

Functional Analysis of Large Conductance Ca^{2+} -Activated K^+ Channels: Ion Flux Studies by Atomic Absorption Spectrometry

Ashutosh S. Parihar,¹ Duncan R. Groebe,² Victoria E. Scott,¹ Jianlin Feng,¹ Xu-Feng Zhang,¹ Usha Warrior,² Murali Gopalakrishnan,¹ and Char-Chang Shieh¹

Abstract: Although techniques such as $^{86}\text{Rb}^+$ flux provide a sensitive measure of K^+ channel activity, the relatively short half-life and high-energy emission, together with the quantities of radioactive material generated, hinder the usefulness of flux-based formats in high throughput screening efforts. This study elaborates on the utilization of flame atomic absorption spectrometry (AAS) techniques for a nonradioactive rubidium efflux assay for large conductance Ca^{2+} -activated K^+ channels (BK_{Ca}) channels. Utilizing human embryonic kidney (HEK293) cells expressing the BK_{Ca} α subunit, a 96-well cell-based nonradioactive rubidium efflux screen for channel openers and inhibitors was established. Known BK_{Ca} channel openers, including NS1608, NS1619, and NS-8, activated rubidium efflux with EC_{50} values ranging from 1 to 4 μM in both radioactive and nonradioactive efflux formats. Compounds such as iberiotoxin, paxilline, and charybdotoxin inhibited rubidium efflux responses evoked by the BK_{Ca} channel opener NS1608 in both radioactive and nonradioactive efflux formats. The IC_{50} values of the inhibitors in AAS format were comparable to those derived from $^{86}\text{Rb}^+$ efflux assays. The present studies show that the pharmacological profiles of BK_{Ca} channels assessed by AAS compare well with those obtained using the $^{86}\text{Rb}^+$ efflux assay, and support the utility of nonradioactive efflux format for higher throughput screening campaigns for novel K^+ channel modulators.

Introduction

VOLTAGE-GATED K^+ CHANNELS serve as attractive drug targets for a wide variety of central nervous system, metabolic, and cardiovascular disorders.¹ These channels represent a large and diverse class of membrane proteins, organized into several families based upon their primary amino acid sequence and biophysical characteristics. In many excitable cell types, including smooth muscles and neurons, activation of K^+ channels generally leads to membrane hyperpolarization and subsequent tissue relaxation or inhibition of action potential generation, whereas inhibition of K^+ channels tends to increase cellular excitability.

Among the K^+ channels, K_{Ca} , K_{ATP} , and KCNQ type

channels have been explored for the development of openers for a variety of disorders, including cardiac ischemia, epilepsy, stroke, hypertension, bladder overactivity, erectile dysfunction, obesity, and diabetes. Although progress in the development of selective K_{Ca} channel openers has somewhat lagged behind that of K_{ATP} channel openers, the unique properties of K_{Ca} channels, particularly that of the large conductance type (KCNMA1 , BK_{Ca}), makes them an appealing target.²⁻⁴ However, more recently, openers of BK_{Ca} channels from diverse structural classes targeted for diverse urological and central nervous system disorders have emerged.⁵⁻⁷ BK_{Ca} channels are sensitive to both membrane potential and intracellular calcium concentrations. Organizationally, these channels contain a six-transmembrane domain-spanning region with a

¹Neuroscience Research and ²Advanced Technology, Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, IL.

ABBREVIATIONS: AAS, atomic absorption spectrometry; BK_{Ca} , large conductance Ca^{2+} -activated K^+ channels; K_{ATP} , ATP-sensitive K^+ channels; K_{Ca} , Ca^{2+} -activated K^+ channels.

pore-forming α subunit, which could be associated with accessory β subunit.⁸ The α subunit of the BK_{Ca} channel was first cloned from *Drosophila melanogaster* and subsequently from other species, including human. A single gene is known to encode the BK_{Ca} α subunit, whereas multiple variants of β -subunits exist. Functional BK_{Ca} channels can be formed solely by expression of the α -subunit at least in recombinant systems.

Over the years, functional analysis of BK_{Ca} channels has primarily used electrophysiological techniques. Although critical to biophysical characterization of channels, the low throughput of this technology has thus far made it impractical for high throughput screening campaigns, despite more recent enhancements in *Xenopus* oocyte multiplex systems and planar array-based recording technologies.^{9–14} Advances in fluorescence-based technology have enabled cellular assays for functional assessment of activity via ion channels using Ca²⁺ indicator dyes such as fura-2, fluo-3, and fluo-4 and by voltage-sensitive dyes.^{15–17} Radioligand binding assays have also been widely used to screen for ion channel modulators; however, radioligand binding formats are limited by the availability and/or affinity of the radioligand. In addition, only compounds that can influence binding of the radioligand can be discovered, thus missing activity of allosteric channel modulators.

