Roles of various phospholipases A_2 in providing lysophospholipid acceptors for fatty acid phospholipid incorporation and remodelling[1]

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In the present study the lysophospholipid sources for arachidonic (AA) and eicosapentaenoic acid (EPA) incorporation into and redistribution within the phospholipids of phorbol-ester-differentiated U937 cells was investigated. Initially, AA incorporated primarily into choline glycerophospholipids (PC), whereas EPA incorporated mainly into ethanolamine glycerophospholipids (PE). Bromoenol lactone (BEL), an inhibitor of the Group VI Ca^{2+}-independent phospholipase A_2 ($iPLA_2$), diminished both lysophosphatidylcholine levels and the incorporation of AA into phospholipids. However BEL had little effect on EPA incorporation. In concanavalin A-activated cells, EPA, but not AA, incorporation was also affected by methyl arachidonyl fluorophosphonate (MAFP), suggesting an additional role for the group IV cytosolic phospholipase A_2. In the activated cells AA and EPA did not compete with each other for incorporation, indicating that the pathways for AA and EPA incorporation are partially different. The AA and EPA initially incorporated into PC slowly moved to PE in a process that took several hours. The transfer of AA and EPA from PC to PE was not inhibited by BEL, MAFP or LY311727 [3-(3-acetamide 1-benzyl-2-ethylindolyl-5-oxy)propanesulphonic acid], raising the possibility that an as-yet-undetermined phospholipase A_2 may be involved in fatty acid phospholipid remodelling. A strong candidate to be involved in these reactions is a novel Ca^{2+}-independent phospholipase A_2 that, unlike all known $iPLA_2$s, is resistant to inhibition by BEL and also to MAFP and LY311727. The enzyme activity cleaves both PC and PE and is thus able to provide the lysoPC and lysoPE acceptors required for the fatty acid acylation reactions.

Key words: arachidonic acid, reacylation, transacylation.

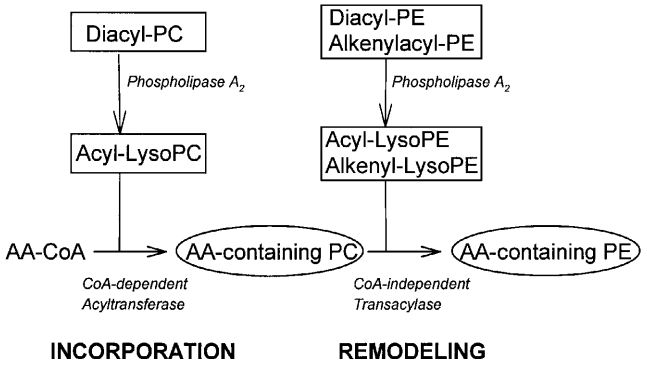
INTRODUCTION

Availability of free arachidonic acid (AA) is widely recognized as a rate-limiting step in the formation of prostaglandins by activated cells [1]. This fatty acid is an intermediate in a reacylation/deacylation cycle of membrane phospholipids, the so-called 'Lands pathway', in which the fatty acid is cleaved from phospholipid by phospholipase A_2 (PLA_2) and reincorporated by acyltransferases [2–4]. Whereas in resting cells reacylation dominates, in stimulated cells the dominant reaction is the deacylation. Nevertheless, increased AA reacylation during cellular activation is still very significant, as manifested by the fact that only a minor portion of the free AA released by PLA_2 is converted into eicosanoids, the remainder being effectively incorporated back into phospholipids [2–4].

AA incorporation into phospholipids is critically dependent on the availability of lysophospholipid acceptors, particularly lysophosphatidylcholine (lysoPC). In many cells, the levels of lysoPC appear to be maintained by the continuing action of the Ca^{2+}-independent Group VI phospholipase A_2 ($iPLA_2$) on cellular phospholipids [5]. Thus a decrease in the activity of the $iPLA_2$ frequently results in the diminished production of lysoPC [6–11] and hence in the inhibition of AA incorporation into phospholipids [6–10].

Once the AA is initially incorporated into lysoPC by the action of CoA-dependent acyltransferases, it is transferred to certain lysophospholipids, particularly the ethanolamine lysophospholipids (lysoPE). Such a transfer is catalysed by the enzyme CoA-independent transacylase (CoA-IT) [12]. Thus, for the AA to be efficiently incorporated into phospholipids, two kinds of lysophospholipid acceptors should be readily available (Scheme 1). The provider of the lysoPE acceptors for the CoA-IT reaction is assumed to be a PLA_2 [12,13], but its identity has not been elucidated.

U937 cells are derived from a human histiocytic lymphoma and differentiate into macrophage-like cells when treated with phorbol esters such as PMA [14,15]. The objective of the present study was to understand better the biochemical features of AA



Scheme 1 AA incorporation into and remodeling within phospholipids

Abbreviations used: AA, arachidonic acid; Con A, concanavalin A; EPA, eicosa-5,8,11,14,17-pentaenoic acid; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; $sPLA_2$, secretory phospholipase A_2; CoA-IT, CoA-independent transacylase; BEL, bromoenol lactone [(*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one]; MAFP, methyl arachidonyl fluorophosphonate; IHP, diethyl 7-(3,4,5-triphenyl-2-oxo-2,3-dihydroimidazol-1-yl)hepatine phosphonate (SK&F 98625); LY311727, 3-(3-acetamide-1-benzyl-2-ethylindolyl-5-oxy)propanesulphonic acid; PC, choline glycerophospholipids; PE, ethanolamine glycerophospholipids; PI, phosphatidylinositol.

