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Inhibition of calcium-independent phospholipase A_2 prevents arachidonic acid incorporation and phospholipid remodeling in $P388D_1$ macrophages

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ABSTRACT **Cellular levels of free arachidonic acid (AA) are controlled by a deacylation/reacylation cycle whereby the fatty acid is liberated by phospholipases and reincorporated by acyltransferases. We have found that the esterification of AA into membrane phospholipids is a Ca^{2+}-independent process and that it is blocked up to 60–70% by a bromo*enol*-lactone (BEL) that is a selective inhibitor of a newly discovered Ca^{2+}-independent phospholipase A_2 (PLA_2) in macrophages. The observed inhibition correlates with a decreased steady-state level of lysophospholipids as well as with the inhibition of the Ca^{2+}-independent PLA_2 activity in these cells. This inhibition is specific for the Ca^{2+}-independent PLA_2 in that neither group IV PLA_2, group II PLA_2, arachidonoyl-CoA synthetase, lysophospholipid:arachidonoyl-CoA acyltransferase, nor CoA-independent transacylase is affected by treatment with BEL. Moreover, two BEL analogs that are not inhibitors of the Ca^{2+}-independent PLA_2—namely a bromomethyl ketone and methyl-BEL—do not inhibit AA incorporation into phospholipids. Esterification of palmitic acid is only slightly affected by BEL, indicating that *de novo* synthetic pathways are not inhibited by BEL. Collectively, the data suggest that the Ca^{2+}-independent PLA_2 in $P388D_1$ macrophages plays a major role in regulating the incorporation of AA into membrane phospholipids by providing the lysophospholipid acceptor employed in the acylation reaction.**

A general feature of membrane phospholipids is the position-specific esterification of the glycerol phosphate backbone with fatty acids. Saturated fatty acids are usually found in the *sn*-1 position whereas polyunsaturated fatty acids such as arachidonic acid (AA) are usually found in the *sn*-2 position. AA is the common precursor of the eicosanoids, a family of biologically active compounds which include the prostaglandins and leukotrienes (for review, see ref. 1). Because cellular AA is almost exclusively found in esterified form, the availability of this fatty acid is critical for eicosanoid biosynthesis (for review, see refs. 1–3). In unstimulated cells, the cellular levels of free AA are primarily controlled by the highly active AA esterification system (3–5). Thus, free AA provided to the cell or liberated by phospholipase A_2 (PLA_2) is rapidly converted to arachidonoyl-CoA by arachidonoyl-CoA synthetase at the expense of ATP and immediately incorporated into phospholipids by CoA-dependent acyltransferases (6, 7).

The process of AA incorporation into phospholipids displays unique features when compared with that of other fatty acids. First, the enzymatic machinery responsible for AA esterification in various cell types appears to be highly selective for this fatty acid (3, 4). Second, the major route for incorporation of AA into phospholipids is not mediated by the *de novo* pathway via acylation of glycerol phosphate and/or dihydroxyacetone phosphate to produce phosphatidic acid (PA), but rather by a deacylation/reacylation cycle (8). It is believed that this cycle is largely responsible for remodeling of cellular phospholipids leading to the selective distribution of AA at the *sn*-2 position (Fig. 1). Accordingly, the fatty acid at the *sn*-2 position of preexisting phospholipids is cleaved by PLA_2, creating a lysophospholipid which is rapidly reesterified with another fatty acid by a CoA-dependent acyltransferase (2–7). Due to the exceedingly high activity of cellular arachidonoyl-CoA synthetase as compared with that of cellular PLA_2, fatty acid activation appears not to limit AA incorporation into phospholipids (9–11). Thus, in resting cells, availability of the lysophospholipid acceptor provided by a PLA_2-like activity is a limiting factor for incorporation of AA into phospholipids.

