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# 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 $\beta$ subunits (KCNMB1–4). In smooth muscle tissues, $\beta$1 is almost exclusively expressed, and presence of this subunit confers unique pharmacological and biophysical properties to the channel. Maxi-K channel

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**ABBREVIATIONS:** Maxi-K, high-conductance, calcium-activated potassium; PDE, phosphodiesterase; CHO, Chinese hamster ovary; $CC_2$-DMPE, *N*-(6-chloro-7-hydroxycoumarin-3-carbonyl)-dimyristoylphosphatidylethanolamine; $DiSBAC_2(3)$, bis-(1,3-diethylthiobarbituric acid)trimethine oxonol; IbTX, iberiotoxin; FRET, fluorescence resonance energy transfer; DMSO, dimethyl sulfoxide; LC/MS, liquid chromatography/mass spectrometry; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; SAR, structure-activity relationship; CV, column volumes; RP-HPLC, reverse-phase high-performance liquid chromatography; DMA, 4-dimethylaminopyridine; TFA, trifluoroacetic acid.

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 hyperexcitability, 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 ~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 >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-hydroxycoumarin-3-carbonyl)-dimyristoylphosphatidylethanolamine ($CC_2$-DMPE), bis-(1,3-diethylthiobarbituric 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, penitrem A, verruculogen, 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 (KCNMA1, 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$H]IbTX-D19C (Garcia et al., 2000). Stable clones expressing the highest [$^3$H]IbTX-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 $^{125}$I-IbTX-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 Iscove's modified Dulbecco's medium, containing 10% heat-inactivated fetal bovine serum, 500 $\mu$g/ml G-418, 1× 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. All liquid handling for the assay was performed on a Matrix PlateMate 2X3 (Thermo Fisher Scientific), unless otherwise noted. Individual test compound stock solutions were prepared in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich), and quarter-log dilutions that extended 10 concentrations were manually prepared in 100% DMSO in a 96-well polypropylene plate (Corning Life Sciences, Lowell, MA). For the FRET assay, $CC_2$-DMPE was mixed with Pluronic F-127, incubated in the dark for 30 min, and then diluted into Dulbecco's phosphate-buffered saline (PBS) supplemented with 10 mM 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× concentration in a buffer containing 140 mM NaCl, 0.1 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES, pH adjusted to 7.4 by the addition of NaOH (low K buffer),

and 4 $\mu$M DiSBAC$_2$(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 $\mu$l of Dulbecco's PBS supplemented with 10 mM HEPES pH 7.4, and 50 $\mu$l 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 $\mu$l of low K buffer, and 25 $\mu$l 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 VIPR 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 $\mu$l of a buffer containing 280 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 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 ($F/F_0$) was calculated as $F/F_0 = [(A_{460}/A_{580})/(I_{460}/I_{580})]$, 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 ml 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 $\mu$l of low K buffer, and 25 $\mu$l 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$^{TETRA}$ instrument (Molecular Devices), illuminated using the 470 to 495 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 $\mu$l of high K buffer was added, and fluorescence emissions were recorded for up to an additional 35 s. The change in fluorescence emission ($F/F_0$) 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% $CO_2$ 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 $\mu$l. 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 $\mu$l/well), and test compounds and controls were added using a 30-nl 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$^{TETRA}$ instrument. After baseline emissions were recorded, 3 $\mu$l 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 GNF bottle valve washer (Genomics Institute of the Novartis Research Foundation, San Diego, CA).

$EC_{50}$ values were calculated as follows: fold increase = control + (max-control)/[1 + ($EC_{50}$/conc)$^n$], where $EC_{50}$ is the concentration that produces 50% of maximal activation and $n$ is the Hill coefficient. $IC_{50}$ 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′ factor was calculated using the following equation:

$$Z' = 1 - \frac{3(\mathrm{SD}c + \mathrm{SD}n)}{|C - N|}$$

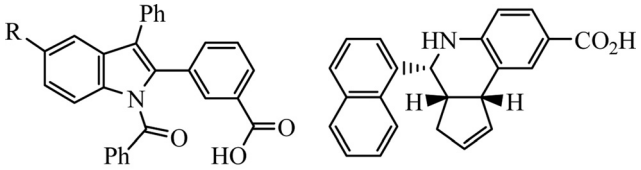
where SD$c$ and SD$n$ are the standard deviation of the Maxi-K channel agonist group (C) and the group in the presence of Maxi-K channel inhibitor (N), and $C$ and $N$ are the means of the two groups, respectively.

**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 benzoyl 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-aminobenzoic acid, naphthalene-1-aldehyde, 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 chromatograph using a chiral column to give individual enantiomers **Y** and **Z**.

