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Bromoenol Lactone Inhibits Magnesium-dependent Phosphatidate Phosphohydrolase and Blocks Triacylglycerol Biosynthesis in Mouse P388D$_1$ Macrophages*

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Bromoenol lactone (BEL) has previously been identified as a potent, irreversible, mechanism-based phospholipase A$_2$ (PLA$_2$) inhibitor that possesses greater than 1000-fold selectivity for inhibition of Ca^{2+}-independent PLA$_2$ (iPLA$_2$) *versus* the Ca^{2+}-dependent ones. Thus, this compound has been used as a selective tool for studies aimed at elucidating the role of iPLA$_2$ in certain cellular functions. Herein we report that BEL also inhibits cellular phosphatidic acid phosphohydrolase (PAP) activity in intact P388D$_1$ macrophages with an IC$_{50}$ of about 8 μM, which is very similar to that previously found for inhibition of iPLA$_2$ under the same experimental conditions. This results in the blockage of the incorporation of exogenous arachidonate and palmitate into diacylglycerol and triacylglycerol. Thus, inhibition of PAP by BEL blocks triacylglycerol biosynthesis in P388D$_1$ cells due to decreased diacylglycerol availability. Because two forms of PAP activity exist in mammalian cells, differential assays were performed to identify which of these forms was inhibited by BEL. The results of these experiments revealed that BEL selectively inhibits the cytosolic, Mg^{2+}-dependent enzyme. No apparent effect of BEL on the membrane-bound Mg^{2+}-independent PAP form could be detected. Collectively, the results reported herein establish that BEL inhibits two cellular phospholipases, namely iPLA$_2$ and Mg^{2+}-dependent PAP, with similar potency. Therefore, the inhibitory effect of BEL on Mg^{2+}-dependent PAP might explain several cellular functions previously attributed to iPLA$_2$.

Current evidence indicates that phospholipase A$_2$ (PLA$_2$)[1] is a major mediator of agonist-induced arachidonic acid (AA) release in most cell types (for review, see Ref. 1). However, the levels of free AA available for eicosanoid synthesis are also controlled by the AA reacylating and remodeling enzymes, *i.e.* arachidonoyl-CoA synthetase, acyltransferases, and transacylases (for review, see Ref. 2). Thus, a major portion of the AA released by the action of one or more PLA$_2$s (3) is rapidly reincorporated into phospholipids (4, 5). This PLA$_2$-counteracting mechanism is thought to efficiently regulate the level of eicosanoids generated during cell activation (2).

Two main pathways exist for the incorporation of AA into phospholipids. One is the *de novo* pathway, *i.e.* the acylation of glycerol-3-phosphate and/or lysophosphatidic acid to yield PA (for review, see Ref. 6), and this leads to the production of AA-containing TAG. The other route involves a deacylation-reacylation cycle of membrane phospholipids and is initiated by PLA$_2$-mediated cleavage of preexisting phospholipids, followed by rapid re-esterification of the lysophospholipid formed with AA by CoA-dependent acyltransferase (2, 7).

Our studies on the mechanisms regulating free AA levels in P388D$_1$ macrophages have established that in these cells, phospholipid deacylation-reacylation reactions constitute the preferred route for incorporation of low amounts (nanomolar range) of free AA into phospholipids (8–10). In addition, a lower affinity, higher capacity pathway, the *de novo* pathway, leads to incorporation of AA into TAG. The latter appears to operate only when high amounts of free AA (micromolar range) are available (10).

