.6) were then detected by LC/MS.

ingly, a comparative study of the lyso-PtdIns species present in resting cells versus cells treated with BEL was carried out. The results are shown in Table 1, and indicate that BEL induced statistically significant decreases in the cellular levels of oleoyl-containing and stearoyl-containing lyso-PtdIns.

Detection of diarachidonoyl phospholipids and neutral lipids

Detection of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol at low levels of exogenous [^{2}H]AA (up to 1 μM) was a somewhat unexpected finding, as generation of

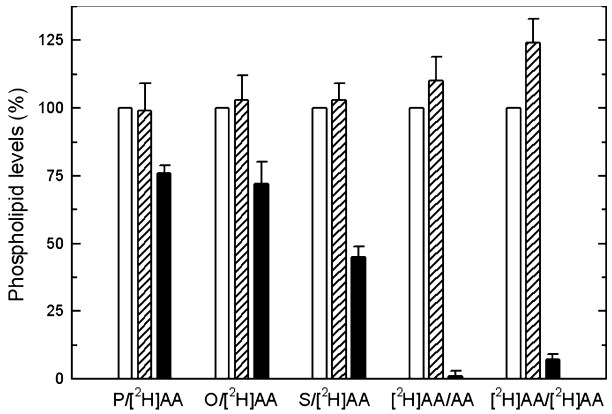

Fig. 5. Effect of PLA_2 inhibitors on the incorporation of [^{2}H]AA into PtdIns molecular species. The U937 cells were either untreated (open bars), treated with 1 μM pyrrophenone (hatched bars), or treated with 10 μM BEL (black bars) for 30 min. They were exposed to 1 μM [^{2}H]AA for 30 min, and the incorporation of [^{2}H]AA into PtdIns species was studied by LC/MS. P/[^{2}H]AA, 1-palmitoyl-2-[^{2}H]AA-glycero-3-phosphoinositol; O/[^{2}H]AA, 1-oleoyl-2-[^{2}H]AA-glycero-3-phosphoinositol; S/[^{2}H]AA, 1-stearoyl-2-[^{2}H]AA-glycero-3-phosphoinositol; [^{2}H]AA/AA, 1-[^{2}H]AA-2-AA-glycero-3-phosphoinositol; [^{2}H]AA/[^{2}H]AA, 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol. Data are expressed as a percentage of the signal detected for each phospholipid species in the absence of inhibitor.

diarachidonoyl lipids is thought to occur through the *de novo* pathway when the levels of available free AA are very high [3]. If 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol was produced *de novo*, one might have expected to detect the appearance of at least diarachidonoyl phosphatidic acid, as this is the immediate precursor of diarachidonoyl-PtdIns via the *de novo* pathway. However, we failed to detect such a phosphatidic acid species at exogenous [^{2}H]AA levels up to 1 μM. We also failed to detect diarachidonoyl-glycerol and 1,2-diarachidonoyl-glycero-3-phosphocholine under these conditions (data not shown). In contrast, when the cells were exposed to high [^{2}H]AA levels (30 μM), conditions under which the *de novo* pathway is known to participate in phospholipid AA incorporation [3], diarachidonoyl phosphatidic acid and diarachidonoyl glycerol (Fig. 6) and diarachidonoyl-glycerophosphocholine (Fig. 7) were all readily detected.

Metabolic fate of [^{2}H]AA-containing PtdIns species

To characterize changes in the distribution of [^{2}H]AA-containing PtdIns species with time, the cells were pulse-labeled with 1 μM [^{2}H]AA for 30 min, after which they were extensively washed with NaCl/P_i containing 1% fatty acid-free BSA to remove the [^{2}H]AA still remaining as free fatty acid. Cell samples were then taken for lipid extraction at different time intervals, and the distribution of [^{2}H]AA among the various PtdIns species was studied. Strikingly, the levels of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol showed a sharp, exponential decrease along the time course of the experiment (Fig. 8). At 3 h, the levels of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol decreased by more than 90%. In contrast, the levels of 1-stearoyl-2-[^{2}H]AA-glycero-3-phosphoinositol and 1-oleoyl-2-[^{2}H]AA-glycero-3-phosphoinositol showed much less pronounced decreases, in agreement with previous findings in human neutrophils [13] (Fig. 8).

