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# Bromoenol Lactone Promotes Cell Death by a Mechanism Involving Phosphatidate Phosphohydrolase-1 Rather than Calcium-independent Phospholipase $A_2$*

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**Lucía Fuentes, Rebeca Pérez, María L. Nieto, Jesús Balsinde‡, and María A. Balboa**

*From the Institute of Molecular Biology and Genetics, University of Valladolid School of Medicine, E-47005 Valladolid, Spain*

**Originally described as a serine protease inhibitor, bromoenol lactone (BEL) has recently been found to potently inhibit Group VI calcium-independent phospholipase $A_2$ ($iPLA_2$). Thus, BEL is widely used to define biological roles of $iPLA_2$ in cells. However, BEL is also known to inhibit another key enzyme of phospholipid metabolism, namely the magnesium-dependent phosphatidate phosphohydrolase-1 (PAP-1). In this work we report that BEL is able to promote apoptosis in a variety of cell lines, including U937, THP-1, and MonoMac (human phagocyte), RAW264.7 (murine macrophage), Jurkat (human T lymphocyte), and GH3 (human pituitary). In these cells, long term treatment with BEL (up to 24 h) results in increased annexin-V binding to the cell surface and nuclear DNA damage, as detected by staining with both DAPI and propidium iodide. At earlier times (2 h), BEL induces the proteolysis of procaspase-9 and procaspase-3 and increases cleavage of poly(ADP-ribose) polymerase. These changes are preceded by variations in the mitochondrial membrane potential. All these effects of BEL are not mimicked by the $iPLA_2$ inhibitor methylarachidonyl fluorophosphonate or by treating the cells with a specific $iPLA_2$ antisense oligonucleotide. However, propranolol, a PAP-1 inhibitor, is able to reproduce these effects, suggesting that it is the inhibition of PAP-1 and not of $iPLA_2$ that is involved in BEL-induced cell death. In support of this view, BEL-induced apoptosis is accompanied by a very strong inhibition of PAP-1-regulated events, such as incorporation of [$^3$H]choline into phospholipids and *de novo* incorporation of [$^3$H]arachidonic acid into triacylglycerol. Collectively, these results stress the role of PAP-1 as a key enzyme for cell integrity and survival and in turn caution against the use of BEL in studies involving long incubation times, due to the capacity of this drug to induce apoptosis in a variety of cells.**

Bromoenol lactone (BEL)[1] is a member of a family of compounds known as haloenol lactones that were first described as suicide substrates of chymotrypsin and related serine proteases (1). Later, this compound was found to potently inhibit myocardial calcium-independent phospholipase $A_2$ ($iPLA_2$) activity (2), a finding that was corroborated by studies utilizing purified $iPLA_2$ from $P388D_1$ macrophages (3). BEL has proven to be exquisitely selective for $iPLA_2$ *versus* other $Ca^{2+}$-dependent $PLA_2$ forms (including both secreted and cytosolic $PLA_2$s) (2, 4). Due to this selectivity, BEL is frequently used to identify $iPLA_2$ roles in cells (5, 6).

However, BEL has also been recently found to inhibit another enzyme of key significance in phosholipid metabolism, namely the magnesium-dependent cytosolic phosphatidate phosphohydrolase, also known as phosphatidate phosphohydrolase-1 (PAP-1) (7). PAP-1 from mammalian sources has yet to be purified, which has considerably hampered the study of biological role(s) of this enzyme in metabolism and cell activation. An $Mg^{2+}$-dependent PAP has been purified from yeast (8), but its relationship, if any, to mammalian PAP-1 remains obscure. PAP-1 is known to be primarily involved in controlling cellular diacylglycerol (DAG) that acts as the precursor for triacylglycerol (TAG) and phosphatidylcholine (PC) biosynthesis in the Kennedy pathway (9, 10). It is likely that PAP-1 also participates in certain signaling events (9, 10).

Apoptosis is a type of cell death that does not involve an inflammatory response and occurs in a tightly controlled manner. In contrast to necrosis, apoptosis involves activation of catabolic mediators and enzymes prior to cytolysis. Many cellular changes that take place during this form of death have an important role in the recognition and elimination of those cells by neighboring phagocyte cells (11, 12). One of these changes is the scrambling of cellular phospholipids (loss of bilayer asymmetry) and phosphatidylserine exposure on the cell surface (13, 14). Apoptosis can be induced in cells by a death receptor (*e.g.* the tumor necrosis factor receptor) or by cytotoxic agents that cause a mitochondria-dependent cascade of events (15, 16). In the death receptor-mediated apoptosis, a death-inducing signaling complex is formed by the death receptors, adapter proteins, and caspases (cysteinyl aspartate-specific proteases) like caspase-8 and caspase-10 (15–18). In the mitochondria-depend-

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‡ To whom correspondence should be addressed: Inst. de Biología y Genética Molecular (IBGM-CSIC), Facultad de Medicina, Universidad de Valladolid, Avenida Ramón y Cajal 7, E-47005 Valladolid, Spain. Tel.: 34-983-423-062; Fax: 34-983-423-588; E-mail: jbalsinde@ibgm.uva.es.

[1] The abbreviations used are: BEL, bromoenol lactone ((*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one); AA, arachidonic acid; DAG, 1,2-diacylglycerol; DAPI, 4′,6-diamidino-2-phenylindole; $PLA_2$, phospholipase $A_2$; $iPLA_2$, calcium-independent phospholipase $A_2$; JC-1, (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide); MAFP, methylarachidonyl fluorophosphonate; PAP-1, magnesium-dependent phosphatidic acid phosphohydrolase-1; PARP, poly(ADP-ribose) polymerase; PC, phosphatidylcholine; TAG, triacylglycerol; PBS, phosphate-buffered saline; Z, benzyloxycaronyl; FMK, fluoromethyl ketone; FITC, fluorescein isothiocyanate.