Incorporation of AA via phospholipid remodeling appears to be strikingly dependent on the generation of lysophospholipid acceptors by the Ca^{2+}-independent PLA$_2$ (iPLA$_2$) (9, 10). This enzyme is irreversibly inhibited by the mechanism-based inhibitor BEL, a compound that shows great selectivity for inhibition of Ca^{2+}-independent *versus* Ca^{2+}-dependent PLA$_2$s (3, 11) and that has no effect on the AA-reacylating enzymes arachidonoyl-CoA synthetase, CoA-dependent acyltransferase, and CoA-independent transacylase (9). Using this inhibitor, we previously demonstrated that P388D$_1$ cell iPLA$_2$ largely regulates AA esterification via phospholipid remodeling by providing the acceptor lysophospholipid used in the reaction (9). Now we have found that BEL also inhibits incorporation of AA into TAG, and that this is due to inhibition of the Mg^{2+}-dependent PA phosphohydrolase (PAP-1) present in these cells, an enzyme that converts PA into diacylglycerol (DAG). Although the finding that BEL blocks PAP-1 does not affect our previous conclusion that iPLA$_2$ is responsible for basal phospholipid remodeling (9), it does raise the possibility that some other biological functions assigned to iPLA$_2$ on the sole basis of sensitivity to BEL (for review, see Ref. 12) may actually be due to PAP-1.

EXPERIMENTAL PROCEDURES

Materials—Murine P388D$_1$ cells were obtained from the American Type Culture Collection (Rockville, MD). Iscove's modified Dulbecco's medium (endotoxin, <0.05 ng/ml) was from Whittaker Bioproducts

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[1] The abbreviations used are: PLA$_2$, phospholipase A$_2$; AA, arachidonic acid; BEL, bromoenol lactone; DAG, 1,2-diacylglycerol; NEM, *N*-ethyl maleimide; PAP, phosphatidic acid phosphohydrolase; PAP-1, Mg^{2+}-dependent phosphatidic acid phosphohydrolase; PAP-2, Mg^{2+}-independent phosphatidic acid phosphohydrolase; iPLA$_2$, Ca^{2+}-independent phospholipase A$_2$; PA, phosphatidic acid; TAG, triacylglycerol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

(Walkersville, MD). Fetal bovine serum was from HyClone (Logan, UT). Nonessential amino acids were from Irvine Scientific (Santa Ana, CA). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (specific activity, 100 Ci/mmol), [9,10-^{3}H]palmitic acid (specific activity, 54 Ci/mmol), 1,2-dipalmitoyl-*sn*-[U-^{14}C]glycerol-3-phosphate (144 mCi/mmol), and [1-^{14}C]palmitoyl-CoA (47 Ci/mmol) were from DuPont NEN. Phorbol 12-myristate 13-acetate, palmitoyl-CoA, glycerol-3-phosphate, bovine serum albumin (fatty acid free), *N*-ethyl maleimide (NEM), and lipid standards were from Sigma. Silica Gel G-60 TLC plates were from Analtech (Newark, DE). Organic solvents (analytical grade) were from Baker (Phillipsburg, NJ) or Fisher. The $iPLA_2$ inhibitor BEL was synthesized in our laboratory by Dr. K. Conde-Frieboes (13).

Cell Culture—P388D_1 cells were maintained at 37 °C in a humidified atmosphere at 90% air and 10% CO_2 in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids. Cells were plated at 10^6/well in 6- or 12-well plastic culture 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 (10 μM, 0.5 μCi/ml) or [^{3}H]palmitic acid (10 μM, 0.5 μCi/ml). When BEL-treated cells were used, they were incubated for 30 min with the indicated concentrations of inhibitor, washed, and overlaid with fresh medium containing the radioactive fatty acid. At the indicated times, supernatants were removed, and the cell monolayers were gently washed with medium containing 5 mg/ml albumin. The cell monolayers were scraped twice with 0.5% Triton X-100, and total lipids were extracted according to the method of Bligh and Dyer (14). Lipids were separated by thin-layer chromatography with *n*-hexane/diethyl ether/acetic acid (70:30:1, v/v/v). This system allows a good resolution among phospholipids, monoacylglycerol, DAG, free fatty acids, and TAG. 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 actetate/isooctane/acetic acid/water (130:20:30:100). Radioactive content in the different lipid classes was quantitated by liquid scintillation counting.

