Identification of Calcium-independent Phospholipase A$_2$ (iPLA$_2$) $\beta$, and Not iPLA$_2$$\gamma$, as the Mediator of Arginine Vasopressin-induced Arachidonic Acid Release in A-10 Smooth Muscle Cells

ENANTIOSELECTIVE MECHANISM-BASED DISCRIMINATION OF MAMMALIAN iPLA$_2$s*

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The agonist-stimulated release of arachidonic acid (AA) from cellular phospholipids in many cell types (e.g., myocytes, $\beta$-cells, and neurons) has been demonstrated to be primarily mediated by calcium-independent phospholipases $\alpha$ (iPLA$_2$s) that are inhibited by the mechanism-based inhibitor (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (BEL). Recently, the family of mammalian iPLA$_2$s has been extended to include iPLA$_2$$\gamma$, which previously could not be pharmacologically distinguished from iPLA$_2$$\beta$. To determine whether iPLA$_2$$\beta$ or iPLA$_2$$\gamma$ (or both) were the enzymes responsible for arginine vasopressin (AVP)-induced AA release from A-10 cells, it became necessary to inhibit selectively iPLA$_2$$\beta$ and iPLA$_2$$\gamma$ in intact cells. We hypothesized that the $R$- and $S$-enantiomers of BEL would possess different inhibitory potencies for iPLA$_2$$\beta$ and iPLA$_2$$\gamma$. Accordingly, racemic BEL was separated into its enantiomeric constituents by chiral high pressure liquid chromatography. Remarkably, (S)-BEL was approximately an order of magnitude more selective for iPLA$_2$$\beta$ in comparison to iPLA$_2$$\gamma$. Conversely, (R)-BEL was approximately an order of magnitude more selective for iPLA$_2$$\gamma$ than iPLA$_2$$\beta$. The AVP-induced liberation of AA from A-10 cells was selectively inhibited by (S)-BEL (IC$_{50}$ ~2 $\mu$M) but not (R)-BEL, demonstrating that the overwhelming majority of AA release is because of iPLA$_2$$\beta$ and not iPLA$_2$$\gamma$ activity. Furthermore, pre-treatment of A-10 cells with (S)-BEL did not prevent AVP-activated MAPK phosphorylation or protein kinase C translocation. Finally, two different cell-permeable protein kinase C activators (phorbol-12-myristate-13-acetate and 1,2-dioctanoyl-sn-glycerol) could not restore the activity of A-10 cells to release AA after exposure to (S)-BEL, thus supporting the downstream role of iPLA$_2$$\beta$ in AVP-activated AA release.

Calcium-independent phospholipases $\alpha_2$ (iPLA$_2$$\alpha_2$)$^1$ constitute an important group of intracellular enzymes that function to hydrolyze esterified fatty acids from membrane phospholipids in response to agonist stimulation, changes in intracellular calcium ion homeostasis, and alterations in cellular energy requirements (for reviews see Refs. 1–3). In earlier studies, we identified a calcium-independent phospholipase $\alpha_2$, which is responsible for arginine vasopressin (AVP)-induced AA release in A-10 cells. Calcium-independent phospholipase $\alpha_2$ (iPLA$_2$$\alpha_2$), unlike calcium-dependent phospholipase $\alpha_2$ (cPLA$_2$$\alpha_2$), is inhibited by the mechanism-based inhibitor (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (rac-BEL). Based upon activity assays, calcium requirements, loss of arachidonoylated phospholipid mass, and inhibition of iPLA$_2$$\beta$ by rac-BEL, a diverse array of cellular processes has been proposed to be regulated by iPLA$_2$$\alpha_2$, including arachidonic acid (AA) release (10–15), cellular proliferation (16), assembly of very low density lipoprotein (17), purinergic receptor-stimulated kallikrein secretion (18), apoptosis (19), endothelial cell platelet-activating factor synthesis (20), and induction of inducible nitric-oxide synthase and nitric oxide production (21).

At present, three distinct subclasses of iPLA$_2$s have been identified at the genetic level (with subsequent confirmation of iPLA$_2$$\alpha_2$ catalytic activity by recombinant technologies) and have been designated iPLA$_2$$\alpha_2$, iPLA$_2$$\beta$, and iPLA$_2$$\gamma_2$, in order of their discovery (22–24). The iPLA$_2$$\alpha_2$s have been categorized based upon their strict conservation of nucleotide-binding (GXXGXXG) and lipase (GXSTG) consensus sequences (Fig. 1). Two of the iPLA$_2$ subclasses, iPLA$_2$$\beta$ and iPLA$_2$$\gamma_2$, have been cloned from mammalian cDNA libraries, whereas the ortholog of iPLA$_2$$\alpha_2$ (patatin), at the time of writing this paper (with 97.8% of the human genome sequenced), has not been identified in mammals. Calcium-independent phospholipase $\alpha_2$$\beta$ contains eight ankyrin-repeat domains, most of which are believed to facilitate intracellular sorting (23, 25, 26) and a CaM-binding domain near the C terminus which binds calcium-activated CaM and regulates enzyme activity (27) (Fig. 1). The binding of CaM to iPLA$_2$$\beta$ results in inhibition of iPLA$_2$$\beta$ activity which is reversible

