Selective, Direct Activation of High-Conductance, Calcium-Activated Potassium Channels Causes Smooth Muscle Relaxation

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ABSTRACT
High-conductance calcium-activated potassium (Maxi-K) channels are present in smooth muscle where they regulate tone. Activation of Maxi-K channels causes smooth muscle hyperpolarization and shortening of action-potential duration, which would limit calcium entry through voltage-dependent calcium channels leading to relaxation. Although Maxi-K channels appear to indirectly mediate the relaxant effects of a number of agents, activators that bind directly to the channel with appropriate potency and pharmacological properties useful for proof-of-concept studies are not available. Most agents identified to date display significant polypharmacy that severely compromises interpretation of experimental data. In the present study, a high-throughput, functional, cell-based assay for identifying Maxi-K channel agonists was established and used to screen a large sample collection (>1.6 million compounds). On the basis of potency and selectivity, a family of tetrahydroquinolines was further characterized. Medicinal chemistry efforts afforded identification of compound X, from which its two enantiomers, Y and Z, were resolved. In in vitro assays, Z is more potent than Y as a channel activator. The same profile is observed in tissues where the ability of either agent to relax precontracted smooth muscles, via a potassium channel-dependent mechanism, is demonstrated. These data, taken together, suggest that direct activation of Maxi-K channels represents a mechanism to be explored for the potential treatment of a number of diseases associated with smooth muscle hyperexcitability.

Introduction
High-conductance, calcium-activated potassium (Maxi-K) channels are widely distributed throughout the body where they contribute to many physiological functions. In epithelial cells, Maxi-K channels regulate electrolyte movement (Pluznick et al., 2003; Bailey et al., 2006; Grimm et al., 2009), whereas in neurons and endocrine cells, the channels participate in action-potential repolarization and can modulate neurotransmitter and hormone release (Brenner et al., 2005). In smooth muscle tissues, Maxi-K channels regulate tone (Ledoux et al., 2006). Activation of Maxi-K channels provides a feedback mechanism to limit muscle contraction by causing smooth muscle hyperpolarization and shortening of action-potential duration, thereby limiting calcium entry through voltage-dependent calcium channels. Maxi-K channels are formed by association of four pore-forming subunits (KCNMA1) with four auxiliary β subunits (KCNMB1–4). In smooth muscle tissues, β1 is almost exclusively expressed, and presence of this subunit confers unique pharmacological and biophysical properties to the channel. Maxi-K channel
dysfunction caused either by genetic ablation of either channel subunit or by the use of selective channel inhibitors leads to increased tone in vascular, corpus cavernosum, and detrusor smooth muscles (Suarez-Kurtz et al., 1991; DeFarias et al., 1996; Brenner et al., 2000; Garcia and Kaczorowski, 2001; Meredith et al., 2004; Thorneloe et al., 2005; Werner et al., 2005, 2008; Brown et al., 2008), and Maxi-K channel knockout mice display hypertension (Brenner et al., 2000; Plüger et al., 2000; Sausbier et al., 2005), erectile dysfunction (Werner et al., 2005), and overactive bladder/incontinence (Meredith et al., 2004). Maxi-K channels appear to mediate indirectly the relaxant effects of a number of vasodilators, including nitric oxide, and recent data suggest that attenuated vasoconstrictor response to norepinephrine during experimental human endotoxemia is due to nitric oxide-mediated activation of Maxi-K channels in the vascular wall (Pickkers et al., 2006). Maxi-K channels are major modulators of bladder function by regulating spontaneous and nerve-evoked contractions, and overactivity of urinary bladder smooth muscle causes urge incontinence (Herrera et al., 2005; Thorneloe et al., 2005). Thus, experimental evidence suggests that Maxi-K channel activators could represent a novel therapy for treatment of smooth muscle disorders, such as hypertension, airway hyperreactivity, urinary incontinence, and erectile dysfunction.

The search for small-molecule Maxi-K channel activators has been and still remains a major topic of interest. For use in proof-of-concept studies, Maxi-K channel activators will need to produce potent and direct channel activation while displaying appropriate selectivity across other families of ion channels. Importantly, such agents should not have significant calcium-entry-blocking or phosphodiesterase (PDE)-inhibitory activities, which could compromise interpretation of pharmacological studies. Unfortunately, many of the small-molecule Maxi-K channel activators reported to date are weak channel activators and possess significant polypharmacy (Nardi et al., 2006; Garcia et al., 2007; Nardi and Olesen, 2008). In some cases, these agents have not been fully characterized at the level of the channel, and their selectivity profiles have not been examined (Gore et al., 2010). In other cases, the mechanism of pharmacological response has not been shown to be associated with a direct effect of the compound on the channel itself (Garcia et al., 2007). Interestingly, lithocholate, a naturally occurring bile acid, has been shown to specifically activate native smooth muscle Maxi-K channels through a mechanism dependent on presence of the \( \beta_1 \) subunit and to reversibly increase the diameter of pressurized resistance cerebral arteries, independent of an intact endothelium (Bukiya et al., 2007, 2009). However, lithocholate is a relatively weak channel activator, displaying an \( EC_{50} \) of \( \approx 45 \mu M \). Thus, the need still exists to identify potent, selective, small-molecule Maxi-K channel activators that could be used to determine the therapeutic utility of activating this target.