[1] This work is dedicated to the memory of Belén Fernández-Boya.

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incorporation into, and remodelling within, phospholipid species of PMA-differentiated U937 cells. To this end, AA incorporation into phospholipids was compared with the incorporation of eicosa-5,8,11,14,17-pentanoic acid (EPA), a $\omega-3$ fatty acid that is usually regarded as a 'competitor' of AA [2].

EXPERIMENTAL

Materials

[5,6,8,9,11,12,14,15-^{3}H]AA (100 Ci/mmol) was from Amersham (Arlington Heights, IL, U.S.A.). [5,6,8,9,11,12,14,15,17,18-^{3}H]-EPA (100 Ci/mmol) was from American Radiolabelled Chemicals (St. Louis, MO, U.S.A.). The bromoenol lactone (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BEL) and methyl arachidonyl fluorophosphonate (MAFP) were from Cayman (Ann Arbor, MI, U.S.A.). The CoA-IT inhibitor diethyl 7-(3,4,5-triphenyl-2-oxo-2,3-dihydroimidazol-1-yl)hepatine phosphonate (IHP; also known as SK&F 98625) [16] was synthesized and generously provided by Dr K. Conde-Frieboes [Eidgenössische Technische Hochschule (ETH), Zürich, Switzerland]. The sPLA$_2$ inhibitor 3-(3-acetamide 1-benzyl-2-ethylindolyl-5-oxy)propanesulphonic acid (LY311727) was generously provided by Dr E. Mihelich (Eli Lilly Co., Indianapolis, IN, U.S.A.). All other reagents were from Sigma (St. Louis, MO, U.S.A.).

Cell culture

U937 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal-calf serum, 2 mM glutamine, penicillin (100 units/ml) and gentamycin (24 μg/ml). The cells were incubated at 37 °C in a humidified atmosphere of CO_2/O_2 (1:19) at a cell density of $(0.5–1)\times10^6$ cells/ml in 12-well plastic culture dishes (Costar). Cell differentiation was induced by treating the cells with 35 ng/ml PMA for 24 h [14,15].

Measurement of [^{3}H]AA incorporation into phospholipids

The PMA-differentiated U937 cells were placed in serum-free medium for 60 min before exposure to exogenous [^{3}H]AA (5 nM; 0.5 μCi/ml). At the indicated times, supernatants were removed and the cell monolayers were scraped twice with 0.1% Triton X-100. Total lipids were extracted and were separated by TLC with n-hexane/diethyl ether/acetic acid (70:30:1, by vol.). For separation of phospholipid classes, a mobile phase consisting of chloroform/methanol/acetic acid/water (25:20:3:0.3, by vol.) was used. This system allows a good resolution between major phospholipids, including phosphatidylinositol (PI) and phosphatidylserine (PS).

Measurement of [^{3}H]fatty acid phospholipid remodeling

For these experiments, the PMA-differentiated U937 cells were pulse-labelled with [^{3}H]AA or [^{3}H]EPA (5 nM; 0.5 μCi/ml) for 30 min at 37 °C. The cells were then washed four times with medium containing 1 mg/ml BSA to remove the non-incorporated label. Afterward the cells were placed in serum-free medium and incubated at 37 °C for the indicated periods of time, and in the absence or presence of the indicated compounds. The lipids were extracted and separated as described above.

Fatty acid release experiments

The cells were labelled with 0.5 μCi/ml [^{3}H]AA or [^{3}H]EPA for 18 h. After this period, the cells were washed and placed in serum-free medium for 1 h before the addition of 50 μg/ml concanavalin A (Con A) in the presence of 0.5 mg/ml BSA. The supernatants were removed, cleared of cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

Lysophospholipid determination

Cells labelled with 0.5 μCi/ml [^{3}H]choline or [^{3}H]ethanolamine for 2 days were used. After the incubations, lipids were extracted with ice-cold butan-1-ol and separated by TLC with chloroform/methanol/acetic acid/water (50:40:6:0.6, by vol.) as a solvent system. Spots corresponding to lysoPC or lysoPE were scraped into scintillation vials and the amount of radioactivity was estimated by liquid scintillation counting.

iPLA$_2$ assay

Briefly, aliquots of PMA-treated U937 cells were incubated for 30 min at 37 °C in 100 mM Hepes, pH 7.5, containing 5 mM EDTA and 100 μM phospholipid substrate (pH 7.5), in a final volume of 250 μl. The substrates utilized in the assay were [^{3}H]AA-labelled PC and [^{3}H]AA-labelled PE, and they were used in the form of sonicated vesicles in buffer. Alternatively, iPLA$_2$ activity was also assayed toward a substrate in the form of mixed micelles of Triton X-100/phospholipid at a molar ratio 4:1. This was carried out exactly as described by Balsinde et al. [6].