Discussion

By utilizing liquid chromatography (LC)/ESI-MS, we identified 1,2-diarachidonoyl-glycero-3-phosphoinositol as an acceptor of [^{2}H]AA within the PtdIns class in U937 cells and peripheral blood monocytes, and determined that its pathway of biosynthesis proceeds via direct acylation of both the *sn*-1 and *sn*-2 positions, and not via the *de novo* pathway. The species is short-lived, more than 90% of it disappearing after only 3 h of exposure of the cells to [^{2}H]AA. These rapid kinetics of synthesis and degradation indicate that 1,2-diarachidonoyl-glycero-3-phosphoinositol acts as a transient acceptor for the incorporation of AA into cellular phospholipids, but does not constitute a stable reservoir of AA under normal equilibrium conditions. On the contrary, 1-stearoyl-2-AA-glycero-3-phosphoinositol and 1-oleoyl-2-AA-glycero-3-phosphoinositol

Table 1. Effect of BEL on lyso-PtdIns levels in resting U937 cells. U937 cells were treated with or without 10 μM BEL for 30 min. Lyso-PtdIns species were detected by LC/MS. *$P < 0.05$ for one-tailed *t*-test.

	Intensity (arbitrary units × 10^{-8})	
Lyso-PtdIns species	Control cells	BEL-treated cells
1-Palmitoyl-2-lyso-glycero-3-phosphoinositol	0.46 ± 0.02	0.41 ± 0.03
1-Oleoyl-2-lyso-glycero-3-phosphoinositol	2.78 ± 0.04	2.16 ± 0.18*
1-Stearoyl-2-lyso-glycero-3-phosphoinositol	2.08 ± 0.06	1.62 ± 0.11*

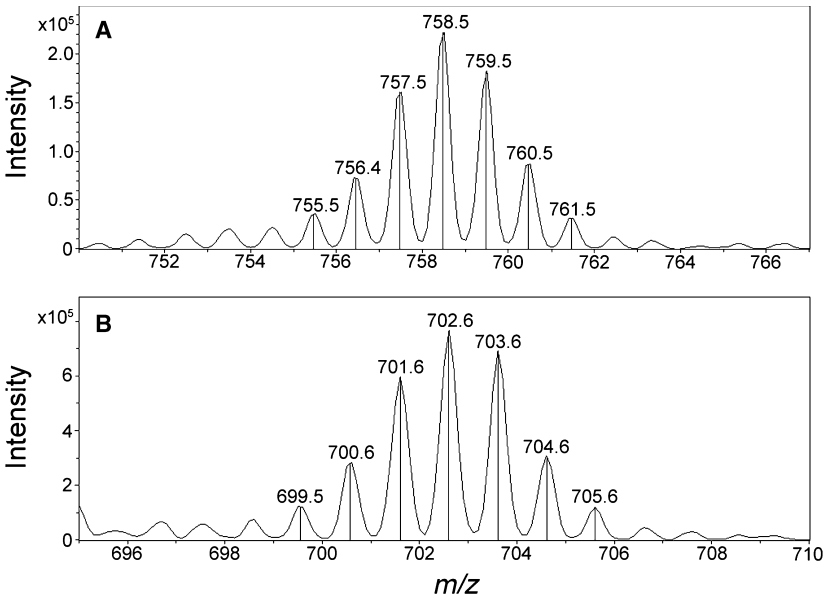

Fig. 6. Detection of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphate and 1-[^{2}H]AA-2-[^{2}H]AA-glycerol in U937 cells. Cells were exposed to 30 μM [^{2}H]AA for 5 min. (A) 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphate was detected in negative mode as $[M–H]^-$. (B) 1-[^{2}H]AA-2-AA-glycerol was detected by LC/MS in positive mode as $[M + Na]^+$.

appear to retain over time a major fraction of the [^{2}H]AA initially incorporated, consistent with their known roles as major stable reservoirs of AA within the PtdIns class [13,33].