Ion flux is a technique that has been successfully used to assess functional ion channel activity more directly, unlike fluorescent indicator measures that use an indirect readout. Typically, this technique utilizes radiotracers such as ²²Na⁺, ⁴⁵Ca²⁺, and ⁸⁶Rb⁺ for studying Na⁺, Ca²⁺, and K⁺ channels, respectively. However, the use of radioisotopes like rubidium itself has limitations for high capacity screening. More recently, AAS techniques have been developed for measuring rubidium efflux via nonselective cation channels and K⁺ channels.^{18–20} AAS uses light absorption to measure the concentration of gas-phase atoms. Analyte atoms are vaporized by high heat, which then absorbs light energy to transition to higher energy levels; the concentration of ions is determined from the amount of light absorption. Although this principle has been well documented, utilization of this technique for pharmacological studies and screening applications has, thus far, been limited. In this study, we have evaluated the profile of channel openers and inhibitors of the BK_{Ca} channels by measuring cation efflux by standard radioactive ⁸⁶Rb⁺ efflux and by AAS determination of nonradioactive rubidium efflux. These studies demonstrate that the pharmacological profile of the agents determined by both radioactive and nonradioactive assessment of cation efflux compare well, and provide further support that AAS provides a reliable alternative for assessment of ion channel activity, with potential application to medium throughput screening assays of K⁺ channel modulators.

Materials and Methods

Expression of BK_{Ca} α subunit and cell culture

Total RNA from human bladder (ABS, Wilmington, DE, U.S.A.) was reverse-transcribed into cDNA using the first-strand cDNA synthesis Superscript kit and primed using random hexamers according to the manufacturer's instructions (GibcoBRL, Gaithersburg, MD, U.S.A.). Oligonucleotides (Genbank accession number AF025999) specific for the BK_{Ca} α subunit were designed and used to amplify the α subunit from human bladder cDNA using the ExpandTM Long Template PCR system (Boehringer Mannheim, Indianapolis, IN, U.S.A.). A single PCR product was obtained for the BK_{Ca} α subunit (3,465 bp), which was subcloned into a mammalian expression vector (pcDNA 3.1-Zeo). The BK_{Ca} α subunit construct was sequenced to confirm identity. HEK293 cells were transfected with the BK_{Ca} α subunit using Lipofectamine as suggested by the manufacturer (Life Technologies, Grand Island, NY, U.S.A.). Forty-eight hours post transfection, antibiotic-resistant clones were selected with 100 μ g/ml zeocin (Invitrogen, Carlsbad, CA, U.S.A.). Clones were subsequently screened by whole-cell patch clamp to identify functional cell lines.

Cells were maintained in Dulbecco's modified Eagle medium (Life Technologies) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, U.S.A.), 5 mM L-glutamine (Invitrogen), and 100 μ g/ml zeocin (Invitrogen). Cells were subcultured in Costar vented cap tissue culture flasks (VWR Scientific Products, West Chester, PA, U.S.A.) at 37°C in an atmosphere of 5% CO₂/95% air. When cells achieved 80–90% confluency, adherent cells were removed from the tissue culture flask with cell dissociation buffer (Invitrogen) and plated on collagen-coated 96-well plates (Biocoat, VWR Scientific Products) for ⁸⁶Rb⁺ efflux and AAS.

Electrophysiological recording

The patch-clamp technique was used to measure membrane currents in whole-cell configurations as previously described.^{21,22} Fire-polished patch electrodes had a resistance of 2–5 M Ω when filled with pipette solution, which contained the following (in mM): KCl, 140; MgCl₂, 1; CaCl₂, 0.1; EGTA, 1; HEPES, 10; pH 7.2 with NaOH, 285 mOsm. The bath solution contained the following (in mM): NaCl, 135; KCl, 5; CaCl₂, 2.5; MgCl₂, 1.2; HEPES, 5; pH 7.4 with NaOH, 310 mOsm. The HEK293 cells transfected with BK_{Ca} α subunits were voltage-clamped at a holding potential of –80 mV. The changes in ionic currents in the presence of compounds were measured at the testing potential from –80 mV to +80 mV for 500 ms. The whole currents were amplified using an Axopatch 200B amplifier (Axon Instruments, Union City, CA, U.S.A.) and

low-pass filtered at 5 kHz (−3 dB, four pole Bessel filter) before digitization by Digidata 1200B at a sampling rate of 10 kHz.

AAS assays

Flame AAS measurements were performed using an Ion Channel Reader 8000 (Aurora Biomed Inc., Vancouver, BC, Canada). Cells were plated at $2.5\text{--}5.0 \times 10^4$ cells/well in 96-well plates for 24–48 h prior to assay. Cells were loaded with 1.0 mM cold RbCl (Sigma Chemical Co., St. Louis, MO, U.S.A.) and incubated for 18–24 h in culture media. In all experiments, the rubidium lamp was selected and the spectrometer on ICR 8000 calibrated to a wavelength of 780 nm, because at this wavelength non-specific absorption does not contribute to the atomic absorption signal. Calibration of the instrument was accomplished using varying concentrations of unlabeled rubidium. The rubidium standards were analyzed prior to each experiment on the spectrometer. The photomultiplier tube was adjusted to a voltage meter reading of 70–80%, and the absorbance integration time was set to 15 s. Samples were diluted with 200 μ l of assay buffer containing the following (in mM): NaCl, 150; CaCl₂, 2; NaH₂PO₄, 0.8; MgCl₂, 1.0; HEPES, 25; pH 7.4. A volume of 200 μ l of the diluted sample was injected into the spectrometer, followed by a 200- μ l wash with assay buffer, and the absorbance and rubidium concentration values were determined.