Measurement of PAP Activity—PAP activity in homogenates from P388D_1 cells was assayed according to the method of Day and Yeaman (15) as modified by Balboa *et al.* (16). The substrate [^{14}C]glycerol-labeled PA was presented as mixed micelles with Triton X-100 at a detergent/phospholipid mole ratio of 10:1. Assays were conducted at 37 °C. The incubation mixture contained in a final volume of 0.1 ml: 100 μM [^{14}C]PA substrate (0.025 μCi/assay), 1 mM Triton X-100, 50 mM Tris-HCl, pH 7.1, 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and the indicated amount of homogenate protein. After the indicated times the reaction was stopped, and [^{14}C]PA and [^{14}C]DAG were separated by thin layer chromatography using the system *n*-hexane/acetic acid/water (70:30:1). When PAP activity was measured in cytosolic fractions, the $MgCl_2$ concentration in the assay was 3 mM (17). When PAP activity was measured in membrane fractions, $MgCl_2$ was omitted from the assays (17). When NEM was used, the samples were incubated with this reagent (8 mM) for 10 min before adding the [^{14}C]PA substrate (17). All assays were conducted under conditions of linearity with respect to time and protein concentration and showed zero order kinetics for the concentration of substrate used.

Other Assays—Glycerol-3-phosphate-acyl-CoA acyltransferase activity was measured as described by Lehner and Kuksis (18) with slight modifications. Briefly, the assay mixture was composed of 50 μM [^{14}C]palmitoyl-CoA (100,000 cpm), 250 μM glycerol-3-phosphate, 1 mM dithiotreitol, 1 mg/ml bovine serum albumin, 70 mM Tris-HCl, pH 8.0, and cell homogenate (up to 50 μg protein) in a final volume of 100 μl. After incubation at 37 °C for 10 min, the reaction was stopped by adding 1 μl of glacial acetic acid and 375 μl of chloroform/methanol (1:2). Chloroform (125 μl) and water (125 μl ml) were added, and the mixture was vortexed vigorously before centrifugation at 1,000 × *g* for 5 min. The organic phase was evaporated and chromatographed on Silica Gel G plates using *n*-hexane/ethyl ether/acetic acid (70:30:1, v/v/v) as the developing solvent. Lyso-PA was scraped off the plate and assayed for radioactivity by liquid scintillation counting.

Lyso-PA-acyl-CoA acyltransferase activity was assayed exactly as described (9), using lyso-PA instead of lyso-PC as an acyl acceptor.

DAG-acyl-CoA acyltransferase activity was measured as described by Lehner and Kuksis (18) with slight modifications. Briefly, the assay mixture was composed of 30 μM [^{14}C]palmitoyl-CoA (100,000 cpm), 150 μM DAG, 0.04% CHAPS, 1 mM dithiotreitol, 2 mg/ml bovine serum albumin, 25 mM Tris-HCl, pH 8.0, and cell homogenate (up to 50 μg of protein) in a final volume of 100 μl. After incubation at 37 °C for 10 min, the reactions were stopped by adding 375 μl of chloroform/methanol (1:2). Chloroform (125 μl) and water (125 μl) were added, and the mixture was vortexed vigorously before centrifugation at 1,000 × *g* for 5 min. The organic phase was evaporated and chromatographed on Silica Gel G plates using *n*-hexane/ethyl ether/acetic acid (70:30:1, v/v/v) as the developing solvent. TAG was scraped off the plate and assayed for radioactivity by liquid scintillation counting.

Data Presentation—Experiments were carried out in duplicate or triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, data are shown as means ± S.E. of at least three different experiments. Error bars are not shown when they are smaller than the symbol size.