In the present study, a novel, high-throughput, functional membrane potential-based cellular assay for identifying Maxi-K channel activators was established and validated. This assay was used to screen a large chemical collection of \( \approx 1.6 \) million compounds, from which a family of tetrahydroquinolines was selected for further characterization. Medicinal chemistry efforts led to the identification of compound X, from which two enantiomers, Y and Z, were resolved. Importantly, Z is more potent as a Maxi-K channel activator than Y and does not possess other known activities that would compromise its use in pharmacological studies. In smooth muscle tissues, these agents relax precontracted preparations in a dose-dependent manner by a potassium-dependent mechanism that is consistent with Maxi-K channel activation. These data strongly support the notion that direct Maxi-K channel activation represents a valid mechanism to be explored for potential treatment of smooth muscle disorders.

**Materials and Methods**

**Materials.** Tissue culture medium and supplements were from Invitrogen (Carlsbad, CA), whereas serum was from Thermo Fisher Scientific (Waltham, MA). N-(6-chloro-7-hydroxyxoumarin-3-carbonyl)-dimystriostoylphosphatidylethanolamine (CC\(_2\)-DMPE), bis-(1,3-diethylylithio)barbituric acid/trimethine oxonol [DiSBAC\(_2\)(3)] and Pluronic acid F-127 were purchased from Invitrogen. FLIPR\(_{TETRA}\) membrane potential blue dye kit was from Molecular Devices (Sunnyvale, CA). Iberiotoxin (IBTx) was purchased from Peninsula Laboratories (Belmont, CA), and paxilline, pentamet, vircuculogen, apamin, and glibenclamide were from Sigma-Aldrich (Saint Louis, MO). Other reagents were obtained from commercial sources and were of the highest purity commercially available.

**Construction of Stable Cell Line Expressing Maxi-K Channels.** Chinese hamster ovary (CHO) cells were stably transfected with the human Slo1 (KCNaM1, KCa1.1, U11058) and \( \beta_1 \) (KCNMB1) subunits of the Maxi-K channel. The gene for the hSlo1 was inserted in the pcDNA3 vector for selection with G-418 (Geneticin), whereas the \( \beta_1 \) subunit gene was cloned in the pIRESpuro vector for selection with puromycin. A Maxi-K hSlo1 stable cell line was first constructed by selecting clones for levels of channel expression in a binding assay with \(^{[3]}\)HIBTX-D19C (Garcia et al., 2000). Stable clones expressing the highest \(^{[3]}\)HIBTX-D19C binding signals were then transfected with \( \beta_1 \) using FuGENE6 (Roche Applied Science, Indianapolis, IN). Cells were grown under selection, and clones were selected on the basis of their ability to bind \(^{[3]}\)HIBTX-D19Y/Y36F (Schmalhofer et al., 2005) and the characteristics of the fluorescence signal generated in the presence of a control Maxi-K channel agonist in a fluorescence resonance energy transfer (FRET) membrane potential-based assay (see below). CHO hSlo1 cells were grown in Iacove’s modified Dubelcco’s medium, containing 10% heat-inactivated fetal bovine serum, 500 \( \mu \)g/ml G-418, \( 1 \times \) penicillin streptomycin glutamine, and were maintained in a humid, 37°C, 10% CO\(_2\) atmosphere. After transfection of the \( \beta_1 \) subunit, growth medium was further supplemented with 15 \( \mu \)g/ml puromycin (Invitrogen).

**Membrane Potential-Based Functional Assay.** CHO cells stably transfected with the human Slo1 and \( \beta_1 \) subunits of the Maxi-K channel were plated using a Thermo Fisher Scientific Matrix WellMate system at 15,000 to 25,000 cells/well in a tissue culture-treated, flat, clear-bottom, black-wall, 384-well plate (BD Biosciences, Franklin Lakes, NJ) in 50 \( \mu l \) of selection medium and were incubated overnight (16–20 h) in a humid, 37°C, 10% CO\(_2\) atmosphere. For the FRET assay, CC\(_2\)-DMPE was mixed with Pluronic F-127, incubated in the dark for 30 min, and then diluted into Dubelcco’s phosphate-buffered saline (PBS) supplemented with 10 \( \mu \)M HEPES pH 7.4 to provide final concentrations of 15 \( \mu \)M CC\(_2\)-DMPE, 0.04% Pluronic F-127 (dye 1). The 10-point concentration-response curves were diluted to the final \( 1 \times \) concentration in a buffer containing 140 mM NaCl, 0.1 mM KC1, 2 mM Ca\(_{2+}\), 1 mM Mg\(_{2+}\), and 20 mM HEPES, pH adjusted to 7.4 by the addition of NaOH (low K buffer),
and 4 μM DiSBAC4(3) (dye 2), maintaining a final DMSO concentration of ≤1%, in a 96-well polypropylene plate (Dot Scientific Inc., Burton, MI). Cell medium was removed, wells were washed once with 50 μl of Dulbecco’s PBS supplemented with 10 mM HEPES pH 7.4, and 50 μl of dye 1 was added to each well and allowed to incubate 45 min in the dark at 25°C. After the incubation period, dye 1 was removed, wells were washed once with 50 μl of low K buffer, and 25 μl of dye 2, with or without test compound, was added and allowed to incubate 30 min in the dark at 25°C. At the end of the 30-min incubation period, the plate was placed in a VPIR II instrument (Aurora Biosciences, San Diego, CA), illuminated at 400 nm, and fluorescence emission was recorded at 460 and 580 nm. After baseline emissions were recorded for 8 s, 25 μl of a buffer containing 280 mM KCl, 2 mM CaCl2, 1 mM MgCl2, and 20 mM HEPES, pH adjusted to 7.4 by the addition of KOH, was added, and fluorescence emissions were recorded for up to an additional 30 s. The change in FRET ratio \(\Delta(F/F_0)\) was calculated as \(\Delta(F/F_0) = \left[ \frac{A_{580}/A_{460}}{I_{580}/I_{460}} \right] \), where \(I\) and \(A\) represent the fluorescence emission at the specified wavelength before and after addition of high K buffer, respectively. \(F\) was calculated by averaging the maximal three readings just after the signal reached a plateau level, usually at 10 to 12 s, and \(F_0\) was calculated by averaging the second through fifth readings, 2 to 5 s.