Radioactive rubidium (⁸⁶Rb⁺) efflux assays

Cells were plated at $2.5\text{--}5.0 \times 10^4$ cells/well in 96-well plates for 24–48 h prior to assay. Cells were loaded with 1.0–2.0 μ Ci/well of the radiotracer ⁸⁶Rb⁺ (NEN Life Science Products, Boston, MA, U.S.A.) and incubated for 18–24 h in culture media. Cells were then washed three times with assay buffer containing the following (in mM) HEPES, 20; NaCl, 120; KCl, 5; CaCl₂, 2; MgCl₂, 1; MgSO₄, 0.4; D-glucose, 20; ouabain, 0.01; and adjusted with NaOH to pH 7.4. Assays were initiated by the addition of appropriate concentrations of test compounds. In cases where inhibitors were assessed, assays were initiated after a 10-min preincubation with the inhibitor followed by a 30-min incubation with the test compounds. For the measurements of ⁸⁶Rb⁺ efflux, supernatants were harvested and saved in 96-well Opti-Plates (Packard Bioscience, Meriden, CT, U.S.A.). Cells were subsequently lysed with 1.0 M NaOH and the supernatants again saved in another 96-well Opti-Plate. EcoLume liquid scintillation fluid (ICN, Costa Mesa, CA, U.S.A.) was added in both sets of supernatants (efflux and lysate), and the plates were counted on a Packard TopCount (PerkinElmer Life Sciences, Downers Grove, IL, U.S.A.). Each test concentration of compounds was tested in duplicate wells.

Data analysis

The basal release was defined as percent Rb⁺ released from cells not exposed to test compounds. The percent rubidium release was defined by measuring the amount of Rb⁺ released in the well after stimulation divided by the total amount of Rb⁺ loaded per well (% rubidium = [buffer of stimulated cells]/[buffer from lysed cells] \times 100). To account for potential variability of cell number per well and loading efficiency, percent rubidium was calculated and normalized per well. Rubidium efflux obtained from both assays (⁸⁶Rb⁺ efflux and AAS) in the presence of test compounds was normalized to the maximal value obtained in the presence of 10 μ M ionomycin and expressed as a percent of control. The concentration-response curves of changes in rubidium efflux from both assays were analyzed by nonlinear regression using Prism (GraphPad Software, Inc., San Diego, CA, U.S.A.) to obtain EC₅₀ or IC₅₀ values. Results are expressed as means \pm SEM.

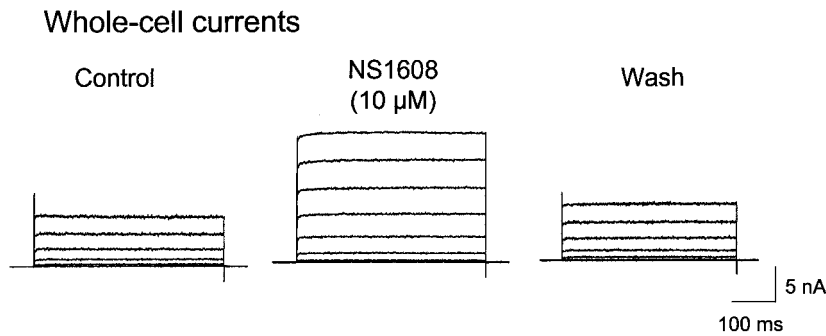
Compounds

NS1619 [1-(2-hydroxy-5-trifluoromethylphenyl)-5-trifluoromethyl-1,3-dihydro-benzimidazol-2-one]tetraethylammonium, charybdotoxin, iberiotoxin, ionomycin, and other chemicals were obtained from Research Biochemicals International/Sigma Chemical Co. NS-8 [2-amino-5-(2-fluorophenyl)-4-methyl-1H-pyrrole-3-carbonitrile], NS1608 [*N*-(3-(trifluoromethyl)phenyl)-*N'*-(2-hydroxy-5-chlorophenyl)urea], the aryl hydroxyoxindole analogue, [3-(5-chloro-2-hydroxyphenyl)-3-hydroxy-6-trifluoromethyl-1,3-dihydroindol-2-one, Compound 1], and CGS-7181 were synthesized at Abbott Laboratories (Abbott Park, IL, U.S.A.). Stock solutions of compounds were prepared in 100% dimethyl sulfoxide and diluted in buffer before use. Paxilline was obtained from Alexis Biochemicals (San Diego, CA, U.S.A.).

Results and Discussion

Functional expression of BK_{Ca} currents in HEK293 transfected with BK_{Ca} α subunit was initially assessed by whole-cell patch-clamp techniques. Cells were voltage-clamped from a holding potential of −80 mV, and ionic currents were measured from a test membrane potential of −80 mV to +80 mV by whole-cell patch clamp. Steep voltage-dependent increases in ionic currents were recorded when test potentials were above −10 mV (Fig. 1). Addition of 10 μ M NS1608 further evoked increases in ionic currents. The mean increase in currents (at +40 mV) evoked by NS1608 was $503.8 \pm 48.0\%$ ($n = 5$; Fig. 1). Charybdotoxin inhibited evoked currents with a mean IC₅₀ value of 9.1 ± 0.1 nM ($n = 5$; Fig. 2). These results demonstrate that the stable cell line expresses functional BK_{Ca} currents.