RESULTS

Effect of BEL on AA Incorporation into TAG—Previous work from this (9, 10) and other laboratories (19, 20) has demonstrated that an important factor that controls the incorporation of AA into the different cellular lipids is the concentration of available free fatty acid. At low, nanomolar levels of free AA, the fatty acid is incorporated almost exclusively into phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, which occurs via deacylation-reacylation reactions (9). No AA is incorporated into PA or neutral lipids (9). In contrast, at high, micromolar levels of AA, abundant incorporation of fatty acid occurs via the *de novo* pathway, which ultimately leads to accumulation of AA in TAG (10, 19, 20). However, even at these high levels of free fatty acid, the preferred pathway for AA incorporation into phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol still appears to be the phospholipid-remodeling pathway (10). Thus, an important yet unresolved question is whether the two routes are linked, *i.e.* whether a precondition for AA incorporation via the *de novo* pathway to occur is that AA incorporation via phospholipid remodeling reaches saturation (2, 10). We hypothesized that this question could be assessed by studying AA incorporation into TAG (*i.e. de novo* pathway) (10) in BEL-treated cells, wherein AA incorporation via phospholipid remodeling is blocked (9). Fig. 1 shows the effect of BEL on the incorporation of exogenous AA (10 μM) into the lipids of P388D_1 cells. In agreement with our previous results (10), the bulk of AA esterified under these concentrations was found in TAG, not in phospholipids. Unexpectedly, preincubation of the cells with 25 μM BEL for 30 min before addition of exogenous AA resulted in inhibition of fatty acid incorporation not only into the major glycerophospholipid classes, such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, but also into DAG and TAG (Fig. 1). In contrast, AA incorporation into PA was enhanced (Fig. 1).

Effect of BEL on PAP Activity of P388D_1 Cells—The above results suggested that BEL blocks an event in the *de novo* biosynthetic route that is upstream of DAG production. Thus, we proceeded to examine the effect of BEL on the four enzyme activities that participate in the *de novo* pathway of glycerolipid synthesis, *i.e.* glycerol-3-phosphate acyltransferase, lyso-PA-acyl-CoA acyltransferase, PAP, and DAG-acyl-CoA acyltransferase. Only PAP activity was inhibited by BEL (Fig. 2).

For the experiments depicted in Fig. 2, BEL was added directly to the assay mixture. However, such an approach does not assure that the observed inhibitory effects of BEL are physiologically relevant in the intact cells. To overcome this problem, PAP activity was also measured in homogenates from BEL-treated cells. Thus, before preparing the homogenates that would serve as a source of enzyme, the cells were pretreated with the indicated concentrations of BEL for 30 min. The dose dependence curve obtained was very similar to that found when BEL was added directly to the assay mixture (*cf* Figs. 2*D* and 3), *i.e.* saturation of inhibition at concentrations

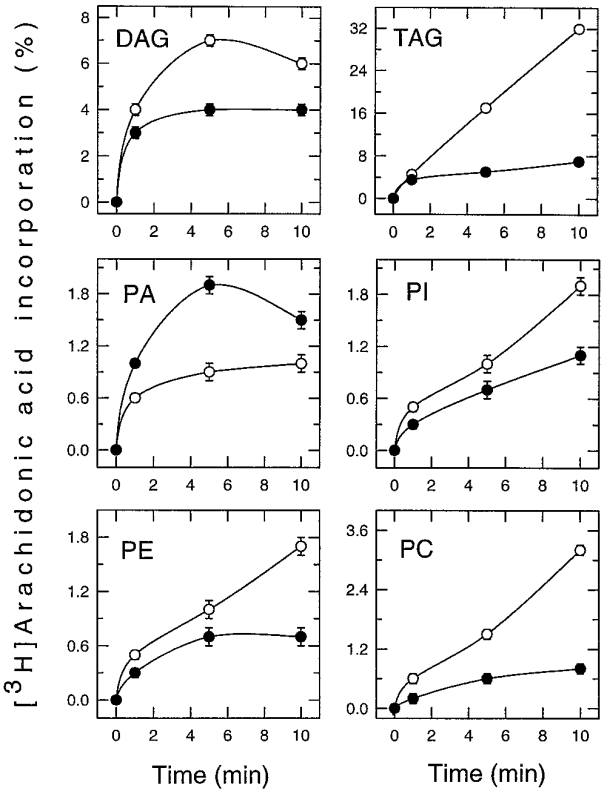

FIG. 1. **Time course of the effect of BEL on the incorporation of [^{3}H]AA into the different lipid classes.** The cells, either untreated (○) or treated with 25 μM BEL for 30 min (●), were exposed to 10 μM [^{3}H]AA (0.5 μCi) for the indicated periods. The ^{3}H radioactivity incorporated into the different lipids was determined as described under "Experimental Procedures" and is expressed as a percentage of the radioactivity originally present in the media.