Preparation of labelled substrates for the iPLA$_2$ assay

[^{3}H]AA-labelled PC and PE were isolated from cellular lipids of U937 cells incubated for 24 h with the corresponding exogenous fatty acids (0.5 μCi/ml). Labelled phospholipids were purified by TLC and tested for purity as described previously [17].

Data presentation

Assays were carried out in duplicate or triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data presented are from representative experiments.

RESULTS

Incorporation of AA and EPA into U937 cell phospholipids

When PMA-differentiated U937 cells were exposed to exogenous [^{3}H]AA, it was rapidly incorporated into phospholipids (Figure 1A). This process was blunted by the presence of the iPLA$_2$ inhibitor BEL in the incubation medium (Figure 1A). Inhibition of the iPLA$_2$ by BEL has previously been observed to diminish [^{3}H]AA incorporation into the phospholipids of a variety of cells of myeloid [6–8] and non-myeloid [9,10] origin. Thus the data suggest that the iPLA$_2$ is also required for [^{3}H]AA incorporation into the phospholipids of PMA-differentiated U937 cells. In agreement with this view, depletion of intracellular Ca^{2+} did not affect AA incorporation into phospholipids (results not shown). Figure 1(B) shows that the incorporation of exogenous [^{3}H]EPA into U937-cell phospholipids was also inhibited by BEL, although to a considerably lesser extent. Addition of the dual cPLA$_2$/iPLA$_2$ inhibitor MAFP to the BEL-treated cells did not further decrease either [^{3}H]AA or [^{3}H]EPA incorporation into phospholipids (results not shown), suggesting that the cPLA$_2$ has no significant role under these conditions.

Reduction of fatty acid incorporation by iPLA$_2$ inhibition is thought to reflect the diminished availability of lysoPC acceptor

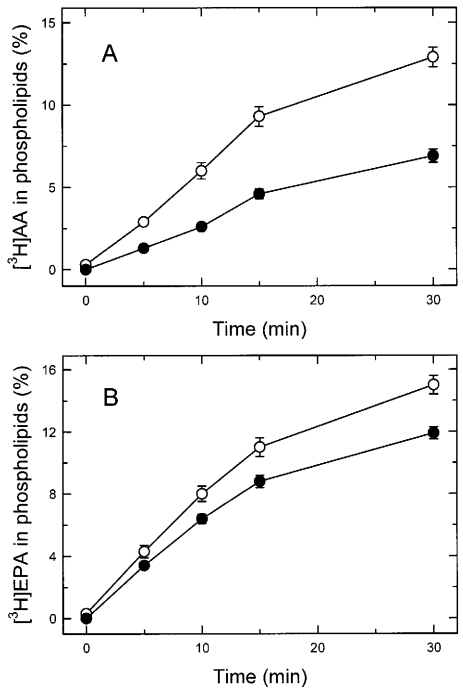

Figure 1 Effect of BEL on the incorporation of AA and EPA into the phospholipids of PMA-differentiated U937 cells

The cells were either untreated (open symbols) or treated with 25 μM BEL for 30 min (closed symbols) and then incubated with [^{3}H]AA (**A**) or [^{3}H]EPA (**B**) for the indicated times. The radioactivity incorporated into phospholipids is given as a percentage of the radioactivity originally present in the incubation medium.

Table 1 Effect of different fatty acids on the incorporation of [^{3}H]AA and [^{3}H]EPA into phospholipids

The cells were exposed to either [^{3}H]AA or [^{3}H]EPA for 30 min in the absence or presence of the indicated fatty acids (0.5 μM). [^{3}H]Fatty acid incorporation into phospholipids is expressed as a percentage of the radioactivity originally present in the incubation medium.

Fatty acid added	Incorporation (%)	
	[^{3}H]AA	[^{3}H]EPA
None (control)	13±1	15±2
Stearic acid	13±1	15±1
Oleic acid	13±1	15±1
Linoleic acid	13±2	15±1
Linolenic acid	12±2	15±2
AA	6±2	10±1
EPA	8±1	7±1

molecules generated by the enzyme activity. In [^{3}H]choline-labelled U937 cells, preincubation with 25 μM BEL induced a decrease of 42±3% (mean±S.E.M., $n = 4$) of the steady-state level of [^{3}H]choline-labelled lysoPC, as measured by TLC. This decrease corresponds reasonably well to the inhibition of AA incorporation into phospholipids. No significant effect of BEL on the cellular levels of lysoPE in [^{3}H]ethanolamine-labelled cells could be detected.

The effect of different fatty acids on AA and EPA incorporation was investigated next. As illustrated in Table 1, [^{3}H]AA incorporation was not affected by the presence of several saturated, mono- or di-unsaturated fatty acids in the incubation medium, but was severely impaired by exogenous AA and EPA. Similar experiments determining the incorporation of [^{3}H]EPA gave essentially the same results, i.e. inhibition was only observed in the presence of exogenous AA or EPA (Table 1). These results are similar to those previously reported in mouse peritoneal macrophages [18] and thus support the existence in PMA-differentiated U937 cells of specific systems for the uptake and acylation of C_{20} polyunsaturated fatty acids.