According to the pioneering work of Chilton & Murphy [3,34], diarachidonoyl phospholipids are generated *de novo* when the cells are exposed to high concentrations of exogenous AA. In this route, a molecule of arachidonoyl-CoA is transferred to the *sn*-1 position of glycerol 3-phosphate. Subsequently, a second molecule of arachidonoyl-CoA is transferred to the *sn*-2 position, thereby yielding diarachidonoyl-phosphatidic acid, which may be dephosphorylated to produce diarachidonoyl-glycerol. These two molecules would act in turn as precursors of various diarachidonoyl phospholipids, in particular 1,2-diarachidonoyl-*sn*-glycero-3-phosphocholine [3,34,35]. Although we have confirmed that this pathway is fully operational in monocytic cells exposed to high concentrations of exogenous AA (30 μM), we have detected an abundance of a previously unidentified phospholipid, namely 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol, under conditions of low exogenous AA availability, which do not favor the incorporation of fatty acids via the *de novo* pathway but via deacylation–reacylation reactions [3]. 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol can be detected in cells at exogenous AA concentrations as low as 160 nM. Using tritiated AA, we have found elsewhere that, at concentrations up to 1 μM, no fatty acid is incorporated into triacylglycerol in human monocytes (A. M. Astudillo & J. Balsinde, unpublished results), indicating that AA incorporation via the *de novo* route does not occur under these conditions.

Direct evidence that 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol is produced via deacylation–reacylation reactions was provided by the use of BEL, a widely used inhibitor of $iPLA_2$ [6,22,23]. BEL decreases cellular lyso-PtdIns levels and almost completely abrogates the appearance of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol, thus suggesting a role for $iPLA_2$-mediated deacylation–reacylation reactions in the biosynthesis of this phospholipid. It is important to note here that BEL was previously demonstrated not to inhibit CoA-dependent acyltransferases, CoA-independent transacylases, and arachidonoyl-CoA synthetase [10], and also not to affect any of the *de novo* biosynthetic enzymes leading to phosphatidic acid synthesis [36]. Collectively, the fact that of all the cellular activities involved in AA phospholipid incorporation, only the lyso lipid-producing $iPLA_2$ is inhibited by BEL, provides strong support for a deacylation–reacylation-based mechanism in 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol synthesis. Also, it is worth mentioning that specific inhibition of $cPLA_2$ by pyrrophenone exerts no effect on 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol synthesis, pointing to the selective involvement of $iPLA_2$-mediated deacylation–reacylation in the process.

Inhibition of $iPLA_2$ not only by BEL but also by specific antisense oligonucleotides leading to reduced incorporation of AA into phospholipids has been previously reported under a variety of conditions [10–12,37]. As a matter of fact, the regulation of