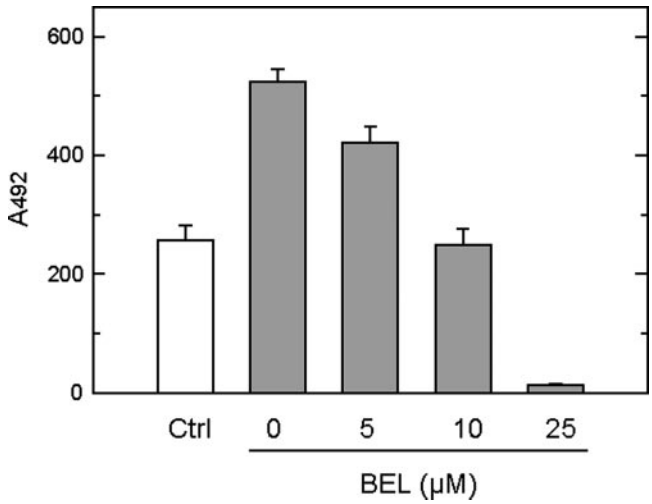


FIG. 1. **Cellular proliferation in the presence of BEL.** U937 cells were grown in triplicates with the indicated amounts of BEL for 24 h and assayed for proliferation as mentioned under "Experimental Procedures." Control (*Ctrl*) indicates the absorbance of cells at time 0, *i.e.* immediately after seeding the cells at the beginning of the experiment.

ent apoptosis, major alterations in mitochondrial membrane function occur (16, 19). It has been found that mitochondrial membrane permeabilization is an early event of apoptosis (14), resulting in the release of proteins from the soluble intermembrane space in a nonspecific fashion. Cytochrome *c* is one of these proteins and, once released to the cytosol, interacts with Apaf-1 and pro-caspase-9 to form a molecular caspase activation complex, the apoptosome, where caspase-9 is in its active form (20). There is also disruption of the mitochondrial inner transmembrane potential that often precedes nuclear DNA degradation, which is considered an irreversible event (21). Caspase-8, caspase-10, and caspase-9 are believed to be the initiator caspases at the top of the caspase signaling cascade that culminates with the cleavage and activation of caspase-3, the principal effector caspase (18).

Recent studies have implicated $iPLA_2$ in stimulus-induced cell proliferation (22, 23). While studying the possible implications of $iPLA_2$ in the serum-induced growth response of different cell lines of myelomonocytic origin, we found that BEL dramatically induces cell death by a mechanism that is clearly identifiable as a mitochondria-dependent apoptosis event. Importantly, the BEL effect appears to be unrelated to $iPLA_2$ inhibition but appears to work through inhibition of PAP-1.

## EXPERIMENTAL PROCEDURES

*Materials*—[5,6,8,9,11,12,14,15-$^3$H]AA (200 Ci/mmol) and [$^3$H]choline chloride (80 Ci/mmol) were purchased from Amersham Ibérica (Madrid, Spain). BEL was from Cayman Chemicals (Ann Arbor, MI). Caspase-9 antibody, caspase-3 antibody, and PARP (poly(ADP-ribose) polymerase) antibody were from Santa Cruz Biotechnology. Oligonucleotides were from MWG-Biotech AG (Ebersberg, Germany). JC-1 was from Molecular Probes. Annexin-V FITC was from Pharmingen. The general caspase inhibitor Z-VAD-FMK was from Calbiochem. All other reagents were from Sigma.

*Cell Culture*—The following cell lines: U937, Jurkat, THP-1, MonoMac, and RAW264.7 were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin 100 μg/ml. MonoMac cells were also supplemented with OPI medium (Sigma). GH3 pituitary cells were cultured in RPMI 1640 medium supplemented with 15% horse serum and 2.5% fetal calf serum. The cells were incubated at 37 °C in a humidified atmosphere of 5% $CO_2$.

*Cell Cycle Analysis*—Cells were incubated for 24 h with or without the inhibitors, washed twice with cold PBS, and fixed with 70% ethanol at 4 °C for 18 h. Cells were then washed and resuspended in PBS with 5 mM EDTA. RNA was removed by digestion with RNase A at room temperature. Staining was achieved by incubation with staining solution (500 μg/ml propidium iodide in PBS containing 5 mM EDTA) for 1 h, and cell cycle analysis was performed by flow cytometry in a Coulter Epics XL-MCL cytofluorometer.

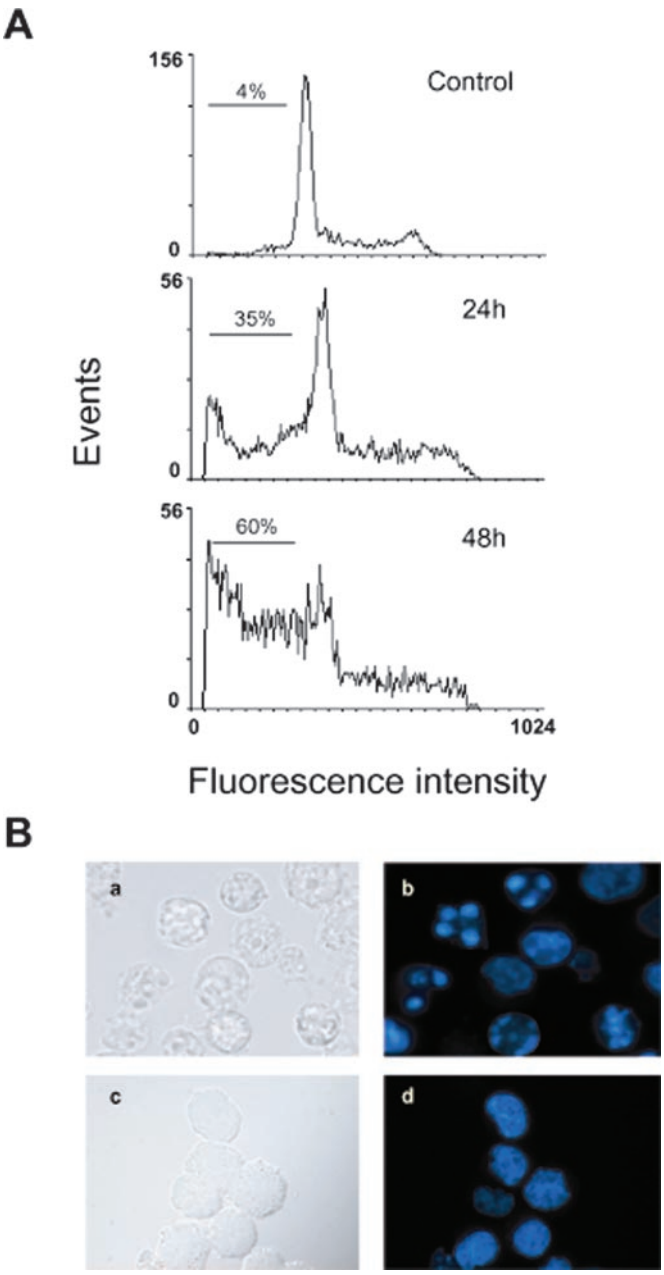


FIG. 2. **Effect of BEL on cell cycle distribution and nuclear morphology in U937 cells.** *A*, the cells were treated with or without 25 μM BEL for the indicated periods of time, harvested, and labeled with 500 μg/ml propidium iodide. Flow cytometry analysis of control cells (no BEL), or treated with BEL for 24 or 48 h, is shown. *B*, cell were treated with (*panels a* and *b*) or without (*panels c* and *d*) 25 μM BEL for 24 h, stained with DAPI, and examined by fluorescence microscopy. *Panels a* and *c* are the Nomarski images, and *panels b* and *d* show the fluorescence of the same cells.