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$^1$ The abbreviations used are: iPLA$_2$, calcium-independent phospholipase $\alpha_2$; AVP, arginine vasopressin; BEL, (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one; Rac-BEL, racemic (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one; NpI6, (E)-6-(iodomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one; cPLA$_2$, cytosolic phospholipase $\alpha_2$; iPLA$_2$$\beta$, calcium-independent phospholipase $\alpha_2$$\beta$; iPLA$_2$$\gamma_2$, calcium-independent phospholipase $\alpha_2$$\gamma_2$; PAP, phosphatidate phosphohydrolase; PMA, phorbol 12-myristate 13-acetate; DOG, 1,2-dioctanoyl-sn-glycerol; AA, arachidonic acid; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; DMM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid; CaM, calmodulin; CAPS, 3-(cyclohexylamino)propanesulfonic acid; HPLC, high pressure liquid chromatography; DAG, diacylglycerol.
through removal of Ca\(^2\+\) and subsequent dissociation of CaM from the C terminus of iPLA\(_2\beta\) (27, 28). In this paradigm, iPLA\(_2\beta\) is regulated through alterations in cellular calcium ion homeostasis and becomes activated after dissociation from its complex with Ca\(^2\+\)/CaM when intracellular calcium stores are depleted by SERCA inhibitors, calcium-ionophores, or agonist stimulation (29, 30). In contrast, the recently identified iPLA\(_2\gamma\) does not bind CaM,\(^2\) and its mechanisms of regulation are unknown at present.

Many prior studies of iPLA\(_2\) have utilized the mechanism-based suicide inhibitor rac-BEL as a pharmacologic tool to identify the type of intracellular phospholipase A\(_2\) involved in many diverse cellular processes. Because rac-BEL inhibits both iPLA\(_2\beta\) and iPLA\(_2\gamma\) at low micromolar concentrations (24, 25, 31, 32), it is impossible to assign rac-BEL-mediated inhibition of AA release to iPLA\(_2\beta\) or iPLA\(_2\gamma\) activities. Accordingly, it became necessary to develop pharmacologic approaches that could discriminate between iPLA\(_2\beta\) and iPLA\(_2\gamma\) to facilitate identification of their biologic roles. In addition, it has been reported previously (33, 34) that high concentrations of BEL partially inhibit the magnesium-dependent cytosolic phosphatidate phosphohydrolase, PAP-1, which converts phosphatidate to diacylglycerol (DAG). In those investigations, it was proposed that PAP-1 inhibition by BEL would prevent activation of protein kinase C leading to attenuated AA release. However, “rescue” experiments in which PKC was exogenously activated by phorbol esters or diacylglycerol analogs after BEL treatment were not reported by the authors to address their hypothesis (33, 34).

In our early experiments we employed rac-BEL to demonstrate a 1000-fold selectivity between iPLA\(_2\alpha\) versus cPLA\(_2\) and sPLA\(_2\) family members (31, 32). Based upon the increasing appreciation of the utility of chiral pharmacologic agents in enhancing the specificity of inhibitors toward targeted biologic processes, we hypothesized that (R)- and (S)-BEL could differentially inhibit iPLA\(_2\beta\) and iPLA\(_2\gamma\) activities. Moreover, we reasoned that development of chiral mechanism-based inhibitors could provide an increased degree of discrimination between specific targeted enzyme systems and those of “nonspecific inhibition.” Here we report the resolution of racemic BEL into its individual enantiomeric constituents by chiral HPLC and the selective inhibition of iPLA\(_2\beta\) by (S)-BEL and iPLA\(_2\gamma\) by (R)-BEL, and we demonstrate that BEL-mediated inhibition of AA release in A-10 cells is likely mediated by iPLA\(_2\beta\) and not due to inhibition of iPLA\(_2\gamma\) or the effects of BEL on MAPK or PKC activation.

\(^2\) C. M. Jenkins and R. W. Gross, unpublished observations.