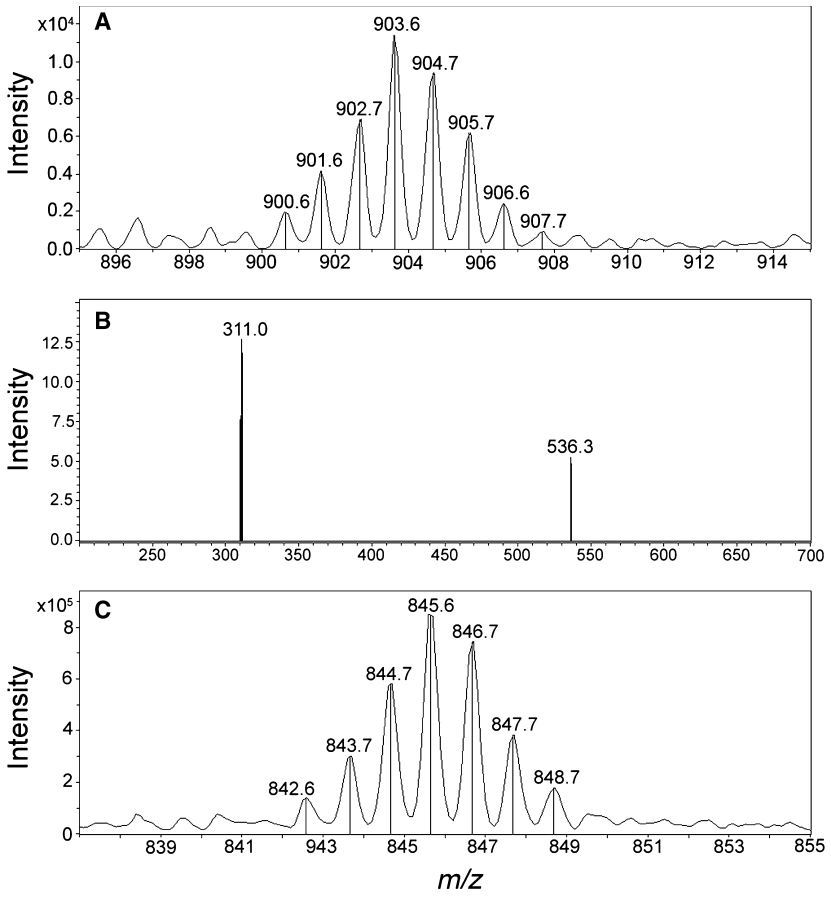

Fig. 7. Detection of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphocholine in U937 cells. U937 cells were exposed to 30 μM [^{2}H]AA for 30 min. (A) Detection of 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphocholine in negative mode as the adduct $[M + CH_3CO_2]^-$. (B) MS/MS/MS analysis of the peak at *m/z* 904.7. This peak lost 74 units in an MS/MS experiment, which corresponds to the sum of the masses of the acetyl and methyl groups. The MS/MS peak at *m/z* 830.5 was isolated again and fragmented, yielding the ions with *m/z* 311 ([^{2}H]AA) and *m/z* 536.3 (produced from the loss of one of the fatty acids). Thus, the compound is identified as 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphocholine. (C) Spectrum of this compound in positive mode, as $[M + H]^+$.

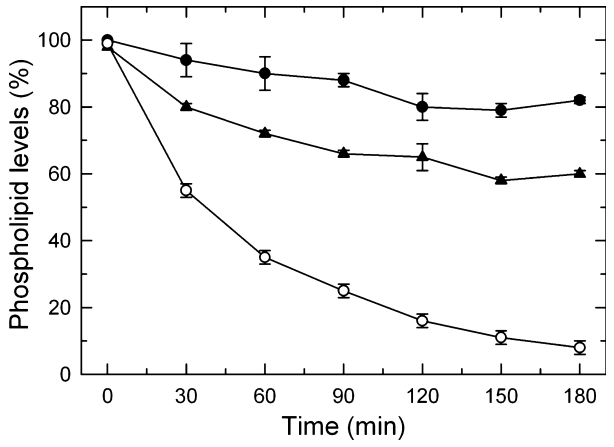

Fig. 8. Metabolism of [^{2}H]AA-containing PtdIns species. The cells were pulse-labeled with 1 μM [^{2}H]AA for 30 min. After extensive washing, the intracellular levels of [^{2}H]AA-containing PtdIns were measured at different times by LC/MS. Black circles: 1-stearoyl-2-[^{2}H]AA-glycero-3-phosphoinositol. Black triangles: 1-oleoyl-2-[^{2}H]AA-glycero-3-phosphoinositol. Open circles: 1-[^{2}H]AA-2-[^{2}H]AA-glycero-3-phosphoinositol. Data are expressed as a percentage of the signal detected for each phospholipid species after washing of the cells (zero time).

lysophospholipid-dependent fatty acid incorporation is one of the earliest roles attributed to this enzyme in cell physiology [38,39]. Although such a role for iPLA$_2$ may occur primarily in cells of myelomonocytic origin [40], our present results obtained by utilizing LC/ESI-MS methodology are consistent with these previous observations and extend them, for the first time, to the metabolism of inositol-containing phospholipids.

At low levels of exogenous [^{2}H]AA, we could not detect accumulation of [^{2}H]AA-containing lyso-PtdIns. Thus, it is not possible for us at this time to define whether recycling of the fatty acid at the *sn*-1 position occurs before or after recycling at the *sn*-2 position. However, it must also be taken into account that recycling at the *sn*-1 and *sn*-2 positions could not necessarily be sequential but rather simultaneous. This would be so because the enzyme that we have identified as controlling these recycling reactions, the BEL-sensitive iPLA$_2$, possesses significant lysophospholipase activity in addition to its intrinsic PLA$_2$ activity [41,42]. Unlike PCs and PEs, PtdIns molecules in mammalian cells do not present ether linkages at the *sn*-1 position; thus, the possibility certainly exists that iPLA$_2$-mediated

hydrolysis of PtdIin in cells gives not a free fatty acid and a 2-lysophospholipid, but rather two fatty acids and glycerophosphoinositol. The direct acylation of glycerophosphoinositol by two fatty acids would re-form PtdIns [43,44]. Given that under our experimental conditions free AA is readily available, a major PtdIns species that would be formed by this route would be 1,2-diarachidonoyl-PtdIns.

