Bromoenol Lactone Inhibits Voltage-Gated Ca^{2+} and Transient Receptor Potential Canonical Channels^[S]

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ABSTRACT

Circulating hormones stimulate the phospholipase $\text{C}\beta$ (PLC)/ Ca^{2+} influx pathway to regulate numerous cell functions, including vascular tone. It was proposed previously that Ca^{2+} -independent phospholipase A_2 (iPLA₂)-dependent store-operated Ca^{2+} influx channels mediate hormone-induced contractions in isolated arteries, because bromoenol lactone (BEL), a potent irreversible inhibitor of iPLA₂, inhibited such contractions. However, the effects of BEL on other channels implicated in mediating hormone-induced vessel contractions, specifically voltage-gated Ca^{2+} ($\text{Ca}_v1.2$) and transient receptor potential canonical (TRPC) channels, have not been defined clearly. Using isometric tension measurements, we found that thapsigargin-induced contractions were ~34% of those evoked by phenylephrine or KCl. BEL completely inhibited not only thapsigargin- but also phenylephrine- and KCl-induced ring contractions, suggesting that $\text{Ca}_v1.2$ and receptor-operated TRPC channels also may be sensitive to BEL. Therefore,

we investigated the effects of BEL on heterologously expressed $\text{Ca}_v1.2$ and TRPC channels in human embryonic kidney cells, a model system that allows probing of individual protein function without interference from other signaling elements of native cells. We found that low micromolar concentrations of BEL inhibited $\text{Ca}_v1.2$, TRPC5, TRPC6, and heteromeric TRPC1–TRPC5 channels in an iPLA₂-independent manner. BEL also attenuated PLC activity, suggesting that the compound may inhibit TRPC channel activity in part by interfering with an initial PLC-dependent step required for TRPC channel activation. Conversely, BEL did not affect endogenous voltage-gated K^+ channels in human embryonic kidney cells. Our findings support the hypothesis that iPLA₂-dependent store-operated Ca^{2+} influx channels and iPLA₂-independent hormone-operated TRPC channels can serve as smooth muscle depolarization triggers to activate $\text{Ca}_v1.2$ channels and to regulate vascular tone.

Introduction

Circulating hormones, such as angiotensin II, histamine, endothelin, and catecholamines, regulate vascular tone. An excessive plasma concentration of these hormones has been associated with chronically elevated blood pressure (Sitter et al., 2004; Harris et al., 2008), a risk factor for stroke, kidney failure, and heart failure. In vascular smooth muscle cells

(Fig. 1A), circulating hormones activate $\text{G}_{q/11}$ protein-coupled receptors that, in turn, stimulate phospholipase $\text{C}\beta$ (PLC) activity. Activated PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol and inositol trisphosphate (IP_3). Whereas diacylglycerol stimulates protein kinase C, IP_3 acts on the IP_3 receptor in the endoplasmic reticulum, an intracellular Ca^{2+} store, and stimulates release of the stored Ca^{2+} . Upon Ca^{2+} store depletion, a plasma membrane store-operated Ca^{2+} influx (SOC) channel is activated. In addition, a debated signal downstream of the PLC pathway stimulates receptor-operated Ca^{2+} -permeable transient receptor potential canonical (TRPC) channels (Hofmann et al., 1999; Clapham, 2003; Beech, 2005; Ramsey et al., 2006). Cation influx via receptor- and store-

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ABBREVIATIONS: PLC, phospholipase $\text{C}\beta$; BEL, bromoenol lactone; $\text{Ca}_v1.2$, dihydropyridine-sensitive L-type voltage-gated Ca^{2+} channels; cPLA₂, Ca^{2+} -dependent cytosolic phospholipase A_2 ; DAG, diacylglycerol; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein; GFP-PH, the phospholipase $\text{C}\delta$ PH domain-green fluorescent protein fusion; HEK, human embryonic kidney; IP_3 , inositol trisphosphate; iPLA₂, Ca^{2+} -independent phospholipase A_2 ; PACOCF₃, palmitoyl trifluoromethyl ketone; PIP_2 , phosphatidylinositol 4,5-bisphosphate; SOC, store-operated Ca^{2+} influx; TRP, transient receptor potential; TRPC, transient receptor potential canonical; NMDG, *N*-methyl-D-glucamine; U73122, 1-[6-[[17 β -methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione.

Fig. 1. Effect of BEL on phenylephrine-, KCl-, and thapsigargin-induced contractions in rat aortic rings. **A**, schematic describing the signaling pathways under investigation (details under *Introduction*). *, chiral center in the structure of BEL. **B**, typical recording demonstrating rat aortic ring contractions induced by KCl and phenylephrine (PE). **C**, comparison of phenylephrine- (10 μ M), KCl- (60 mM), and thapsigargin-induced (1 μ M) contractions in denuded rat aortic rings. Isradipine (Isr) concentration was 10 μ M. *, significant difference between the tested groups, $p < 0.05$. **D**, a representative trace showing the effect of 25 μ M BEL on thapsigargin- and KCl-induced contractions in intact aortic rings. *, significant difference between the tested groups, $p < 0.05$. **E**, comparison of thapsigargin- and KCl-induced rat aortic ring contractions in the presence and absence of 25 μ M BEL in intact aortic rings. Inset, acute applications of BEL inhibited thapsigargin-induced contractions. **F**, dose-response curves for phenylephrine-induced contractions in the absence and presence of BEL in intact aortic rings. **G**, comparison of contractions induced by 10 μ M phenylephrine in the presence of various concentrations of BEL. The solid line represents the fit of the data to the four-parameter logistic function. The mean values are plotted in **C** and **E** to **G**. The vertical bars show S.E.M. The number of experiments is indicated in parentheses. N, tension in newtons; L, the ring length in millimeters.

operated channels depolarizes smooth muscle cells. Smooth muscle cell depolarization, in turn, activates dihydropyridine-sensitive L-type voltage-gated Ca^{2+} ($\text{Ca}_v1.2$)

channels (Catterall, 2000; Moosmang et al., 2003) that provide further Ca^{2+} entry into the cells, thus resulting in smooth muscle cell contraction.