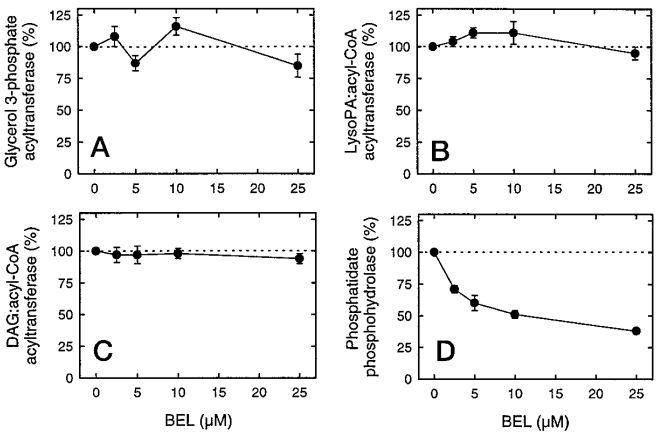

FIG. 2. **Effect of BEL on glycerolipid biosynthetic enzyme activity in homogenates from P388D$_1$ cells.** *A,* glycerol 3-phosphate acyltransferase; *B*, lyso-PA-acyl-CoA acyltransferase; *C*, DAG-acyl-CoA acyltransferase; *D*, phosphatidate phosphohydrolase. The homogenates were incubated with the indicated amounts of BEL for 10 min; then the various substrates were added, and the assays were conducted as described under "Experimental Procedures."

above 25 μM and an IC_{50} of about 8 μM. Importantly, the IC_{50} for PAP inhibition by BEL in intact cells is very similar to that previously found for inhibition of cellular $iPLA_2$ under the same experimental conditions (7 μM; Ref. 9).

Effect of BEL on Palmitic Acid Incorporation into the Lipids of P388D$_1$ Cells—For the sake of comparison, we also studied the effect of BEL on the incorporation of palmitic acid into the lipids of P388D$_1$ macrophages (Fig. 4). Unlike AA, palmitic acid entry into phospholipids and neutral lipids takes place mainly

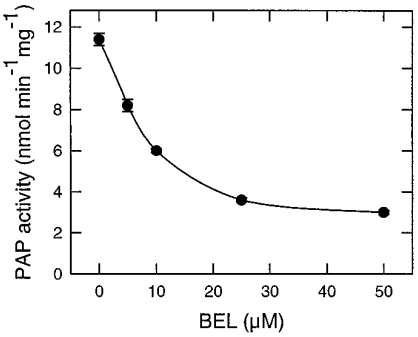

FIG. 3. **PAP activity in homogenates from BEL-treated P388D$_1$ cells.** P388D$_1$ cells were preincubated with the indicated BEL concentrations for 30 min. Afterward, homogenates were prepared, and PAP activity was determined as described under "Experimental Procedures."

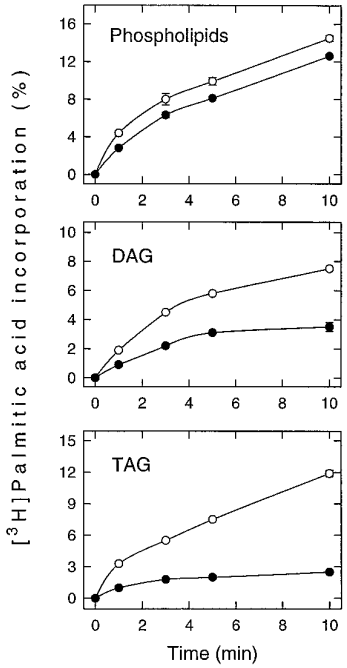

FIG. 4. **Time course of the effect of BEL on the incorporation of [^{3}H]palmitic acid into different lipid classes.** The cells, either untreated (○) or treated with 25 μM BEL for 30 min (●), were exposed to 10 μM [^{3}H]palmitic acid (0.5 μCi) for the indicated periods. The ^{3}H radioactivity incorporated into the different lipids was determined as described under "Experimental Procedures" and is expressed as a percentage of the radioactivity originally present in the media.