Kainu *et al*. [45] have recently described a methodological approach to specifically deliver defined phospholipid species into cells. Using this method, Kainu *et al*. [45] characterized the metabolic pathways for fatty acid recycling in ethanolamine and serine phospholipids in BHK21 and HeLa cells. Following this approach, work is currently in progress in our laboratory to achieve the delivery of 1,2-diarachidonoyl-*sn*-glycero-3-phosphoinositol and its two related lyso forms into U937 cells. We expect that this strategy will allow us to clarify the steps involved in the biosynthesis and catabolism of this unusual phospholipid in human monocytes.

Experimental procedures

Reagents

Cell culture medium was from Invitrogen Life Technologies (Carlsbad, CA, USA). Deuterated AA ([^{2}H]AA) was from Sigma-Aldrich (Madrid, Spain). Unlabeled lipids were from Avanti Polar Lipids (Alabaster, AL, USA). BEL was from Cayman Chemical (Ann Arbor, MI, USA). Chloroform, methanol and water solvents (HPLC grade) were from Riedel-de-Häen (Seelze, Germany). Hexane (HPLC grade), ammonium hydroxide (30%) and acetic acid were from Merck (Darmstadt, Germany). All other reagents were from Sigma-Aldrich. Pyrrophenone was kindly provided by T. Ono (Shionogi Research Laboratories, Osaka, Japan).

Cell culture

U937 cells were generously provided by P. Aller (Centro de Investigaciones Biológicas, Madrid, Spain). The cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin [46]. The cells were incubated at 37 °C in a humidified atmosphere of CO_2 (5%). To induce a monocyte-like phenotype, the cells were incubated in the presence of 1.3% dimethylsulfoxide for 3 days. For experiments, 4×10^6 cells were placed in 2 mL of serum-free medium for 2 h, and then exposed to exogenous [^{2}H]AA. After 30 min, the cells were harvested by centrifugation at 300 ***g*** for 5 min. Where indicated, inhibitors (1 μM pyrrophenone, 10 μM BEL) were added 30 min before the [^{2}H]AA. [^{2}H]AA was dissolved in ethanol. The final concentration of this solvent after addition to the cells was 0.1%.

Human monocytes were obtained from buffy coats of healthy volunteer donors obtained from the Centro de Hemoterapia y Hemodonación de Castilla y León (Valladolid, Spain). Briefly, the buffy coats (200 mL) were diluted 1 : 1 with NaCl/P$_i$, layered over a cushion of Ficoll-Paque Plus (GE Healthcare, Chalfont St Giles, UK), and centrifuged at 750 ***g*** for 30 min. The mononuclear cellular layer was then recovered and washed with NaCl/P$_i$, resuspended in RMPI-1640 supplemented with 2 mM L-glutamine and 40 mg·mL^{-1} gentamycin, and allowed to adhere to plastic in sterile dishes for 2 h. Non-adherent cells were removed by extensive washing with NaCl/P$_i$. Monocytes remained attached to the plastic culture dishes, and were used for experiments on the following day.

LC/MS

For HPLC separation of lipids, a Hitachi LaChrom Elite L-2130 binary pump was used, together with a Hitachi Autosampler L-2200 (Merck). The HPLC system was coupled on-line to a Bruker esquire6000 ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). In all cases except for diacylglycerol determination, the HPLC effluent was split, and 0.2 mL·min^{-1} entered the ESI interface of the mass spectrometer. For diacylglycerol, 0.05 mL·min^{-1} was introduced into the ESI chamber. The nebulizer was set to 30 lb·inch^{-2}, the dry gas to 8 L·min^{-1}, and the dry temperature to 350 °C. The MS spectra were identified by comparison with previously published databases [47,48].

Analysis of PtdIns and PC species

Total lipid content corresponding to 2×10^6 cells was extracted according to Bligh & Dyer [49]. After evaporation of the organic solvent under vacuum, the lipids were redissolved in methanol/water (9 : 1), and stored under nitrogen at −80 °C until analysis. The column was a Supelcosil LC-18 (5 μm particle size, 250 × 2.1 mm) (Sigma-Aldrich) protected with a Supelguard LC-18 20 × 2.1 mm guard cartridge (Sigma-Aldrich). Chromatographic conditions were adapted from those described by Igbavboa *et al*. [50]. Briefly, the mobile phase was a gradient of solvent A (methanol/water/n-hexane/30% ammonium hydroxide, 87.5 : 10.5 : 1.5 : 0.5, v/v), and solvent B (methanol/n-hexane/30% ammonium hydroxide, 87.5 : 12 : 0.5, v/v). The gradient was started at 100% solvent A, and was then decreased linearly to 65% solvent A in 20 min, to 10% in 5 min, and to 0% in another 5 min. The flow rate was 0.5 mL·min^{-1}; 80 μL of the lipid extract was injected. PtdIns species were detected in negative ion mode with the capillary current set at +3500 V over the initial 21 min. PC