*Measurement of Apoptosis*—Apoptosis was analyzed by labeling with the annexin-V FITC apoptosis detection kit (Pharmingen), which recognizes phosphatidylserine exposure on outer leaflet of the plasma membrane. Apoptosis was also evaluated by DAPI staining. For this purpose, cells were fixed with 4% paraformaldehyde and then permeabilized by 0.5% Triton-X-100 in PBS. Cells were then stained for 30 min with DAPI (1 μg/ml) and analyzed by fluorescence microscopy to assess chromatin condensation. Experiments utilizing $iPLA_2$ antisense oligonucleotides were carried out exactly as described previously (24). Briefly, 1 μg/ml antisense or sense oligonucleotides were mixed with 10 μM LipofectAMINE, and complexes were allowed to form at room temperature for 30 min. The complexes were then added to the cells, and

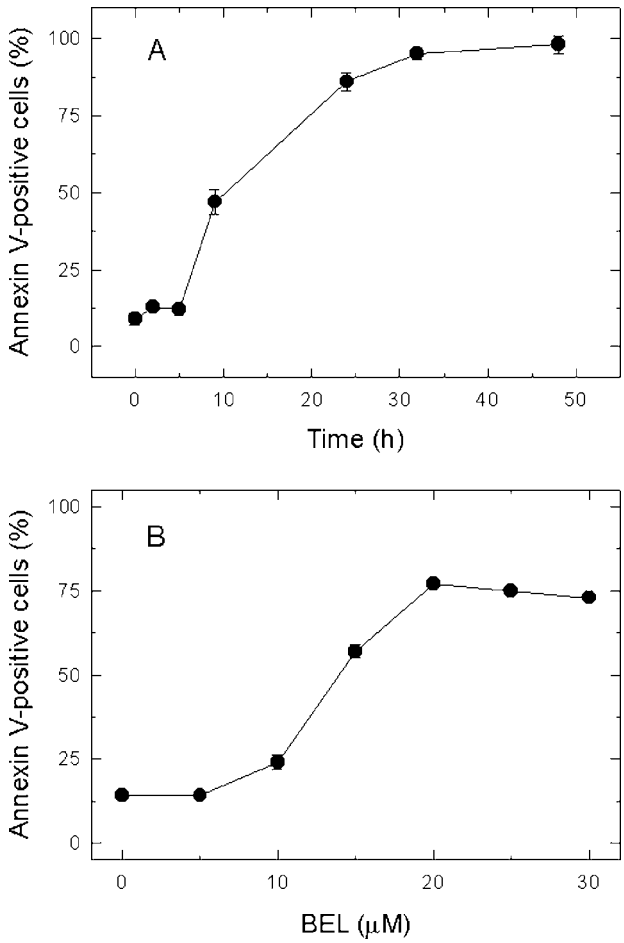


FIG. 3. **Staining of U937 cells with annexin-V.** The cells were treated with or without BEL, stained with annexin-V FITC, and analyzed by flow cytometry. *A*, percentage of annexin-V-positive cells as a function of time of incubation with BEL. *B*, percentage of annexin-V-positive cells as a function of the concentration of BEL used (24-h incubations).

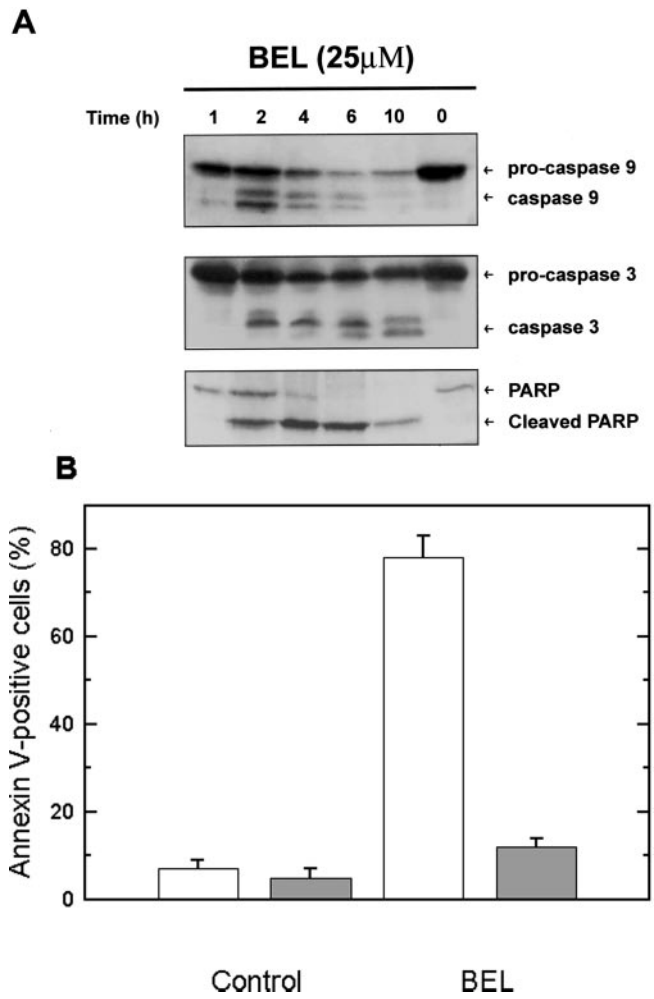


FIG. 4. **Analysis of caspases in BEL-treated U937 cells.** *A*, the cells were treated with 25 μM for the time indicated, harvested, and analyzed by immunoblot for the intact and cleaved forms of caspase-9, caspase-3, and PARP. *B*, the cells were treated with 25 μM BEL as indicated, in the absence (*open bars*) or presence (*gray bars*) of the caspase inhibitor Z-VAD-FMK (20 μM) for 24 h. Afterward, the cells were stained with annexin-V FITC and analyzed by flow cytometry.

the incubations were allowed to proceed under standard cell culture conditions for 48 h.