A



B

I-V curve

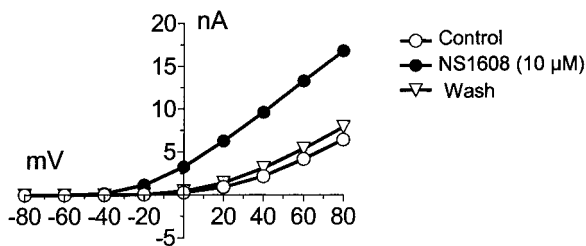


FIG. 1. NS1608 evoked increases in currents from HEK293 cells expressing BK_{Ca} α subunit. Whole-cell currents were recorded at test potentials from -80 mV to $+80$ mV from the holding potential of -80 mV in control, in the presence of NS1608 (10μ M), and following washout (A). The I-V curve was generated by plotting steady-state currents versus test potential (B). In control, evoked currents showed a steep voltage dependency with measurable currents starting from -10 mV. As depicted, NS1608 further evoked reversible increases in BK_{Ca} currents. \circ , control; \bullet , NS1608 (10μ M); ∇ , wash.

As noted previously, rubidium (Rb^+) functions as a surrogate permeant ion through K^+ channels and other nonselective cation channels. To evaluate the interactions of reference K^+ channel openers and inhibitors, rubidium efflux was assessed in the stably transfected HEK293 cell line identified above using both radioactive and non-radioactive formats. Typically, flux responses exhibited a consistent three- to four-fold window over basal levels in both $^{86}\text{Rb}^+$ flux and AAS assays. In both cases, when experiments were carried out with transfected and untransfected HEK293 cells, increases in Rb^+ flux were observed in transfected cells only (data not shown). Furthermore, the evoked increases in Rb^+ flux by the opener, NS1608, were found to be sensitive to iberiotoxin, charybdotoxin, and paxilline, suggesting that the flux is mediated through BK_{Ca} channel activation (Fig. 3).

In addition to NS1608, various structurally diverse BK_{Ca} openers, including aryl pyrrole NS-8 and benzimidazolone NS1619 (Fig. 4), were assessed in flux assays. Addition of openers evoked concentration-dependent efflux, under resting conditions without further manipulations. The significant flux observed under normal physiological conditions could possibly be attributed to the robust expression of BK_{Ca} currents, which generate outward currents of 10 nA or greater at $+80$ mV. Although the opening probability of BK_{Ca} channels is low at membrane potential ranging from -20 to -40 mV, the high expression of BK_{Ca} channels may compensate, enabling sufficient channel activity to evoke Rb^+ flux. Indeed, little or no Rb^+ flux was observed in the cell lines ex-

pressing low levels of BK_{Ca} channels (data not shown). The openers tested evoked concentration-dependent increases in radioactive Rb^+ efflux within the concentration range 0.01 – 100μ M with EC_{50} values ranging from $0.98 \pm 0.30 \mu$ M to $4.29 \pm 0.59 \mu$ M (Table 1, Fig. 5).

As shown in Fig. 3, known blockers such as iberiotoxin, paxilline, and charybdotoxin attenuated opener-evoked efflux responses. To determine the potency of blockers to inhibit Rb^+ efflux, cells were preincubated

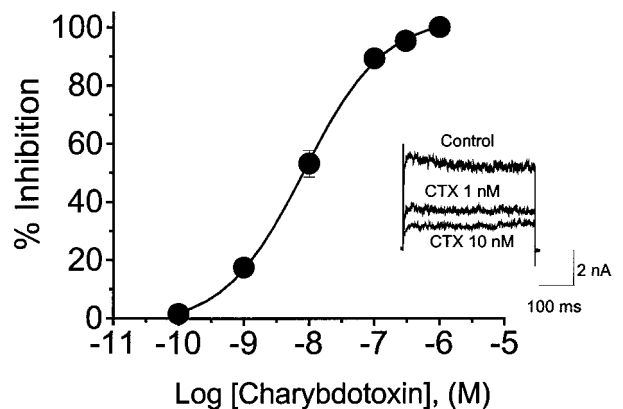


FIG. 2. Concentration-dependent inhibition of BK_{Ca} currents by charybdotoxin. Currents were recorded at a test potential of $+80$ mV from a holding potential of -80 mV. Charybdotoxin inhibited evoked currents with an IC_{50} value of 9.1 ± 0.1 nM ($n = 5$). The inset shows sample traces of whole-cell currents recorded in the absence and presence of 1 nM and 10 nM charybdotoxin (CTX) (as indicated).