species were then detected over the elution interval from 21 to 35 min in positive ion mode as $[M + H]^+$ ion with the capillary current set at −4000 V. Assessment of PC species in negative mode was carried out with postcolumn addition of acetic acid at a flow rate of 100 μL·h^{-1} as $[M + CH_3CO_2]^-$ adducts.

Analysis of lyso-PtdIns and phosphatidic acid

The sample was homogenized in 0.5 mL of water/6 M HCl (19 : 1), and lipids were extracted two times with 0.5 mL of water-saturated n-butanol [51,52]. After evaporation of the organic solvent under vacuum, the lipids were redissolved in chloroform and stored under nitrogen at −80 °C until analysis. A normal phase Supelcosil LC-Si 3 μm 150 × 3 mm column protected with a Supelguard LC-Si 20 × 3 mm guard cartridge column was used. The flow rate was 0.5 mL·min^{-1}; 80 μL of the lipid extract was injected. Separation solvents were: chloroform/methanol/30% ammonium hydroxide (75 : 24.5 : 0.5, v/v) (solvent A), and chloroform/methanol/water/30% ammonium hydroxide (55 : 39.5 : 5.5 : 0.5, v/v) (solvent B). The gradient was started with 100% solvent A, and switched to 50% in 2 min. This percentage was maintained for 8 min, and was then changed to 0% solvent A in 2 min. Lyso-PtdIns and phosphatidic acid species were detected in negative mode as $[M–H]^-$ ions by MS.

Diacylglycerol determination

The cells were resuspended in 0.5 mL of methanol/0.1 M HCl (1 : 1), and the lipids were extracted twice with 0.5 mL of chloroform. After evaporation of the solvent under vacuum, the lipids were redissolved in methanol/water (9 : 1), and stored under nitrogen at −80 °C until analysis. A Supelcosil LC-18, 5 μm particle size, 250 × 2.1 mm column protected with a Supelguard LC-18 20 × 2.1 mm guard cartridge (Sigma-Aldrich) was used to separate diacylglycerol species. The gradient was started at 100% solvent A (methanol/water/1.3 M sodium acetate, 87.5 : 12.5 : 0.05, v/v), and switched linearly to solvent B (methanol/n-hexane/1.3 M sodium acetate, 87.5 : 12.5 : 0.05, v/v) in 10 min. The flow rate was 0.5 mL·min^{-1}, and 40 μL was injected. The diacylglycerol species were detected in positive ion mode as $[M + Na]^+$ over the *m/z* 520–750 range.

Data presentation

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 ± standard error.