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**Experimental Procedures**

**Materials**—BEL, phorbol 12-myristate 13-acetate (PMA), and 1,2-dioctanoyl-sn-glycerol (DOG) were obtained from Calbiochem. A Chirex HPLC column composed of a stationary phase of (R)-phenylglycine linked through an amide linkage to 3,5-dinitrobenzoic acid was purchased from Phenomenex. 3-Fold-crystallized α-chymotrypsin, N-succinyl-Ala-Ala-Pro-7-ami-d0-4-methylcoumarin, fatty acid-free bovine serum albumin (low endotoxin), antibodies against PKC\(_\beta\) and α- and other reagents were obtained from Sigma. AVP was purchased from Bachem. HPLC-grade organic solvents and channeled LK60 silica gel 60 Å thin layer chromatography plates (Whatman) were obtained from Fisher. Enhanced chemiluminescence (ECL) reagents and film were purchased from Amersham Biosciences. t-A-1-Palmitoyl-2-(1\(^{-}\)\(^{14}\)C)arachidonylphosphatidylcholine, t-A-1-palmitoyl-2-(1\(^{-}\)\(^{14}\)C)oleoylphosphatidylcholine, [5,6,8,9,11,12,14,15\(^{-}\)\(^{14}\)H]arachidonic acid, and t-A-dipalmito-oyl(1\(^{-}\)\(^{14}\)C)phosphatic acid were purchased from PerkinElmer Life Sciences. A-10 cells derived from rat aortic smooth muscle were obtained from the ATCC and cultured as described previously (10). R- and S-enantiomers of (R)-6-(iodomethylene)-3-(1-naphthalenyl)-2H-tetrahydropyran-2-one (αNp6f) were generous gifts of John A. Katzenellenbogen (University of Illinois, Urbana). Anti-active MAPK (pTEpY) and anti-ERK/2 antibodies were obtained from Promega. Antibodies against PKCs and PKC\(_\gamma\) were kindly provided by David A. Ford (St. Louis University, St. Louis). Antibodies against PKC\(_\gamma\) were obtained from BD Transduction Laboratories.

**Recombinant iPLA\(_2\) Enzymes**—Recombinant iPLA\(_2\beta\) was expressed and purified from SF9 cells as described previously (35). Recombinant full-length iPLA\(_2\gamma\) was expressed in SF9 cells, and the membrane fraction was washed and isolated as described previously (24). Inactivation of BEL—BEL enantiomers were resolved by HPLC utilizing a Chirex column of 3,5-dinitrobenzoyl-\(\alpha\)-chymotrypsin (2\(^{-}\)\(^{14}\)C)-phosphatidylcholine, N-succinyl-Ala-Ala-Pro-7-ami-d0-4-methylcoumarin attached to a silica matrix as the stationary chiral phase. The chiral column was equilibrated with hexane/dichloroethane/ethanol (150:15:1), and optical enantiomers were eluted isocratically at a flow rate of 2 ml/min. Elution of BEL racemates from the column was monitored by UV absorbance at 280 nm. Peaks corresponding to the R- and S-enantiomers were collected, dried under \(\text{N}_2\), and stored at \(-20^\circ\text{C}\). The concentration of BEL for each experiment was determined spectrophotometrically based on UV absorbance (\(\epsilon_{280} = 6130\ \text{cm}^{-1}\ \text{M}^{-1}\) in acetonitrile).

**Inhibition of α-Chymotrypsin by BEL**—The kinetics of α-chymotrypsin inactivation by (R)-, (S)-, and rac-BEL were performed similar to methods described previously (36). The concentration of α-chymotrypsin (\(M_0 = 25,000\)) was determined using an \(A_{\text{in}} = 20\) (1-cm path length) at 280 nm (37). Briefly, 1 ml of α-chymotrypsin (2 \(\mu\)M) was incubated with up to a 5-fold molar excess of (R)-, (S)-, or rac-BEL dissolved in acetonitrile or vehicle alone for 5 min at 22 °C in 0.1 M sodium phosphate buffer, pH 7.2. A 10-μl aliquot of the reaction was diluted 1000-fold in 10 ml of 0.1 M sodium phosphate buffer, pH 7.2, containing 100 mM hydrazine to deacetylate transiently inactive BEL-α-chymotrypsin complexes. Following incubation at 22 °C for 1 h, chymotrypsin activity in each diluted sample was measured utilizing a SPECTRAMAX GeminiXS microplate spectrophotometer with N-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin (50 \(\mu\)M) as substrate for 1 min at 25 °C. Excitation and emission wavelengths were 380 and 460 nm, respectively.

**Assay of iPLA\(_2\beta\) and iPLA\(_2\gamma\) Inhibition by BEL**—Purified recombinant iPLA\(_2\beta\) or SF9 cell membranes containing recombinant iPLA\(_2\gamma\)