The role of $\text{Ca}_v1.2$ channels in regulating vascular tone is widely accepted, and inhibitors of $\text{Ca}_v1.2$ channels have been used as antihypertensive drugs for decades. However, less is known about the contributions of SOC and TRPC channels to hormone-activated Ca^{2+} influx in vascular smooth muscle cells. SOC channels are formed by the Orai proteins (Orai1–Orai3) (Hogan et al., 2010). Such SOC channels are highly Ca^{2+} selective (Dietrich et al., 2010a). However, the existence of a nonselective SOC channel in vascular smooth muscle cells also has been described, suggesting some heterogeneity of vascular SOC channels (Bolotina and Csutora, 2005; Li et al., 2008). Receptor-operated TRPC channels are highly homologous to the *Drosophila* transient receptor potential (TRP) channels that play a role in phototransduction, a PLC-dependent process (Liu et al., 2007). There are seven members in the TRPC subfamily, which are subdivided further into TRPC1/4/5 and TRPC3/6/7 subgroups (Clapham, 2003; Ramsey et al., 2006) on the basis of sequence homology. Smooth muscle cells predominantly express TRPC1 and TRPC6 channels (Albert et al., 2009; Dietrich et al., 2010a), and up-regulation of TRPC6 channel expression has been implicated in the pathogenesis of some types of hypertension (Yu et al., 2004).

The lack of selective antagonists for SOC and receptor-operated TRPC channels has slowed the progress of identifying the role of these channels in the hormone-activated contractions of blood vessels. The findings that Ca^{2+} -independent phospholipase A_2 (iPLA $_2$) is activated upon store depletion and plays a key role during SOC channel activation (Fig. 1A) (Wolf and Gross, 1996; Wolf et al., 1997; Seegers et al., 2002) prompted the use of a specific inhibitor of iPLA $_2$, E-6-(bromoethylene)tetrahydro-3-(1-naphthyl)-2H-pyran-2-one (bromo-enol lactone, BEL; Fig. 1A), for defining SOC channel functions in various physiological and pathophysiological systems. BEL first was described as a selective mechanism-based inhibitor of iPLA $_2$ (Balsinde et al., 1995). BEL is a “suicide” substrate of this enzyme, because its cleavage product covalently binds to a cysteine residue within the enzyme’s catalytic pocket to irreversibly inhibit iPLA $_2$ activity. BEL exhibits a >1000-fold selectivity for iPLA $_2$ over the Ca^{2+} -dependent cytosolic PLA $_2$ (cPLA $_2$) (Hazen et al., 1991). Because complete inhibition of iPLA $_2$ was achieved only at a concentration of ~ 10 μ M (Balsinde et al., 1995), BEL is used commonly at concentrations of 3 to 25 μ M or even higher (Jörs et al., 2006; Park et al., 2008; Johnson et al., 2009; McElroy et al., 2009; Ratz et al., 2009; Wong et al., 2010).

Phenylephrine is a potent vasoconstrictor and acts on the $\text{G}_{q/11}$ protein-coupled α_1 adrenoceptor in aortic, mesenteric, cerebral, and carotid smooth muscle cells to stimulate PLC-dependent Ca^{2+} influx. It has been proposed that phenylephrine-induced Ca^{2+} influx is mediated solely by iPLA $_2$ -dependent SOC channels, because BEL pretreatment totally eliminated phenylephrine-induced contractions in mesenteric, cerebral, and carotid arteries (Park et al., 2008). Conversely, another report indicated that BEL did not affect phenylephrine contractions but inhibited acetylcholine-induced endothelium-dependent relaxation (Seegers et al., 2002). To clarify these discrepancies and establish if BEL inhibits other proteins involved in Ca^{2+} homeostasis, we

assessed the effect of BEL on phenylephrine-, thapsigargin-, and KCl-induced responses in isolated rat aortic rings and investigated the effects of BEL on $\text{Ca}_v1.2$ and TRPC channels heterologously expressed in human embryonic kidney (HEK) cells. Our data indicate that BEL inhibits $\text{Ca}_v1.2$ channels and interferes with the receptor-operated mode of TRPC channel activation by inhibiting PLC activity.

Materials and Methods

Isometric Tension Measurements. The animal protocol was approved by the Institutional Animal Care and Use Committee at the Indiana University School of Medicine. All of the experiments were carried out in accordance with the Declaration of Helsinki and/or with the *Guide for the Care and Use of Laboratory Animals* as adopted by the National Institutes of Health (Institute of Laboratory Animal Resources, 1996). Sprague-Dawley rats (3–6 weeks old) were purchased from Taconic Farms (Germantown, NY). The thoracic aorta was isolated quickly and immersed in ice-cold phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO). After the removal of the adventitia, aortic segments were cut into 3-mm rings and mounted in organ baths (Emka, Falls Church, VA) containing oxygenated Krebs buffer maintained at 37°C. Both denuded and intact rings were used during the experiments. The composition of the Krebs buffer was as follows: 131.5 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 1.2 mM NaH_2PO_4 , 1.2 mM MgCl_2 , 25 mM NaHCO_3 , and 10 mM glucose. Optimal length was determined by assessing contraction with 60 mM KCl added to the bath. Passive tension was increased in gram increments until there was <10% change in active tension developed in response to KCl. All of the drugs were added directly to the organ baths.

HEK Cell Culture and Transfections. HEK cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. Cells were split one to two times per week. For patch-clamp experiments, HEK cells were seeded on poly-L-lysine-coated glass coverslips and transfected using the Lipofectamine LTX reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's recommendations. The following cation channel clones were studied: TRPC1 (GenBank accession number NM_011643), TRPC5 (GenBank accession number NM_009428.2), TRPC6 (GenBank accession number FJ205713), and $\text{Ca}_v1.2$ (GenBank accession number NM_001136522). $\text{Ca}_v1.2$ channels were co-expressed with β_{1a} and $\alpha_2\text{-}\delta$ subunits (GenBank accession numbers: NM_001082279 and 012919, respectively).

Patch-Clamp Electrophysiology. In all of the electrophysiological experiments, the whole cell patch-clamp technique was used. Currents were recorded using an Optopatch amplifier (Cairn Research, Kent, UK) and digitized at a sampling rate of 1 kHz. The pCLAMP 10 software package was used for data analyses. The standard external solution contained: 145 mM NaCl, 2.5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, and 5.5 mM glucose (pH 7.2). The intracellular solution contained the following: 125 mM CsMeSO₃, 3.77 mM CaCl_2 , 2 mM MgCl_2 , 10 mM EGTA (yielding free $[\text{Ca}^{2+}] = 100$ nM), and 10 mM HEPES (pH 7.2). The standard intracellular solution was supplemented with 2 mM ATP and did not contain CaCl_2 for recording currents via voltage-gated Ca^{2+} channels. The NMDG⁺ solution contained: 150 mM NMDG-Cl, 10 mM HEPES, and 5.5 mM glucose (pH 7.2). The composition of the Ba^{2+} extracellular solution was as follows: 135 mM *N*-methyl-D-glucamine (NMDG)-Cl, 10 mM BaCl_2 , 10 mM HEPES, and 5.5 mM glucose (pH 7.2). We used a 100- μl perfusion chamber equipped with a manifold allowing the application of various compound-containing extracellular solutions directly into the bath. In this chamber, a full exchange of the bath solution can be achieved within ~10 to 20 s at a perfusion rate of 3 ml/min.