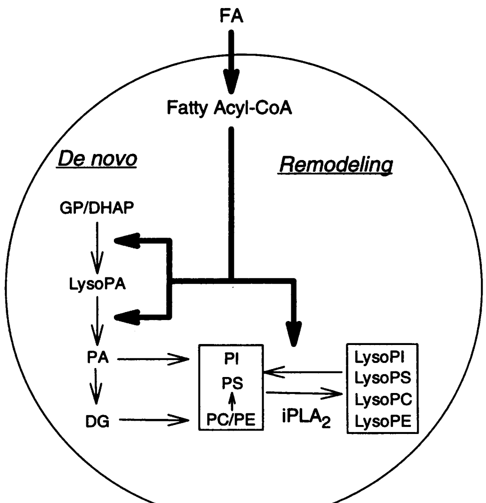

FIG. 1. *De novo* and remodeling pathways for incorporation of free fatty acid (FA) into phospholipids. In the *de novo* pathway, FA is incorporated via fatty acyl-CoA into glycerol phosphate (GP) or dihydroxyacetone phosphate (DHAP) and into the resulting lysophosphatidic acid (lysoPA) by fatty acyl-CoA acyltransferases to form phosphatidic acid (PA). In macrophages, the PA can be converted to phosphatidylinositol (PI) or can be converted to diacylglycerol (DG), which is the precursor for phosphatidylcholine (PC) and phosphatidylethanolamine (PE), which in turn form phosphatidylserine (PS). In contrast, in the remodeling pathway, preformed PI, PS, PC, or PE is acted on by the Ca^{2+}-independent PLA_2 ($iPLA_2$) to produce lysoPI, lysoPS, lysoPC or lysoPE; these can be reacylated by acyltransferases using fatty acyl-CoA.

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Abbreviations: AA, arachidonic acid; BEL, bromo*enol*lactone [(*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one]; BMK, bromomethyl ketone [6-bromo-2-(1-naphthyl)-5-oxohexanoic acid]; MeBEL, methyl-BEL [(*E*)-6-(1-bromoethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one]; PLA_2, phospholipase A_2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid.
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We have examined the esterification of AA into phospholipids in murine macrophage-like P388D$_1$ cells. Evidence is presented that a recently characterized Ca^{2+}-independent PLA$_2$ (12) plays a hitherto unrecognized role in this process by providing the lysophospholipid used in AA esterification. This PLA$_2$ thereby may play a major role in the remodeling of membrane phospholipids.

EXPERIMENTAL PROCEDURES

Materials. Mouse P388D$_1$ cells were obtained from the American Type Culture Collection. Iscove's modified Dulbecco's medium (endotoxin, <0.05 ng/ml) was from BioWhittaker. Fetal bovine serum was from HyClone. Nonessential amino acids were from Irvine Scientific. [5,6,8,9,11,12,14,15-^{3}H]AA (100 Ci/mmol; 1 Ci = 37 GBq), [9,10-^{3}H]palmitic acid (54 Ci/mmol), [*methyl*-^{3}H]choline chloride (79 Ci/mmol), 1-palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (60 mCi/mmol), 1-*O*-[1′,2′-^{3}H]hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine (60 Ci/mmol), and 1-[1-^{14}C]palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine (60 mCi/mmol) were from New England Nuclear. Quin-2 tetrakis(acetoxymethyl) ester (AM) and bovine serum albumin (fatty acid-free) were from Sigma. Silica gel G-60 TLC plates were from Analtech. Organic solvents (analytical grade) were from Baker or Fisher. The Ca^{2+}-independent PLA$_2$ inhibitor (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (bromo*enol*lactone, BEL) and its analogs 6-bromo-2-(1-naphthyl)-5-oxohexanoic acid (bromomethyl ketone, BMK) and (*E*)-6-(1-bromoethylene)tetrahydro-3-(1-naphthalenyl)-2*H*-pyran-2-one (methyl-BEL, MeBEL) were synthesized in our laboratory as described (13).

Cell Culture. P388D$_1$ cells were maintained at 37°C in a humidified atmosphere of 90% air and 10% CO_2 in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml) streptomycin (100 μg/ml), and nonessential amino acids. Adherent cells were selected by passage of only adherent cells. The cells used in the experiments reported below were between passages 15 and 30. Cells were plated at 10^6 per well in six-well plates, allowed to adhere overnight, and used for experiments the following day. All experiments were conducted in serum-free Iscove's modified Dulbecco's medium.