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**Fig. 1.** The iPLA\(_2\) gene family and sequence alignment of iPLA\(_2\) nucleotide and lipidase consensus motifs. Members of the iPLA\(_2\) gene family (α, β, and γ) are aligned according to their nucleotide-binding motifs (diagonal bars) and lipidase consensus sites (filled bars). Calcium-independent phospholipase A\(_2\) (iPLA\(_2\)) contains eight ankyrin repeat domains (gray bars) and a calmodulin-binding domain (CaM) near the C terminus (horizontal bars).
were incubated with (R)-BEL, (S)-BEL, racemic BEL, or ethanol vehicle for 3 min at 22 °C in the presence of 100 mM Tris-HCl, pH 7.0, and 4 mM EGTA (for iPLA2β) or 100 mM Tris-acetate, pH 8.0, and 4 mM EGTA (for iPLA2γ). The concentration of BEL used for each experiment ranged from 0 to 16 µM. 1-α,3β-4-cholesteryl monooleate was final concentration) or 1-α,3β-4-cholesteryl monooleate (5 μM final concentration) in ethanol was then added to each sample and incubated at 37 °C for 2 min. Reactions were terminated by addition of radiolabeled products into butanol, and reactants and products were separated by thin layer chromatography using Whatman LKGD 60-Å Silica Gel plates with petroleum ether/ethyl ether/ethyl acetate (70/30/1) as the mobile phase. Regions corresponding to the migration of a fatty acid standard visualized by iodine staining were scraped into vials, and radioactivity was quantified by scintillation spectrometry.

Quantification of [3H]Arachidonic Acid Liberation from A-10 Phospholipids—Rat aortic smooth muscle A-10 cells, cultured in 60-mm dishes (2.5 x 10⁶ cells/dish), were radiolabeled with 0.5 μCi of [3H]arachidonic acid per dish as described previously (29). Cells were washed once with DMEM containing 0.25% fatty acid-free bovine serum albumin followed by two washes with DMEM alone. Cells were then incubated with the indicated concentrations of (R)-, (S)-, rac-BEL, or ethanol vehicle (0.1% final concentration) in DMEM for 20 min. This medium was removed, and the cells were then incubated with DMEM containing 0.25% ethanol or ethanol vehicle (0.1% final concentration) for 5 min. After brief sonication utilizing a Vibra-Cell VC40 sonicator (5 times with 1-s pulses at 30% power), the lysed cell suspension was centrifuged at 100,000 x g for 30 min to separate cytosolic and membrane fractions. In some experiments, A-10 cells were washed and pretreated with BEL or ethanol vehicle (as described above for experiments examining [3H]IgA liberation) before isolation of cell homogenates. Each fraction (40 μl) was preincubated with BEL (up to 200 μM) or ethanol vehicle at 22 °C for 5 min in the presence of 50 mM Tris-HCl, pH 7.2, containing 10 mM β-mercaptoethanol, 2 mM MgCl₂, and 1 mM EGTA (90 μl final volume). Dipalmitoylphosphatidic acid (100 μM final concentration containing 0.05 μCi of 1-α,3β-dipalmitoyl-sn-[14C]glycerol chloride) phosphatidic acid per reaction in the presence of 1 mM Triton X-100 was added to each sample and incubated at 37 °C for 2 min. Reactions were terminated with 900 μl of 5% acetic acid and extracted into chloroform by the method of Bligh and Dyer (38) prior to separating cytosolic and membrane fractions. The chemical structures of (R)- and (S)-BEL are as indicated above the chromatogram.

Measurement of Cytosolic and Membrane-bound Phosphatidate Phosphohydrolase Activities—A-10 cells were grown to confluence, washed twice in ice-cold phosphate-buffered saline, and harvested in ice-cold PBS, the cells were collected by scraping into 20 mM CAPS, pH 11, for ECL Western analysis. After blocking with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 5% non-fat dry milk for 2 h, primary rabbit polyclonal antibodies against phospho-YEP and dephosphorylated MAPK diluted 1:5000 in PBS containing 5% BSA were incubated with the blot for 1 h. After washing with TBS-T, the blots were incubated with ice-cold PBS containing 0.25% glutaraldehyde for 15 min as described previously (40). Washed, and incubated with a protein A-peroxidase conjugate diluted (1:5000) in TBS-T containing 5% BSA for 1 h. Immunoreactive bands were visualized by ECL as described by the manufacturer (Amersham Biosciences). Determination of PKC Translocation—Confluent A-10 cells in 10-cm dishes were washed twice with DMEM without serum, followed by incubation with either 5 µM (S)-BEL or ethanol vehicle in DMEM for 15 min at 37 °C. This medium was then removed, and DMEM with or without 1 µM AVP was incubated with the cells for 5 min. After washing with ice-cold PBS, the cells were collected by scraping into 20 mM Tris-HCl, pH 7.4, containing 0.33 M sucrose, 0.5 mM EDTA, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 5 µg/ml leupeptin and were lysed by three cycles of flash freezing with liquid nitrogen and thawing. Each sample was then further homogenized utilizing a Teflon homogenizer before isolating the low speed pellet (1,000 x g), membrane (100,000 x g pellet), and cytosol (100,000 x g supernatant) fractions. The protein concentrations of the fractions were determined utilizing the bicinchoninic acid (BCA) assay (Pierce) with BSA as a standard. Samples were electrophoresed and subjected to ECL Western analysis utilizing rabbit polyclonal antibodies against PKCα and PKCδ as described above for MAPK phosphorylation. For blots incubated with mouse monoclonal antibodies against PKCδ and PKCε, an anti-mouse IgG (Fab-specific)-peroxidase conjugate was utilized in place of the protein A-peroxidase conjugate.