In $\text{Ca}_v1.2$ channel-transfected HEK cells, voltage-gated calcium channels were stimulated by 200-ms voltage ramps from –100 to

+100 mV from a holding potential of –60 mV. Ba^{2+} was used as a surrogate for Ca^{2+} to prevent Ca^{2+} -induced Ca^{2+} channel inactivation, a characteristic property of $\text{Ca}_v1.2$ channels. To determine the effect of BEL on $\text{Ca}_v1.2$ channels, we compared the peak amplitudes of Ba^{2+} currents recorded during the voltage ramps in BEL- or dimethyl sulfoxide (DMSO)-pretreated $\text{Ca}_v1.2$ channel-expressing HEK cells. The I_{Ba} density was calculated by dividing the peak Ba^{2+} current by the cell capacitance. To determine the voltage dependence of the steady-state inactivation for $\text{Ca}_v1.2$ channels, we used a standard protocol consisting of a 5-s voltage prepulse to various potentials from –80 to +30 mV with 10-mV increments, followed by a 5-ms repolarization to –80 mV, and a 50-ms test pulse to +10 mV with a 15-s interval between episodes (Fig. 4A). We fitted the data to a Boltzmann-charge-voltage function $f(V) = C + I_{\text{max}}/(1 + e^{[(V_{0.5} - V)/V_C]})$, where $V_{0.5}$ is the voltage of half-maximum inactivation) using the Levenberg-Marquardt search method and the sum of squared errors as the minimization approach (pCLAMP 10 software). All of the electrophysiological experiments were performed at room temperature (22–23°C).

Fluorescence Imaging. A monochromator-based imaging system (TILL-Photonics, Martinsreid, Germany) equipped with a DU885 charge-coupled device camera (Andor Technology plc, South Windsor, CT) was used to monitor the fluorescence of green fluorescent protein (GFP) and fura-2. The GFP fluorescence was excited at 488 nm, whereas fura-2 fluorescence was excited at 345 and 380 nm. Emitted light was collected with a 510-nm long-pass filter. Data were analyzed using TILLvisION software.

PLC Activity Assay. HEK cells were seeded in 12-well plates, grown to 60% confluence, and transiently transfected (2 μg per well) with the histamine receptor type 1 cDNA using Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Cells were incubated overnight with 5 $\mu\text{Ci/ml}$ [*myo*-³H]inositol (American Radiolabeled Chemicals, St. Louis, MO) to label the membrane inositol lipids. The cells were washed three times with the standard external solution and incubated for 30 min in the presence of 10 mM LiCl (Jenkinson et al., 1994) to block inositol monophosphate hydrolysis, with or without the indicated concentrations of BEL or 25 μM 1-[6-[[17 β -methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (U73122), a well established inhibitor of PLC (Calbiochem, San Diego, CA). The cells then were challenged with 40 μM histamine for 30 min, and the reaction was terminated by buffer aspiration and addition of ice-cold trichloroacetic acid (0.5 M). Water-soluble [³H]inositol-containing components then were extracted by addition of tri-*n*-octylamine/1,1,2-trichlorofluoroethane (1:1 ratio), and [³H]inositol phosphates were separated by ion exchange chromatography (Berridge et al., 1982) using Dowex resin (Sigma-Aldrich) in the formate form. Glycerophospholipids were removed by elution with 40 ml of 0.1 M ammonium formate/0.1 M formic acid. The inositol phosphate species then were eluted with 10 ml of 1.2 M ammonium formate/0.1 M formic acid. Ultima-Flo (PerkinElmer Life and Analytical Sciences, Waltham, MA) was added to the eluate, and ³H content was determined using liquid scintillation counting. Data are expressed as the percentage of histamine response in the absence of inhibitors after the subtraction of basal counts.

BEL Pretreatment. Before BEL (a racemic mixture; Sigma-Aldrich) pretreatment, rat aortic rings or HEK cells were washed three times with the standard extracellular/bath solution to remove any residues of the serum. BEL was dissolved in DMSO. The stock solution of BEL was stored in small aliquots at –80°C. A fresh aliquot was used for each experiment. BEL was diluted in an appropriate volume of the standard extracellular/bath solution just before each treatment. For vehicle control experiments, the same volume of pure DMSO was added to the standard extracellular solutions. All of the pretreatments were carried out with 100 nM to 100 μM BEL at 37°C for 30 min in the dark. After BEL pretreatment, aortic rings or HEK cells were washed three times with the standard extracellular/bath solution and used immediately for isometric tension or patch-

clamp experiments. In some experiments, BEL was added acutely to the bath during a recording.

IC₅₀ Values. To determine the IC₅₀ values, the normalized data were fitted to a four-parameter logistic function: $F(x) = \min + (\max - \min)/(1 + 10^{(\log IC_{50} - x) \times \text{Hill slope}})$, where min indicates the minimum of $F(x)$, max indicates the maximum of $F(x)$, IC₅₀ indicates median inhibitory concentration, and Hill slope characterizes the slope of the curve at its midpoint.

Statistical Analysis. Data sets were compared using the Student's *t* test followed by the Mann-Whitney rank sum test or one-way analysis of variance followed by Dunn's post hoc test. The significance level was set to 0.05. Data were expressed as mean $\pm$ S.E.M.

Results

BEL Inhibits Thapsigargin-, Phenylephrine-, and KCl-Induced Contractions. Using isometric tension measurements, we determined that KCl and phenylephrine stimulated strong contractions (1.9 ± 0.4 and 2.1 ± 0.5 g, respectively; Fig. 1, B and C) in intact and denuded rat aortic rings. Isradipine (10 μ M; Sigma-Aldrich), a dihydropyridine derivative that potently inhibits voltage-gated Ca_v1.2 channels, significantly decreased phenylephrine-induced contractions by 67% (Fig. 1C), indicating that voltage-gated Ca²⁺ channels mediate a significant portion of Ca²⁺ entry necessary for generating aortic ring contractions. After rat aortic rings were incubated with 25 to 30 μ M BEL for 30 min, KCl- and phenylephrine-induced contractions were decreased drastically (0.1 ± 0.1 and 0.03 ± 0.01 g, respectively; Fig. 1, D–F). Figure 1F shows the dose-response curves for phenylephrine-induced contractions in rat aortic rings pretreated with various concentrations of BEL. BEL (25 μ M) markedly and irreversibly decreased phenylephrine-induced contractions at all of the tested phenylephrine concentrations. To quantify the effects of various BEL concentrations on 10 μ M phenylephrine-induced contractions, the data were fitted to the four-parameter logistic function (under *Materials and Methods*). The fit yielded an IC₅₀ value of 7.0 ± 0.1 μ M with a Hill slope of -1.4 ± 0.1 (Fig. 1G).