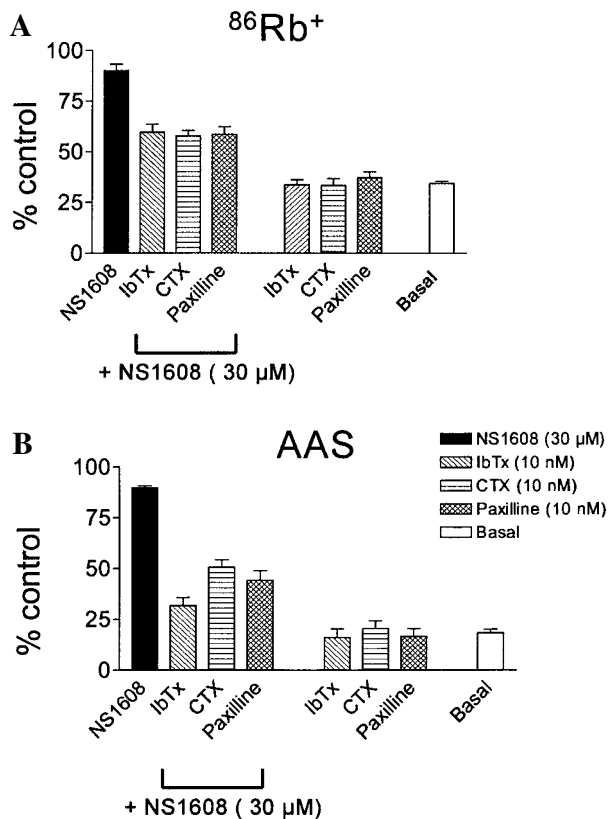


FIG. 3. Analysis of cation flux using ⁸⁶Rb⁺ and AAS. Radioactive (A) and nonradioactive (B) Rb⁺ flux assays were used to characterize BK_{Ca} channels expressed in HEK293 cells. NS1608 stimulated increases in Rb⁺ flux, which was three- to fourfold higher than basal flux (nonstimulated). Increases in Rb⁺ flux by NS1608 were sensitive to inhibition by iberiotoxin (IbTx), charybdotoxin (CTX), and paxilline.

with various concentrations of blockers prior to challenge with NS1608. Under these conditions, iberiotoxin, paxilline, and charybdotoxin all induced a concentration-dependent inhibition of Rb⁺ efflux (Table 1). The IC₅₀ value of iberiotoxin to inhibit Rb⁺ flux through BK_{Ca} channels obtained from this study is comparable to that in a previous report.¹⁸ The indole alkaloid, paxilline, previously shown to enhance toxin binding,²³ also inhibited efflux with an IC₅₀ value of 7.59 ± 0.24 nM.

The same set of openers and blockers was used to characterize the pharmacology of efflux measured by AAS. Again, in good agreement with radioactive efflux results, the EC₅₀ values for these compounds to evoke Rb⁺ efflux ranged from 0.91 ± 0.42 μM to 1.14 ± 0.31 μM (n = 5). Preincubation of cells with 100 nM iberiotoxin or 100 nM charybdotoxin prior to the addition of NS1608 (30 μM) completely abolished efflux responses as measured by AAS. Further, to assess the potency of blockers for inhibition of Rb⁺ flux, various concentrations of inhibitors were tested. Iberiotoxin, paxilline, and charybdotoxin all evoked concentration-dependent inhibition of Rb⁺ efflux elicited by NS1608 (Table 1). Iberiotoxin and charybdotoxin inhibited efflux with IC₅₀ values of 12.3 ± 0.25 nM and 24.0 ± 0.21 nM. As summarized in Table 1, the potencies of openers and inhibitors obtained from AAS measurements compare well with those measured using conventional radioactive ⁸⁶Rb⁺ efflux assay. Previous studies have demonstrated the roles of the β1 subunit in gating and pharmacological properties of BK_{Ca} channels.^{2,3,8} It is to be noted that although the cell line used in this study expresses only the BK_{Ca} α-subunit, the assay, in princi-

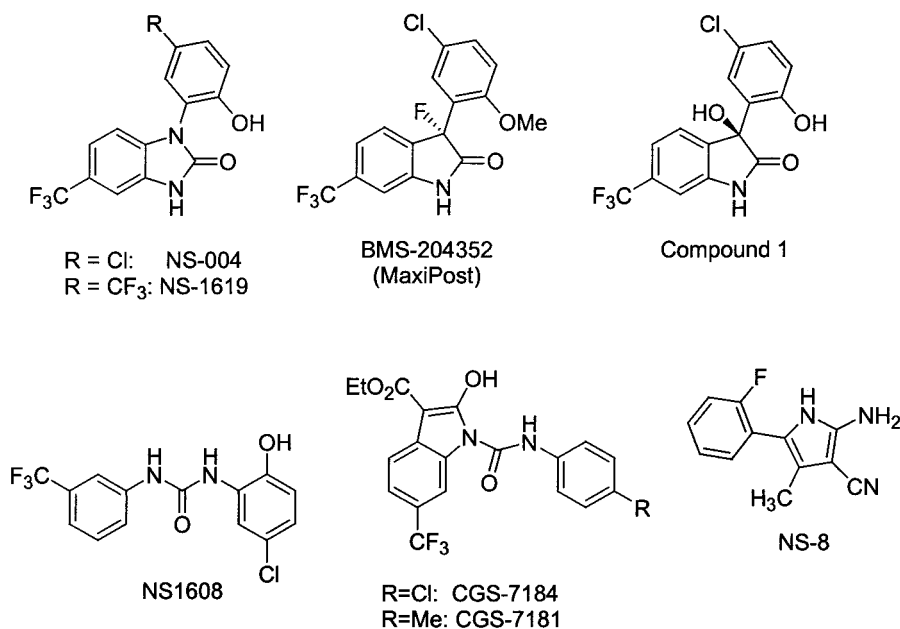


FIG. 4. Structures of selected prototypical BK_{Ca} opener standards.