For the single-dye assay, one bottle of Molecular Devices FLIPR Membrane Potential Blue dye (no. R8042) was resuspended in 25 μl of low K buffer (blue dye buffer). The 10-point concentration-response curves were diluted to the final 1× concentration in blue dye buffer maintaining a final DMSO concentration of ≤1% in a 96-well polypropylene plate (Dot Scientific Inc.). Cell selection medium was removed, wells were washed once with 50 μl of low K buffer, and 25 μl blue dye buffer, with or without test compound, was added and allowed to incubate 30 min in the dark at 25°C. At the end of the 30-min incubation period, the plate was placed in a FLIPR\textsuperscript{RTETRA} instrument (Molecular Devices), illuminated using the 470 to 495 nm light-emitting diode module, and fluorescence emission was recorded using the 565–625 nm filter. Light-emitting diode intensity was set at 100%, camera gain was varied between 80 and 120, and read rate was 1.5 Hz. After baseline emissions were recorded for approximately 5 s, 25 μl of high K buffer was added, and fluorescence emissions were recorded for up to an additional 35 s. The change in fluorescence emission \(\Delta(F/F_0)\) was calculated by averaging the maximal five readings after the signal reached a plateau level, usually at 30 to 35 s \((F)\) and by averaging the initial three readings usually from 1 to 5 s \((F_0)\).

For the single-dye assay in 1536 wells, cells were seeded in a tissue culture-treated 1536-well plate and were incubated overnight in a humid, 37°C, 10% CO\textsubscript{2} atmosphere. On the day of the experiment, the growth media were washed off using low K buffer, with a final residual well volume of 2 to 3 μl. One bottle of Molecular Devices FLIPR Membrane Potential Blue dye was resuspended in 75 ml of low K buffer. The dye was added to cells (4 μl/well), and test compounds and controls were added using a 30-μl pin tool addition, followed by a 40-min incubation protected from light at ambient temperature \((23–25°C)\). The microplate was then read on the FLIPR\textsuperscript{RTETRA} instrument. After baseline emissions were recorded, 3 μl of the high K buffer was added, and fluorescence was recorded for up to an additional 40 s. All cell plating, washing, and dispensing used the GFN bottle valve washer (Genomics Institute of the Novartis Research Foundation, San Diego, CA).

EC\textsubscript{50} values were calculated as follows: fold increase = control + (max-control)/[1 + (EC\textsubscript{50}/conc)\(n\)], where EC\textsubscript{50} is the concentration that produces 50% of maximal activation and \(n\) is the Hill coefficient. IC\textsubscript{50} values for inhibition were determined according to the Hill equation from dose-response curves, where all parameters were left unconstrained. To evaluate the quality of the data, the Z\textsuperscript{\textlangle} factor was calculated using the following equation:

\[
Z^\text{\textlangle} = 1 - \frac{3\text{SDc} + \text{SDn}}{C - N}
\]

where SDc and SDn are the standard deviation of the Maxi-K channel agonist group (C) and the group in the presence of Maxi-K channel inhibitor (N), and C and N are the means of the two groups, respectively.

**Synthesis of Compounds A, B, X, Y, and Z.** The synthetic routes to compounds A, B, and X are shown in Fig. 1. A Fisher indole synthesis using an appropriate phenylhydrazine Ca or Cb and keto acid D afforded the 2-aryl indole intermediate E. Acylation of E using excess benzoil chloride gave a mixed anhydride intermediate F, which was not isolated and selectively hydrolyzed directly to provide the final product A or B. The racemic compound X was prepared in one step from 4-amino benzoic acid, naphthalene-1-sulfonyl chloride, and freshly cracked cyclopentadiene via a Diels-Alder reaction of an iminium intermediate as the major endo-adduct. Compound X was resolved by supercritical fluid chromatography using a chiral column to give individual enantiomers Y and Z.

**3-(Fluoro-3-phenyl-1H-indol-2-yl)benzoic Acid (Ea).** A mixture of 4-fluorophenylhydrazine hydrochloride (Ca, 537 mg, 3.3 mmol), keto acid D (721 mg, 3.00 mmol), sodium acetate (328 mg, 4.0 mmol), and 6 μl acetic acid was heated in a capped test tube in a 120°C oil bath with magnetic stir for 6 h. Following the same workup procedure as described for Eb, a 61% yield of the title compound Ea was isolated. Alternatively, using the procedure for Eb below employing anhydrous zinc chloride and starting from 4-fluorophenylhydrazine hydrochloride as the starting material, Ea was obtained in 62% yield. Liquid chromatography/mass spectrometry (LC/MS): 3.48 min (m/z 332.1).

**3-(Benzyl-5-fluoro-3-phenyl-1H-indol-2-yl)benzoic Acid (A).** Using the procedure for B below and starting from Ea, A was obtained in 70–92% yields. LC/MS, 3.80 min (m/z 436.0, 418.0).