Thapsigargin, an irreversible inhibitor of the sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase pump that causes Ca²⁺ store depletion and vascular SOC channel activation, also evoked contractions of rat aortic rings that were 34% of those observed with phenylephrine in denuded rings (0.6 ± 0.6 g; Fig. 1C). Notably, thapsigargin-induced contractions developed more slowly (Fig. 1E, inset) than phenylephrine- or KCl-induced contractions. In agreement with previous reports (Park et al., 2008), BEL completely and irreversibly blocked thapsigargin-induced contractions (Fig. 1, D and E).

BEL Inhibits Ca_v1.2 Channels. Thus, we found that the isradipine-sensitive voltage-gated Ca²⁺ channels mediated a significant portion of phenylephrine-induced contractions (see above and Fig. 1C). KCl depolarizes smooth muscle cells in the vascular wall and stimulates voltage-gated Ca²⁺ channels in a hormone-independent manner. KCl-induced (60 mM) ring contractions were potently inhibited in BEL-pretreated rings (Fig. 1E). We determined that BEL inhibited KCl-induced contractions with an IC₅₀ value of 16.3 ± 1.3 μ M and a Hill slope of -1.7 ± 0.2 (Fig. 2). Because the removal of BEL from the bath did not restore the KCl-induced contractions in BEL-treated rat aortic rings, we concluded that BEL irreversibly inhibited KCl-induced contractions (Fig. 2C). In the presence of 30 μ M BEL, the residual

Fig. 2. Effect of BEL on KCl-induced rat aortic ring contractions. A, a control experiment with rings treated with DMSO. KCl (60 mM) stimulated reproducible ring contractions. B, BEL pretreatment markedly inhibits KCl-induced contractions. A representative recording is shown. Inset, the percentage of inhibition of KCl-induced contractions in rings pretreated with various concentrations of BEL. C, the removal of BEL from the recording bath does not restore the ability of rings to contract upon 60 mM KCl stimulation. N, tension in newtons; L, the ring length in millimeters.

KCl-induced contractions were not sustained, as was seen in control rings, but decayed slowly to the baseline (Fig. 2C).

We then hypothesized that dihydropyridine-sensitive, voltage-gated Ca²⁺ channels may be a target of BEL. Vascular smooth muscle cells express Ca_v1.2 channels (under *Introduction*). To investigate whether BEL affects Ca_v1.2 channels, we heterologously coexpressed the Ca_v1.2 (α_{1c-b}) channel together with β_{1a} and $\alpha_2\delta$ auxiliary subunits in HEK cells and investigated the channel's sensitivity to BEL pretreatments. In these experiments, we used Ba²⁺ instead of Ca²⁺ in the extracellular solution to reduce the Ca²⁺-dependent inactivation of Ca_v1.2/ β_{1a} / $\alpha_2\delta$ channels (the Ba²⁺ extracellular solution, under *Materials and Methods*). Voltage-gated Ba²⁺ currents were recorded during a single 200-ms voltage ramp recorded 2 min after establishing the whole-cell configuration. No voltage-activated Ba²⁺ currents were observed in nontransfected HEK cells (Fig. 3A). Conversely, the Ca_v1.2/ β_{1a} / $\alpha_2\delta$ channel-transfected cells exhibited large voltage-gated Ba²⁺ currents with a peak current density of -34.4 ± 4.6 pA/pF activated during voltage ramps from -100 to $+100$ mV in DMSO (vehicle)-pretreated cells (Fig. 3). The current-voltage relationships of the Ba²⁺ currents

Fig. 3. Effect of BEL on heterologously expressed $\text{Ca}_v1.2$ channels in HEK cells. **A**, averaged leak-subtracted current-voltage relationships obtained in $\text{Ca}_v1.2$ channel-expressing (DMSO- or 25 μM BEL-pretreated) or untransfected (control) HEK cells. The gray areas indicate the S.E.M. values. **B**, $\text{Ca}_v1.2$ channel current densities recorded in transfected HEK cells pretreated with various concentrations of BEL (open circles). The solid line represents the fit of the data to the four-parameter logistic function. The holding potential was -60 mV. The peak current values were measured during voltage ramps from -100 to $+100$ mV. The extracellular solution contained 10 mM BaCl_2 . The inset shows a comparison of Ba^{2+} current densities in transfected HEK cells pretreated with DMSO or 25 μM BEL *, significant difference between the DMSO and BEL-treated groups, $p < 0.05$.

reached a maximum amplitude at 6.1 ± 2.0 mV (Fig. 3A). BEL (1 μM) pretreatment reduced the Ba^{2+} currents through $\text{Ca}_v1.2/\beta_{1a}/\alpha_2\text{-}\delta$ channels (-26.0 ± 4.6 pA/pF), and 25 μM BEL pretreatment almost completely eliminated the Ba^{2+} currents through $\text{Ca}_v1.2/\beta_{1a}/\alpha_2\text{-}\delta$ channels (-4.3 ± 1.2 pA/pF; Fig. 3, A and B). In the $\text{Ca}_v1.2/\beta_{1a}/\alpha_2\text{-}\delta$ channel-expressing HEK cells pretreated with 25 μM BEL, the current-voltage relationships of the Ba^{2+} currents reached a maximum amplitude at 15.8 ± 2.2 mV (Fig. 3A). A fit of the BEL dose-inhibition curve to the four-parameter logistic function yielded an IC_{50} value of 7.6 ± 0.3 μM with a Hill slope of -1.4 ± 0.1 (Fig. 3B).