*iPLA$_2$ Assay*—Aliquots of cell homogenates were incubated for 2 h at 37 °C in 100 mM Hepes (pH 7.5) containing 5 mM EDTA and 100 μM labeled phospholipid substrate (1-palmitoyl-2-[$^3$H]palmitoylglycero-3-phosphocholine; specific activity, 60 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) in a final volume of 150 μM. The phospholipid substrate was used in the form of sonicated vesicles in buffer.

*Choline Incorporation into Phosphatidylcholine*—Cells ($0.5 \times 10^6$ cells/ml) were treated with 25 μM BEL at different time points, and 2 μCi/ml [*methyl*-$^3$H]choline chloride was added for the last hour. Cells were then washed and lipids were extracted according to Bligh and Dyer (25). Lipids were separated by thin-layer chromatography with chloroform/methanol/acetic acid/water (50:40:6:0.6) as a solvent system. Spots corresponding to phosphatidylcholine were scraped into scintillation vials, and the amount of radioactivity was estimated by liquid scintillation counting.

*AA Incorporation into TAG*—Cells ($0.5 \times 10^6$ cells/ml) were treated with 25 μM BEL or vehicle for 2 h. Cells were then exposed to 10 μM [$^3$H]AA (0.5 μCi/ml) for different periods of time. Cells were washed, the lipids were extracted according to Bligh and Dyer (25), and the lipids in the chloroform phase were separated by thin-layer chromatography with hexane/diethyl ether/acetic acid (70:30:1) as a mobile phase. The spots corresponding to TAG were scraped into scintillation vials, and the amount of radioactivity was estimated by liquid scintillation counting.

*Proliferation Assay*—The CellTiter96 Aqueous One solution cell proliferation assay (Promega) was used, and the manufacturer's instructions were followed. Briefly, cells (10,000 cells per well) were seeded in 96-well plates treated with vehicle or different concentrations of BEL. After 24 h, formazan product formation was assayed by recording the absorbance at 490 nm in a 96-well plate reader.

*Immunoblot Analyses*—Cells were lysed in an ice-cold lysis buffer and 50 μg of cellular protein from each sample were separated by standard 10% SDS-PAGE and transferred to nitrocellulose membranes. Dilution of both primary and secondary antibodies was made in PBS containing 5% defatted dry milk and 0.1% Tween 20. After 1-h incubation with the corresponding primary antibody at 1:1000, blots were washed 4 times and the secondary peroxidase-conjugated antibody was added for another hour. Immunoblots were developed using the Amersham ECL system.

*Mitochondrial Depolarization Measurements*—After drug treatment for 24 h, cells were incubated with 7.5 μM JC-1 at room temperature for 15 min in the dark. Depolarization was measured as decay of red fluorescence by flow cytometry analysis.

*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

*Effect of BEL on Cell Growth*—Previous studies have demonstrated that inhibition of iPLA$_2$ results in a decreased proliferative response of the cells to mitogens (22, 23). We began the current study by investigating the effect of the iPLA$_2$ inhibitor BEL on the basal growth of U937 promonocytes (*i.e.* that normally induced by the serum present in the culture