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References

1 Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* **294**, 1871–1875.
2 Lands WEM (2000) Stories about acyl chains. *Biochim Biophys Acta* **1483**, 1–14.
3 Chilton FH, Fonteh AN, Surette ME, Triggiani M & Winkler JD (1996) Control of arachidonate levels within inflammatory cells. *Biochim Biophys Acta* **1299**, 1–15.
4 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.
5 Schaloske RH & Dennis EA (2006) The phospholipase A_2 superfamily and its group numbering system. *Biochim Biophys Acta* **1761**, 1246–1259.
6 Balsinde J, Balboa MA, Insel PA & Dennis EA (1999) Regulation and inhibition of phospholipase A_2. *Annu Rev Pharmacol Toxicol* **39**, 175–189.
7 Balsinde J, Winstead MV & Dennis EA (2002) Phospholipase A_2 regulation of arachidonic acid mobilization. *FEBS Lett* **531**, 2–6.
8 Balsinde J & Balboa MA (2005) Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A_2 in activated cells. *Cell Signal* **17**, 1052–1062.
9 Balboa MA & Balsinde J (2006) Oxidative stress and arachidonic acid mobilization. *Biochim Biophys Acta* **1761**, 385–391.
10 Balsinde J, Bianco ID, Ackermann EJ, Conde-Frieboes K & Dennis EA (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.
11 Balsinde J, Balboa MA & Dennis EA (1997) Inflammatory activation of arachidonic acid signaling in murine $P388D_1$ macrophages via sphingomyelin synthesis. *J Biol Chem* **272**, 20373–20377.
12 Pérez R, Melero R, Balboa MA & Balsinde J (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.
13 Chilton FH & Murphy RC (1986) Remodeling of arachidonate-containing phosphoglycerides within the human neutrophil. *J Biol Chem* **261**, 7771–7777.
14 Fonteh AN & Chilton FH (1992) Rapid remodeling of arachidonate from phosphatidylcholine to phosphatidylethanolamine pools during mast cell activation. *J Immunol* **148**, 1784–1791.
15 Boilard E & Surette ME (2001) Anti-CD3 and concanavalin A-induced human T cell proliferation is associated with an increased rate of arachidonate-phospholipid remodeling. Lack of involvement of group IV and group VI phospholipase A2 in remodeling and increased susceptibility of proliferating T cells to CoA-independent transacyclase inhibitor-induced apoptosis. *J Biol Chem* **276**, 17568–17575.
16 Balsinde J, Barbour SE, Bianco ID & Dennis EA (1994) Arachidonic acid mobilization in $P388D_1$ macrophages is controlled by two distinct Ca^{2+}-dependent phospholipase A_2 enzymes. *Proc Natl Acad Sci USA* **91**, 11060–11064.
17 Pérez R, Matabosch X, Llebaria A, Balboa MA & Balsinde J (2006) Blockade of arachidonic acid incorporation into phospholipids induces apoptosis in U937 promonocytic cells. *J Lipid Res* **47**, 484–491.
18 Harkewicz R, Fahy E, Andreyev A & Dennis EA (2007) Arachidonate-derived dihomoprostaglandin production observed in endotoxin-stimulated macrophage-like cells. *J Biol Chem* **282**, 2899–2910.
19 Hsu FF & Turk J (2000) Characterization of phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate by electrospray ionization tandem mass spectrometry: a mechanistic study. *J Am Soc Mass Spectrom* **11**, 986–999.
20 Ono T, Yamada K, Chikazawa Y, Ueno M, Nakamoto S, Okuno T & Seno K (2002) Characterization of a novel inhibitor of cytosolic phospholipase $A_2\alpha$, pyrrophenone. *Biochem J* **363**, 727–735.
21 Ghomashchi F, Stewart A, Hefner Y, Ramanadham S, Turk J, Leslie CC & Gelb MH (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.
22 Hazen SL, Zupan LA, Weiss RH, Getman DP & Gross RW (1991) Suicide inhibition of canine myocardial cytosolic calcium-independent phospholipase A_2. Mechanism-based discrimination between calcium-dependent and -independent phospholipases A2. *J Biol Chem* **266**, 7227–7232.
23 Balsinde J & Dennis EA (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.
24 Balboa MA & Balsinde J (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.
25 Pérez R, Balboa MA & Balsinde J (2006) Involvement of group VIA calcium-independent phospholipase A_2 in macrophage engulfment of hydrogen peroxide-treated U937 cells. *J Immunol* **176**, 2555–2561.
26 Fuentes L, Pérez R, Nieto ML, Balsinde J & Balboa MA (2003) Bromoenol lactone promotes cell death by a mechanism involving phosphatidate phosphohydrolase-1 rather than calcium-independent phospholipase A_2. *J Biol Chem* **278**, 44683–44690.
27 Balboa MA, Sáez Y & Balsinde J (2003) Calcium-independent phospholipase A_2 is required for lysozyme secretion in U937 promonocytes. *J Immunol* **170**, 5276–5280.
28 Balboa MA, Pérez R & Balsinde J (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.
29 Balboa MA, Pérez R & Balsinde J (2008) Calcium-independent phospholipase A_2 mediates proliferation of human promonocytic U937 cells. *FEBS J* **275**, 1915–1924.
30 Balsinde J, Balboa MA, Yedgar S & Dennis EA (2000) Group V phospholipase A_2-mediated oleic acid mobilization in lipopolysaccharide-stimulated $P388D_1$ macrophages. *J Biol Chem* **275**, 4783–4786.
31 Balsinde J, Balboa MA & Dennis EA (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.
32 Balsinde J, Balboa MA, Insel PA & Dennis EA (1997) Differential regulation of phospholipase D and phospholipase A_2 by protein kinase C in $P388D_1$ macrophages. *Biochem J* **321**, 805–809.
33 Murthy PP & Agranoff BW (1982) Stereospecific synthesis and enzyme studies of CDP-diacylglycerols. *Biochim Biophys Acta* **712**, 473–483.
34 Chilton FH & Murphy RC (1987) Stimulated production and natural occurrence of 1,2-diarachidonoylglycerophosphocholine in human neutrophils. *Biochem Biophys Res Commun* **145**, 1126–1133.
35 Kuwae T, Schmid PC & Schmid HH (1996) Alterations of fatty acyl turnover in macrophage glycerolipids induced by stimulation. Evidence for enhanced recycling of arachidonic acid. *Biochim Biophys Acta* **1344**, 74–86.
36 Balsinde J & Dennis EA (1996) Bromoenol lactone inhibits magnesium-dependent phosphatidate