We next investigated whether BEL affected the voltage dependence of the steady-state inactivation of $\text{Ca}_v1.2/\beta_{1a}/\alpha_2\text{-}\delta$ channels. Figure 4 shows the Ba^{2+} currents in DMSO- or 1 μM BEL-pretreated cells recorded using the standard protocol to assess the steady-state inactivation. Fits of the Ba^{2+} current decay data to the standard two-exponential function yielded the following time constants: 1) the τ_{fast} constants

Fig. 4. Effect of BEL on the voltage dependence of the steady-state inactivation of $\text{Ca}_v1.2$ channels. **A**, representative traces and the steady-state inactivation protocol used in these experiments. The traces were recorded in cells pretreated either with DMSO or 1 μM BEL. The scaled Ba^{2+} current from the BEL panel is shown in the DMSO panel (gray trace) to demonstrate the faster decay rate of Ba^{2+} currents in BEL-pretreated cells. **B**, comparison of the steady-state inactivation curves obtained in either DMSO- or 1 μM BEL-pretreated $\text{Ca}_v1.2$ channel-transfected HEK cells. The solid line represents the fit of the data to the Boltzmann charge-voltage function.

were 237.5 ± 48.5 ms (DMSO) and 88.4 ± 4.6 ms (BEL); and 2) the τ_{slow} constants were 1428.4 ± 314.4 ms (DMSO) and 406.0 ± 29.2 ms (BEL). Thus, the BEL pretreatment significantly accelerated both the fast and the slow components of the Ba^{2+} currents (Fig. 4A). The fact that the Ba^{2+} currents decayed much faster in the BEL-pretreated cells is consistent with our finding that BEL pretreatment facilitated the rate of ring relaxation in KCl-precontracted aortic rings (Fig. 2, B and C). The steady-state inactivation curves were fitted to the Boltzmann charge-voltage function as described under *Materials and Methods*. The fits yielded the following values for the voltages of half-maximum inactivation: -21.5 ± 0.6 mV in DMSO-pretreated $\text{Ca}_v1.2/\beta_{1a}/\alpha_2\text{-}\delta$ channel-expressing cells ($n = 4$) and -39.2 ± 1.6 mV in BEL-pretreated $\text{Ca}_v1.2/\beta_{1a}/\alpha_2\text{-}\delta$ channel-expressing cells ($n = 3$; Fig. 4B).

BEL Inhibits TRPC Channels. Smooth muscle cells also express TRPC1, TRPC5, and TRPC6 channels (Albert et al., 2009; Edwards et al., 2010), which have been implicated in

mediating receptor-operated contractions of blood vessels. Because BEL inhibits hormone-induced vessel contractions, we hypothesized that the drug also may block hormone-activated TRPC channels. To test this hypothesis, we expressed TRPC channels in HEK cells and assessed the ability of BEL to modulate the channel activity. No histamine-operated currents were observed in untransfected HEK cells (Supplemental Fig. 1) (Schaefer et al., 2000; Obukhov and Nowycky, 2004, 2008). In HEK cells expressing TRPC6 channel and the H1 histamine receptor (Fig. 5A), histamine activated robust inward currents exhibiting a mean current density of -24.8 ± 17.2 pA/pF with an S-shaped current-voltage relationship, a feature of TRPC6 channels (Hofmann et al., 1999). These currents were abolished in the solution containing bulky NMDG⁺ as the only cation, indicating that they are carried by small cations (Fig. 5A). Pretreatment with BEL at 25 and 100 μ M concentrations dose-dependently inhibited histamine-elicited currents in TRPC6 channel-expressing HEK cells (-4.5 ± 8.7 and -0.5 ± 0.6 pA/pF, respectively) measured at a holding potential of -60 mV

Fig. 5. Effect of BEL on TRPC6 channel currents in HEK cells expressing TRPC6 channels and the H1 histamine receptor. A, representative traces of histamine-activated currents recorded in the absence or presence of 25 and 100 μ M BEL. The holding potential was -60 mV. The insets on the right show current-voltage relationships obtained during voltage ramps from -100 to $+100$ mV in each experiment. B, comparison of the mean current densities of histamine-activated and spontaneous currents in BEL- or DMSO-pretreated TRPC6 channel-expressing HEK cells. Inset, TRPC6 channel current densities recorded in TRPC6 channel-expressing cells pretreated with various concentrations of BEL. The solid line represents the fit of the data to the four-parameter logistic function *, significant difference between the DMSO (open bar) and BEL-treated groups, $p < 0.05$. SA, spontaneous activity.

(Fig. 5). A fit of the dose-inhibition curve to the four-parameter logistic function yielded an IC₅₀ value of 8.1 ± 1.6 μ M with a Hill slope of -1.6 ± 0.6 (Fig. 5B, inset). Transiently expressed TRPC6 channels exhibited slight spontaneous activity (Fig. 5A, indicated as SA), which was noted as small currents with S-shaped TRPC6 channel-like current-voltage relationships in the absence of histamine. Interestingly, spontaneous activity of TRPC6 channels (-1.7 ± 1.9 pA/pF) was not affected significantly by BEL pretreatments at either 25 or 100 μ M concentrations (Fig. 5B). This suggests that an upstream molecule involved in TRPC6 channel activation rather than the channel itself most likely was inhibited by BEL pretreatment.

We also investigated whether homomeric TRPC5 and heteromeric TRPC1–TRPC5 channels (TRPC1 and TRPC5 channel cDNA were a gift from Dr. Michael Schaefer, Rudolf-Boehm-Institut für Pharmakologie und Toxikologie, Leipzig, Germany) are sensitive to BEL pretreatment. These channels also were found in some vascular smooth muscle cells (Albert et al., 2009). The current density of histamine-activated TRPC5 channel currents was much larger than that of TRPC1–TRPC5 channel currents (-80.7 ± 45.9 and -12.8 ± 7.1 pA/pF, respectively). TRPC5 channel currents exhibited

Fig. 6. Effect of BEL on TRPC5 channel currents in HEK cells expressing TRPC5 channels and the H1 histamine receptor. A, representative traces of histamine-activated currents recorded in the absence or presence of 100 μ M BEL. The holding potential was -60 mV. The inset shows the current-voltage relationship obtained during a voltage ramp from -100 to $+100$ mV. B, comparison of BEL effects on histamine-activated and spontaneous currents in TRPC5 channel-expressing HEK cells. Inset, normalized TRPC5 channel current densities recorded in TRPC5 channel-expressing HEK cells pretreated for 30 min with various concentrations of BEL. The solid line represents the fit of the data to the four-parameter logistic function *, significant difference between the DMSO (open bar) and BEL-treated groups, $p < 0.05$. SA, spontaneous activity.

Fig. 7. Effect of BEL on heteromeric TRPC1-TRPC5 channel currents in HEK cells expressing TRPC1 and TRPC5 channels and the H1 histamine receptor. **A**, representative traces of histamine-activated currents recorded in the absence or presence of 100 μM BEL. The holding potential was -60 mV. The inset shows the TRPC1-TRPC5 channel current-voltage relationship obtained during a voltage ramp from -100 to $+100$ mV. **B**, summary of BEL effects on histamine-activated and spontaneous currents in TRPC1-TRPC5 channel-expressing HEK cells. Inset, normalized heteromeric TRPC1-TRPC5 channel current densities recorded in TRPC1-TRPC5 channel-expressing cells pretreated with various concentrations of BEL. The solid line represents the fit of the data to the four-parameter logistic function * , significant difference between the DMSO (open bar) and BEL-treated groups, $p < 0.05$. SA, spontaneous activity.