RESULTS

Separation of BEL Enantioomers—Because BEL contains a chiral center at C-2 (Fig. 2), we hypothesized that (R)-BEL and (S)-BEL might have different potencies and/or selectivities for iPLA2β and iPLA2γ so that individual enantiomers of BEL could be exploited to identify the roles of iPLA2β and iPLA2γ in agonist-stimulated AA release in intact cells. Accordingly, a chiral HPLC column was used to separate (R)-BEL and (S)-BEL from rac-BEL (Fig. 2). Separation of the BEL enantiomers under the conditions employed resulted in resolution of two major UV-absorbing peaks with an Rₑ difference of ~2 min. The first peak eluted at 18.8 min (Peak A), and the second peak eluted at 20.5 min (Peak B). For the purpose of this report, the retention times (RT) of Peak A and Peak B were 18.8 and 20.5 min, respectively. The percent composition of Peak A was 82.5% (Peak A) and 17.5% (Peak B) of the total area. Integration of the areas of each peak was identical (within 1%), suggesting that the enantiomers of BEL had been separated. Proton NMR data demonstrated peaks with the anticipated chemical shifts and coupling constants as described previously (data not shown) (41). Moreover, electrospray ionization-mass spectrometric analysis of the major peaks confirmed the purity of the isolated enantiomers.
terial in each peak revealed the presence of a lithiated doublet at 323.3 and 325.3 daltons (consistent with the presence of a bromine atom) which was of equal intensity for each moiety. Re-injection of Peak A or Peak B onto the chiral column demonstrated that each purified moiety eluted at its previous retention time with negligible amounts of contaminating material demonstrating that, as expected, no equilibration had occurred. Finally, both peaks co-eluted utilizing a non-chiral C18 HPLC column (data not shown).

Identification of the Absolute Chirality of the BEL Enantiomers—To determine the absolute chirality of the resolved BEL enantiomers, synthetic enantiomers of αNp6 of known chirality were chromatographed separately and together on the chiral stationary phase. Under these conditions, (S)-αNp6 (R_T = 19.5 min) eluted prior to (R)-αNp6 (R_T = 21.4 min) with approximately the same degree of separation (R_{RT} = 0.911) as the BEL enantiomers. Chymotrypsin has been identified previously as a suitable target for aromatic haloenol lactones resulting in its mechanism-based inhibition as detailed by careful kinetic analyses by Katzenellenbogen (41–44). In prior studies, (R)-BEL was determined to be a more efficient inhibitor of chymotrypsin than (S)-BEL in comparison to its chiral counterpart (36). To substantiate further the absolute stereochemistry of the BEL enantiomers resolved by chiral HPLC and to confirm the ability of the resolved enantiomers to inhibit selectively chymotrypsin activity, increasing concentrations of (R)-BEL, (S)-BEL, or rac-BEL were incubated with chymotrypsin and diluted in buffer as described under “Experimental Procedures.” Activity assays were performed using the fluorogenic chymotrypsin substrate N-succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin as described under “Experimental Procedures.”

Under the conditions employed, (R)-BEL stoichiometrically and irreversibly inhibited chymotrypsin, whereas (S)-BEL was considerably less potent (Fig. 3). These results confirm that both peaks are distinct enantiomers of BEL and substantiate, by independent criteria, the assigned absolute stereochemistry of the peaks eluting from the chiral HPLC column.

Enantiomeric Selective Inhibition of iPLA2β and iPLA2γ—Previous experiments with iPLA2β and iPLA2γ have established that iPLA2β and iPLA2γ are inhibited by racemic BEL with an IC_{50} range of 200 nM for iPLA2β and ~3 μM for iPLA2γ (24, 31). Accordingly, we next examined the ability of the resolved enantiomers of BEL to inhibit iPLA2β and iPLA2γ. For these experiments, iPLA2β or iPLA2γ was preincubated with (R)-BEL, (S)-BEL, rac-BEL, or ethanol vehicle alone for 3 min followed by measurement of remaining enzymatic activity utilizing a radiolabeled phospholipid substrate. Remarkably, (S)-BEL selectively inhibited iPLA2γ 10-fold more potently than (R)-BEL (Fig. 4A). In stark contrast, (R)-BEL selectively inhibited iPLA2γ ~10-fold more potently than (S)-BEL (Fig. 4B). As anticipated, the inhibitory potency of racemic BEL was intermediate of that of (R)-BEL and (S)-BEL. Thus, (S)-BEL is a more potent inhibitor of iPLA2γ, whereas (R)-BEL is a more potent inhibitor of iPLA2β.