Fatty acid incorporation into the phospholipids of activated cells

For these experiments, the PMA-differentiated U937 cells were stimulated by 50 μg/ml Con A [14,15] in the presence of [^{3}H]AA or [^{3}H]EPA. Cellular activation resulted in an elevated capacity of the cells to import both [^{3}H]AA and [^{3}H]EPA into phospholipids (Table 2), which is in agreement with previous reports on other cell types [19–22].

Pretreatment of the cells with BEL resulted in an expected decrease of the incorporation of both fatty acids into phospholipids (Table 2). Importantly, addition of the dual $cPLA_2$/$iPLA_2$ inhibitor MAFP to the BEL-treated cells resulted in further inhibition of the [^{3}H]EPA incorporation, but no detectable effect on [^{3}H]AA incorporation (Table 2). Since, in these experiments, the $iPLA_2$ was already inhibited by BEL, any additional effect of MAFP on fatty acid incorporation would be due to $cPLA_2$ inhibition. Thus the data suggest that [^{3}H]EPA incorporation into the phospholipids of activated cells may depend on both $iPLA_2$ and $cPLA_2$, whereas [^{3}H]AA incorporation appears to depend only on the $iPLA_2$.

Table 2 also shows the effect of exogenous fatty acids on the incorporation of [^{3}H]AA or [^{3}H]EPA into the phospholipids of activated cells. In contrast with the results for unstimulated cells described above, the addition of exogenous EPA to Con A-stimulated cells did not blunt the incorporation of [^{3}H]AA into phospholipids. In a similar manner, exogenous AA did not block [^{3}H]EPA incorporation. Thus, in activated cells, AA and EPA utilize different routes for incorporation into cellular phospholipids.

Table 2 Effect of cellular activation by ConA on AA and EPA incorporation into phospholipids

The cells were exposed to either [^{3}H]AA or [^{3}H]EPA for 30 min in the absence or presence of 50 μg/ml Con A and in the absence or presence of the indicated inhibitors. BEL and MAFP were used at 25 μM, and exogenous unlabelled AA and EPA were used at 0.5 μM. [^{3}H]Fatty acid incorporation into phospholipids is expressed as a percentage of the radioactivity originally present in the incubation medium.

Fatty acid	Incorporation (%)					
	None	Con A	Con A + BEL	Con A + BEL + MAFP	Con A + AA	Con A + EPA
[^{3}H]AA	12±1	20±3	10±3	11±2	8±1	21±2
[^{3}H]EPA	16±2	25±3	19±1	15±1	22±2	13±3

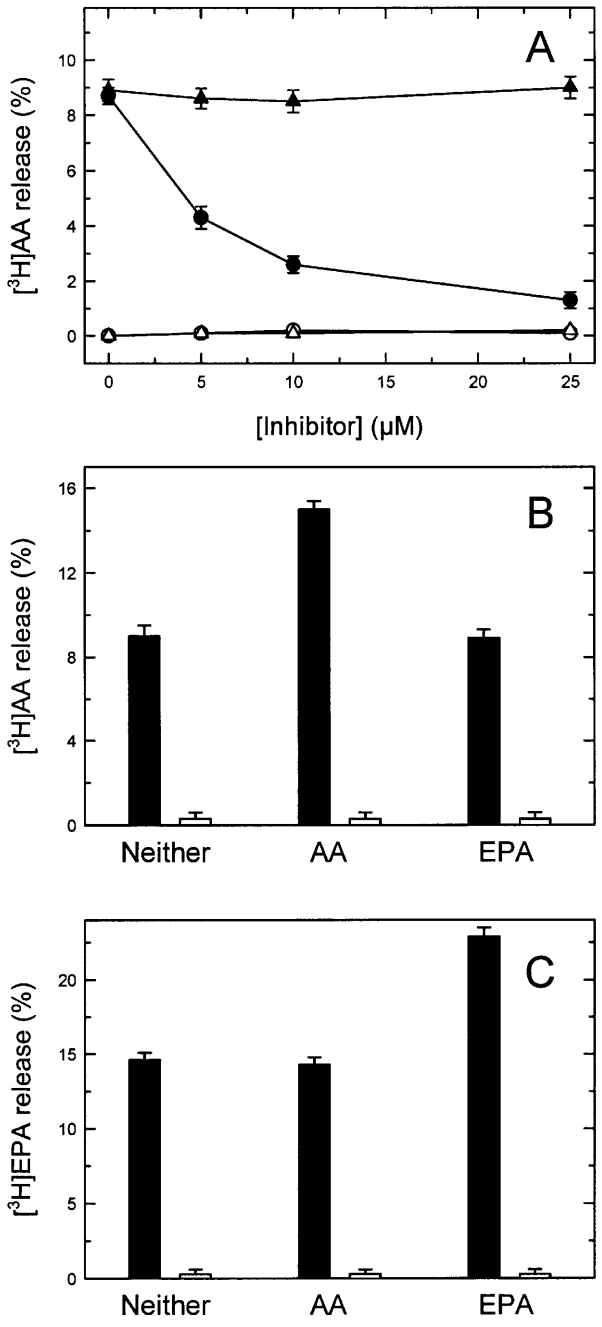

Figure 2 Con A-stimulated release of AA and EPA from PMA-differentiated U937 cells