Measurement of [^{3}H]AA and [^{3}H]Palmitic Acid Incorporation into Cellular Phospholipids. P388D$_1$ cells were placed in serum-free medium for 30–60 min before exposure to exogenous [^{3}H]AA (5 nM; 0.5 μCi/ml) or [^{3}H]palmitic acid (10 nM; 0.5 μCi/ml). At the indicated times, supernatants were removed and the cell monolayers were gently washed with medium containing albumin at 5 mg/ml (14). The cell monolayers were scraped twice with 0.05% Triton X-100, and total lipids were extracted according to Bligh and Dyer (15). Lipids were separated by TLC with *n*-hexane/diethyl ether/acetic acid (70:30:1 by volume). In this system, phospholipids remain at the origin of the plate. For separation of phospholipid classes, plates that had been sprayed with 1% potassium oxalate were run twice, using the upper phase of a system consisting of ethyl acetate/isooctane/acetic acid/water (130:20:30:100 by volume) (16). This system allowed a good resolution among major phospholipid classes. Radioactive content in the different lipid classes was quantitated by liquid scintillation counting. For preparation of Ca^{2+}-depleted cells, cells were incubated with 1 mM EGTA and 40 μM quin-2 AM in a Ca^{2+}-free medium for 60 min at 37°C (17), washed twice, and treated with [^{3}H]AA as described above. When BEL was used, it was added to the cells 30 min before addition of [^{3}H]AA or [^{3}H]palmitic acid.

Assay for Ca^{2+}-Independent PLA$_2$. Aliquots of P388D$_1$ cell homogenates were incubated for 30 min at 40°C in 100 mM Hepes, pH 7.5/5 mM EDTA/0.8 mM ATP/400 μM Triton X-100/100 μM 1-palmitoyl-2-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine, in a final volume of 500 μl. The substrate was used in the form of mixed micelles of Triton X-100/phospholipid at a molar ratio 4:1, obtained by a combination of heating (above 40°C), vortex mixing, and water bath sonication until the solution clarified (12). At the end of the reaction, the radiolabeled fatty acid product was extracted by the modified Dole assay procedure (12).

Measurement of LysoPC Levels. For the measurement of lysoPC, cells were incubated with [^{3}H]choline at 1 μCi/ml for 3 days. After the cells were treated with the indicated BEL doses for 30 min, cellular lipids were extracted with ice-cold 1-butanol as described (18), and separated by TLC, with chloroform/methanol/acetic acid/water (50:40:6:0.6 by volume) as a solvent system. Spots corresponding to lysoPC were scraped and assayed for radioactivity by liquid scintillation counting.

Other Methods. Group IV PLA$_2$ and group II PLA$_2$ activities were assayed exactly as described (19). Arachidonoyl-CoA synthetase was measured as described by Wilson *et al.* (20). In brief, the assay mixture was composed of 20 mM $MgCl_2$, 10 mM ATP, 1 mM coenzyme A, 1 mM 2-mercaptoethanol, 130 μM [^{3}H]AA, 100 mM Tris·HCl (pH 8.0), and cell homogenate (up to 100 μg of protein) in a total volume of 0.15 ml. After incubation at 37°C for 10 min, the reaction was terminated by the addition of 2.25 ml of 2-propanol/heptane/2 M sulfuric acid (40:10:1 by volume). Heptane (1.5 ml) and water (1 ml) were added and the mixture was vortexed vigorously before centrifugation at 1000 × *g* for 5 min. The aqueous phase was extracted twice with 2 ml of heptane containing nonradioactive AA at 4 mg/ml, and a 1-ml aliquot of the aqueous phase was used for radioactivity determination by liquid scintillation counting.

Arachidonoyl-CoA:lysophospholipid acyltransferase activity was measured as described by Lands *et al.* (21). The assay mixture was composed of 50 μM arachidonoyl-CoA, 50 μM of 1-[1-^{14}C]palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine, 50 mM Tris·HCl (pH 7.5), and cell homogenate (up to 100 μg of protein) in a final volume of 0.15 ml. After incubation at 37°C for 10 min, the reaction was stopped by the addition of 0.56 ml of chloroform/methanol (1:2 by volume). Chloroform (0.19 ml) and water (0.19 ml) were added and the mixture was vortexed vigorously before centrifugation at 1000 × *g* for 5 min. The organic phase was evaporated and chromatographed on Silica gel G plates with chloroform/methanol/acetic acid/water (50:25:8:4 by volume) as the developing solvent. PC and lysoPC were scraped off the plate and assayed for radioactivity by liquid scintillation counting.