Identification of iPLA2β and Not iPLA2γ as the Mediator of AVP-induced AA Release in A-10 Cells—Upon stimulation with...
Selective Inhibition of iPLA$_2$$\beta$ and iPLA$_2$$\gamma$

AVP, A-10 smooth muscle cells rapidly released a relatively large percentage (~5%) of their esterified arachidonic acid (10). Previous studies (10, 29) have demonstrated that pretreatment of the cells with 2–5 μM BEL inhibits ~60–80%, respectively, of AVP-induced arachidonic acid release. Moreover, the absence of extracellular calcium ion (incubations performed in the presence of EGTA in the media) or the presence of intracellular calcium ion chelators (e.g. BAPTA) does not affect AVP-induced AA release in A-10 cells (29). Because combined incubations with EGTA and BAPTA completely ablated FURA-2-observable increases in intracellular calcium ions, these results further implicated the involvement of a calcium-independent phospholipase A$_2$ in this process. However, since both iPLA$_2$$\beta$ and the newly identified iPLA$_2$$\gamma$ are both inhibited by rac-BEL (and are calcium-independent), the identity of the iPLA$_2$-mediating AA release in AVP-stimulated A-10 cells was unknown. To address this issue, we exploited the selectivity of (S)-BEL and (R)-BEL for inhibition of iPLA$_2$$\beta$ and iPLA$_2$$\gamma$, respectively, to determine the type of iPLA$_2$-catalyzing AVP-induced release of [H]$\text{arachidonic acid}$ from A-10 cells. As demonstrated previously, A-10 cells stimulated with AVP resulted in a substantial increase in the amount of nonesterified [H]$\text{arachidonic acid}$ relative to control cells incubated with vehicle alone (Fig. 5). This AVP-induced increase in non-esterified [H]$\text{arachidonic acid}$ was significantly reduced in the presence of low concentrations of rac-BEL (1 and 5 μM, p < 0.01 and p < 0.001, respectively) (Fig. 5). Importantly, (S)-BEL (1 μM) significantly inhibited AA release (40% inhibition, p < 0.01) and 5 μM (S)-BEL largely attenuated AVP-induced AA release (80% inhibition, p < 0.001). In sharp contrast, (R)-BEL is virtually ineffective in inhibiting AVP-induced AA release from A-10 cells under similar conditions (Fig. 5). Thus, iPLA$_2$$\beta$, and not iPLA$_2$$\gamma$, is the likely mediator of AA release in this system.

**Confirmation of the Lack of Effects of BEL on Processes Typically Associated with cPLA$_2$$\alpha$ Activation—Activation of cPLA$_2$$\alpha$ in most systems depends on the concomitant activation of MAPK, PKC, and increases in intracellular [Ca$^{2+}$]$^-$ (45–47).**

In previous studies (29) we demonstrated that BEL does not inhibit AVP-induced increases in [Ca$^{2+}$]$^-$, and that ablation of changes in [Ca$^{2+}$]$^-$ by BAPTA does not attenuate AA release. Recently, Dennis and co-workers (33, 34) have suggested that cytosolic phosphatidate phosphohydrolase (PAP-1) in some cell types may be a target for BEL and that the resulting inhibition of PAP-1 would result in diminished levels of diacylglycerol produced from phosphatidic acid, thereby attenuating PKC activation precluding cPLA$_2$$\alpha$ activation and AA release. To address this possibility, we first examined the effects of rac-BEL on A-10 cell PAP activities in cytosol and membrane fractions (Fig. 6A) as well as in intact cells (Fig. 6B). These experiments consistently demonstrated the lack of any effect of BEL on either the cytosolic (PAP-1) or the membrane-bound (PAP-2) forms of A-10 cell phosphatidate phosphohydrolase at concentrations up to 200 μM BEL (Fig. 6A). Furthermore, homogenates from intact A-10 cells previously exposed to up to 100 μM racemate BEL did not inhibit total phosphatidate phosphohydrolase activity in comparison to ethanol-treated controls (Fig. 6B). Next, we examined whether activation of PKC by exogenous addition of either PMA or DOG could rescue AA release after BEL pretreatment. Neither PMA nor DOG could restore the ability of (S)-BEL treated A-10 cells to release arachidonic acid, thereby demonstrating that BEL is likely inhibiting arachidonic acid release in a manner that is no longer responsive to PKC activation (i.e. irreversible covalent modification of iPLA$_2$) (Fig. 7). A-10 cells contain at least four PKC isofoms, PKC$_{\alpha}$, PKC$_{\delta}$, PKC$_{e}$, and PKC$_{\gamma}$, by Western blot analysis (Fig. 8A); however, no bands corresponding to PKC$_{\beta_1}$, PKC$_{\beta_2}$, PKC$_{\gamma}$, PKC$_{\eta}$, or PKC$\xi$ could be visualized in previous work. According to the results published by the authors, we treated A-10 cells with AVP and determined whether (S)-BEL could inhibit PKC translocation to the membrane fraction. Stimulation of A-10 cells with AVP causes translocation of PKC$_{\delta}$ and PKC$_{e}$ from the cytosol to the membrane fraction, but neither PKC$_{\alpha}$ nor PKC$_{\gamma}$ undergo AVP-induced translocation in A-10 cells (Fig. 8A). Pretreatment of A-10 cells with 5 μM (S)-BEL, which causes almost complete inhibition of AA release, does not affect the translocation of either PKC$_{\delta}$ or PKC$_{e}$ (Fig. 8A). Finally, AVP-induced phosphorylation of ERK2 is not affected by the presence of 5 μM (R)-, (S)-, or rac-BEL (Fig. 8B). Collectively, these results demonstrate the following: 1) neither PAP-1 nor PAP-2 is a target for BEL in A-10 smooth muscle cells; 2) BEL does not appreciably affect PKC$_{\delta}$ and PKC$_{e}$ translocation or MAPK phosphorylation in A-10 cells; and 3) iPLA$_2$$\beta$ is likely responsible for the large majority of arachidonic acid release from A-10 cells.