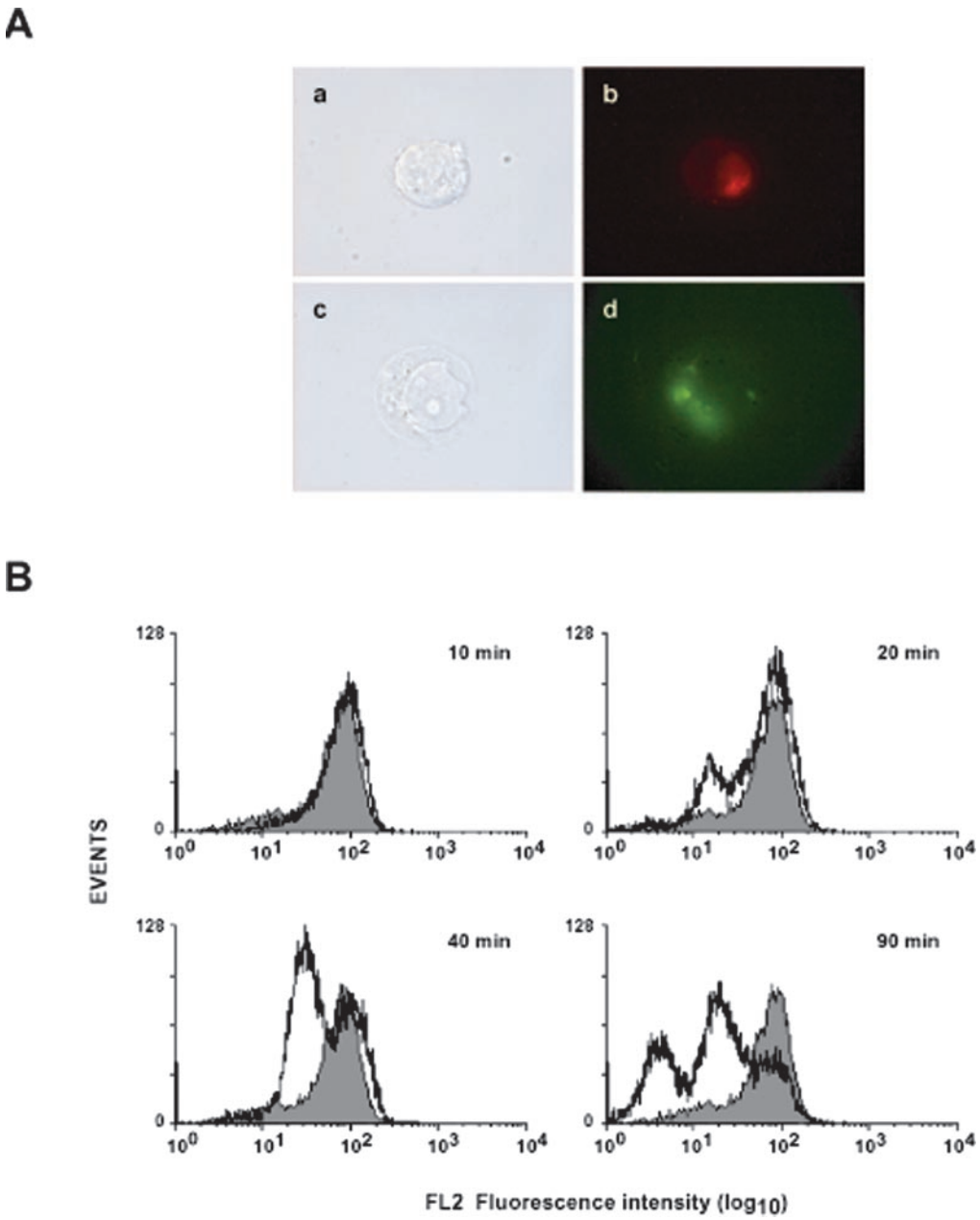


FIG. 5. **Mitochondrial depolarization in BEL-treated U937 cells.** *A*, the cells were treated with or without 25 $\mu$M BEL, stained with JC-1, and analyzed by fluorescence microscopy (*A*) or flow cytometry (*B*). *A*, *panels a* and *b*, control untreated cells; *panels c* and *d*, BEL-treated cells. *Panels a* and *c*, Nomarski images; *panels b* and *d*, fluorescence emitted by the cells. *B*, decay of JC-1 red fluorescence at different time points after BEL treatment. *Gray peaks* show the fluorescence of control untreated cells, and *white peaks* show the fluorescence of BEL-treated cells.

medium, in the absence of any other mitogenic stimulus). The continued presence of BEL for 24 h in the culture medium of U937 cells resulted in a marked inhibition of cell growth, as assessed by a colorimetric method (Fig. 1). At concentrations between 10 and 25 $\mu$M BEL, the reduction of growth was so intense that the absorbance readings fell well below the absorbance of control, BEL-untreated cells at time 0 (*i.e.* the absorbance reading that is obtained immediately after seeding the cells at the beginning of the experiment). This indicated that the number of live cells present in the BEL-treated wells at the end of the experiment was much less than that seeded at the beginning of the experiment. The experiment depicted in Fig. 1 was also repeated with other human monocyte-like cell lines such as MonoMac and THP-1 and also with the T-lymphocyte-like cell line Jurkat. Identical results to those shown in Fig. 1 were obtained.

*Effect of BEL on Cell Cycle and DNA*—To analyze in more detail the effect of BEL on the U937 cells, cellular DNA was analyzed by propidium iodide staining. As shown in Fig. 2*A*, BEL clearly induced subdiploid events, characteristic of apoptotic cells. The percentage of apoptotic cells increased from 4% in control cells to 35% at 24 h, and 65% at 48 h. Typical apoptotic nuclei, exhibiting highly fluorescent, condensed chromatin, were also demonstrated by DAPI staining (Fig. 2*B*).

*Effect of BEL on Annexin-V Labeling*—The above data indicated that there is cell damage after BEL treatment and that cellular DNA breakdown occurs. These events are characteristic of cell death by apoptosis. To further explore this possibility, experiments were carried out to study annexin-V labeling to the BEL-treated cells. Fig. 3*A* shows the time course of the effect of BEL on phosphatidylserine exposure on the outer membrane of the cells. Phosphatidylserine externalization is an early event in apoptosis, and as such, it was significantly detected after 9 h of treatment with BEL. Fig. 3*B* shows the

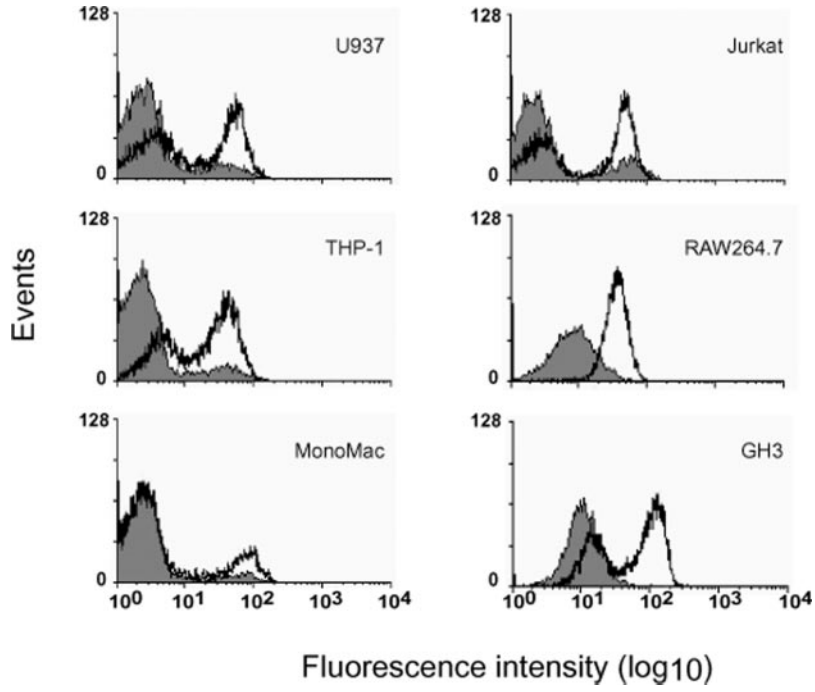


FIG. 6. **Annexin-V FITC labeling of different cell types after a BEL treatment.** The indicated cell types were treated with or without 25 mM BEL for 24 h and stained with annexin-V FITC as described under "Experimental Procedures." Labeling obtained after BEL treatment (*open traces*) is compared with that of cells treated with vehicle alone (*gray-filled traces*).