S-shaped current-voltage relationships, whereas TRPC1-TRPC5 channel current-voltage relationships were outwardly rectifying. Both TRPC5 and TRPC1-TRPC5 channel currents also were abolished in the solution containing NMDG^+ as the only cation, indicating that the currents are carried by small cations (Figs. 6 and 7). BEL (25–100 μM) pretreatments significantly inhibited histamine-activated TRPC5 and TRPC1-TRPC5 channel currents as summarized in Figs. 6B and 7B. Fits of the dose-inhibition curves to the four-parameter logistic function yielded IC_{50} values of 10.6 ± 0.5 and 7.2 ± 0.7 μM with Hill slopes of -2.4 ± 0.2 and -2.2 ± 0.4 for TRPC5 and TRPC6 channels, respectively (Figs. 6B and 7B, insets). The Hill coefficient value of ~ 2 suggests that there may be some cooperativity of BEL effects. As with TRPC6 channels, the spontaneous activity of TRPC5 channels was not affected by 25 μM BEL, yet, unlike TRPC6 channels, a higher concentration of BEL (100 μM) slightly potentiated the spontaneous activity of TRPC5 channels (Fig. 6B). Spontaneous currents through the TRPC1-TRPC5 heteromeric channel were inhibited significantly by 100 μM BEL (Fig. 7B).

Potent inhibition of TRPC channel currents was observed after a 30-min preincubation with BEL. We also examined whether an acute application of 100 μM BEL affects TRPC channel currents (Fig. 8). We found that acutely applied 100 μM BEL facilitated the rate of natural decay of the histamine-activated TRPC5 and TRPC6 channel currents. TRPC channel currents decayed to the steady-state level within 1 to 2 min in the presence of BEL. To determine the rate of solution exchange in our perfusion chamber, we determined how fast removing the current carriers from the bath, by substitution of the NMDG^+ solution for the standard extracellular solution, would eliminate the histamine-activated inward currents in TRPC channel-expressing HEK cells. We found that the histamine-activated inward currents were eliminated completely in NMDG^+ solution in much less time (~ 10 – 20 s; Figs. 5A, 6A, and 8) than we observed during the acute application of 100 μM BEL (Fig. 8). This suggests that BEL may inhibit TRPC channel activity within a 5-min interval.

BEL Inhibits TRPC Channels in an iPLA_2 -Independent Manner. Because BEL is reported to be a specific iPLA_2 inhibitor, we reasoned that PLA_2 may be required for TRPC channel activation. This hypothesis was supported by the fact that lysophospholipids, the products of PLA_2 , were reported to stimulate some TRPC channels under certain conditions (Fig. 1) (Beech et al., 2009). Therefore, we next

Fig. 8. Effect of the acute application of BEL on TRPC channel currents in HEK cells expressing TRPC channels and the H1 histamine receptor. **A**, representative trace of histamine-activated currents in a HEK cell expressing TRPC6 channels is shown. Histamine (His; 40 μM) was added at the times indicated by the upper black horizontal bar. BEL (100 μM) was added to the bath at the times indicated by the gray horizontal bar and gray circles within the trace, whereas the NMDG^+ solution, containing no small cations, was added at the times indicated by the lower black horizontal bar and open circles within the trace. The holding potential was -60 mV. **B**, comparison of the current decay rates observed in the presence of either NMDG^+ (see Figs. 3A and 4A) or 100 μM BEL * , significant difference between the NMDG^+ and BEL-treated groups, $p < 0.05$.

investigated whether other $iPLA_2$ inhibitors affect TRPC channel activity. We used a potent, broad-spectrum $cPLA_2$ and $iPLA_2$ inhibitor, palmitoyl trifluoromethyl ketone (PACOCF₃). To replicate the BEL protocol, we preincubated the TRPC channel-expressing cells with 100 μ M PACOCF₃ for 30 min at 37°C before obtaining whole-cell patch-clamp recordings and then applied histamine in the presence of 100 μ M PACOCF₃. In control experiments, we preincubated the TRPC channel-expressing cells with DMSO (vehicle) for 30 min at 37°C, and then the TRPC channel currents were stimulated with histamine in the presence of DMSO. Figure 9 shows that the amplitudes of histamine-activated TRPC channel currents recorded in the presence or absence of 100 μ M PACOCF₃ were not significantly different.

BEL Does Not Inhibit Voltage-Gated Potassium K_V Channels in HEK Cells. Previous studies demonstrated that BEL inhibits $iPLA_2$ -dependent SOC channels (Park et al., 2008). Data from the present investigation clearly show that BEL also blocks voltage-gated Ca^{2+} channels and TRPC channels. Thus, it is possible that BEL is a broad-spectrum cation channel inhibitor. To test this hypothesis, we investigated whether BEL inhibits activation of potassium K_V channels in HEK cells. We found that 100 μ M BEL did not significantly alter the amplitude of endogenous K_V channels in HEK cells (Fig. 10). The lack of BEL effects on potassium channels suggests that BEL may be specific for Ca^{2+} -permeable channels such as SOC, $Ca_v1.2$, and TRPC channels.

BEL Inhibits PLC Activity. Spontaneous TRPC5 and TRPC6 channel currents were less sensitive to BEL than receptor-operated TRPC5 and TRPC6 channel currents, suggesting that a molecular element upstream of TRPC channel activation may be a target of BEL. Our finding that PACOCF₃ does not affect histamine-activated TRPC channel currents suggests that $iPLA_2$ is not required for the receptor-operated mode of TRPC channel activation under conditions that prevent store depletion. Another element that is activated upstream of TRPC channels in the presence of circulating hormones is PLC (Clapham, 2003; Ramsey et al., 2006). Importantly, PLC belongs to the same superfamily of phospholipases as $iPLA_2$, a known target of BEL. Therefore, we reasoned that BEL also may inhibit PLC activity. To investigate the effect of BEL on PLC activity, we used a combination of fluorescence imaging techniques that allowed for the recording of PLC-dependent increases in intracellular Ca^{2+} levels and simultaneous tracking of histamine-activated translocation of a molecular PIP_2/IP_3 probe. The ratio-metric Ca^{2+} imaging approach was used due to changes in HEK cell morphology in the presence of histamine. To track PIP_2 turnover in live cells, we used the phospholipase C δ PH domain-GFP fusion (GFP-PH) developed by Várnai and Balla (1998). The schematics in Fig. 11, A and B, show that GFP-PH binds PIP_2 located in the plasma membrane of unstimulated HEK cells. Upon cleavage of PIP_2 by PLC, GFP-PH translocates to the cytosol together with IP_3 , thus enabling the visualization of the PLC activity by tracking the distribution of GFP-PH fluorescence in live cells. We transfected HEK cells with the H1 histamine receptor and a 10 times lower amount of GFP-PH cDNA (a gift from Dr. Tamas Balla, National Institute of Child Health and Human Development, Bethesda, MD) and investigated whether BEL pretreatment alters the ability of GFP-PH to translocate to the cytosol in response to H1 histamine receptor stimulation.