**DISCUSSION**

Genetic approaches have now demonstrated the presence of two types of iPLA$_2$ activities present in the human genome (iPLA$_2$$\beta$ and iPLA$_2$$\gamma$) which are both inhibited by rac-BEL (24, 31, 32). Accordingly, all prior experiments demonstrating inhibition of arachidonic acid release by rac-BEL cannot discriminate between hydrolysis catalyzed by iPLA$_2$$\beta$ or that mediated by iPLA$_2$$\gamma$. Virtually nothing is known about the regulation of iPLA$_2$$\gamma$ or its potential role in agonist-stimulated eicosanoid release. In this work, we 1) resolve rac-BEL by chiral HPLC; 2) 

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3 D. A. Ford, C. M. Jenkins, and R. W. Gross, unpublished observations.
assign the absolute stereochemistry of the resolved enantiomers by two independent techniques; 3) demonstrate a 10-fold selectivity of (S)-BEL for inhibition of iPLA2β and a 10-fold selectivity of (R)-BEL for inhibition of iPLA2γ; 4) demonstrate that (S)-BEL inhibits that vast majority of AVP-induced AA liberation in A-10 cells, whereas (R)-BEL does not; 5) provide evidence that BEL-mediated inhibition of AA release in A-10 cells is not mediated through inhibition of either membrane-bound or cytosolic phosphatidate phosphohydrolases; and 6) demonstrate that treatment of A-10 cells with (S)-BEL does not attenuate PKC translocation or MAPK activation after AVP stimulation. Collectively, these results, in combination with prior work (see below), demonstrate that AVP-stimulated AA release in A-10 cells is likely mediated by iPLA2β and not iPLA2γ, cPLA2α, or chymotrypsin-like proteases.

The utilization of chiral pharmacologic agents instead of racemic mixtures has increasingly been appreciated to enhance the potency of inhibitors toward targeted processes and markedly reduce toxicity and nonspecific inhibition mediated by interactions with non-targeted systems. Because enzymes possess multiple chiral centers, the interaction between a chiral inhibitor and one or more optically active centers at or near the enzyme active site results in diastereotropic interactions that possess different physical properties and spatial relationships for each diastereotropic pair. In the case of mechanism-based inhibitors such as BEL, these diastereotropic interactions are anticipated to 1) alter binding, 2) modify the rate of formation of the acyl-enzyme intermediate, and 3) alter the covalent trapping of the α-halomethyl ketone in the acyl enzyme by nucleophiles at or near the active site. In this study, we have exploited diastereotropic interactions between individual enantiomers of BEL and the known mammalian iPLA2s (i.e. iPLA2β and iPLA2γ) to achieve a remarkable specificity for inhibition of iPLA2β by (S)-BEL and iPLA2γ by (R)-BEL. Moreover, we demonstrated that proteases with similar stereochemical relationships as chymotrypsin are more likely to be inhibited by (R)-BEL than (S)-BEL, thereby further increasing the utility of mechanism-based inhibition to gain insight into the types of phospholipases A2 mediating AA release in mammalian cells.

We specifically point out that, as with any pharmacologic compound, unanticipated effects on non-targeted systems may occur with increasing likelihood at high concentrations of inhibitor. Mammalian cells have in excess of 30,000 genes which after splicing and post-translational modification give rise to well over 10^5 and perhaps as many as 10^6 different chemical
moieties. Of course, it is impossible to test every compound with each of these chemical moieties in each different microenvironment in the cell in which pharmacologic agents might interact. Indeed, at high enough concentrations in aqueous systems, virtually any low molecular weight organic compound will interact with a diverse array of proteins due to hydrophobic effects alone. That is precisely why it is important to examine biologic effects elicited by pharmacologic agents at or near their effective inhibitory concentrations in intact cells as was determined in isolated purified systems. In the case of BEL, some investigators have employed 50–100 μM BEL which exceeds the effective inhibitory concentration of BEL for the known mammalian iPLA₂βs by almost 2 orders of magnitude. Accordingly, these experiments must be interpreted with caution given the IC₅₀ of rac-BEL for iPLA₂β and iPLA₂γ is in the 0.5–3 μM range. Moreover, the mere exposure of cells to high concentrations of organic compounds (50–100 μM) is likely to perturb the fragile order of the membrane microenvironment and, in the case of investigating membrane-related phenomena, may have effects that are independent of interactions with targeted enzyme systems alone. Indeed, we have observed cell death employing 100 μM BEL which is almost certainly independent of the effects of BEL on targeted processes.