(**A**) Effect of BEL and MAFP on AA release. The cells were treated with the indicated concentrations of MAFP (circles) or BEL (triangles) for 30 min before the addition of 50 μg/ml Con A (closed symbols), and the incubations proceeded for 30 min. Open symbols denote incubations in the absence of Con A. (**B**) Effect of exogenous fatty acids on Con A-stimulated [^{3}H]AA release. (**C**) Effect of exogenous fatty acids on Con A-stimulated [^{3}H]EPA release. The cells were incubated in the absence (open bars) or presence of 50 μg/ml Con A (closed bars) and in the presence of the indicated exogenous fatty acid added at 0.5 μM (see the abscissa).

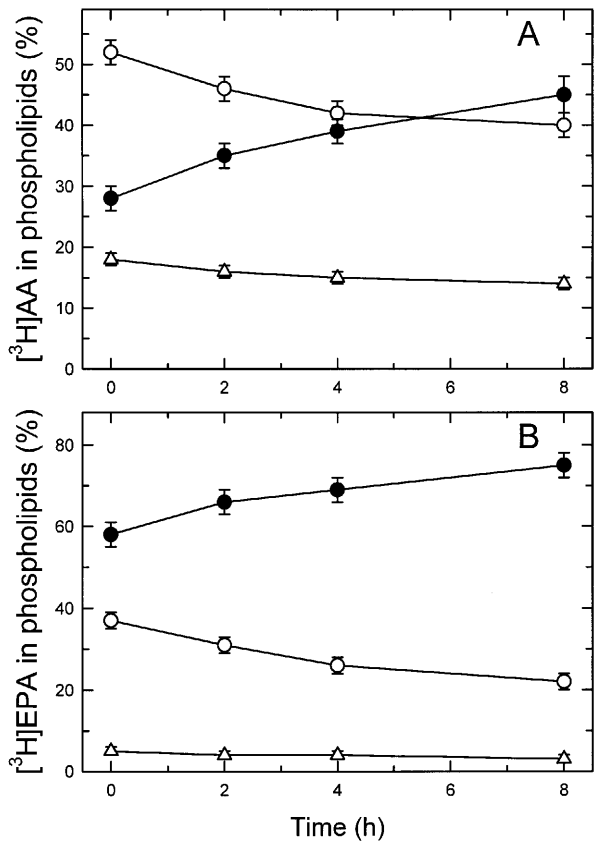

Figure 3 Distribution of [^{3}H]AA or [^{3}H]EPA among phospholipids

The cells were pulse-labelled with [^{3}H]AA (**A**) or [^{3}H]EPA (**B**) for 30 min, washed and then incubated without label for different periods of time. ○, PC; △, PI; ●, PE. The radioactivity incorporated into phospholipids is given as a percentage of the radioactivity present in phospholipids.

Fatty acid release from U937 cell phospholipids

To further substantiate the above findings, experiments utilizing cells labelled overnight with either [^{3}H]AA or [^{3}H]EPA were also conducted. In these experiments, the release of either [^{3}H]AA or [^{3}H]EPA was measured in the presence or absence of unlabelled exogenous fatty acid. Stimulation of the [^{3}H]AA-labelled PMA-differentiated cells with Con A resulted in a significant release of radiolabel to the incubation medium (Figure 2A). Such a release was inhibited by MAFP, but not by BEL, suggesting that, in common with many other cells of myelomonocytic origin [23–28], it is cPLA$_2$, not iPLA$_2$, that is the enzyme responsible for the release (Figure 2A).

When the experiments were conducted in the presence of excess unlabelled AA, the release of endogenous [^{3}H]AA was appreciably increased (Figure 2B). This indicates that the unlabelled exogenous fatty acid effectively competed with the [^{3}H]AA released by the cells for reacylation back into phospholipids. Under the same conditions however, exogenous EPA did not raise the Con A-stimulated [^{3}H]AA release (Figure 2B). At the concentration used (0.5 μM), neither fatty acid raised the basal level of [^{3}H]AA. This indicates that the exogenous fatty acids did not stimulate the cells on their own, nor did they compete for reacylation with the low quantities of [^{3}H]AA released under basal conditions (results not shown).

Results similar to those described above for [^{3}H]AA were obtained when EPA was used instead (Figure 2C). [^{3}H]EPA release was increased by the presence of exogenous EPA, but not by the presence of exogenous AA (Figure 2C), thus supporting the existence of separate pathways for AA and EPA incorporation in activated cells.