via *de novo* reactions (6–8). In accord with this, BEL severely impaired incorporation of this fatty acid into DAG and TAG (Fig. 4). Interestingly, although AA incorporation into phospholipids was also strongly blunted by BEL (75–85%; Fig. 1), palmitic acid incorporation into phospholipids was only slightly affected (10–20%) by the inhibitor (Fig. 4). Dose-response measurements of the specific products into which palmitic acid was incorporated revealed that the slight inhibitory effect of BEL was due to a decreased labeling of phosphatidylcholine and phosphatidylethanolamine that was partially offset by an increased labeling of PA (Fig. 5). Labeling of phosphatidylinositol was not significantly affected by BEL (Fig. 5).

BEL Selectively Blocks Mg^{2+}-dependent PAP—PAP activity exists in two distinct forms in mammalian cells. The first one, designated PAP-1, is a Mg^{2+}-dependent, cytosolic activity that is inhibited by NEM. The second, PAP-2, is a Mg^{2+}-independent, membrane-bound activity that shows no sensitivity to NEM (for review, see Ref. 21). Based on these biochemical properties, differential assays were performed to identify which

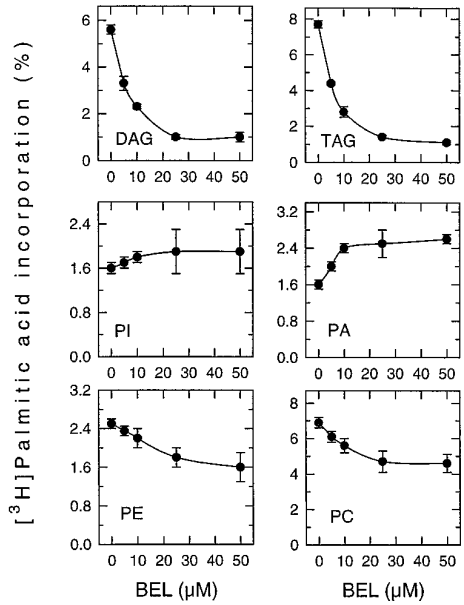

FIG. 5. **Dose dependence of the effect of BEL on the levels of [^{3}H]palmitic acid-containing lipids.** The cells, preincubated for 30 min with the indicated BEL concentrations, were exposed to 10 μM [^{3}H]palmitic acid (0.5 μCi) for 10 min. The ^{3}H radioactivity incorporated into the different lipid classes was determined as described under "Experimental Procedures" and is expressed as a percentage of the radioactivity originally present in the media.

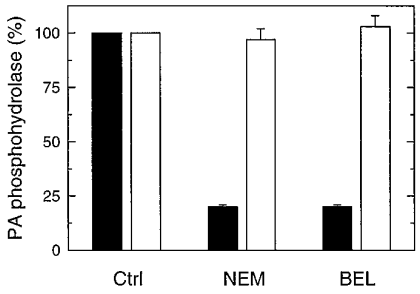

FIG. 6. **Effect of BEL on PAP activity in subcellular fractions of P388D$_1$ cells.** PAP activity in cytosolic (■) or membrane (□) fractions was determined. The samples were preincubated with either 25 μM BEL, 8 mM NEM, or neither (*Ctrl*) for 10 min before adding the substrate, as indicated. Assays using cytosolic fractions were conducted in the presence of 3 mM $MgCl_2$; assays using membrane fractions were conducted in the absence of $MgCl_2$.

of the two PAP forms was inhibited by BEL. PAP-2 was assayed using membrane fractions obtained after ultracentrifugation of the cell homogenates at 39,000 rpm for 60 min in a Ti-50 rotor. The assays were carried out in the absence of $MgCl_2$ and in the presence of 1 mM EGTA and 1 mM EDTA. The substrate [^{14}C]glycerol-labeled PA was presented as mixed micelles with Triton X-100 at a detergent/phospholipid mole ratio of 10:1. Confirming that under these conditions only PAP-2 was being measured, PAP activity was not affected if the membrane fraction was treated with 8 mM NEM for 10 min before adding the substrate (Fig. 6). Interestingly, PAP-2 activity was unchanged by the presence of 25 μM BEL in the assay mixture (Fig. 6).