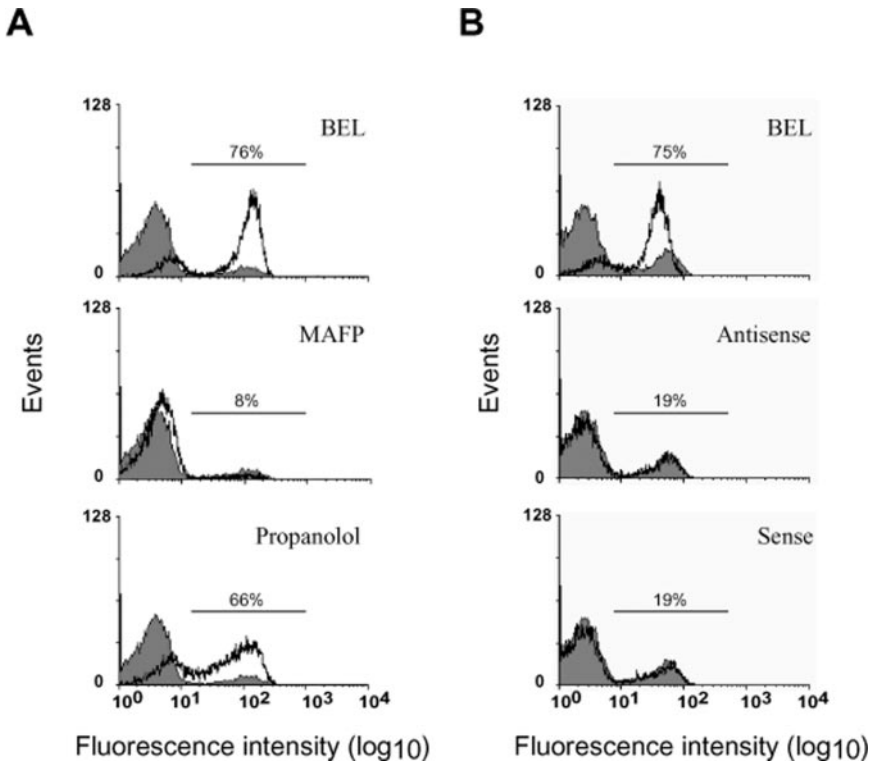


FIG. 7. **Effect of $iPLA_2$ and PAP-1 inhibitors on annexin-V FITC staining of the U937 cells.** *A*, the cells were treated with vehicle, 25 $\mu$M BEL, 25 $\mu$M MAFP, or 150 $\mu$M propranolol for 24 h. *B*, the cells were treated with vehicle, 25 $\mu$M BEL, an $iPLA_2$ antisense oligonucleotide (*Antisense*) or the sense oligonucleotide control (*Sense*) for in the presence of LipofectAMINE for 24 h. Afterward, the cells were labeled with annexin-V FITC and analyzed by flow cytometry. In all panels, labeling obtained after each treatment (*open traces*) is compared with that of cells treated with vehicle alone (*gray-filled traces*).

concentration dependence of the BEL effect on phosphatidylserine exposure. Significant increases in annexin-V labeling were already observed at BEL concentrations as low as 10 $\mu$M.

*BEL Effect on Caspase Breakdown*—One of the signature events of cell death by apoptosis is the activation of a cascade of proteolytic enzymes, the so-called caspases, which are synthesized as zymogens (procaspases) and undergo proteolytic maturation. Hydrolysis of procaspase-9 was detected in BEL-treated U937 cells (Fig. 4*A*). The caspase-9 active fragments of 35/37 kDa could be detected as early as 1 h after treatment with BEL (Fig. 4*A*). Caspase-9 is the initiator caspase implicated in mitochondria-dependent apoptosis (26). The cleaved forms of caspase-3 could also be detected at 2 h (Fig. 4*A*), suggesting that apoptotic signals brought about by BEL activate the effector caspase-3 via a mitochondrial pathway.

To confirm caspase-3 activation in BEL-induced apoptosis, cleavage of PARP was evaluated. PARP is known to be a caspase-3 substrate *in vivo* (27). Cells not treated with BEL expressed the intact PARP 112-kDa polypeptide (Fig. 4*A*). In the BEL-treated cells a digestion fragment of 85 kDa was already detected at 2 h (Fig. 4*A*).

Experiments were also carried out in the presence of Z-VAD-FMK, a general irreversible caspase inhibitor (28). In the presence of inhibitor, cell surface labeling with annexin-V was

greatly diminished in the BEL-treated cells (Fig. 4*B*), thus confirming the involvement of caspases in the process under study.

*BEL Effects on Mitochondria*—Early apoptotic pathways converge on mitochondrial membranes to induce their permeabilization. The outer membrane becomes protein-permeable, and the inner membrane keeps retaining matrix proteins but can dissipate the mitochondrial transmembrane potential (21). To confirm that BEL induces cell death through a mitochondrial pathway, changes in the mitochondria inner membrane potential were assessed. The dye JC-1 is commonly used to monitor these changes (29). As shown in Fig. 5*A*, control cells are stained *red*, whereas in the BEL-treated cells the staining shifts to *green*, which indicates a change in the mitochondrial membrane potential of these cells (19). Flow cytometry analyses of BEL-treated cells at different time points revealed a decrease on red fluorescence that was already evident at 20 min (Fig. 5*B*). Collectively, these data suggest the involvement of mitochondria in BEL-induced apoptosis.

*Effects of BEL on Other Cell Types*—To assess whether BEL-induced apoptosis was a general effect, several cell lines of different lineages were assayed for annexin-V binding after a 24-h treatment with BEL (Fig. 6). These cells included U937, MonoMac, and THP-1 (human monocyte) and RAW264.7 (murine macrophage), Jurkat (human T-lymphocyte), and GH3 (human pituitary). Although the extent of the effects observed varied from cell to cell, BEL treatment promoted significant increases in annexin-V binding in all cell types under study, indicating that apoptosis was triggered in all of them.

*Effect of Other iPLA$_2$ Inhibition Strategies on Annexin-V Labeling*—Since BEL inhibits both iPLA$_2$ and PAP-1, it is important to determine which of these two enzymes is involved in BEL-induced apoptosis. Thus, different pharmacological approaches were undertaken to discriminate between the two enzymes. In the first series of experiments, the cells were treated with MAFP, an unspecific inhibitor of PLA$_2$s that, like iPLA$_2$, utilize a catalytic serine (5). As shown in Fig. 7, treatment of the cells with 25 $\mu$M MAFP did not change annexin-V labeling of the cell surface. *In vitro* measurements confirmed that both MAFP and BEL strongly inhibited the iPLA$_2$ activity of U937 cell homogenates under these conditions (Fig. 8*A*).

The effect of an iPLA$_2$ antisense oligonucleotide on annexin-V labeling was evaluated next. The antisense oligonucleotide used is the human counterpart of the murine one that we and others (24, 29–32) have successfully employed elsewhere. In the U937 cells, this antisense produced a 70–80% decrease of both immunoreactive iPLA$_2$ protein and cellular iPLA$_2$ activity (Fig. 8*B*). Under these conditions, the iPLA$_2$ antisense did not affect annexin-V labeling of the U937 cell surface (Fig. 7). Collectively, these data indicate that iPLA$_2$ inhibition does not lead to apoptotic cell death.