1H nuclear magnetic resonance (NMR) (acetone-d\textsubscript{6}, 500 MHz) δ: 7.86 (br s, 1 H), 7.76 (d, 8.0 Hz, 1 H), 7.74 (dd, 9.2 and 4.6 Hz), 7.37 (m, 2 H), 7.30 to 7.44 (m, 8.0 Hz, 2 H), 7.32 to 7.47 (m, 6 H), 7.29 (dd, 2.5 and 9.1 Hz, 1 H), 7.26 (dd, 7.7 and 7.6 Hz, 1 H), 7.17 (dd, 2.5, 9.2, and 9.5 Hz, 1 H), 1.71 (m, 2.5 Hz, 2 H).

**3-(Phenyl-1H-indol-2-yl)benzoic Acid (Eb).** A 100-μl round-bottom flask was charged with phenylhydrazine Cb (95%, 685 mg, 6.34 mmol), keto acid D (1.201 g, 5.00 mmol), and 5 μl of acetic acid. This mixture was heated in a 120°C oil bath under nitrogen for 30 min with magnetic stir before anhydrous zinc chloride (1.363 g, 10.00 mmol) was added. After additional 4.5 h of heating, the reaction mixture was cooled and loaded directly onto a 100-g silica gel column with the aid of some 1:1 dichloromethane and acetic acid mixture. The column was eluted with 2 column volumes (CV) of hexanes followed by 10 CV of –0 to 100% gradient of ethyl acetate in hexanes. The solvents were pooled and evaporated from fractions containing the desired product, and the resulting crude product was dissolved in 3.2 dioxane-water and purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) on a C-18 column using –30 to 100% MeCN gradient in water with 0.1% v/v trifluoroacetic acid (TFA). After lyophilization, this provided 1.098 g of Eb (70%), which was pure on the basis of analytical RP-HPLC and LC/MS: 3.44 min (m/z 314.1). 1H NMR (CDCl\textsubscript{3}, 500 MHz): δ: 8.38 (br s, 1 NH), 8.28 to 8.29 (m, 1 H), 8.045 (d, 8.0 Hz, 1 H), 8.045 (d, 8.0 Hz, 1 H), 8.015 (d, 8.0 Hz, 1 H), 7.71 (d, 8.0 Hz, 1 H), 7.62 (d, 7.8 Hz, 1 H), 7.50 (d, 8.0 Hz, 1 H), –7.39 to 7.47 (m, 10 H).

**Scheme 1.** Chemical structures of compounds synthesized in Fig. 1.
5 H), −7.33 to 7.36 (m, 1 H), −7.29 to 7.32 (m, 1 H), and −7.19 to 7.22 (m, 1 H).

3-(1-Benzoyl-3-phenyl-1H-indol-2-yl)benzoic Acid (B). A 250-ml round-bottom flask was charged with Eb (470 mg, 1.50 mmol) and 15 ml of anhydrous dichloromethane followed by triethylamine (1.25 ml, 911 mg, 9.0 mmol) and 4-dimethylaminopyridine (9.8 mg, 0.080 mmol). After sitting at room temperature for 3 days, this mixture was heated in a 45°C oil bath under nitrogen for 1.5 h and evaporated to remove all volatiles. The residue was dissolved in 30 ml 2:1 dioxane-water. More 4-dimethylaminopyridine (303 mg, 2.5 mmol) was added to this solution, and the mixture was heated at 45°C for 3 h. After cooling the reaction mixture and acidifying with an ice-cold mixture of 1.0 ml TFA in 4 ml of 1:1 dioxane-water, the resulting clear solution was stirred at room temperature for 5.5 h during which a precipitate formed. The solid was filtered and washed with acetonitrile. This crude solid product was resolved into enantiomers Y and Z by chiral HPLC using supercritical fluid chromatograph on a 300 × 25 mm ChiralPak-AD (Daicel Chemical Industries, Ltd., Osaka, Japan) using 4:6 ratio of CO2 and MeOH at 100 ml/min, 100 Bar, and 35°C column temperature. The fast-eluting isomer is Y (less active) and the slower-eluting enantiomer is Z. Y: 1H NMR (DMSO-d6, 400 MHz) δ 8.81 (d, 8.1 Hz, 1 H), 7.81 (d, 7.6 and 1.7 Hz, 1 H), 7.72 (d, 8.2 Hz, 1 H), 7.61 (d, 7.1 Hz, 1 H), 7.50 (s, 1 H), 7.36–7.44 (m, 4 H), 6.65 (d, 8.4 Hz, 1 H), 6.04 (s, 1 H), 5.77 (d, 1.5 Hz, 1 H), 5.39 (d, 4.8 Hz, 1 H), 5.28 (d, 2.2 Hz, 1 H), 4.07 (d, 8.4 Hz, 1 H), −2.97 to 3.03 (m, 1 H), −2.23 to 2.30 (m, 1 H), −1.20 to 1.26 (m, 1 H). Mass spectrometry (electrospray ionization) m/z (M + 1) = 342; Z: 1H NMR (DMSO-d6, 400 MHz) δ 12.0 (br s, 1 H), 8.11 (d, 8.0 Hz, 1 H), 7.82 (dd, 7.5 and 1.7 Hz, 1 H), 7.72 (d, 8.2 Hz, 1 H), 7.60 (d, 7.1 Hz, 1 H), 7.48 (d, 8.4 Hz, 1 H), 7.35 to 7.44 (m, 4 H), 6.68 (d, 8.4 Hz, 1 H), 6.21 (s, 1 H), 5.77 (d, 1.7 Hz, 1 H), 5.40 (d, 4.5 Hz, 1 H), 5.30 (d, 2.4 Hz, 1 H), −2.96 to 3.02 (m, 1 H), −2.21 to 2.28 (m, 1 H), and −1.21 to 1.27 (m, 1 H). In contrast to that of Y, this NMR sample showed a water peak at 3.17 ppm. Mass spectrometry (electrospray ionization) m/z (M + 1) = 342.