PAP-1 activity was assayed using cytosolic fractions, and the assays contained 3 mM $MgCl_2$ (plus 1 mM EGTA and 1 mM EDTA). The substrate was presented as mixed micelles composed of PA/Triton X-100 at a mole ratio 10:1.[2] PAP activity in the cytosolic fraction used was inhibited up to 80% by 8 mM NEM, indicating the presence of small amounts of PAP-2 in this fraction (Fig. 6). Inclusion of 25 μM BEL in the assays led to the same degree of inhibition as that observed for NEM, *i.e.* 80%. Thus, BEL completely inhibits PAP-1 activity, and the 20% BEL-insensitive PAP activity in this fraction corresponds to the contaminating PAP-2. Collectively, the results from Fig. 6, showing differential effects of BEL on the Mg^{2+}-dependent and -independent PAPs, likely explain the lack of total inhibition observed when whole cell homogenates were used as the enzyme source (Figs. 2 and 3).

DISCUSSION

The results presented in this paper demonstrate that, besides inhibiting iPLA$_2$ (10), BEL also blocks PAP in P388D$_1$ macrophage-like cells. The inhibition of PAP activity by BEL in intact cells has an IC_{50} of 8 μM, which is almost identical to that previously found for the inhibition of iPLA$_2$ by BEL under the same experimental conditions (10). However, concentrations of BEL higher than 25 μM completely inhibited cellular iPLA$_2$ (10), whereas complete inhibition of cellular PAP was not reached at 50 μM BEL. At these BEL concentrations, cellular PAP activity was inhibited by about 70%. However, mammalian cells contain two completely different types of PAP activity (for review, see Ref. 21). The first one, designated PAP-1, is a Mg^{2+}-dependent enzyme, and the other form, designated PAP-2, has no such a requirement. PAP-1 is cytosolic and translocates to the endoplasmic reticulum, whereas PAP-2 is an integral plasma membrane protein. Moreover, PAP-1 is sensitive to NEM and other sulfhydryl group reagents, whereas PAP-2 is insensitive to these reagents (21).

Taking advantage of these biochemical differences, it was possible to perform assays that could distinguish between the two PAP activities. By doing so, we have found that BEL selectively blocks PAP-1 but has no effect on PAP-2.[3] Thus, these differential effects on the Mg^{2+}-dependent and -independent PAPs may explain the lack of 100% inhibition of cellular PAP activity by BEL.

BEL is a suicide inhibitor, which means that the enzyme has to first act on the compound for the inhibitory species to be produced. In the case of BEL, this is achieved by enzymatic rupture of the lactone ring present in the molecule (23). PAP-1 is a phosphomonoesterase, not an acyl esterase. Therefore, it would not be surprising if the inhibition of PAP-1 by BEL is not the result of an irreversible covalent modification, as might be expected from previous studies with the iPLA$_2$ (13).

PAP-1 has long been thought to be involved in phospholipid biosynthesis (21). Consistent with this notion, we have found that inhibition of PAP-1 by BEL leads to a strong reduction of DAG and TAG synthesis by the P388D$_1$ cells. On the other hand, PAP is also known to play a key role in receptor-mediated intracellular signaling (21). PAP, acting on the PA produced by either receptor-activated phospholipase D or *de novo* synthesis, induces a delayed elevation of cellular DAG levels, which may mediate sustained cellular responses (21). Although it appears likely that PAP-2 is the enzyme primarily involved in intracellular signaling (24), a role for PAP-1 in these events is possible as well, especially in those cases in which the phospholipid biosynthetic route does contribute to receptor-mediated DAG elevations (21, 25–27). Thus, the fundamental aspect of our observation that BEL similarly affects iPLA$_2$ and PAP-1

[2] Triton X-100 has been reported to severely inhibit PAP-1 under certain experimental conditions (17). In agreement with the results by Day and Yeaman (15), from whom our PAP assay was adapted, PAP-1 activity was higher if measured with PA and Triton X-100 mixed micelles than with pure PA vesicles.