TABLE 1. POTENCIES OF OPENERS AND INHIBITORS OF RUBIDIUM FLUX FROM HEK293 CELLS EXPRESSING BK_{Ca} α SUBUNIT

| | Compound | Efflux format | |
|----------------------------------------|------------------------|-----------------------------------------------|----------------------|
| | | Radioactive (⁸⁶ Rb ⁺) | Nonradioactive (AAS) |
| Opener (EC ₅₀ , μ M) | NS1608 | 2.41 \pm 0.68 | 1.14 \pm 0.31 |
| | NS1619 | 3.78 \pm 0.35 | 1.02 \pm 0.30 |
| | NS-8 | 4.26 \pm 0.24 | 0.93 \pm 0.19 |
| | Aryl oxindole analogue | 4.29 \pm 0.59 | 0.91 \pm 0.42 |
| | (Compound 1) CGS-7181 | 0.98 \pm 0.30 | 1.02 \pm 0.26 |
| Inhibitor (IC ₅₀ , nM) | Iberiotoxin | 1.53 \pm 0.28 | 12.3 \pm 2.51 |
| | Charybdotoxin | 4.84 \pm 0.26 | 24.0 \pm 2.11 |
| | Paxilline | 4.11 \pm 0.14 | 7.59 \pm 0.24 |

For inhibition studies, efflux was evoked by addition of 30 μ M NS1608. Values indicated are means \pm SEM of three to five observations. For structures, see Fig. 4.

ple, could be extended to functional and pharmacological studies of various BK_{Ca} $\beta\alpha$ combinations.

In contrast to the selective and high-affinity interaction of toxins such as the peptidyl blocker iberiotoxin, compounds that open BK_{Ca} channels are largely weak and not very selective. Benzimidazolone analogues such as NS-4 and NS1619 are earlier known openers of BK_{Ca} channels. NS1619 activates BK_{Ca} currents at 10–30 μ M in vascular and nonvascular smooth muscle, although over similar concentration ranges, the compound also inhibits delayed rectifiers and calcium currents in certain

tissues. NS1608 is a diphenylurea analogue that enhances BK_{Ca} activity by shifting current activation to more negative potentials at micromolar concentrations. Other known BK_{Ca} openers reported include glycosylated triterpene activators such as dehydrosoyasaponin-I. Several of the earlier known BK_{Ca} channel openers are also known to possess ancillary pharmacology, which limits their utility as therapeutic agents or as probes to study the *in vivo* therapeutic relevance of BK_{Ca} channels.¹ The aryl pyrrole NS-8 has been reported to activate BK_{Ca} currents in guinea pig bladder cells, relax guinea pig bladder strips,