Automated Electrophysiology Assay. Maxi-K currents were recorded using the IonWorks Quattro system (Molecular Devices) in Population Patch Clamp mode as described previously (Ratliff et al., 2008). CHO cells stably expressing Maxi-K α and β subunits were grown as described above. After dispensing cells into the patch plates, seal resistance of cells was measured for each well, and cells were perforated by incubation with 10 µg/ml amphotericin B (Sigma-Aldrich), which was dissolved in the internal solution composed of 76 K2SO4, 20 KCl, 1 MgCl2, and 5 mM HEPES, pH 7.4. The bath solution consisted of Dulbecco’s PBS (Mediatech, Herndon, VA). Cells were voltage clamped at −90 mV, and a 10-mV depolarizing pulse was applied to calculate a linear leak correction that was applied to the recorded currents. Cells were depolarized in two steps, to +50 mV for 200 ms and to +80 mV for 200 ms, and currents were sampled at 2.5 kHz. After a control measurement, compound (or vehicle) was added for 7.5 min, and a second voltage step protocol was applied. Ten-point concentration dilution series were created by
serial diluting a 10 mM DMSO stock 1:3 in DMSO. The upper final concentration applied to cells was 31.6 μM. The final concentration of DMSO (0.33%) had no effect on control current recordings and was identical in all wells including control wells. Wells with equivalent seal resistances less than 20 MΩ and precompound currents less than 0.3 nA at +80 mV or displaying equivalent seal resistance decreases by more than 30% after compound addition were excluded from analysis. High concentrations of Maxi-K channel agonists may activate channels at the holding potential and interfere with linear leak corrections, which may limit measurements for potent activators at high concentrations. Peak current amplitudes at each voltage were exported to Excel (Microsoft Corp., Redmond, WA) and sorted using a template that gathered precompound and postcompound replicates from the 384-well dataset and translated them back to the 96-well compound plate. Data were then expressed as a ratio of current in the presence of compound to control currents in the same well. Igor Pro (WaveMetrics, Lake Oswego, OR) software was used for graphing and fitting of the data. Titrations data were fit by the Hill equation: fold increase = control + (max-control)/(1 + (EC50/ conc)H/nH), where EC50 is the concentration that produces 50% of maximal activation and nH is the Hill coefficient.

Other Assays. The activity of test compounds on the voltage-gated sodium channel Nav1.5, and L-type calcium channel Cav1.2 was determined in functional assays, as described previously (Felix et al., 2004; Abbadie et al., 2010). The interaction of compounds with the hERG channel was evaluated in a [35S]MK-499 binding assay to membranes prepared from HEK293 cells expressing hERG as described previously (Schmalhofer et al., 2010). Compound Z was evaluated in EMD Milli- pore’s Cardiac Profiler panel (Millipore Corporation, Billerica, MA) (Kaczorowski et al., 2011) consisting of Kv4.3/KChIP2, Kv1.5, KCNQ1/ minK, hERG, HCN4, and Kir2.1 channels (http://www.millipore.com/life_sciences/flux/id/ion/jon/tab1=2/tab1=3-tab2=1). In vitro assays for a panel of PDE enzymes (PDE1-P6) were performed by MDS Pharma Services (King of Prussia, PA).

Isometric Tension Recordings. Experiments were performed at 37°C on isolated urinary bladder strips and thoracic aorta rings from adult rats. De-endothelialized aorta rings were used in the same experiments. Animals were kept following the precepts of humane care, in rooms with temperature control and light/dark cycle and were asphyxiated by CO2 inhalation. The preparations were mounted under 1-g tension in organ baths containing modified Krebs-Henseleit solution (120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.1 mM MgCl2, 15 mM NaHCO3, 1.2 mM NaH2PO4, 11 mM glucose, and 10 mM HEPES). The pH of this solution after equilibration with 95% O2 and 5% CO2 was 7.3 at 37°C. An isometric high K solution (120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1.1 mM MgCl2, 15 mM NaHCO3, 1.2 mM NaH2PO4, 11 mM glucose, and 10 mM HEPES) was used to induce depolarization signal suggesting that the open probability of Maxi-K channels under resting calcium levels is not high enough for the channel to significantly affect the resting potential of the cells (Fig. 2, A and B). However, when cells are preincubated in the presence of a Maxi-K channel agonist, such as compound B, addition of the high potassium solution generates a fluorescence signal that is not observed when cells are also incubated in the presence of the selective Maxi-K channel inhibitor paxilline (Fig. 2, A and B). In both assays, the increase in fluorescence signal occurs in a concentration-dependent manner and in the FRET assay displays EC50 values of 0.63 ± 0.13 (n = 3) or 1.32 ± 0.35 μM (n = 16) for compound A and B, respectively (Fig. 2C). Both assays are robust, reproducible, and operate with high Z’ factors (>0.7) in 384-well format. However, to screen the large Merck sample collection (>1.6 million compounds) in 1536-well format, we elected to use the single fluorescence dye system because of ease of operation and a limited number of wash steps. Compounds were tested at a final concentra-
Fig. 2. Maxi-K channel agonist assays. CHO cells stably expressing the human Slo1 and β1 subunits of the Maxi-K channel were preincubated with the FRET-based membrane-potential-sensing dyes (A) or a single-membrane-potential-reporting dye (B) as described under Materials and Methods. Cells were also incubated in the absence (○) or presence of compound B (○) or compound B and the Maxi-K channel inhibitor paxilline (△). Upon recording the emission of the dyes, a high-potassium solution was added, and fluorescence was monitored for an additional period of time. In A, the fluorescence emission ratio of CC2-DMPE and DiSBAC4(3) is illustrated. C, CHO cells stably expressing the human Slo1 and β1 subunits of the Maxi-K channel were preincubated with the FRET-based membrane-potential-sensitive dyes, in the absence or presence of increasing concentrations of either compound A (○) or B (●). Error bars (S.D.) are indicated. From these experiments, EC50 values of 0.63 ± 0.13 μM (n = 3) and 1.32 ± 0.35 μM (n = 16) for compounds A and B, respectively, were determined.