Fig. 9. A broad-spectrum PLA_2 inhibitor, PACOCF₃ (100 μ M), does not affect TRPC channel currents. A, histamine-activated currents in TRPC channel-expressing HEK cells. Histamine, NMDG⁺, and PACOCF₃ were added at the times indicated by the horizontal bars. Representative traces are shown. The insets show corresponding current-voltage relationships and the structure of PACOCF₃. B, comparison of averaged peak currents recorded in the presence or absence of PACOCF₃ for each TRPC channel is shown.

Figure 11, C and E, and Supplemental Fig. 2 show that histamine application induced robust translocation of GFP-PH to the cytosol in control HEK cells. Pretreatments with 25 μ M BEL significantly inhibited GFP-PH translocation (Fig. 11G). Histamine-activated Ca^{2+} release from the intracellular Ca^{2+} store was not decreased significantly in this case (Fig. 11F). These data suggest that reduced IP_3 production is still able to trigger IP_3 -dependent Ca^{2+} release from intracellular stores (Fig. 11F). We speculate that this

Fig. 10. Voltage-gated K⁺ channels are not affected by BEL in HEK cells. A, representative current-voltage relationships of voltage-gated K⁺ channels are shown. The K⁺ currents were acquired during 200-ms voltage ramps in untransfected HEK cells pretreated with 100 μ M BEL (gray) or DMSO (black). B, comparison of mean current densities measured at +100 mV of endogenous voltage-gated K⁺ currents activated with voltage ramps from -100 to +100 mV in HEK cells pretreated with 100 μ M BEL or DMSO.

increase in Ca²⁺ release is likely subsequently amplified by Ca²⁺-induced Ca²⁺ release. Figure 11, H and I, show that 100 μ M BEL almost completely abolished both hormone-induced Ca²⁺ release and GFP-PH translocation. HEK cells pretreated with 100 μ M BEL exhibited slightly elevated levels of intracellular Ca²⁺. This is consistent with our electrophysiological data indicating that HEK cells pretreated with 100 μ M BEL had increased leak currents. The GFP-PH translocation data were used only for qualitative assessments of PLC-induced PIP₂ hydrolysis, and the exact IC₅₀ values for BEL were not calculated due to changes in cell morphology that resulted in brighter GFP fluorescence in images acquired during histamine application compared with that in control images.

Our fluorescence imaging experiments suggested that micromolar concentrations of BEL inhibit PLC activity. To exclude the possibility that BEL interferes with GFP-PH binding to PIP₂ and to obtain a quantitative assessment of BEL effects on PLC activity, we used a direct assay of receptor-stimulated inositol phosphate generation. This biochemical assay of PLC activity measures the release of [³H]inositol phosphates into the cytosol of prelabeled HEK cells. Our data show that BEL inhibited histamine-induced inositol phosphate production in a concentration-dependent manner (Fig. 12). Data fit to the four-parameter logistic function yielded an IC₅₀ value of 364.0 ± 34.2 nM and a Hill slope of -0.8 ± 1.2 . Inhibition was partial, with maximum inhibition of 35% with 100 μ M BEL. Partial inhibition (45%) also was observed with the classic PLC inhibitor U73122 in this assay. Thus, full inhibition of histamine-induced inositol phosphate formation was not achieved in this cell system. This may reflect the large receptor reserve due to the overexpressed histamine receptor (under *Discussion*). Taken together, these data demonstrate the ability of BEL to significantly inhibit PLC activity.

Discussion

Our major finding is that BEL potently inhibits the recombinant voltage-gated Ca_v1.2/ β_{1a} / α_{2-8} channel and three TRPC channels (TRPC5, TRPC6, and TRPC1-TRPC3) but not voltage-gated potassium channels endogenously expressed in HEK cells. BEL inhibited TRPC channel activity in an iPLA₂-independent manner. We were surprised to find that BEL inhibited only receptor-operated activity of TRPC channels but not the spontaneous activity of the channels.

This suggests that the compound acts on a molecular target upstream of TRPC channels. While unraveling the mechanism of this effect, we found that BEL potently inhibits PLC (IC₅₀ value of 384 nM), which is required for receptor-operated TRPC channel activation (Clapham, 2003; Ramsey et al., 2006).

Ca²⁺ influx is crucial for vessel constriction. Compelling evidence indicates that, in vascular smooth muscle cells, store depletion induces iPLA₂ activity. This leads to the stimulation of store-operated Ca²⁺-permeable channels (Wolf and Gross, 1996; Wolf et al., 1997; Seegers et al., 2002; Smani et al., 2004; Bolotina and Csutora, 2005). Because BEL, a selective inhibitor of the cytosolic β -isoform of iPLA₂ (Jenkins et al., 2003), totally inhibited store-operated channel activation, it has emerged as a vital tool for revealing the role of SOC channels during contractile responses in various vascular beds. BEL totally inhibited phenylephrine-induced constrictions in cerebral and mesenteric arteries. Therefore, it has been proposed that the iPLA₂-dependent activation of the SOC channel is the major pathway mediating phenylephrine-induced Ca²⁺ influx in these arteries (Park et al., 2008). However, the precise role of iPLA₂ in mediating vessel contractions is still debated, because isolated pressurized mesenteric arteries from iPLA₂(-/-) knockout mice exhibited increased contractions to phenylephrine compared with the mesentery from the wild-type mice (Dietrich et al., 2010b). Conversely, it was found that endothelial SOC channel-dependent acetylcholine-induced dilation was reduced in the iPLA₂ knockout model. Notably, the fact that iPLA₂ is predominantly expressed in endothelial cells but not in smooth muscle cells (Wong et al., 2010) is consistent with the observation that the genetic ablation of iPLA₂ does not inhibit phenylephrine-induced vessel contractions.