[3] We have found that BEL partially inhibits (60–70%) a pure Mg^{2+}-dependent PAP from yeast (microsomal 104-kDa isoform) (22). However, it is not known how similar the yeast PAP is to the P388D$_1$ cell PAP-1.

in intact cells is that, where a role for $iPLA_2$ in a given cellular process has been attributed on the sole basis of its inhibition by BEL, a role for PAP-1 must also be considered. An example of this may be the regulation of AA release in agonist-stimulated cells. Based on sensitivity to BEL, a number of studies have suggested a major role for the $iPLA_2$ in receptor-mediated AA release in certain cell types (28–33). However, only in a few of these studies, involvement, either direct or indirect, of DAG in the response was examined.

DAG can influence receptor-mediated AA release in at least two different ways. In the first, DAG may itself serve as a substrate for AA release through the sequential action of DAG and monoacylglycerol lipases (34). Although the amount of AA released by the DAG deacylation pathway may not be significant in certain cell types, there are some examples in which a role for this pathway has been established. For instance, Konrad *et al.* (35) have provided evidence of the involvement of the DAG lipase pathway in AA release in glucose- and carbachol-stimulated pancreatic islets. In stark contrast with these data, Ramanadham *et al.* (28) proposed $iPLA_2$ as the major mediator of AA release in this same system, on the basis of inhibition of the response by BEL. The studies reported in this article might help explain this discrepancy if, besides $iPLA_2$, BEL was inhibiting DAG generation in the studies by Ramanadham *et al.* (28).

DAG may also be involved in regulating stimulus-induced AA release indirectly, by activating protein kinase C. The key regulatory role that protein kinase C exerts on receptor-coupled AA release has been clearly established in a wide variety of cell types with many different agonists (36). Based on the results reported here, it cannot be ruled out that BEL, by inhibiting PAP-1, may act to lower intracellular DAG levels and consequently to blunt protein kinase C activation. This in turn could reduce PLA_2 activation and hence AA release (36).

Despite these complications in the use of BEL, there are studies that have used BEL to assign a role for $iPLA_2$ in AA release in which the conclusions are likely correct, because confirmatory evidence was provided by other approaches, *e.g.* the use of Ca^{2+}-depleted cells to demonstrate that AA release is truly a Ca^{2+}-independent process (32). Furthermore, when BEL has been used to study the role of $iPLA_2$ in processes that are not affected by variations in DAG levels, the conclusions have remained valid. That is the case for AA remodeling into the phospholipids of resting $P388D_1$ cells via reacylation-deacylation reactions (9). This process, independent of DAG, is regulated by a PLA_2 that we have identified as the $iPLA_2$ on the basis of: (i) the calcium-independent nature of the response; (ii) the correlative inhibition by BEL of endogenous $iPLA_2$ activity, steady-state levels of lysophospholipid, and AA incorporation into phospholipids; and (iii) the inhibition of AA incorporation into phospholipids by palmitoyl trifluoromethyl ketone, another $iPLA_2$ inhibitor that is structurally unrelated to BEL but protects against BEL inhibition by binding at the same site (9, 10, 37).

In conclusion, the present studies demonstrate inhibition by BEL of PAP-1, a key enzyme in glycerolipid metabolism, and identify one functional sequel of the inhibition of DAG production by BEL, *i.e.* the reduction of AA accumulation into TAG. The latter process is thought to represent a homeostatic mechanism that either protects the cells from extremely high concentrations of AA or recaptures AA released intracellularly during conditions in which massive PLA_2 activation occurs (2, 38). Moreover, AA incorporation into TAG is associated with changes in the morphology of the cells (39) and constitutes an important step in the chain of reactions leading to remodeling and redistribution of AA among cellular compartments (19, 20, 38, 39). Thus the current studies establish that the effect of BEL on cellular phospholipid metabolism is not limited to inhibition of deacylation reactions catalyzed by $iPLA_2$ but is more complex and also involves reduction of cellular DAG levels.

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