**3-(5-Fluoro-3-phenyl-1*H*-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 ml 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-(1-Benzoyl-5-fluoro-3-phenyl-1*H*-indol-2-yl)benzoic Acid (A).** Using the procedure for **B** below and starting from Ea, **A** was obtained in ~86 to 92% yields. LC/MS, 3.80 min (*m/z* 436.0, 418.0). $^1$H nuclear magnetic resonance (NMR) (acetone-$d_6$, 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 ($J_{H\text{-}F}$) Hz, 1 H], ~7.63 to 7.65 (m, 2 H), ~7.44 to 7.48 (m, 2 H), ~7.37 to 7.41 (m, 2 H), ~7.32 to 7.36 (m, 6 H), 7.29 [dd, 2.5 and 9.1 ($J_{H\text{-}F}$) Hz, 1 H], 7.26 (dd, 7.7 and 7.6 Hz, 1 H), 7.17 (ddd, 2.5, 9.2, and 9.2 Hz, 1 H).

**3-(3-Phenyl-1*H*-indol-2-yl)benzoic Acid (Eb).** A 100-ml 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 ml 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). $^1$H NMR ($CDCl_3$, 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), 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,




**A**, R=F
**B**, R=H

**X**, racemic
**Y**, enantiomer A
**Z**, enantiomer B

**Scheme 1.** Chemical structures of compounds synthesized in Fig. 1.

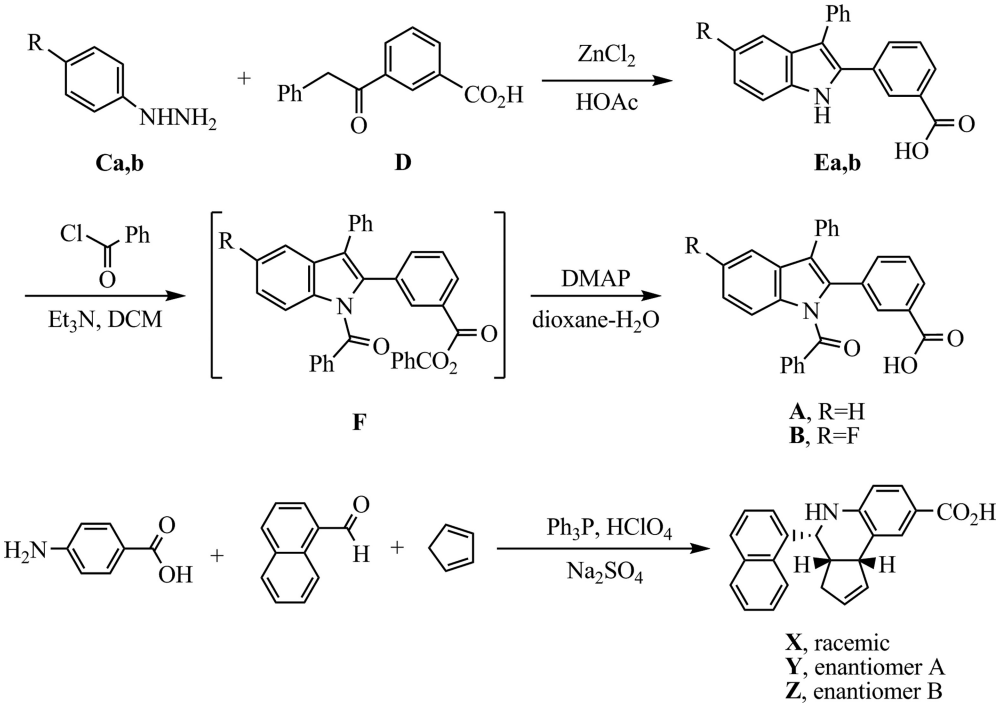



**Fig. 1.** Synthetic routes to compounds **A**, **B**, **X**, **Y**, and **Z**.

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-1*H*-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 reaction mixture was purified directly by preparative RP-HPLC on a C-18 column using ~40 to 100% MeCN gradient in water with 0.1% (v/v) TFA to give 510.3 mg of **B** as yellowish solid after lyophilization. The color of this product can be reduced by purifying it again on silica gel using ~0 to 100% gradient of ethyl acetate in hexanes to afford 474.5 mg of **B** as yellowish solid (75%). LC/MS 3.76 min (*m/z* 296.1, 418.2, 400.2). $^1$H NMR (acetone-$d_6$, 500 MHz) δ: 7.88 (s, 1 H), 7.76 (d, 7.8 Hz, 1 H), ~7.64 to 7.68 (m, 3H), 7.63 (dd, 6.9 and 1.6 Hz, 1H), 7.48 (dd, 7.5 Hz, 1 H), 7.43 (d, 7.3 Hz, 1 H), ~7.30 to 7.40 (m, 9 H), and 7.24 (dd, 7.7 and 7.8 Hz, 1 H).