Table 3 Distribution of [^{3}H]AA and [^{3}H]EPA among phospholipid classes

PMA-differentiated U937 cells were exposed to [^{3}H]AA for 30 min, washed and then incubated for a further 8 h period without the label in the absence or presence of the indicated compounds. Lipids were extracted and the amount of [^{3}H]AA or [^{3}H]EPA in each phospholipid class was determined. Data are given as percentages of the radioactivity found in cellular phospholipids.

	Radioactivity (%)		
Fatty acid	PC	PI	PE
[^{3}H]AA			
Control	40±2	14±2	46±2
+IHP (50 μM)	49±2	17±1	34±2
+BEL (25 μM)	39±2	14±1	47±1
+MAFP (25 μM)	40±3	13±1	48±2
+LY311727 (25 μM)	40±2	14±1	46±1
[^{3}H]EPA			
Control	22±1	3±1	75±3
+IHP (50 μM)	34±2	6±1	60±3
+BEL (25 μM)	23±2	3±1	74±3
+MAFP (25 μM)	20±2	3±1	76±6
+LY311727 (25 μM)	20±2	2±1	78±4

Fatty acid redistribution within phospholipids

In the following series of experiments, the redistribution of AA and EPA among phospholipids was examined. For these studies, the cells were pulse-labelled with [^{3}H]AA or [^{3}H]EPA for 30 min. After removal of exogenous label and continued culture for 8 h, label distribution within phospholipid classes was determined.

During the 30 min pulse, [^{3}H]AA was incorporated mainly in PC (52%), followed by PE (30%) and PI (18%). No other phospholipid incorporated significant amounts of label. Interestingly, the pattern of distribution of [^{3}H]EPA within phospholipids was markedly different. PE incorporated the majority of this fatty acid (58%), followed by PC (37%) and PI (5%).

During the 8 h incubation period without label, a time-dependent decline in the [^{3}H]AA content in PC and a concomitant increase in PE occurred. The ^{3}H content in PI changed little along the time course (Figure 3A). Despite the marked losses of [^{3}H]AA from PC, this phospholipid still contained fatty acid quantities similar to those in PE, which contrasts with the situation in other cells of myelomonocytic origin [8,16,22,29–31], where the overwhelming amount of AA resides in PE. The rate of [^{3}H]AA redistribution between phospholipids did not appreciably change if the experiments were conducted in the absence of Ca^{2+} in the incubation medium. Cellular stimulation with Con A did not significantly accelerate the response (results not shown).

Figure 3(B) shows the remodelling of [^{3}H]EPA among phospholipids. A slow, time-dependent decline of [^{3}H]EPA in PC and a concomitant increase in PE again occurred, while changes in PI were undetectable. As previously observed for [^{3}H]AA, the distribution of [^{3}H]EPA between PC and PE was not significantly affected by cellular treatment with Con A nor was it affected by Ca^{2+} depletion.

The redistribution of AA and EPA from PC to PE is a hallmark of the CoA-IT-driven phospholipid remodelling [12], and, as such, was significantly inhibited by the specific inhibitor IHP [16] (Table 3). CoA-IT catalyses the CoA-independent transfer of AA from a phospholipid donor (AA-containing PC) to a lysophospholipid acceptor (usually lysoPE, acyl and alkenyl species) [12,13]. To study the origin of the lysophospholipid acceptors used in the CoA-IT-driven reaction, the cells were incubated with different PLA_2 inhibitors during the 8 h remodelling period. The inhibitors used were the $iPLA_2$ inhibitor BEL (up to 25 μM), the dual $iPLA_2$/$cPLA_2$ inhibitor MAFP (up to 25 μM) and the secretory PLA_2 inhibitor LY311727 (up to 50 μM). None of those compounds exerted a significant effect on the movement of [^{3}H]AA or [^{3}H]EPA from PC to PE (Table 3). No known mammalian form of PLA_2 is resistant to all the three PLA_2 inhibitors listed in Table 3 [32,33]. Hence, if the provider of lysoPE for the CoA-IT-catalysed reaction is a PLA_2, it must be of an as-yet-undescribed type.

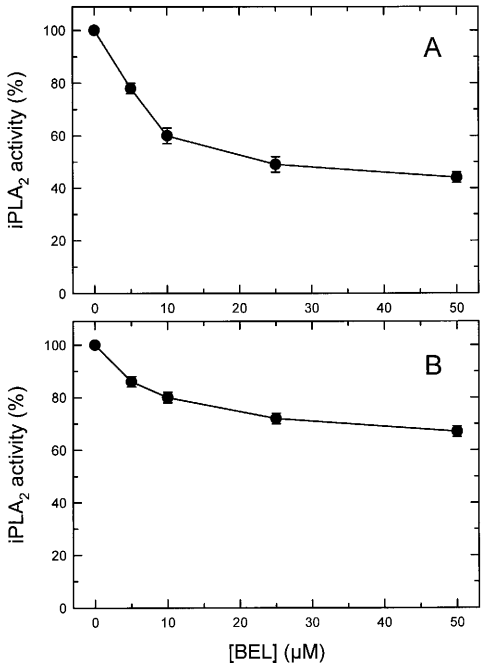

Figure 4 $iPLA_2$ activity of U937 cells

Homogenates were prepared from cells treated with the indicated concentrations of BEL for 30 min, and endogenous $iPLA_2$ activity was assayed using PC (**A**) or PE (**B**) as substrates. Data are expressed as a percentage of activity in the absence of BEL.