CoA-independent transacylase activity was measured as described by Venable *et al.* (22). The assay mixture was composed of 120 mM NaCl, 2 mM EGTA, 5 μM 1-*O*-[^{3}H]hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso platelet-activating factor, lysoPAF), 100 mM Tris·HCl (pH 7.5), and cell homogenate (up to 100 μg of protein) in a final volume of 0.2 ml. In this assay system, the lysophospholipid acceptor for the acylation reaction (lysoPAF) is added to the assay mixture and the phospholipid donor is provided by the homogenate. After incubation at 37°C for 5 min, the reaction was stopped by the addition of 0.75 ml of chloroform/methanol (1:2). Chloroform (0.25 ml) and water (0.25 ml) were added and the mixture was vortexed vigorously before centrifugation at 1000 × *g* for 5 min. The organic phase was evaporated and chromatographed on Silica gel G plates with chloroform/methanol/acetic acid/water (50:25:8:4 by volume) as the developing solvent. PC and lysoPAF were cut out of the plate and assayed for radioactivity by liquid scintillation counting.

Protein concentration was determined by the method of Bradford (23), with bovine serum albumin as a standard.

Data Presentation. Assays were carried out in triplicate. Each set of experiments was repeated at least three times with similar results. The data presented are from representative experiments.

RESULTS AND DISCUSSION

Ca^{2+}-Independent AA Incorporation into $P388D_1$ Cell Phospholipids. Much attention has been focused on the mechanisms regulating free AA availability during cellular activation. Recent studies have identified at least two distinct PLA_2 enzymes that may play a role in this process by directly generating free AA via their hydrolytic action on membrane phospholipids. These are the 85-kDa group IV PLA_2 and the 14-kDa group II PLA_2 (for review, see ref. 24). Although these two PLA_2 enzymes differ notably in their physical and catalytic properties, both require Ca^{2+} for activity under physiological conditions (24). Not surprisingly, receptor activation of AA release via either or both of these enzymes is strongly dependent on Ca^{2+} availability (25).

However, the release of AA is not the only cellular event being regulated by PLA_2 enzymes. Other aspects of phospholipid metabolism—particularly fatty acid incorporation into and remodeling among phospholipids—have long been thought to depend on a PLA_2 whose identity has remained unknown (3–8). The process of AA incorporation into phospholipids dominates over AA release in unstimulated cells; hence the overwhelming amount of cellular AA is found in esterified form, not as a free fatty acid.

We have previously shown that $P388D_1$ cells avidly take up free [^{3}H]AA from the incubation medium and incorporate it very quickly into their membrane phospholipids (25). In these cells, PLA_2-mediated release of AA from stimulated cells is dependent on the presence of extracellular Ca^{2+} (25). In contrast, in unstimulated cells, AA incorporation into cell phospholipids does not require extracellular Ca^{2+} (Fig. 2). The extent of [^{3}H]AA acylation in Ca^{2+}-free, EGTA-containing medium was the same as that with Ca^{2+} even up to 30 min, conditions which have been shown to cause a progressive depletion of the intracellular Ca^{2+} in $P388D_1$ cells (about 70% loss of intracellular Ca^{2+} after 30 min; see ref. 26). To confirm that incorporation was also independent of intracellular Ca^{2+}, the cells were depleted of their intracellular Ca^{2+} stores by treating them with 40 μM quin-2 AM plus 1 mM EGTA in a Ca^{2+}-free medium. This treatment buffers the intracellular Ca^{2+} concentration at very low levels ($\approx$10 nM) (17, 26). With Ca^{2+}-depleted cells, both the kinetics and magnitude of [^{3}H]AA incorporation into phospholipids remained the same as those shown in Fig. 2. These findings demonstrate that AA esterification is not affected by either extracellular or intracellular Ca^{2+} depletion, suggesting that the PLA_2 responsible for supplying the lysophospholipid acceptor for AA esterification is Ca^{2+}-independent.

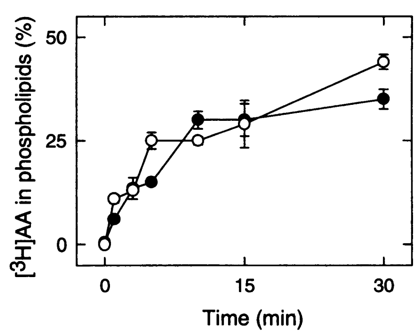

FIG. 2. Effect of extracellular Ca^{2+} depletion on the incorporation of [^{3}H]AA into $P388D_1$ cell phospholipids. $P388D_1$ cells were incubated in serum-free medium for 30–60 min and then exposed to exogenous [^{3}H]AA (5 nM; 0.5 μCi/ml) for the indicated periods of time in medium containing 1.3 mM $CaCl_2$ (●) or Ca^{2+}-free medium containing 1 mM EGTA (○). [^{3}H]AA incorporated into phospholipids is expressed as a percentage of the radioactivity originally present in the medium.