**(3*aS*,4*R*,9*bR*)- and (3*aR*,4*S*,9*bS*)-4-(Naphthalen-1-yl)-3*a*,4,5,9*b*-tetrahydro-3*H*-cyclopenta[*c*]quinoline-8-carboxylic Acid (X, Racemate) (Nagarajan et al., 2001).** Triphenyl phosphine (230 mg, 0.875 mmol) and perchloric acid (70%, 0.076 ml, 126 mg, 0.875 mmol) were premixed either neatly or in a small amount of acetonitrile. This mixture was added to a solution of 4-aminobenzoic acid (300 mg, 2.19 mmol), 1-naphthaldehyde (360 mg, 2.19 mmol), freshly cracked cyclopentadiene (289 mg, 4.38 mmol), and anhydrous sodium sulfate (311 mg, 2.19 mmol) in 9 ml of dry acetonitrile. The resulting clear solution was stirred at room temperature for 5.5 h during which a precipitate formed. The solid was collected by filtration and washed with acetonitrile. This crude solid product was dissolved in 1,4-dioxane, loaded on a 100-g silica gel column, and eluted with 10 CV gradient of ~0 to 100% ethyl acetate in hexanes. The fractions containing the desired product were combined and evaporated to give 610 mg **X**. LC-MS: 3.40 min. (*m/z* 342.04). $^1$H NMR (1,4-dioxane-$d_8$, 500 MHz) δ: 10.17 (v br s, 1 H), 8.25 (d, 8.4 Hz, 1 H), 7.94 (d, 8.0 Hz, 1 H), ~7.84 to 7.85 (m, 2 H), 7.78 (s, 1 H), 7.64 (dd, 8.2 and 1.4 Hz, 1 H), ~7.52 to 7.59 (m, 3 H), 6.70 (d, 8.5 Hz, 1 H), 5.98 (br s, 1 H), 5.61 (d, 4.8 Hz, 1 H), 5.56 (d, 2.5 Hz, 1 H), 5.23 (s, 1 H), 4.25 (d, 8.4 Hz, 1 H), ~3.27 to 3.32 (m, 1 H), ~2.55 to 2.60 (m, 1 H), and ~1.65 to 1.61 (m, 1 H).

**(3*aS*,4*R*,9*bR*)- and (3*aR*,4*S*,9*bS*)-4-(Naphthalen-1-yl)-3*a*,4,5,9*b*-tetrahydro-3*H*-cyclopenta[*c*]quinoline-8-carboxylic Acid (Y and Z, Enantiomers).** The racemic compound **X** 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 $CO_2$ 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**: $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 8.11 (d, 8.1 Hz, 1 H), 7.81 (dd, 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**: $^1$H NMR (DMSO-$d_6$, 400 MHz) δ 12.0 (br, 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 (s, 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, 4.9 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 β1 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 $K_2SO_4$, 20 KCl, 1 $MgCl_2$, 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 $\mu$M. 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. Titration data were fit by the Hill equation: fold increase = control + (max-control)/[1 + ($EC_{50}$/conc)$^{nH}$], where $EC_{50}$ is the concentration that produces 50% of maximal activation and $n_H$ 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 [$^{35}$S]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 Millipore'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/flx4/ld_ion&tab1=2#tab1=3:tab2=1). In vitro assays for a panel of PDE enzymes (PDE1–PDE6) 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 $CO_2$ 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 $CaCl_2$, 1.1 mM $MgCl_2$, 15 mM $NaHCO_3$, 1.2 mM $NaH_2PO_4$, 11 mM glucose, and 10 mM HEPES). The pH of this solution after equilibration with 95% $O_2$ and 5% $CO_2$ was 7.3 at 37°C. An isometric high K solution was prepared by increasing the KCl concentration to 80 mM and reducing the NaCl concentration to 45 mM. Isometric tension was recorded using force-displacement transducers (Grass FT-03; Grass Instruments Co., Quincy, MA) coupled to a polygraph. Integration of the isometric tension data were used to quantify drug effects on the spontaneous myogenic activity of detrusor muscle strips pretreated with 0.1 $\mu$M carbachol (Suarez-Kurtz et al., 1991). The zero level for integration was set at 5% of the average amplitude of the spontaneous tension oscillations, and 10-min periods were integrated at various times during the experiments. The data are expressed relative to the "basal" tension integral, measured 20 to 30 min after the initial exposure to carbachol. Aorta rings precontracted with 1 $\mu$M phenylephrine were used to quantitate the relaxing effect of compounds **X**, **Y**, and **Z**. Stock solutions of **X**, **Y**, **Z**, and glibenclamide were prepared in DMSO, and all subsequent dilutions were made in the experimental saline solutions. The highest bath concentration of DMSO was 0.2%, which has no effect on the functional parameters under study. Stock solutions of IbTX and apamin were made in 100 mM NaCl solution containing 0.1% bovine serum albumin, whereas 4-aminopyridine was stored in aqueous solution. Pooled data from $n$ identical experiments are presented as means ± S.E.