Fig. 3. Tetrahydroquinoline Maxi-K channel activators. A, CHO cells stably expressing the human Slo1 and β1 subunits of the Maxi-K channel were preincubated with the FRET-based membrane-potential-sensitive dyes, in the absence or presence of increasing concentrations of either compound X (●), Y (○), or Z (△). Data are normalized to the maximal fluorescence increase of 10 μM compound B. The increase in fluorescence is concentration-dependent and displays EC50 values of 270 ± 65 nM (n = 12), 3117 ± 540 nM (n = 6), and 109 ± 22 nM (n = 6) for compound X, Y, and Z, respectively. Error bars (S.D.) are indicated. B and C, CHO cells stably expressing the human Slo1 and β1 subunits of the Maxi-K channel were preincubated with 10 μM either compound B (B) or compound Z (C) in the absence or presence of increasing concentrations of either paxilline (●), penitrem A (○), or verruculogen (△). Error bars (S.D.) are indicated. Data are presented as a percentage of inhibition of FRET signal relative to an untreated control. IC50 values: β, 188 nM (B), 546 nM (C); β, 32 nM (B), 30 nM (C); △, 7 nM (B), 15 nM (C).

Maxi-K channel inhibitors paxilline, penitrem A, or verruculogen, and as expected, the fluorescence signal is attenuated in a concentration-dependent manner in the presence of the Maxi-K channel inhibitors paxilline, penitrem A, or verruculogen, which in the presence of 10 μM X display IC50 values of 188, 32, and 7 nM, respectively (Fig. 3B). In functional assays, X is not a potent inhibitor of the voltage-gated sodium channel Nav1.5 (21 and 38% inhibition at 30 μM (n = 2)] and inhibits the L-type Cav1.2 channel with an IC50 of 14.32 ± 0.65 μM (n = 4). In addition, X has no significant effect (7% inhibition at 30 μM) on [36S]MK499 binding to the hERG channel and inhibits PDE enzymes with the following potencies: PDE1, 11.5 μM; PDE3, 32.8 μM; PDE4, 87.2 μM; PDE5, 24.7 μM; and PDE6, 75.8. Overall, X is more potent than either A or B and also possesses a better separation of activity against the L-type Cav1.2 channel and PDE enzymes. Interestingly, the two resolved enantiomers of X, compounds Y and Z (Fig. 1), display significantly different potencies as Maxi-K channel activators with EC50 values of 3117 ± 540 (n = 6) and 109 ± 22 nM (n = 6), respectively. In both cases, fluorescence signals were not observed in the presence of a Maxi-K channel inhibitor. As an example, the fluorescence signal at 10 μM Z is attenuated in a concentration-dependent manner in the presence of paxilline, penitrem A, or verruculogen with IC50 values of 346, 30, and 15 nM, respectively (Fig. 3C). In the functional L-type Cav1.2 channel assay, Y and Z inhibit with IC50 values of 15.7 and 10.6 μM, respectively. Compound Z was evaluated using electrophysiology protocols in the EMD Millipore’s Cardiac Profiler panel consisting of functional assays monitoring Kv4.3/KChIP2, Kv1.5, KCNQ1/minK, hERG, HCN4, or Kir2.1 channels. Z was tested at concentrations of up to 30 μM [eight-point concentration-response curves, 14 nM to 30 μM, against each channel (n = 5–8)]. There were no significant effects at concentrations up to 10 μM for each channel, although a ~28%
increase in current was observed for the KCNQ1/mink channel at 10 μM. At 30 μM, inhibition by Z was as follows (in percentage): Kv4.3/KChIP2 (34), Kv1.5 (55), hERG (19), HCN4 (29), and Kir2.1 (18). As observed above, 30 μM Z increased KCNQ1/mink currents by ~50%. The profile of these compounds, in particular compound Z, as Maxi-K channel agonists and their selectivity profiles provide an experimental paradigm with which to test the contribution of the Maxi-K channel in cell physiology and, especially, in those situations where Cav1.2 channels may also be present. Although an independent study has reported the identification of tetrahydroquinolines as Maxi-K channel agonists, the molecular pharmacological characterization of these compounds was limited, and no selectivity data were disclosed (Gore et al., 2010). For instance, in our SAR studies, compound 31 from Gore et al. (2010) was independently prepared and characterized. This compound and its two resolved enantiomers display EC50 values of 490, 830, and 120 nM, respectively, in the FRET-based assay, but compound 31 was found to inhibit PDE enzymes with the following potencies: PDE1, 11.5 μM; PDE3, 34.5 μM; PDE4, 2.6 μM; PDE5, 1.9 μM; and PDE6, 4.1 μM. Thus, the overall profile of compound 31, in particular its PDE5 inhibitory activity, is not appropriate for using this agent as a selective Maxi-K agonist in smooth muscle studies.