Identification of U937 cell $iPLA_2$ activity

Figure 4(A) shows that, when using [^{3}H]AA-containing PC as a substrate, the PMA-differentiated U937 cells exhibited significant $iPLA_2$ activity that could be inhibited by BEL in a dose-dependent manner. Interestingly, a significant portion of the $iPLA_2$ activity (40–50%) was found to be resistant to BEL. This suggests that PMA-differentiated U937 cells express both BEL-sensitive and -insensitive $iPLA_2$ activities. This is a striking finding, in view of previous reports showing that all $iPLA_2$ types cloned so far are completely supressed by BEL [34–37] and that enzymic assays utilizing whole-cell homogenates have generally shown that virtually all the measurable $iPLA_2$ activity is BEL-sensitive [6,38–42]. As a positive control for the results shown in Figure 4(A), $iPLA_2$ assays were conducted under conditions that duplicated those used in [6] to demonstrate complete inhibition of cellular $iPLA_2$ activity by BEL in homogenates from $P388D_1$ macrophages. Specifically, $iPLA_2$ was measured toward a substrate in the form of mixed micelles of Triton X-100/phospholipid at a molar ratio 4:1 [6]. Under these conditions, the $iPLA_2$ activity measured was 3–5-fold lower than that observed toward

pure phospholipid vesicles. Inclusion of 25 μM BEL in the assay mixture resulted in a 40–50 % inhibition, which is consistent with the results obtained with the vesicle assay. Thus, regardless of the assay system utilized, substantial BEL-independent $iPLA_2$ activity could be observed in the U937 cell homogenates. Replacing the EDTA by $CaCl_2$ (2 mM) in both the vesicle and the mixed-micelle assays increased PLA_2 activity by 1.5–2-fold.

Figure 4(B) shows the $iPLA_2$ activity of PMA-differentiated U937 cells as assayed towards [^{3}H]AA-containing PE. Inhibition in the presence of BEL was again detected, although in this case the majority of the activity was BEL-independent. Thus PMA-differentiated U937 cells possess the capacity to generate significant amounts of lysoPE in a BEL-independent manner. Importantly, inclusion of MAFP or LY311727 in the assays had no measurable effect on enzyme activity. When similar studies were conducted with [^{3}H]EPA-containing PC and PE and substrates, the results were essentially the same as those shown in Figure 4 (results not shown).

DISCUSSION

Unlike saturated fatty acids, AA and other polyunsaturated fatty acids such as EPA do not generally enter cellular phospholipids via direct acylation of glycerol phosphate/dihydroxyacetone or lysophosphatidic acid (i.e. the *de novo* pathway), but rather do it at a later stage, via direct acylation of pre-existing lysophospholipid acceptors. Since lysophospholipids are produced by the hydrolytic action of PLA_2 on phospholipids, this class of enzymes necessarily play a key role in AA incorporation into phospholipids.

Previous studies demonstrated that AA incorporation into the phospholipids of murine peritoneal macrophages does not require Ca^{2+}, which suggested the involvement of an $iPLA_2$-like activity [22]. Such an activity was later identified to belong to the Group VI $iPLA_2$ in studies carried out with the $P388D_1$ macrophage-like cell line [6,7]. These findings were later confirmed by several workers in human neutrophils [8], rat submandibular ductal cells [9] and rat uterine stromal cells [10]. Importantly, however, the contribution of Group VI $iPLA_2$ to maintaining the lysophospholipid pool that facilitates AA incorporation appears to largely depend on cell type. On the basis of BEL inhibition studies, the $iPLA_2$ contribution ranges from ≈ 90 % in rat submandibular ductal cells [9], 50–60 % in phagocytic cells [6–8] to only 20–25 % in rat uterine stromal cells [10]. Altogether, these findings suggest that, in addition to the contribution of the $iPLA_2$, the cells possess other mechanisms to generate and maintain the appropriate levels of lysophospholipid acceptors. This notion was highlighted by a recent report in rat pancreatic islets [11], where $iPLA_2$ inhibition by BEL did not result in diminished AA incorporation into phospholipids. Rat pancreatic islets, however, maintain cellular lysophospholipid levels at very high levels and, unlike submandibular ductal cells, macrophages, neutrophils and stromal cells, AA incorporation in these cells depends on Ca^{2+} [11]. These two differential findings might help to explain why the $iPLA_2$ appears not to play a role in AA incorporation into islet phospholipids. Nevertheless, $iPLA_2$ is estimated to contribute to at least 25 % of the very high steady-state lysophospholipid levels in pancreatic islets [11]. This suggests that, despite the apparent lack of involvement of Group VI $iPLA_2$ in AA incorporation reactions in these cells, and in addition to any as-yet-unclear signalling role [43], the enzyme still plays a significant housekeeping role in islets.