## Results

**Identification of Maxi-K Channel Agonists.** A substructure search of the Merck sample collection against a core structure from a series of Maxi-K channel agonists reported in the literature (Lee et al., 2005), and subsequent testing of six compounds identified compound **B** as a novel Maxi-K channel agonist. Subsequent structure activity relationship (SAR) studies afforded compound **A** (Fig. 1). Both compounds increased Maxi-K channel activity in electrophysiological recordings, as well as in a high-throughput membrane potential-based assay designed to identify Maxi-K channel inhibitors (Doherty et al., 2009). Although the potency of these compounds as Maxi-K channel activators is moderate (i.e., channel activity increases ~14- and 5-fold in the presence of 10 $\mu$M of compound **A** or **B**, respectively), their use in pharmacological proof of concept studies aimed at defining the contribution of Maxi-K channel activation to smooth muscle relaxation is compromised by the presence of L-type calcium-channel Cav1.2 blocking (**A**, < 10 $\mu$M; **B**, ~10–30 $\mu$M), and PDE inhibitory activities [compound **A** ($IC_{50}$): PDE1, 1.7 $\mu$M; PDE2, 33.3 $\mu$M; PDE3, 12 $\mu$M; PDE4, 1.2 $\mu$M; PDE5, 7.4 $\mu$M; PDE6, 6.5 $\mu$M; compound **B** ($IC_{50}$ or inhibition at 10 $\mu$M): PDE1, 0.5 $\mu$M; PDE2, 7% inhibition at 10 $\mu$M; PDE3, 29% inhibition at 10 $\mu$M; PDE4, 0.9 $\mu$M; PDE5, 28% inhibition at 10 $\mu$M; PDE6, 41% inhibition at 10 $\mu$M). Given these data and the fact that potent and selective Maxi-K channel activators have not been disclosed, we screened a large small molecule sample collection (>1.6 million compounds) to identify compounds with an improved pharmacological profile.

Because the assay window of the Maxi-K channel inhibitor assay was not optimal for identifying Maxi-K channel agonists, we established a new cell-based high-throughput assay that could satisfy this quest. For this purpose, a CHO cell line stably expressing the human Slo1 and $\beta$1 subunits of the Maxi-K channel was constructed. The presence of Maxi-K channels with appropriate biophysical and pharmacological properties was confirmed in electrophysiological recordings of channel activity. When these cells are loaded with membrane potential indicator dyes, such as either a pair of FRET dyes or a single fluorescence dye, addition of a high potassium solution to the cells does not generate any significant 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 $EC_{50}$ values of 0.63 ± 0.13 ($n$ = 3) or 1.32 ± 0.35 $\mu$M ($n$ = 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-

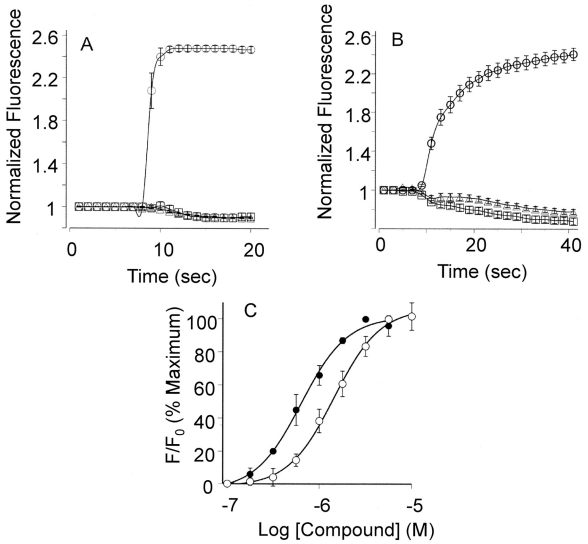



**Fig. 2.** Maxi-K channel agonist assays. CHO cells stably expressing the human Slo1 and $\beta$1 subunits of the Maxi-K channel were preincubated with the FRET-based membrane-potential-sensing dyes (A) or a single-membrane-potential-reporting dye (B) as described under *Materials and Methods*. Cells were also incubated in the absence (□) or presence of compound **B** (○) or compound **B** and the Maxi-K channel inhibitor paxilline (Δ). 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 $CC_2$-DMPE and $DiSBAC_2(3)$ is illustrated. C, CHO cells stably expressing the human Slo1 and $\beta$1 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, $EC_{50}$ values of 0.63 ± 0.13 ($n$ = 3) and 1.32 ± 0.35 $\mu$M ($n$ = 16) for compounds **A** and **B**, respectively, were determined.