**Effects of Maxi-K Channel Agonists in Electrophysiological Assays.** Compounds Y and Z increased currents through Maxi-K channels recorded in an automated electrophysiology assay. Whole-cell recordings were made from CHO cells stably expressing Maxi-K α and β1 subunits, as described under experimental procedures. Maxi-K currents were activated by voltage steps from a holding potential of −80 to +50 mV and +80 mV (Fig. 4, inset). Application of 0.56 μM Y (Fig. 4A) or Z (Fig. 4B) caused clear increases in outward currents at both voltages. Currents measured during voltage steps to +80 mV in control conditions were larger and served as more stable comparators for compound effects than currents measured at +50 mV. Therefore, effects of Y and Z on Maxi-K currents during voltage steps to +80 mV were calculated and plotted in Fig. 4C as normalized increases in current amplitudes. Increases in current amplitudes for both compounds were fit with Hill equations yielding EC50 values for Z (0.44 μM) and Y (1.5 μM), and similar maximal increases in current amplitude of 5.0- and 6.1-fold, respectively, were seen. Similar values were obtained in replicate experiments for Z (EC50 = 0.53 ± 0.22 μM; maximal increase = 5.6 ± 0.3-fold; n = 3) and Y (EC50 = 2.04 ± 0.74 μM; maximal increase = 5.7 ± 0.9-fold; n = 3). The effects of compound Z on Maxi-K currents from CHO cells stably transfected with the α subunit were evaluated by manual patch clamp electrophysiology. Maxi-K currents increased in a concentration-dependent manner in the presence of Z, displaying an EC50 value of 2.3 μM with a maximal increase in current amplitude of ~11-fold (data not shown). Thus, activation of Maxi-K currents by Z appears to occur through an interaction with the α subunit of the channel.

**Maxi-K Channel Agonists Relax Smooth Muscle Preparations.** Figure 5A shows the experimental protocol used to assess the effects of compounds X, Y, and Z on the phenylephrine-induced contractures of aorta rings. After 20
to 30 min of exposure to phenylephrine, one of the test compounds was added to the bathing medium in increasing concentrations (0.1–60 μM) at 60-min intervals. Dose-dependent relaxation was observed, with the average IC50 values being 6.7, 22.2, and 3.24 μM for X, Y, and Z, respectively. Deendothelialization of the aorta rings had negligible or no effect on the relaxation induced by compounds Y and Z (Fig. 5B). Addition of the selective Maxi-K channel blocker IbTX (100 nM) to the bathing medium completely reversed the relaxing effects of Z (Fig. 5A). Pretreatment of aorta rings with IbTX (100 nM) elicited negligible tension per se but prevented the relaxing effects of compound Z (3 μM; Fig. 6A). By contrast, the relaxing effect of compound Z was not affected by pretreatment of the aorta rings with a cocktail containing apamin (a selective blocker of the small conductance, Ca2+-activated K+ channel), glibenclamide (a blocker of KATP channels), and 4-aminopyridine (a blocker of voltage-gated K+ channels). Nevertheless, the relaxation induced by compound Z under these experimental conditions was reversed by subsequent addition of IbTX to the bathing medium (Fig. 6B). Compounds Y and Z at 10 μM caused no relaxation of contractures elicited by 80 mM KCl in aorta rings (data not shown), suggesting that relaxation of the phenylephrine-induced contractions by these compounds is not due to calcium channel-blocking activity.

The protocol used to assess the effects of compounds Y and Z on the carbachol-induced motility of detrusor muscle is shown in Fig. 7A with data for Z. After 30 min of exposure to 0.1 μM carbachol, addition of Z to the bathing medium caused a progressive reduction in detrusor muscle motility, which reached a stable value within 60 min. The inhibitory effect of Y and Z at 10 μM (Fig. 7A) on detrusor motility effect was reversed by 100 nM IbTX. Data from similar experiments were used to construct the concentration-response curve shown in Fig. 7B, from which average IC50 values of 25.3 and 3.6 μM were obtained for Y and Z, respectively. In contrast to IbTX, neither apamin (100 nM) nor glibenclamide (10 μM) was capable of antagonizing the relaxant effects of Y and Z in detrusor muscle (data not shown).
the different experimental paradigms: for Z, EC_{50} was 0.11 in the FRET assay, 0.53 for increasing Maxi-K current amplitudes in CHO cells, 3.24 for inducing relaxation of phenylephrine-induced contractures of aorta rings, and 3.6 for reducing the motility of detrusor muscle strips stimulated with carbachol. The corresponding EC_{50} values for Y were 3.11, 2.04, 22.2, and 25.3 \mu M. The larger EC_{50} values in the smooth muscle experiments, compared with the FRET assay and electrophysiology measurements in isolated cells, may be accounted for by diffusion barriers across the smooth muscle tissues (Suarez-Kurtz et al., 1991).

As an indication of specificity, compound Z is selective as a modulator of Maxi-K channels when compared with its effects on six other unrelated K channels, including Kv4.3/KChIP2, Kv1.5, KCNQ1/minK, hERG, HCN4, and Kir2.1. In electrophysiology assays probing for activity against these targets, there was little to modest effects of this agent at concentrations up to 10 to 30 \mu M. Moreover, in pharmacological assays monitoring smooth muscle contractility, there were apparently no stimulatory effects of Z on either KCa2.X or KATP channels at high compound test concentrations.