Role of Ca^{2+}-Independent PLA_2. We recently purified a Ca^{2+}-independent PLA_2 from the cytosol of $P388D_1$ cells (12). This enzyme does not show preference for phospholipids containing AA; in fact, it prefers palmitoyl over arachidonoyl residues (12). The Ca^{2+}-independent PLA_2 from $P388D_1$ macrophages is potently and irreversibly inhibited by the mechanism-based inhibitor BEL (13). This compound manifests >1000-fold selectivity for inhibition of Ca^{2+}-independent PLA_2 enzymes versus the Ca^{2+}-dependent PLA_2 enzymes (27). BEL inhibits the purified Ca^{2+}-independent PLA_2 from $P388D_1$ cells in a concentration-dependent manner, with half-maximal inhibition occurring at 60 nM after a 5-min preincubation at 40°C (13).

Measurements of Ca^{2+}-independent PLA_2 activity in homogenates prepared from cells treated with variable amounts of BEL for 30 min demonstrate a dose-dependent inhibition of enzyme activity, complete inhibition being reached at BEL concentrations higher than 25 μM (Fig. 3*A*). Cells treated with

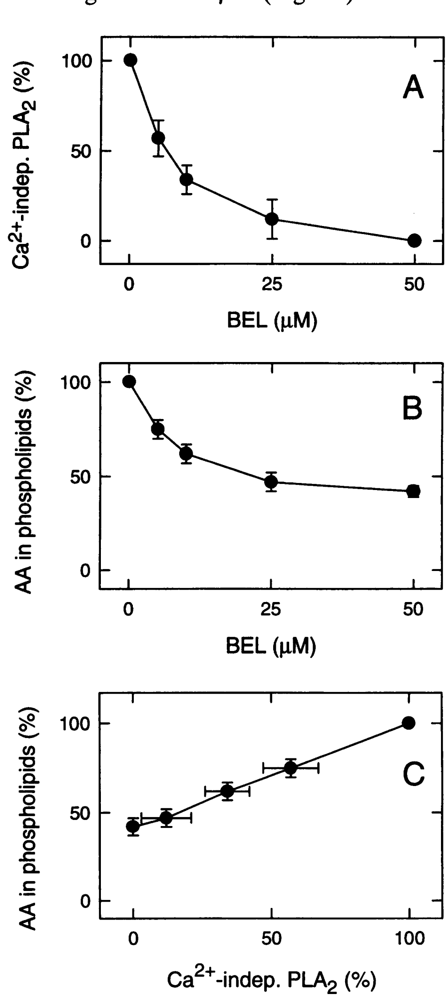

FIG. 3. Inhibition by BEL of both endogenous Ca^{2+}-independent PLA_2 activity and AA esterification into phospholipids. (*A*) Homogenates were prepared from cells treated with the indicated concentration of BEL for 30 min, and endogenous Ca^{2+}-independent PLA_2 activity was assayed and is expressed as a percentage of activity in the absence of BEL. (*B*) Cells were pretreated with the indicated concentration of BEL for 30 min and then incubated with exogenous [^{3}H]AA (5 nM; 0.5 μCi/ml) for 10 min in Ca^{2+}-containing medium. The ^{3}H radioactivity incorporated into phospholipids is given as a percentage of the radioactivity incorporated in the absence of inhibitor. (*C*) Plot of [^{3}H]AA esterification (taken from *B*) versus Ca^{2+}-independent PLA_2 activity (taken from *A*) at each BEL concentration.

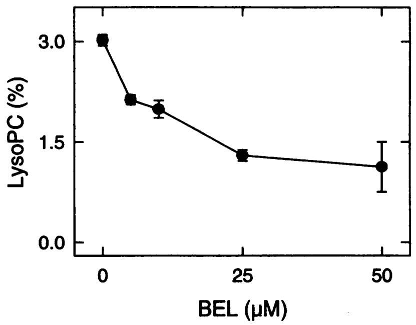

FIG. 4. Effect of BEL on lysoPC levels. Cells prelabeled with [^{3}H]choline were treated with the indicated BEL concentration for 30 min and the cellular amount of lysoPC was determined by TLC. Radioactivity in lysoPC is expressed as a percentage of total [^{3}H]choline incorporated into phospholipids.