phosphohydrolase and blocks triacylglycerol biosynthesis in mouse P388D_1 macrophages. *J Biol Chem* **271**, 31937–31941.
37 Daniele JJ, Fidelio GD & Bianco ID (1999) Calcium dependency of arachidonic acid incorporation into cellular phospholipids of different cell types. *Prostaglandins* **57**, 341–350.
38 Balsinde J & Dennis EA (1997) Function and inhibition of intracellular calcium-independent phospholipase A_2. *J Biol Chem* **272**, 16069–16072.
39 Winstead MV, Balsinde J & Dennis EA (2000) Calcium-independent phospholipase A_2: structure and function. *Biochim Biophys Acta* **1488**, 28–39.
40 Bao S, Bohrer A, Ramanadham S, Jin W, Zhang S & Turk J (2006) Effects of stable suppression of Group VIA phospholipase A_2 expression on phospholipid content and composition, insulin secretion, and proliferation of INS-1 insulinoma cells. *J Biol Chem* **281**, 187–198.
41 Lio YC & Dennis EA (1998) Interfacial activation, lysophospholipase and transacylase activity of group VI Ca^{2+}-independent phospholipase A_2. *Biochim Biophys Acta* **1392**, 320–332.
42 Lio YC, Reynolds LJ, Balsinde J & Dennis EA (1996) Irreversible inhibition of Ca^{2+}-independent phospholipase A_2 by methyl arachidonyl fluorophosphonate. *Biochim Biophys Acta* **1302**, 55–60.
43 Waite M & van Deenen LLM (1967) Hydrolysis of phospholipids and glycerides by rat-liver preparations. *Biochim Biophys Acta* **137**, 498–517.
44 Balsinde J, Diez E, Schüller A & Mollinedo F (1988) Phospholipase A_2 activity in resting and activated human neutrophils. Substrate specificity, pH dependence, and subcellular localization. *J Biol Chem* **263**, 1929–1936.
45 Kainu V, Hermansson M & Somerharju P (2008) Electrospray ionization mass spectrometry and exogenous heavy isotope-labeled lipid species provide detailed information on aminophospholipid acyl chain remodeling. *J Biol Chem* **283**, 3676–3687.
46 Balsinde J & Mollinedo F (1991) Platelet-activating factor synergizes with phorbol myristate acetate in activating phospholipase D in the human promonocytic cell line U937. Evidence for different mechanisms of activation. *J Biol Chem* **266**, 18726–18730.
47 Pulfer M & Murphy RC (2003) Electrospray mass spectrometry of phospholipids. *Mass Spectrom Rev* **22**, 332–364.
48 Murphy RC (2002) *Mass Spectrometry of Phospholipids: Tables of Molecular and Product Ions*. Illuminati Press, Denver, CO.
49 Bligh EG & Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.
50 Igbavboa U, Hamilton J, Kim HY, Sun GY & Wood WG (2002) A new role for apolipoprotein E: modulating transport of polyunsaturated phospholipid molecular species in synaptic plasma membranes. *J Neurochem* **80**, 255–261.
51 Diez E, Balsinde J, Aracil M & Schüller A (1987) Ethanol induces release of arachidonic acid but not synthesis of eicosanoids in mouse peritoneal macrophages. *Biochim Biophys Acta* **921**, 82–89.
52 Balsinde J, Fernández B, Solís-Herruzo JA & Diez E (1992) Pathways for arachidonic acid mobilization in zymosan-stimulated mouse peritoneal macrophages. *Biochim Biophys Acta* **1136**, 75–82.