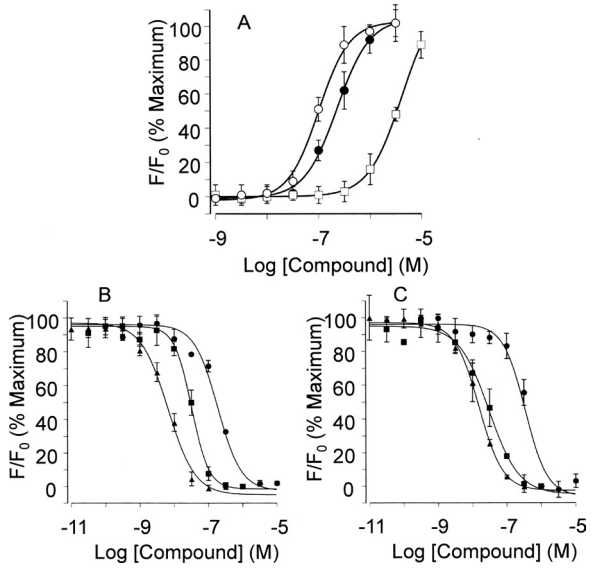



**Fig. 3.** Tetrahydroquinoline Maxi-K channel activators. A, CHO cells stably expressing the human Slo1 and $\beta$1 subunits of the Maxi-K channel were preincubated with the FRET-based membrane-potential-sensitive dyes, in the absence or presence of increasing concentrations of either compound **X** (●), **Y** (□), or **Z** (○). Data are normalized to the maximal fluorescence increase of 10 $\mu$M compound **B**. The increase in fluorescence is concentration-dependent and displays $EC_{50}$ 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 $\beta$1 subunits of the Maxi-K channel were preincubated with 10 $\mu$M either compound **X** (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. $IC_{50}$ values: ●, 188 nM (B), 346 nM (C); ■, 32 nM (B), 30 nM (C); ▲, 7 nM (B), 15 nM (C).

tion of 6 $\mu$M. Approximately 5300 compounds that increased the maximal signal by >50% relative to 1.8 $\mu$M of compound **A** were selected for reconfirmation and further evaluation. These compounds were retested in triplicate at 6 $\mu$M in the absence or presence of a Maxi-K channel inhibitor to identify those with appropriate pharmacological behavior. From the reconfirmation set, ~1400 compounds displayed fluorescence signal increases that were prevented by a Maxi-K channel blocker. These compounds were tested for Maxi-K channel activation by automated electrophysiology in IonWorks and were also subjected to detailed concentration-response curves in the Maxi-K channel agonist membrane potential-based assay. A number of hits that significantly increased Maxi-K currents with $EC_{50}$ values <10 $\mu$M were identified and subjected to selectivity measurements against other ion channels, such as Cav1.2, Nav1.5, and hERG, as well as for PDEs' inhibitory activity.

On the basis of screening and counter-screening studies, a family of tetrahydroquinolines, together with a half dozen other series, was selected for exploratory medicinal chemistry. SAR studies in the tetrahydroquinoline series afforded the identification of the more potent and selective Maxi-K channel agonist, compound **X** (Fig. 1). In the FRET-based assay, **X** displays an $EC_{50}$ of 270 ± 65 nM ($n$ = 12) (Fig. 3A), 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 $\mu$M **X** display $IC_{50}$ 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 $\mu$M ($n$ = 2)] and inhibits the L-type Cav1.2 channel with an $IC_{50}$ of 14.32 ± 0.65 $\mu$M ($n$ = 4). In addition, **X** has no significant effect (7% inhibition at 30 $\mu$M) on [$^{35}$S]MK499 binding to the hERG channel and inhibits PDE enzymes with the following potencies: PDE1, 11.5 $\mu$M; PDE3, 32.8 $\mu$M; PDE4, 87.2 $\mu$M; PDE5, 24.7 $\mu$M; 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 $EC_{50}$ 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 $\mu$M **Z** is attenuated in a concentration-dependent manner in the presence of paxilline, penitrem A, or verruculogen with $IC_{50}$ values of 346, 30, and 15 nM, respectively (Fig. 3C). In the functional L-type Cav1.2 channel assay, **Y** and **Z** inhibit with $IC_{50}$ values of 15.7 and 10.6 $\mu$M, 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 $\mu$M [eight-point concentration-response curves, 14 nM to 30 $\mu$M, against each channel ($n$ = 5–8)]. There were no significant effects at concentrations up to 10 $\mu$M for each channel, although a ~28%

increase in current was observed for the KCNQ1/mink channel at 10 $\mu$M. At 30 $\mu$M, 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 $\mu$M **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 $EC_{50}$ 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 $\mu$M; PDE3, 34.5 $\mu$M; PDE4, 2.6 $\mu$M; PDE5, 1.9 $\mu$M; and PDE6, 4.1 $\mu$M. 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 $\alpha$ and $\beta$1 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 $\mu$M **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 $EC_{50}$ values for **Z** (0.44 $\mu$M) and **Y** (1.5 $\mu$M), 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** ($EC_{50}$ = 0.53 ± 0.22 $\mu$M; maximal increase = 5.6 ± 0.3-fold; $n$ = 3) and **Y** ($EC_{50}$ = 2.04 ± 0.74 $\mu$M; 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 $\alpha$ subunit were evaluated by manual patch clamp electrophysiology. Maxi-K currents increased in a concentration-dependent manner in the presence of **Z**, displaying an $EC_{50}$ value of 2.3 $\mu$M 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 $\alpha$ 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