BEL under the conditions specified above and exposed to exogenous [^{3}H]AA (5 nM) showed a dose-dependent inhibition of [^{3}H]AA incorporation into phospholipids (Fig. 3*B*). The same experiment carried out on Ca^{2+}-depleted cells—i.e., cells treated with 40 μM quin-2 AM plus 1 mM EGTA in a Ca^{2+}-free medium for 1 hr (17)—gave a similar inhibition of [^{3}H]AA esterification. Pretreatment with BEL concentrations higher than 25 μM did not result in further inhibition of [^{3}H]AA esterification. The inhibition by BEL of AA esterification was not due to drug-induced cytotoxic effects, as judged by trypan blue exclusion, or to the loss of cells from the culture dish, as determined by a protein assay according to Bradford (23).

Inhibition by BEL of AA esterification into phospholipids directly correlated with the inhibition of endogenous Ca^{2+}-independent PLA_2 activity (Fig. 3*C*). In agreement with these data, BEL decreased the steady-state level of lysoPC in a dose-dependent and saturable manner (Fig. 4). Importantly, pretreatment with BEL at doses higher than 25 μM did not result in further inhibition of lysoPC levels, demonstrating saturation of inhibition through BEL-sensitive pathways at the 65% level. This is the same saturating level observed for inhibition of [^{3}H]AA esterification (compare Fig. 3*B* and Fig. 4).

In addition to the Ca^{2+}-independent PLA_2, P388D$_1$ macrophages contain two other PLA_2 activities—namely, group IV and group II (19). Measurements of these two PLA_2 activities were conducted in homogenates from BEL-treated cells. No effect of BEL was detected at any concentration tested (Table 1).

Collectively, the data in Figs. 3 and 4 and Table 1 suggest that the inhibition of Ca^{2+}-independent PLA_2 by BEL results in a decreased steady-state level of lysophospholipid in the cells and hence in a decreased incorporation of [^{3}H]AA into phospholipids. To further assess BEL specificity, we tested the effect of two inactive BEL analogs, BMK and MeBEL, on AA incorporation into P388D$_1$ cell phospholipids. These two compounds do not inhibit the Ca^{2+}-independent PLA_2 from P388D$_1$ cells (ref. 13; E.J.A. and E.A.D., unpublished data) and did not affect AA esterification into phospholipids (data not shown).

***De Novo* Pathway and Other Possibilities.** The lack of 100% inhibition by BEL on both lysophospholipid levels and [^{3}H]AA esterification under our conditions most likely reflects some experimental limitation such as the inability of BEL to reach all the Ca^{2+}-independent PLA_2 while the cells are intact. It is also possible that the remaining BEL-insensitive 35% of AA esterification may be modulated by an alternative pathway such as the recently described CoA-dependent transacylation reaction (28), the CoA-independent acylation reaction catalyzed by 2-acylglycerophosphoethanolamine acyltransferase/acyl-[acyl-carrier-protein]synthetase (29), or the *de novo* biosynthetic pathway (30) (see Fig. 1). In this regard, Lapetina *et al.* (31) have shown that horse neutrophils transiently incorporate appreciable amounts of [^{3}H]AA into PA, raising the possibility that in this cell type, a significant portion of the AA esterification into phospholipids arises from *de novo* synthesis. To address this latter possibility in the P388D$_1$ cells, we determined the phospholipid products into which [^{3}H]AA is incorporated and the influence of BEL on this profile. The results are shown in Table 2. BEL treatment did not significantly affect the profile of AA incorporation into phospholipids, suggesting that BEL does not effect further AA remodeling reactions among phospholipids (see below). Also, it is interesting that under the experimental conditions employed, the triacylglycerol incorporated <2% of total [^{3}H]AA, which is consistent with a remodeling pathway dominating rather than the *de novo* pathway. Blank *et al.* (32) found similar limited incorporation of [^{3}H]AA into triacylglycerols in HL-60 granulocytes.