Dennis and co-workers have contended that high concentrations of rac-BEL (~ 25 μM) effectively inhibit magnesium-dependent cytosolic phosphatidate phosphohydrolase (PAP-1) in mouse P388D₁ macrophages (33) and human amnionic WISH cells (34). The authors argue that inhibition of PAP-1 would be expected to cause a deficiency in DAG, thus blunting PKC activation and possibly activation of cPLA₂α by MAPK. However, BEL has been subsequently shown not to affect PMA-induced translocation of PKC (or PKC catalytic activity) in P388D₁ macrophages (15) or MAPK phosphorylation in WISH cells (34), rat neutrophils (48), and A-10 smooth muscle cells (this work). It should be mentioned that in their investigations, Balsinde and Dennis (33) did not observe any effect of BEL on PAP-2, the phosphatidate phosphohydrolase isofrom which is believed to be involved in lipid signal transduction pathways (49–51). This absence of PAP-2 inhibition by HEL has since been observed in McA-RH7777 rat hepatoma cells (17), pancreatic islet cells (52), and A-10 smooth muscle cells (this work). Furthermore, cytosolic PAP-1 activity in McA-RH7777 cells is not significantly inhibited by 100 μM BEL (17). A second possible effect of inhibited PAP-catalyzed DAG production, as described by Balboa et al. (34), is that the phospholipid substrate will be in a suboptimal environment because DAG has been demonstrated to alter membrane bilayers by creating more distance between phospholipid head groups, thereby making the phospholipid ester linkages more susceptible to PLA₂-mediated hydrolysis. However, we have found that rac-BEL does not inhibit release of inositol bisphosphate or inositol 1,4,5-trisphosphate in A-10 cells (10), and Akiba et al. (15) have found that BEL (up to 5 μM) does not significantly affect levels of diacylglycerol or phosphatidic acid formed in P388D₁ macrophages upon stimulation with zymosan.

The identification of chiral specificity of individual enantiomers of BEL to inhibit the known mammalian iPLA₂βs extends the utility of mechanism-based inhibitors in the study of agonist-mediated AA release. The experiments described herein allow, for the first time, assignment of AVP-induced AA release in A-10 cells to iPLA₂β and not iPLA₂γ. The inhibition of AA release by (S)-BEL and not (R)-BEL excludes participation of chymotrypsin or chymotrypsin-like proteases in these processes. Finally, the utilization of chiral mechanism-based inhibitors reduces potential nonspecific complications through comparisons of the effects of specific optical antipodes on the observed end points (i.e. AA release) with their in vitro potency in purified systems. We point out that assignment of specific enzymes as effectors of AA release requires detailed concurrent consideration of biochemical, pharmacologic, and genetic perturbations on the observed process. In the case of AVP-induced AA release from A-10 cells, we have demonstrated the following: 1) that concentrations of (S)-BEL near the IC₅₀ for iPLA₂β markedly attenuate AA release in intact A-10 cells whereas (R)-BEL does not (this study); 2) that BEL-mediated inhibition of AA release occurs in the presence of normal increases in [Ca²⁺], (29) and cannot be rescued by exogenous activation of PKC by PMA or DOG (this study); 3) that AVP-mediated AA release in A-10 cells is not affected by removal of calcium ions from the external media or by effective buffering of internal calcium concentration by BAPTA-AM (entry of extracellular calcium

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4 J. J. Lehman and R. W. Gross, unpublished observations.
and increases in $[Ca^{2+}]$, are each thought to be necessary for cPLA$_2$ activation (53–55); and 4) that many other enzymes thought to be necessary or associated with AA release are not inhibited by the concentrations of BEL employed. For example, enzymes that participate in signal transduction cascades that are known not to be inhibited by the concentrations of BEL employed include phosphatidylinositol-specific phospholipase C (10), phospholipase D (17), protein kinase A (56), and channels that mediate $Ca^{2+}$ release from intracellular stores (10). Of course, as with any other process, we cannot rule out the cell-specific inducible knockouts of iPLA2 and increases in $[Ca^{2+}]$-BEL.}

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Identification of Calcium-independent Phospholipase A$_2$ (iPLA$_2$) $\beta$, and Not iPLA$_2$$\gamma$, as the Mediator of Arginine Vasopressin-induced Arachidonic Acid Release in A-10 Smooth Muscle Cells: ENANTIOSELECTIVE MECHANISM-BASED DISCRIMINATION OF MAMMALIAN iPLA$_2$s

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