For validating the concept that activation of Maxi-K channels may be therapeutically relevant, selective modulators of these channels are required to prevent other mechanisms from contributing to the pharmacological phenotype of interest. For example, blockade of L-type Cav1.2 channels, which contributes to smooth muscle relaxation, is a common feature of several Maxi-K agonists. However, in the case of the novel compound Z, there is a clear dissociation of effects in vitro assays between Maxi-K and L-type Cav1.2 channels. Thus, in the functional L-type Cav1.2 channel assay, Z displays an IC_{50} value of 10.6 \mu M. This value, compared with an EC_{50} of 0.11 \mu M for Maxi-K activation in the FRET assay, provides approximately 2 orders of magnitude selectivity for the Maxi-K channel. This difference is also reflected in the 70-fold greater potency of the racemic compound X as Maxi-K activator (EC_{50} = 0.27 \mu M) versus L-type Cav1.2 channel inhibition (IC_{50} = 14.3 \mu M) but is much attenuated in the case of enantiomer Y (EC_{50} = 3.11 \mu M versus IC_{50} = 15.7 \mu M; 5-fold difference). Compound X was also found to be a weak inhibitor of the voltage-gated sodium channel, Nav1.5, and of PDE enzymes, including PDE5 (IC_{50} = 24.7 \mu M); this latter activity could also contribute to smooth muscle relaxation.

Consistent with the selectivity of enantiomer Z for Maxi-K channels, the fluorescence signal elicited by this compound in the FRET-based assay was attenuated by nanomolar concentrations of the selective Maxi-K channel antagonists, paxitoline, penitrem A, and verruculogen. Accordingly, another selective Maxi-K channel blocker, IbTX, reversed the relaxing effects of Z in two smooth muscle paradigms, aorta rings precontracted with phenylephrine and detrusor muscle strips stimulated with carbachol. By contrast, neither apamin, a selective blocker of the small conductance, calcium-activated K channel, nor glibenclamide, a selective inhibitor of ATP-activated K channels, modified the relaxing effect of Z activated K channels, modified the relaxing effect of Z.

**Discussion**

The search for Maxi-K channel openers is an ongoing effort at both academic and pharmaceutical institutions. Unlike Maxi-K channel blockers, some of which display high selectivity and potency (Calderone, 2002; Zeng et al., 2008), the situation is quite opposite when considering activators of the channel. In general, both potency and specificity have been a issue with previously reported Maxi-K channel openers, an activity and potency (Calderone, 2002; Zeng et al., 2008), the Maxi-K channel blockers, some of which display high selectivity (Suarez-Kurtz et al., 1991).

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be accounted for by selective activation of Maxi-K channels in the muscle fibers. On the basis of an independent study reported previously (Gore et al., 2010) and the results disclosed in this manuscript, additional SAR studies are warranted with the tetrahydroquinoline structural class to determine whether further enhancements in potency and selectivity are possible that could aid in the identification of a putative drug development candidate.

Unlike lisocholesterol, whose mechanism of Maxi-K channel activation depends on the presence of the β1 subunit (Bukiya et al., 2007, 2009), compound Z activates Maxi-K channels in the absence or presence of β1 subunit. Whether the mechanism of action of compounds, such as Z, represents a liability given the wide distribution of Maxi-K channels across the body requires further in vivo evaluation studies. Properties such as pharmacokinetics, protein binding, and tissue distribution will be important determinants in the efficacy and safety profiles of individual drug development candidates. Even for Maxi-K channel activators, which are selective for α/β1 channels, their clinical utility will depend on their efficacy on the tissue of interest versus other mechanistic-based safety issues, such as hypotension caused by activation of the channels present in the vascular smooth muscle compartment. The physicochemical properties of these agents will, therefore, be critical to determine the clinical utility of Maxi-K channel activators.

In summary, the tetrahydroquinoline family of Maxi-K channel activators has been identified after screening a large sample collection using a functional membrane potential-based fluorescent assay. Compound X and its resolved enantiomers, Y and Z, are easily prepared synthetically and activate Maxi-K channels in vitro assays. Importantly, Z is more potent than Y as a Maxi-K channel activator and also displays selectivity for other targets, such as a variety of K channels, Cav1.2 and PDEs, which could complicate the interpretation of pharmacological results with smooth muscle preparations and in vivo data. Compound Z is more potent than Y in relaxing precontracted aortic or detrusor smooth muscle strips. Relaxation by Z appears to occur through a Maxi-K channel dependent mechanism because the effect is reversed in the presence of the selective Maxi-K channel blocker IbTX. In addition, Z has no effect on contractions elicited by 80 mK KCl, suggesting that calcium channel block does not contribute to the observed smooth muscle relaxation. All these data, taken together, strongly suggest that direct activation of Maxi-K channels represents a viable mechanism for treating a number of diseases associated with smooth muscle hyperexcitability, such as hypertension, bladder incontinence, and erectile dysfunction.

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Conducted experiments: Ponte, Schmalhofer, Shen, Dai, Stevenson, Sur, Shah, and Shu.

Contributed new reagents or analytic tools: Shah.

Performed data analysis: Ponte, McManus, Schmalhofer, Shen, Dai, Stevenson, Sur, Shah, Kiss, Shue, Nargund, Suarez-Kurtz, and Garcia.

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References


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