*Role of PAP-1*—The effect of propranolol, a PAP-1 inhibitor that is structurally unrelated to BEL (33) was studied next. Fig. 7 shows that propranolol mimicked the BEL effects on cells and significantly increased annexin-V labeling of the U937 cell surface. PAP-1 is a cytosolic enzyme that produces the DAG moieties utilized for the biosynthesis of PC and TAG in the Kennedy pathway (9, 10). It is well known that perturbation of PC homeostasis induces growth arrest and cell death (24). Fig. 9 shows that BEL treatment of the cells resulted in a time-dependent decrease in the incorporation of [$^3$H]choline into PC, indicating that cellular PC biosynthesis is depressed in the presence of BEL. When propranolol was used instead, essentially the same results were obtained (Fig. 9). *In vitro* measurements confirmed that both BEL and propranolol strongly inhibited PAP-1 activity in the cytosolic fraction of U937 cell homogenates (75 ± 8% inhibition over control for cells treated with 25 $\mu$M BEL and 72 ± 3% inhibition over control for cells treated with 150 $\mu$M propranolol; means ± S.E., $n = 4$).

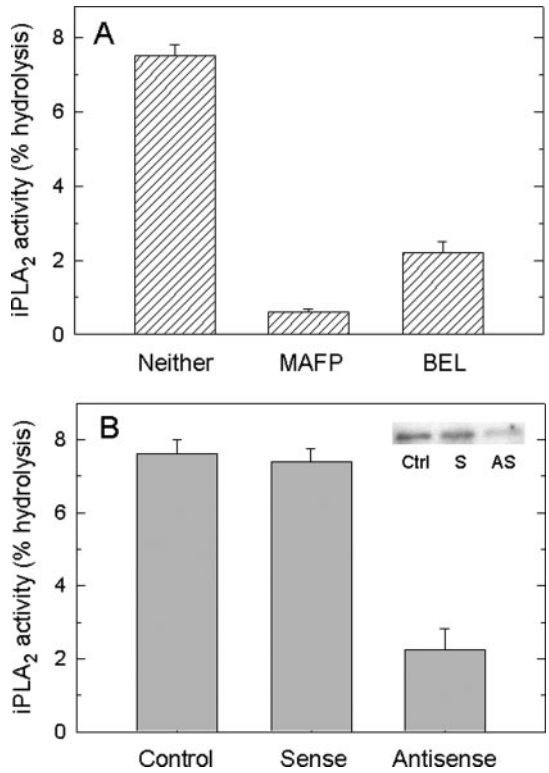


FIG. 8. **Inhibition of iPLA$_2$ in U937 cells.** *A*, effect of treating the cells with 25 $\mu$M MAFP or 25 $\mu$M BEL on the iPLA$_2$ activity of U937 cell homogenates. *B*, effect of an iPLA$_2$ antisense oligonucleotide on iPLA$_2$ activity and iPLA$_2$ protein expression (*inset*).

When cells are exposed to high, micromolar levels of exogenous fatty acids, incorporation into cellular lipids mainly occurs via the *de novo* pathway, which ultimately leads to accumulation of these fatty acids into TAG (7, 35). We have previously shown that this process is strikingly dependent on a functional PAP-1 that provides the DAG precursors for TAG synthesis (7, 35). Fig. 9 shows that the capacity of the cells to incorporate [$^3$H]AA (10 $\mu$M) into TAG is greatly diminished by both BEL and propranolol, indicating again that the Kennedy pathway is blocked under these conditions.

## DISCUSSION

In the present work we have found that BEL, a compound that is widely used as an iPLA$_2$ inhibitor, induces death in a number of cellular types. Staining of nuclei in BEL-treated cells with DAPI demonstrates features of apoptosis, a finding that is corroborated by flow cytometry studies with propidium iodide-labeled cells. In accord with these results, BEL induces an increase in the exposure of phosphatidylserine on the outer surface of the cells, as well as cleavage of caspase-3 and caspase-9. The involvement of caspases in BEL-induced cell death was confirmed by studies utilizing the general caspase inhibitor Z-VAD-FMK.

The finding that caspase-9 cleavage occurs is suggestive of the involvement of mitochondria. Assessment of changes in the mitochondria inner membrane potential with the dye JC-1 indicate that BEL induces apoptosis by activating the mitochondrial cell death pathway (36).

Importantly, none of the aforementioned effects of BEL could be reproduced by MAFP, another iPLA$_2$ inhibitor, or by treating the cells with a specific iPLA$_2$ antisense oligonucleotide, conclusively ruling out the involvement of iPLA$_2$ in BEL-in-