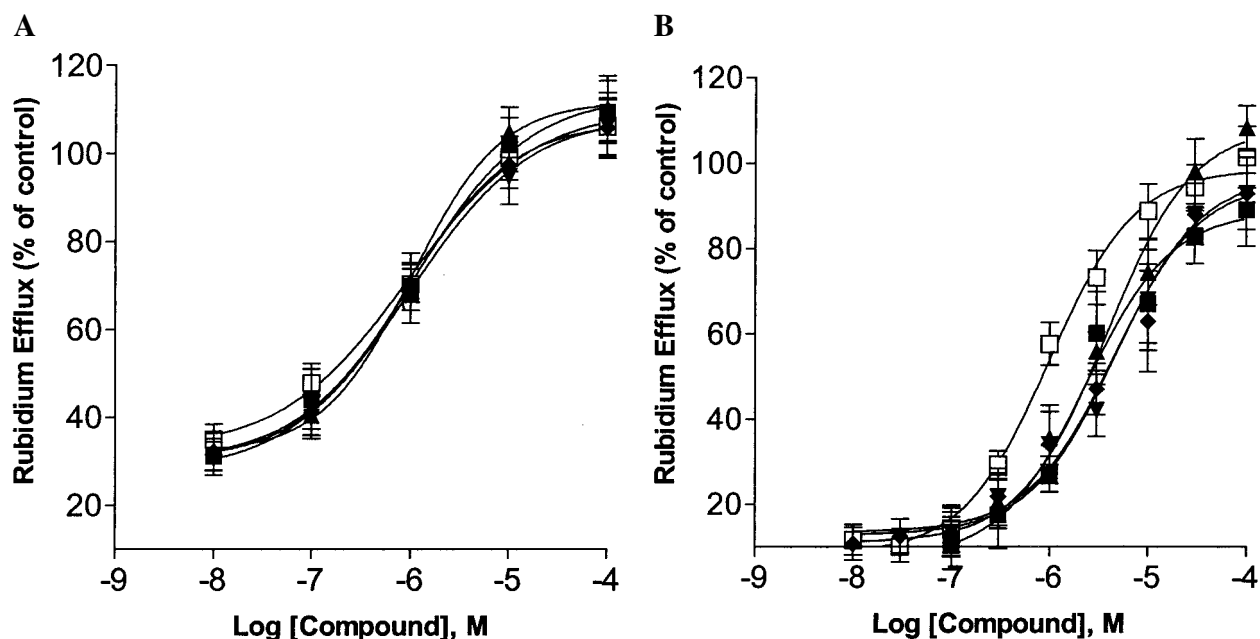


FIG. 5. Concentration-dependent increases in rubidium efflux as measured by flame AAS (A) and by radioactivity (⁸⁶Rb⁺, B). Cells were loaded with rubidium as described under Materials and Methods, and the effects of test compounds, were assessed during 30-min incubation. Efflux was assessed using AAS or counting as appropriate. Potency values of the compounds are summarized in Table 1. ■, NS1608; ▲, NS1619; ▼, NS-8; ◆, Compound 1; □, CGS-7181.

and suppress the excitability of dorsal root ganglion neurons. Novel aryl oxindole openers where the oxindole moiety was replaced for the benzimidazolone have been reported. Analogues such as BMS-204352 and BMS-191011 have emerged and been shown to have neuroprotective properties in animal models of stroke.^{24,25} More recently, arylquinolinone BK_{Ca} openers have been shown to relax corporal smooth muscle and to be efficacious in enhancing intracavernosal pressure in animal models of erectile function.⁵ Nonetheless, improvements in potency and, importantly, selectivity continue to be sought for these agents as efforts continue toward further development as tools or therapeutic agents.

Of various screening technologies currently available, cation flux assays provide a simple, yet reasonably reliable, means of assessing ligand interaction with ion channels. Although radioactive materials such as ⁸⁶Rb⁺ provide a sensitive measure of K⁺ channel activity, its profile, including a short half-life and a high-energy emission along with the quantities of radioactivity generated, somewhat limits its usefulness in high throughput screening efforts. Our current studies show that the pharmacological profile of openers and blockers, assessed by AAS assays utilizing the Aurora Biomed Ion Channel Reader, is comparable to that derived by radioactive ⁸⁶Rb⁺ efflux assay, and further supports the utility of the nonradioactive efflux assay for high throughput screening campaigns of K⁺ channel active compounds.