In an attempt to gain further insight into the mechanisms regulating lysophospholipid levels in phagocytic cells, the features of AA incorporation into and remodelling within the phospholipids of PMA-differentiated U937 cells have been studied and compared with those of EPA. U937 cells are derived from a histiocytic lymphoma and differentiate into macrophage-like cells when treated with PMA [14,15]. U937 cells are known to express $cPLA_2$ [44–46] and $iPLA_2$ [27] and have also recently been reported to express secretory PLA_2 ($sPLA_2$) activity and immunoreactive protein after differentiation with PMA [47]. The present data indicate that, in these cells, AA incorporation into phospholipids of both resting and Con A-activated, PMA-differentiated U937 cells exhibits features that are similar to those of other phagocytic cells such as $P388D_1$ macrophages and neutrophils [6–8], i.e. it is Ca^{2+}-independent and BEL-inhibitable. Importantly, BEL reduces AA incorporation only partially, suggesting the existence of Ca^{2+}-independent pathway(s) for AA incorporation into phospholipids that may not involve the BEL-sensitive Group VI $iPLA_2$.

The above view is further supported by the experiments studying the incorporation of EPA into phospholipids. While Ca^{2+}-independent EPA incorporation is only marginally affected by BEL. EPA is preferentially incorporated into PE over PC, which is at variance with the profile of AA incorporation. However, both fatty acids compete with each other for incorporation into the phospholipids of resting cells, indicating that the two fatty acids share, at least in part, a common pathway for incorporation. This situation contrasts with the one seen in Con A-activated cells, where AA and EPA do not compete with each other for incorporation into phospholipids. Of note, EPA incorporation into the phospholipids of activated cells is sensitive to MAFP, suggesting the involvement of Group IV $cPLA_2$ in the process. It is therefore likely that the increased lysophospholipid availability produced as a consequence of the stimulus-induced $cPLA_2$ activation plays a role in EPA incorporation. In accord with this, the PE pools appear to be preferential targets for $cPLA_2$ phospholipolysis in stimulated phagocytes [23,48]. On the other hand, the stimulated incorporation of AA still occurs preferentially into PC and is MAFP-independent. Thus $cPLA_2$ would provide an additional supply of lysoPE acceptors to be used by EPA, but not by AA, and the existence of such an alternative route for EPA incorporation may explain why, in the activated cells, EPA does not compete with AA. Whether such a difference also reflects the involvement of other systems, such as acyl-CoA transferases specific for $\omega-3$ versus $\omega-6$ fatty acids [2,49], is unknown at present and will be the subject of future studies.

After the initial incorporation of AA mostly into PC, a slow transfer of the fatty acid occurs towards PE. Despite PE being already the major EPA-containing phospholipid at the beginning of the incubations, a transfer of EPA from PC to PE is observed as well. These changes have been well documented in other cell systems and reflect the remodelling action of the CoA-IT on cellular phospholipids [12].

A PLA_2 is strikingly involved in the CoA-IT-driven remodelling reactions by providing the lysoPE acceptors utilized in the transacylation reaction [12,13]. In the present study the nature of such a PLA_2 was investigated by measuring the transfer of AA and EPA from PC to PE in the presence of different PLA_2 inhibitors. However, none of the inhibitors tested, i.e. BEL, MAFP and LY311727, exert any detectable effect on the response. This unexpected finding suggests that the PLA_2 involved in this pathway may not be any of the previously identified PLA_2 types [32,33], and is in accord with a recent report in peripheral-blood T lymphocytes [50]. The Ca^{2+}-independent nature of the response strongly suggests the role of an $iPLA_2$-like activity, different from the Group VI enzyme.

In keeping with the above notion, studies with cell homogenates have revealed that iPLA$_2$ activity of PMA-differentiated U937 cells exhibits a significant component that is resistant to BEL, and this component is more evident when PE is used as a substrate for the assay. This finding is consistent with the possibility that the BEL-insensitive iPLA$_2$ activity of PMA-differentiated U937 cells prefers PE over PC. It is noteworthy that the incorporation of EPA into phospholipids is only slightly affected by BEL and that this fatty acid is incorporated mainly into PE lipids. If a PE-preferring iPLA$_2$ were to mediate most of the incorporation of EPA into phospholipids, one would expect the production of lysoPE to be essentially BEL-independent, as indeed it is. Altogether, the results stress that the PMA-differentiated U937 cells contain a distinctive PLA$_2$-like activity capable of generating lysophospholipid acceptors for incorporation into, and remodelling among, phospholipids under conditions where all other known PLA$_2$s are not involved. Clearly, much work is yet to be done to clarify the mechanisms responsible for lysophospholipid level maintenance, but the current studies have begun to dissect the pathways and enzymes involved, and provide the fundamentals for future research aimed at understanding the role of this novel iPLA$_2$-like activity in cellular phospholipid homoeostasis.

This work was supported in part by grant BMC2001-2244 from the Spanish Ministry of Science and Technology.

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Received 23 January 2002/25 February 2002; accepted 4 April 2002