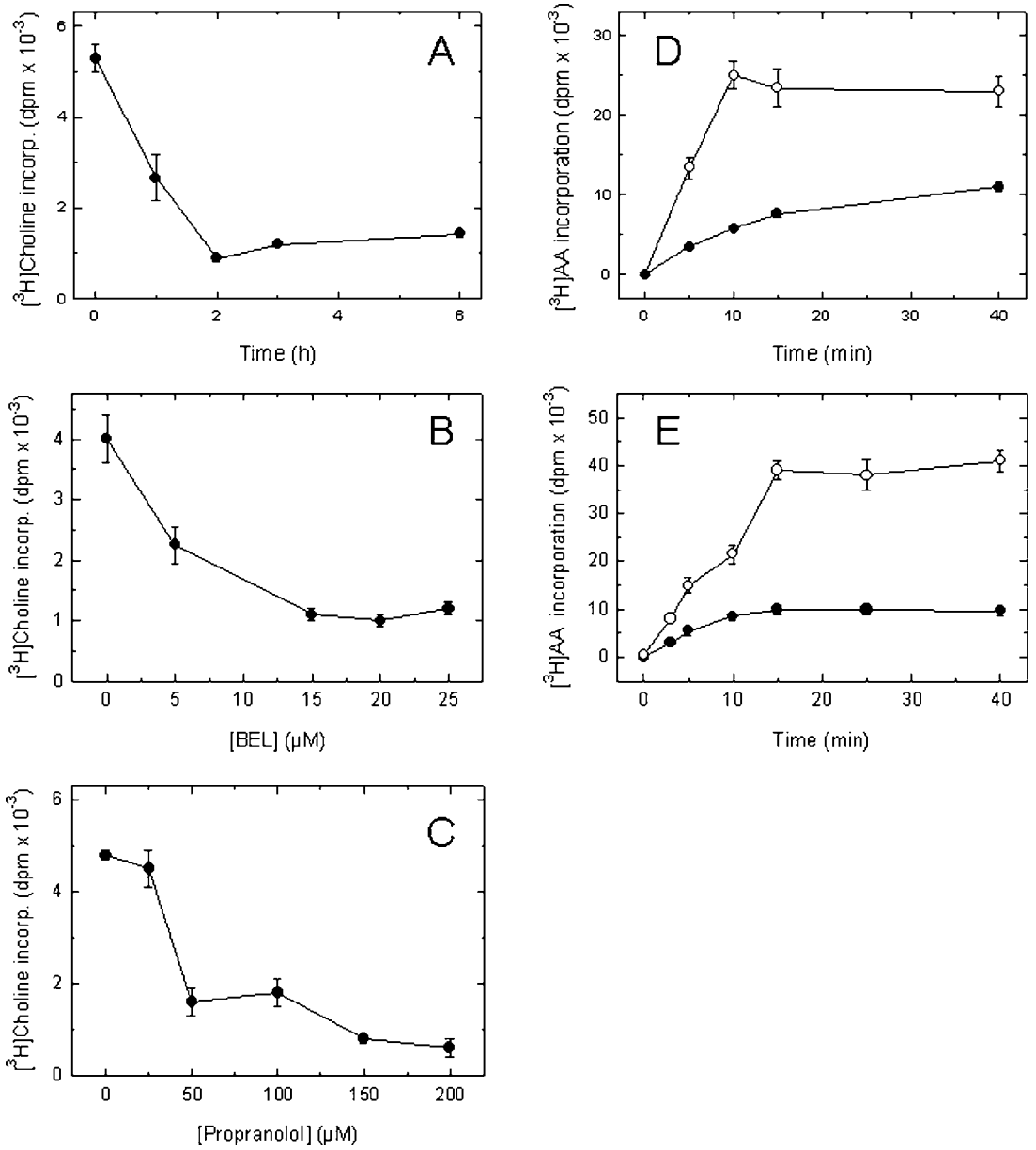


FIG. 9. **Functional consequences of PAP-1 inhibition in U937 cells.** *A*, [$^3$H]Choline incorporation (*incorp.*) into PC in U937 cells. The cells were treated with 25 $\mu$M BEL for the indicated periods of time. 2 $\mu$Ci of [$^3$H]Choline was added for the last hour of incubation. Radioactivity incorporated into PC was measured as described under "Experimental Procedures." *B* and *C*, dose response of the effects of BEL and propranolol on [$^3$H]choline incorporation. *D* and *E*, [$^3$H]AA incorporation into TAG in U937 cells. The cells were treated with either 25 $\mu$M BEL (*D*) or 150 $\mu$M propranolol (*E*) (*closed circles*) or vehicle (*open circles*) for 2 h and then exposed to 10 $\mu$M [$^3$H]AA (0.5 $\mu$Ci/ml) for the indicated periods of time. Radioactivity incorporated into TAG was measured as described under "Experimental Procedures."

duced apoptosis. It is worth mentioning here that $iPLA_2$ has been reported to be responsible for fatty acid liberation and, hence, destruction of membrane phospholipid during Fas-induced apoptosis of U937 cells (37, 38). Whether these actions of $iPLA_2$ during Fas-induced apoptosis are cause or consequence of the apoptotic process itself is not clear at this time. Our studies on different experimental settings appear to suggest that mitochondria-driven apoptosis can be triggered in the absence of $iPLA_2$ activity.

BEL-induced apoptosis was mimicked by the PAP-1 inhibitor propranolol (33), suggesting therefore that the effects of BEL may involve PAP-1 inhibition. PAP-1 is the enzyme that dephosphorylates phosphatidic acid to yield DAG. This is a key reaction in cellular phospholipid metabolism, because it provides the DAG necessary to maintain homeostatic PC biosynthesis in the cells. Thus, inhibition of PAP-1 will result in diminished cellular PC levels, a circumstance that we have confirmed to occur in the BEL-treated cells. PAP-1 inhibition under these conditions is also manifested by strong inhibition of fatty acid incorporation into TAG (7).

Perturbation of PC homeostasis is known to induce growth arrest and apoptotic cell death (34). Evidence that reduced PC synthesis alone can cause apoptosis was first reported in a cell line with a temperature-deficient mutant of CTP:phosphocholine cytidylyltransferase, the enzyme that produces CDP-choline to be used for PC synthesis (39). More recently, synthetic analogues of lyso-PC that function as inhibitors of PC synthesis have been documented to induce apoptosis in fibroblast-like cells (40). In agreement with all of these results, defective PAP-1 activity pinpoints a molecular basis that can explain BEL-induced apoptosis, *i.e.* diminished homeostatic lipid synthesis. Under these conditions, an hypothetical role for ceramides also merits consideration. Decreased synthesis of both DAG and PC in BEL-treated cells should necessarily

impact negatively on sphingomyelin synthesis via the sphingomyelin synthase pathway, ultimately leading to early elevations of ceramide within the cells. Although the mechanism by which ceramide signals apoptosis remains a matter of controversy, ceramide has been found to increase during the initial phases of apoptosis (41, 42). Interestingly, natural ceramides appear to be able to increase the permeability of mitochondrial outer membranes, thus permitting the release of cytochrome *c* and other small proteins to the cytosol (43).

Given the powerful, $iPLA_2$-independent effects of BEL described in this study, caution should be exercised when using this drug to study possible $iPLA_2$ roles in apoptosis or other long term cellular responses. In addition to BEL, other inhibitors and/or alternative techniques to abolish $iPLA_2$ should be utilized to complement the results obtained with BEL. It is important to note that even at times where no morphological alterations are readily visible, there is a cascade of biochemical events already going on in the cells, namely mitochondrial depolarization and the release and activation of cell death effectors. Moreover, BEL effects on lipid metabolism are readily detectable within 1-h exposure to the drug, the time at which both PC and TAG synthesis is severely depressed. Studies are currently under way to explore the effects of BEL derivatives with lower toxicity and to what extent can these compounds and other PAP-1 inhibitors be thought of as potential antitumoral agents.

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