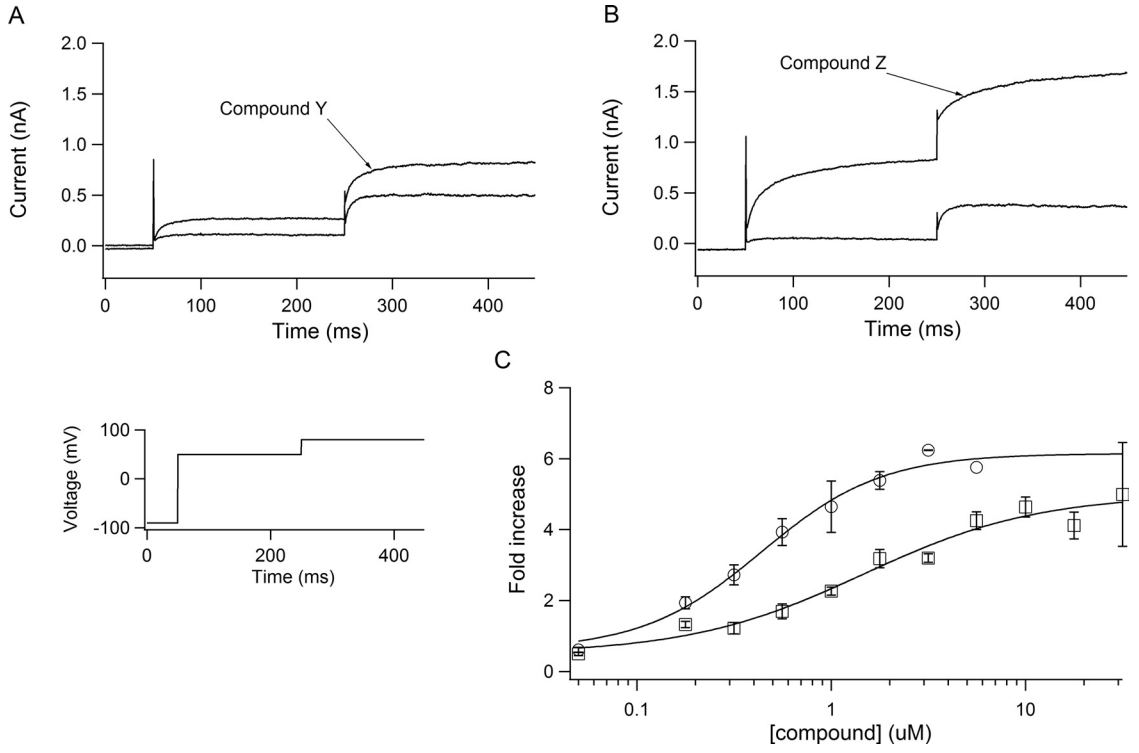



**Fig. 4.** Electrophysiological analysis of tetrahydroquinoline Maxi-K channel activators. Representative recordings are shown from individual wells showing increases in Maxi-K currents after exposure to compound **Y** (A) and compound **Z** (B) using the stimulus voltage protocol shown in the inset. Currents are shown before and after addition of compound **Y** (A) or compound **Z** (B). C, peak current amplitudes measured at +80 mV in the presence of compound **Y** (□) and compound **Z** (○) were normalized to current amplitudes in control for each well, and mean values ± S.E.M. are plotted versus compound concentration. Values for a set of control wells in which buffer containing 0.33% DMSO was added are plotted at 0.05 $\mu$M compound concentration and were used to constrain the base values of the fitted Hill equation (see *Materials and Methods*).