Our data also indicate that BEL inhibits SOC channels in rat aortic rings. However, we found that BEL also inhibits other receptor-operated channels that can mediate receptor-operated Ca²⁺ influx in aortic rings. In addition, submicromolar concentrations of BEL inhibited PLC activity (Fig. 12). Because both TRPC and SOC channels are PLC dependent, PLC inhibition may effectively prevent TRPC and SOC channel activation in intact vessels. However, we found that BEL inhibits TRPC channels with an IC₅₀ value that is ~ 17 times higher than that for PLC. One potential explanation for this, which also can explain the lower sensitivity of histamine-induced Ca²⁺ signals (Fig. 11, D-I), is that there is a large histamine receptor reserve in these cells. This would make it

Fig. 11. Effects of BEL on histamine-activated translocation of the GFP-PH. A, the schematic shows the GFP-tagged PH domain of phospholipase C δ (GFP is shown as a green circle) bound to PIP₂ located in the plasma membrane of unstimulated HEK cells. B, the schematic shows that, upon cleavage of PIP₂ by histamine-stimulated PLC, GFP-PH translocates to the cytosol together with IP₃, thus enabling the visualization of PLC activity by tracking the fluorescence of GFP-PH in live cells. The filled blue rectangle represents the closed IP₃-receptor (A), whereas the open rectangle (B) depicts the open IP₃-receptor. C, representative images demonstrating GFP-PH translocation induced by histamine where 1 is the control, 2 is recorded at the peak of the histamine response, and 3 is recorded during the decaying phase of the histamine response. Histamine application resulted in visible changes in HEK cell morphology. This caused the GFP epifluorescence signal to appear brighter in the presence of histamine than that in the control. Therefore, these data were used only as a qualitative assessment of PLC activity. The regions of interest used for image analysis are indicated as yellow squares in the cytosolic part of the cell. D–I, intracellular Ca²⁺ level increases measured using fura-2 and GFP-PH translocation measured by imaging the GFP fluorescence in control (D and E), 25 μM BEL (F and G), and 100 μM BEL (H and I), respectively.

necessary to inhibit a very large proportion of PLC before there is a significant effect on the downstream signaling steps. Another potential consideration is that TRPC channel

Fig. 12. The receptor-stimulated inositol phosphate turnover assay. The PLC assay was used to assess the effect of BEL on PLC activity. The solid line represents the fit of the data points to the four-parameter logistic function. The data points represent the mean percentage of inhibition of 40 μM histamine-activated PLC activity by various concentrations of BEL. The vertical bars show S.E.M. The triangle shows the mean percentage of inhibition of histamine-induced PLC activity in the presence of 25 μM U73122, a common inhibitor of PLC.

activation involves integration of multiple signaling pathways (Schaefer et al., 2000), some of which may be less sensitive to BEL. The discrepancy in IC₅₀ values between assays therefore may arise from differences in BEL efficacies for individual PLC subtypes, signaling pathways, and the histamine receptor reserve.

The mechanism by which BEL inhibits PLC is not yet clear. It is possible that BEL may be a weak substrate of PLC, as is the case for iPLA₂ (Song et al., 2006). Therefore, BEL may be cleaved in the PLC catalytic center. In such a case, a product of BEL cleavage may bind covalently to a residue within the catalytic center of PLC, thus inactivating the enzyme. Further studies will be required to test this hypothesis.

Our data indicate that BEL also inhibited KCl-induced contractions in the rat aorta. This finding contradicts the results of Park et al. (2008) and Guo et al. (2003), who found that BEL does not inhibit KCl-induced Ca²⁺ level increases and contractions of cerebral and mesenteric arteries. It is not clear why KCl-induced contractions of the rat aorta were more sensitive to BEL than cerebral and mesenteric arteries. However, our data on BEL inhibition of KCl responses in isometric tension aortic ring experiments (Figs. 1 and 2) are consistent with the fact that BEL potently inhibited heterologously expressed Ca_v1.2/β_{1a}/α₂-δ channels in HEK cells (Fig. 3). Ca_v1.2 channels are implicated in mediating Ca²⁺ influx in vascular smooth muscle cells. BEL pretreatment accelerated both fast and slow components of the Ca_v1.2 channel current decay. In addition, BEL pretreatment caused an 18-mV leftward shift of the steady-state inactivation curve compared with that of control cells (Fig. 4B), suggesting that the compound may decrease the steady-state availability of Ca_v1.2/β_{1a}/α₂-δ channels for activation at physiological potentials. At this time, it remains unclear whether BEL directly acts on the pore-forming Ca_v1.2 channel subunit or one of the Ca_v1.2 channel auxiliary subunits (β or α₂-δ).

Our findings that BEL inhibits heterologously expressed TRPC channels are consistent with the results of Jörs et al. (2006), who found that 25 μM BEL inhibited *Drosophila* TRPγ heterologously expressed in HEK cells. TRPγ is highly

homologous to mammalian TRPC channels and is activated by a signal downstream of PLC. However, Jörs et al. (2006) attributed the effect of BEL on $\text{TRP}\gamma$ to the inhibition of iPLA_2 . In our experiments, we found that $100\ \mu\text{M}$ PACOCF₃, a potent, broad-spectrum cPLA_2 and iPLA_2 inhibitor, did not affect histamine-activated currents through TRPC5, TRPC6, and TRPC1–TRPC5 channels heterologously expressed in HEK cells (Fig. 7), suggesting that it is highly unlikely that PLA_2 is required for TRPC channel activation. Data in this study showing that BEL inhibits PLC and TRPC channel activation argue against the proposal that SOC channels are the only Ca^{2+} influx channel involved in hormone-activated contractions in blood vessels. In fact, it is most likely that TRPC channels also contribute to hormone-induced vessel contractions.

BEL possesses a chiral center (see the BEL structure shown in Fig. 1A) and may exist as two enantiomeric isoforms, namely, *S*-BEL or *R*-BEL. We used a racemic mixture of BEL during the above-described experiments. However, BEL enantiomers may exhibit different potencies for their newly identified target channels. Future studies will be needed to determine whether individual BEL enantiomers can preferentially inhibit $\text{Ca}_v1.2$ channels, TRPC channels, or PLC activity.

In conclusion, we demonstrated that BEL potently inhibits two types of Ca^{2+} -permeable cation channels, namely, $\text{Ca}_v1.2$ and TRPC channels, in an iPLA_2 -independent manner. The inhibitory effect of BEL on TRPC channels most likely is achieved by inhibition of PLC activity. We propose that, besides the iPLA_2 -dependent SOC channels, several iPLA_2 -independent hormone-operated TRPC channels also can serve as hormone-operated smooth muscle depolarization triggers to activate $\text{Ca}_v1.2$ channels and to regulate vascular tone.

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Authorship Contributions

Participated in research design: Thomas, Sturek, Tune, and Obukhov.

Conducted experiments: Chakraborty, Berwick, and Bartlett.

Contributed new reagents or analytic tools: Kumar.

Performed data analysis: Chakraborty, Berwick, and Bartlett.

Wrote or contributed to the writing of the manuscript: Thomas, Sturek, Tune, and Obukhov.

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