References

- Coghlan MJ, Carroll WA, Gopalakrishnan M: Recent developments in the biology and medicinal chemistry of potassium channel modulators: update from a decade of progress. *J Med Chem* 2001;44:1627–1653.
- Calderone V: Large-conductance, Ca²⁺-activated K⁺ channels: function, pharmacology and drugs. *Curr Med Chem* 2002;9:1385–1395.
- Gribkoff VK, Starrett JE Jr, Dworetzky SI: Maxi-K potassium channels: form, function, and modulation of a class of endogenous regulators of intracellular calcium. *Neuroscientist* 2001;7:166–177.
- Shieh C-C, Coghlan M, Sullivan JP, Gopalakrishnan M: Potassium channels: molecular defects, diseases, and therapeutic opportunities. *Pharmacol Rev* 2000;52:557–593.
- Hewawasam P, Fan W, Ding M, Flint K, Cook D, Goggins GD, Myers RA, Gribkoff VK, Boissard CG, Dworetzky SI, Starrett JE Jr, Lodge NJ: 4-Aryl-3-(hydroxyalkyl)quinolin-2-ones: novel maxi-K channel opening relaxants of corporal smooth muscle targeted for erectile dysfunction. *J Med Chem* 2003;46:2819–2872.
- Hewawasam P, Erway M, Moon SL, Knipe J, Weiner H, Boissard CG, Post-Munson DJ, Gao Q, Huang S, Gribkoff VK, Meanwell NA: Synthesis and structure–activity relationships of 3-aryloxindoles: a new class of calcium-dependent, large conductance potassium (maxi-K) channel openers with neuroprotective properties. *J Med Chem* 2002;45:1487–1499.
- Hewawasam P, Erway M, Thalody G, Weiner H, Boissard CG, Gribkoff VK, Meanwell NA, Lodge N, Starrett JE Jr: The synthesis and structure–activity relationships of 1,3-diaryl-1,2,4-(4H)-triazol-5-ones: a new class of calcium-dependent, large conductance, potassium (maxi-K) channel opener targeted for urge urinary incontinence. *Bioorg Med Chem Lett* 2002;12:1117–1120.
- Wallner M, Meera P, Toro L: Calcium-activated potassium channels in muscle and brain. In: *Potassium Ion Channels: Molecular Structure, Function, and Diseases*. Kurachi Y, Jan LY, Lazdunski M (eds). Current Topics in Membranes 46:117–140, 1999.
- Schroeder K, Neagle B, Trezise DJ, Worley J: IonWorks HT: A new high-throughput electrophysiology measurement platform. *J Biomol Screen* 2003;8:50–64.
- Asmild M, Oswald N, Krzyzkowski KM, Friis S, Jacobsen RB, Reuter D, Taboryski R, Kutchinsky J, Vestergaard RK, Schroeder RL, Sorensen CB, Bech M, Korsgaard MPG, Willumsen NJ: Upscaling and automation of electrophysiology: toward high throughput screening in ion channel drug discovery. *Receptors Channels* 2003;9:49–58.
- Schnizler K, Kuester M, Methfessel C, and Fejtl M: The Robococyte: automated cDNA/mRNA injection and subsequent TEVC recording on *Xenopus* oocytes in 96-well microtiter plates. *Receptors Channels* 2003;9:41–48.
- Trumbull JD, Maslana ES, McKenna DG, Nemcek TA, Niforatos W, Pan JY, Parihar AS, Shieh C-C, Wilkins JA, Briggs CA, Bertrand D: High throughput electrophysiology using a fully automated, multiplexed recording system. *Receptors Channels* 2003;9:19–28.
- Kiss L, Bennett PB, Uebele VN, Koblan KS, Kane SA, Neagle B, Schroeder K: High throughput ion-channel pharmacology: planar-array-based voltage clamp. *Assay Drug Dev Technol* 2003;1:127–135.
- Klemic KG, Klemic JF, Reed MA, Sigworth FJ: Micro-molded PDMS planar electrode allows patch clamp electrical recordings from cells. *Biosens Bioelectron* 2002;17:597–604.
- Falconer M, Smith F, Surah-Narwal S, Congrave G, Liu Z, Hayter P, Ciaramella G, Keighley W, Haddock P, Waldron G, Sewing A: High-throughput screening for ion channel modulators. *J Biomol Screen* 2002;7:460–465.
- Worley JF, Main MJ: An industrial perspective on utilizing functional ion channel assays for high throughput screening. *Receptors Channels* 2002;8:269–282.
- Whiteaker KL, Gopalakrishnan SM, Groebe D, Shieh C-C, Warrior U, Burns DJ, Coghlan MJ, Scott VE, Gopalakrishnan M: Validation of FLIPR membrane potential dye for high throughput screening of potassium channel modulators. *J Biomol Screen* 2001;6:305–312.
- Terstappen GC: Functional analysis of native and recombinant ion channels using a high-capacity nonradioactive rubidium efflux assay. *Anal Biochem* 1999;272:149–155.
- Tang W, Kang J, Wu X, Rampe D, Wang L, Shen H, Li Z, Dunnington D, Garyantes T: Development and evaluation of high throughput functional assay methods for hERG potassium channel. *J Biomol Screen* 2001;6:325–331.
- Scott CW, Wilkins DE, Trivedi S, Crankshaw DJ: A medium throughput functional assay of KCNQ2 potassium channels using rubidium efflux and atomic absorption spectrometry. *Anal Biochem* 2003;319:251–257.
- Shieh C-C, Feng J, Buckner SA, Brioni JD, Coghlan MJ, Sullivan JP, Gopalakrishnan M: Functional implication of spare ATP-sensitive K⁺ channels in bladder smooth muscle cells. *J Pharmacol Exp Ther* 2001;296:669–675.
- Shieh C-C, Gopalakrishnan M: Electrophysiological anal-

- ysis of ATP-sensitive potassium channels in mammalian cells and *Xenopus* oocytes. *Curr Protocols Pharmacol* 2003;11.6.1–11.6.25.
23. Molinari EJ, Sullivan JP, Wan YP, Brioni JD, Gopalakrishnan M: Characterization and modulation of [¹²⁵I]iberiotoxin-D19Y/Y36F binding in the guinea-pig urinary bladder. *Eur J Pharmacol* 2000;388:155–161.
 24. Gribkoff VK, Starrett JE Jr, Dworetzky SI, Hewawasam P, Boissard CG, Cook DA, Frantz SW, Heman K, Hibbard JR, Huston K, Johnson G, Krishnan BS, Kinney GG, Lombardo LA, Meanwell NA, Molinoff PB, Myers RA, Moon SL, Ortiz A, Pajor L, Pieschl RL, Post-Munson DJ, Signor LJ, Srinivas N, Taber MT, Thalody G, Trojnacki JT, Wiener H, Yeleswaram K, & Yeola SW: Targeting acute ischemic stroke with a calcium-sensitive opener of maxi-K potassium channels. *Nat Med* 2001;7:471–477.
 25. Hewawasam P, Ding M, Chen N, King D, Knipe J, Pajor L, Ortiz A, Gribkoff VK, Starrett J: Synthesis of water-soluble prodrugs of BMS-191011: a maxi-K channel opener targeted for post-stroke neuroprotection. *Bioorg Med Chem Lett* 2003;13:1695–1698.

Address reprint requests to:

Murali Gopalakrishnan, Ph.D.

Neuroscience Research

Department R47W, Building AP9A-3

Global Pharmaceutical Research and Development

Abbott Laboratories

100 Abbott Park Road

Abbott Park, IL 60064-6125

E-mail: murali.gopalakrishnan@abbott.com