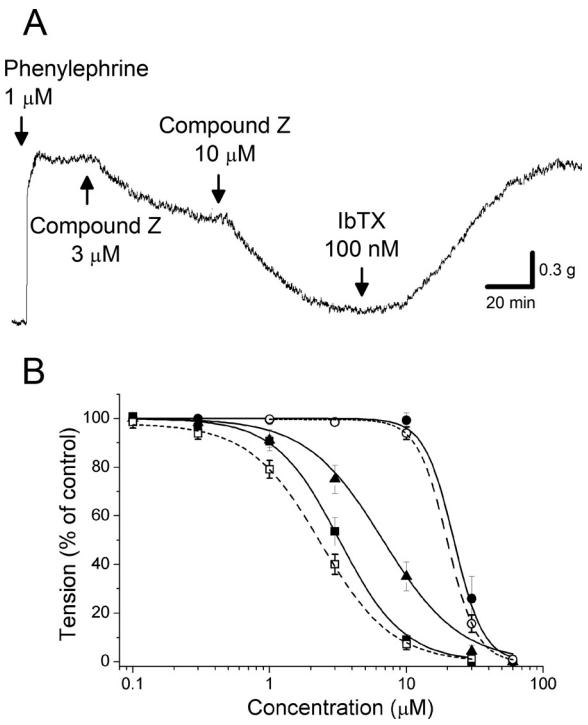



**Fig. 5.** Effects of compounds **X**, **Y**, and **Z** on the contractures elicited by phenylephrine (1.0 $\mu$M) in rat thoracic aorta rings. A, experimental protocol, showing the relaxing effect of increasing concentrations of compound **Z** and the reversal of this effect by 100 nM IbTX. B, data from four to six similar experiments were used to construct the concentration-effect curve for compounds **X** (▲), **Y** (●), and **Z** (■) in aorta rings with intact endothelium (solid lines) or mechanically de-endothelialized (dashed lines).

to 30 min of exposure to phenylephrine, one of the test compounds was added to the bathing medium in increasing concentrations (0.1–60 $\mu$M) at 60-min intervals. Dose-dependent relaxation was observed, with the average $IC_{50}$ values being 6.7, 22.2, and 3.24 $\mu$M for **X**, **Y**, and **Z**, respectively. De-endothelialization 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 $\mu$M; 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, $Ca^{2+}$-activated $K^+$ channel), glibenclamide (a blocker of $K_{ATP}$ 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 $\mu$M 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 $\mu$M 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 $\mu$M (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 $IC_{50}$ values of 25.3 and 3.6 $\mu$M were obtained for **Y** and **Z**, respectively. In contrast to IbTX, neither apamin (100 nM) nor glibenclamide (10 $\mu$M) was capable of antagonizing the relaxant effects of **Y** and **Z** in detrusor muscle (data not shown).

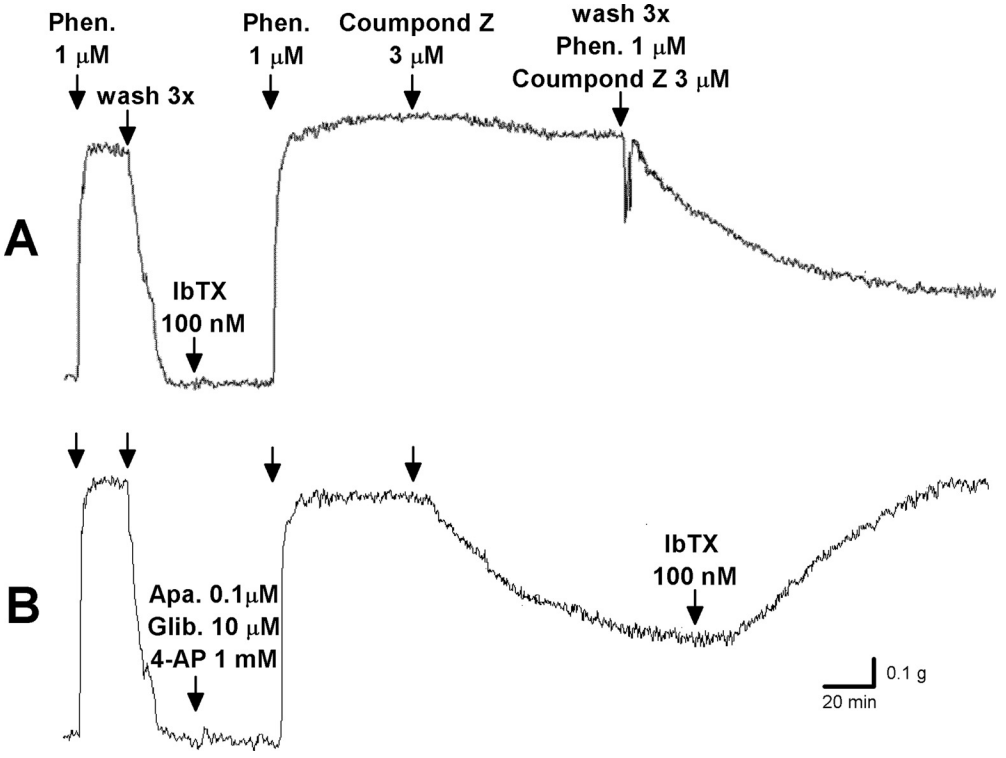



**Fig. 6.** Effects of pretreatment with K channel blockers on the relaxation induced by compound **Z** in aorta rings contracted with phenylephrine (1.0 $\mu$M). A and B, data from two adjacent rings, which were initially contracted with 1.0 $\mu$M phenylephrine (Phen). After washout and complete relaxation, ring A was exposed to 100 nM IbTX (top tracing), and ring B was exposed to a cocktail containing 100 nM apamin (Apa.), 10 $\mu$M glibenclamide (Glib.), and 1 mM 4-aminopyridine (4-AP). After 30 min, a sustained contracture was elicited by 1.0 $\mu$M Phen. in both rings. Addition of compound **Z** (3.0 $\mu$M) relaxed ring B, but not ring A. Removal of IbTX restored the relaxing effect of compound **Z** (A), whereas addition of IbTX reversed the relaxing effect of compound **Z** (B).