We failed to detect significant labeling of PA with [^{3}H]AA both in the presence or absence of BEL. Under exactly the same conditions, the incorporation of [^{3}H]palmitic acid into PA was readily detectable (Table 2). Together, these data suggest that the contribution of the *de novo* synthetic pathway to AA incorporation into phospholipids is minor when compared with the BEL-sensitive route. In contrast, the finding that [^{3}H]palmitic acid is readily incorporated into PA, along with the fact that this incorporation is even enhanced in BEL-treated cells, is fully consistent with the idea that *de novo* biosynthesis is a major pathway for the incorporation of palmitic acid into phospholipids. The relative enhancement of PA labeling with [^{3}H]palmitic acid in BEL-treated cells would be expected if BEL had no effect on the *de novo* pathway but impaired fatty acid esterification via direct acylation of lysophospholipids. Consistent with this view, BEL exerts only slight inhibitory effects on the incorporation of [^{3}H]palmitic acid into phospholipids (Table 2), giving further support to the notion that BEL does not impair *de novo* phospholipid synthesis.

To further confirm that the reduction of AA esterification by BEL is actually due to inhibition of the Ca^{2+}-independent PLA_2 and not the result of unexpected BEL effects on the AA

Table 1. Effect of BEL on other PLA_2 activities and on the AA reacylating enzymes of P388D$_1$ cells

BEL, μM	Group IV PLA_2, pmol/(min·mg)	Group II PLA_2, % hydrolysis	Arachidonoyl-CoA synthetase, nmol/(min·mg)	Acyl-transferase, nmol/(min·mg)	CoA-independent transacylase, pmol/(min·mg)
0	233 ± 15	8.2 ± 0.4	10.4 ± 0.9	3.3 ± 0.1	115 ± 3
5	ND	7.8 ± 0.3	10.2 ± 1.0	3.4 ± 0.1	ND
15	207 ± 12	8.2 ± 0.6	11.3 ± 1.0	3.2 ± 0.2	130 ± 23
25	219 ± 6	7.2 ± 0.2	11.8 ± 0.6	3.0 ± 0.2	131 ± 2
50	260 ± 13	8.1 ± 0.4	12.6 ± 0.1	3.3 ± 0.1	131 ± 4

Homogenates were prepared from cells treated with the indicated concentration of BEL for 30 min. Group IV PLA_2 activity was assayed by using mixed vesicles of PC/diacylglycerol at a molar ratio 2:1, in the presence of 2-mercaptoethanol (19). Group II PLA_2 activity was measured by the *Escherichia coli* assay (19). Arachidonoyl-CoA synthetase, lysophospholipid:arachidonoyl-CoA acyltransferase, and CoA-independent transacylase were determined as described in the text. ND, not determined.

Table 2. Effect of BEL on the profile of fatty acid incorporation into phospholipids

Phospholipid	$[^3H]$AA, %		$[^3H]$Palmitic acid, %	
	− BEL	+ BEL	− BEL	+ BEL
PC	45 ± 1	44 ± 4	62 ± 4	55 ± 6
PE	27 ± 3	22 ± 3	17 ± 1	11 ± 2
PI/PS	28 ± 4	34 ± 7	9 ± 3	12 ± 2
PA	<1	<1	13 ± 2	21 ± 3

$P388D_1$ cells were pretreated with 50 μM BEL for 30 min prior to incubation with exogenous $[^3H]$AA (5 nM; 0.5 μCi/ml) or $[^3H]$palmitic acid (10 nM; 0.5 μCi/ml) for 10 min. Data are given as a percentage of the total radioactivity found in all phospholipid classes at 10 min. Untreated cells and BEL-treated cells incorporated into phospholipids 29 ± 2% and 14 ± 2% of the $[^3H]$AA originally present in the media, and 15 ± 1% and 13 ± 1% of the $[^3H]$palmitic acid, respectively.

reacylating enzymes, enzymatic assays were conducted with homogenates from cells that had previously been preincubated with BEL (0–50 μM) for 30 min. Arachidonoyl-CoA synthetase, arachidonoyl-CoA:lysophospholipid acyltransferase, and CoA-independent transacylase activities were measured according to Wilson *et al.* (20), Lands *et al.* (21), and Venable *et al.* (22), respectively. No effect of BEL on these enzyme activities was detected at any concentration tested (Table 1).

Collectively, the results of this study demonstrate that AA esterification into $P388D_1$ macrophages is a Ca^{2+}-independent process and lead to the identification of a Ca^{2+}-independent PLA_2 as an enzyme responsible for regulating this process under resting conditions. Due to the extraordinary importance of acylation reactions in keeping the intracellular amounts of free AA at very low levels (3–8), the Ca^{2+}-independent PLA_2 appears to be a key enzyme in regulating the basal levels of eicosanoids synthesized by $P388D_1$ cells.

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