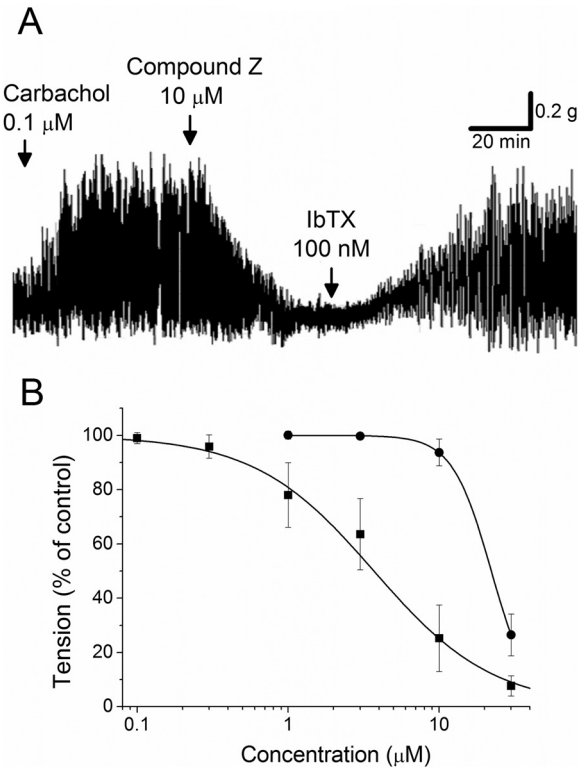



**Fig. 7.** Effects of compounds **Y** and **Z** on the motility of detrusor muscle strips stimulated with 0.1 μM carbachol. A, experimental protocol showing the relaxing effect of 10 μM compound **Z**, and the reversal of this effect by 100 nM IbTX. B, data from four to six similar experiments were used to construct the concentration-effect curve for compounds **Y** (●) and **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 an issue with previously reported Maxi-K channel openers, limiting their use in paradigms aimed at validating the concept of direct channel activation for therapeutic utility. The present study represents a departure from this pattern, through the disclosure of compound **Z**, a novel, potent, and selective Maxi-K channel activator. Compound **Z** is a member of the tetrahydroquinoline class of Maxi-K channel activators. Although several tetrahydroquinoline Maxi-K channel agonists have been independently reported (Gore et al., 2010), detailed information on the interaction of these compounds with the channel, as well as their selectivity profile, mainly their calcium channel blocking and PDEs' inhibitory activities, was not disclosed. Identification of compound **Z** was the final result of high-throughput screening of a large sample collection (>1.6 million compounds) using a novel membrane potential-based functional assay, combined with exploratory medicinal chemistry. In the FRET-based assay, **Z** was 30-fold more potent as a Maxi-K channel activator than its enantiomer, compound **Y**, and 3-fold more potent than the racemate, **X**. The greater potency of **Z**, relative to its enantiomer, was confirmed, albeit attenuated, in the smooth muscle contractility studies and electrophysiology recordings. The $EC_{50}$ values for each compound also varied across 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 μM. 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 μM. 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 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 μM. This value, compared with an $EC_{50}$ of 0.11 μM 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 μM) versus L-type Cav1.2 channel inhibition ($IC_{50}$ = 14.3 μM) but is much attenuated in the case of enantiomer **Y** ($EC_{50}$ = 3.11 μM versus $IC_{50}$ = 15.7 μM; 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 μM); 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, paxilline, 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** on detrusor muscle. The observation that **Z** has no relaxing effect on the contractures elicited by 80 mM KCl in aorta rings provides indirect evidence that blockade of L-type calcium channels does not contribute to the smooth muscle relaxing effects of **Z**, reported here. Collectively, the results in smooth muscle preparations support the conclusion that the relaxing effect of **Z** may

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 lithocholate, whose mechanism of Maxi-K channel activation depends on the presence of the $\beta$1 subunit (Bukiya et al., 2007, 2009), compound **Z** activates Maxi-K channels in the absence or presence of $\beta$1 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 $\alpha/\beta$1 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 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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#### Authorship Contributions

*Participated in research design:* Ponte, McManus, Schmalhofer, Shen, Stevenson, Kiss, Shu, Doherty, Nargund, Kaczorowski, Suarez-Kurtz, and Garcia.

*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.

*Wrote or contributed to the writing of the manuscript:* Ponte, McManus, Schmalhofer, Shen, Kaczorowski, Suarez-Kurtz, and Garcia.

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