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RESEARCH PAPER

A-272651, a nonpeptidic blocker of large-conductance Ca^{2+}-activated K^+ channels, modulates bladder smooth muscle contractility and neuronal action potentials

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Background and Purpose: The large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}, $K_{Ca}1.1$) links membrane excitability with intracellular Ca^{2+} signaling and plays important roles in smooth muscle contraction, neuronal firing, and neuroendocrine secretion. This study reports the characterization of a novel BK_{Ca} channel blocker, 2,4-dimethoxy-N-naphthalen-2-yl-benzamide (A-272651).
Experimental Approach: $^{86}Rb^+$ efflux in HEK-293 cells expressing BK_{Ca} was measured. Effects of A-272651 on BK_{Ca} α- and BK_{Ca} αβ1-mediated currents were evaluated by patch-clamp. Effects on contractility were assessed using low-frequency electrical field stimulated pig detrusor and spontaneously contracting guinea pig detrusor. Effects of A-272651 on neuronal activity were determined in rat small diameter dorsal root ganglia (DRG).
Key Results: A-272651 (10 μM) inhibited $^{86}Rb^+$ efflux evoked by NS-1608 in HEK-293 cells expressing BK_{Ca} currents. A-272651 concentration-dependently inhibited BK_{Ca} currents with IC_{50} values of 4.59 μM (Hill coefficient 1.04, measured at +40 mV), and 2.82 μM (Hill coefficient 0.89), respectively, for BK_{Ca} α and BK_{Ca} αβ1-mediated currents. Like iberiotoxin, A-272651 enhanced field stimulated twitch responses in pig detrusor and spontaneous contractions in guinea pig detrusor with EC_{50} values of 4.05 ± 0.05 and 37.95 ± 0.12 μM, respectively. In capsaicin-sensitive DRG neurons, application of A-272651 increased action potential firing and prolonged action potential duration.
Conclusions and Implications: These data demonstrate that A-272651 modulates smooth muscle contractility and neuronal firing properties. Unlike previously reported peptide BK_{Ca} blockers, A-272651 represents one of the first small molecule BK_{Ca} channel blockers that could serve as a useful tool for further characterization of BK_{Ca} channels in physiological and pathological states.
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Keywords: A-272651; potassium channel; large-conductance Ca^{2+}-activated K^+ channel; bladder; BK_{Ca}; dorsal root ganglia; smooth muscle; sensory neurons; detrusor

Abbreviations: BK_{Ca}, large-conductance Ca^{2+}-activated K^+ channel; BZD, benzodiazepine receptor; DRG, dorsal root ganglia; hCB_2, human cannabinoid receptor; IbTx, iberiotoxin; IK_{Ca}, intermediate-conductance Ca^{2+}-activated K^+ channel; $^{86}Rb^+$, rubidium; SK_{Ca}, small-conductance Ca^{2+}-activated K^+ channel

Introduction

Ca^{2+}-activated K^+ channels (K_{Ca}), which are Ca^{2+}-sensitive and voltage-gated, play important roles in regulating the activity of smooth muscle tissues including that of the urinary bladder. On the basis of Ca^{2+}-sensitivity, voltage dependency and conductance, three subtypes of K_{Ca} channels – large-conductance Ca^{2+}-activated K^+ channel ($K_{Ca}1.1$; BK_{Ca}), intermediate-conductance ($K_{Ca}3.1$; IK_{Ca}) and small-conductance ($K_{Ca}2.1$–$K_{Ca}2.3$; SK_{Ca}) channels have been described (Toro *et al.*, 1998; Stocker, 2004; Turner and Shieh, 2006). In smooth muscles such as those from the urinary bladder, BK_{Ca} channels regulate resting membrane potential and initiate action potential repolarization to limit contraction frequency and amplitude, thus playing significant roles in cholinergic and purinergically mediated

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contractility (Heppner *et al*., 1997; Werner *et al*., 2007). Smooth muscle BK_{Ca} channels are composed of the pore-forming *KCMA1* (α-subunit) and *KCMB1* encoding an auxiliary β1-subunit that modulates kinetics properties and Ca^{2+}/voltage-sensitivity (Hanner *et al*., 1997; Toro *et al*., 1998). In β1-subunit knockout mice where BK_{Ca} activities in bladder smooth muscle cells were greatly reduced, this smooth muscle displayed elevated phasic contraction amplitude and decreased frequency compared to bladder smooth muscle strips from the wild-type mice (Petkov *et al*., 2001). Likewise, ablation of the α-subunit leads to enhanced myogenic and nerve-mediated contractility and increased urination frequency (Meredith *et al*., 2004; Thorneloe *et al*., 2005). In contrast, injection with *KCMA1* gene into obstructed rats ameliorated the hyperactivity of the urinary bladder (Christ *et al*., 2001). These results suggest that BK_{Ca} channels play significant roles in the regulation of phasic activity of the urinary bladder.

In addition to smooth muscle contractility, BK_{Ca} channels also play important roles in action potential duration and shaping spiking pattern of neurons in hippocampus (Shao *et al*., 1999) and dorsal root ganglia (DRG) (Scholz *et al*., 1998; Zhang *et al*., 2003). In mice lacking BK_{Ca} channels, cerebellar ataxia in the form of abnormal conditioned eye-blink reflex, abnormal locomotion and pronounced deficiency in motor coordination were observed (Sausbier *et al*., 2004). Lack of precise timing frequency in inner hair cells and reduction in maximum spike rates in auditory nerve fibres were also observed in the mice with ablation of the BK_{Ca} α-subunit that leads to progressive hearing loss (Ruttiger *et al*., 2004; Oliver *et al*., 2006).

Although gene knockout experiments have indicated a clear role of BK_{Ca} channels in smooth muscle and neuronal function, pharmacological tools for the study of BK_{Ca} channels have thus far been limited. Compounds initially claimed as BK_{Ca} channel openers are relatively weak or are known to possess ancillary pharmacology, which limits their utility as probes to study therapeutic relevance of BK_{Ca} channels (Edwards *et al*., 1994; Schroder *et al*., 2001). Earlier known openers include the glycosylated triterpene activator, dehydrosoyasaponin-I extracted from the medicinal herb, *Desmodium adscendens* (McManus *et al*., 1993) and several benzimidazolone analogues such as NS-004 and NS-1619 (Olesen *et al*., 1994), which stimulate BK_{Ca} activity leading to membrane hyperpolarization. More recently, the BK_{Ca} channel-opening activity of several fluorooxindole, bisphenol, quinolinone, triazolone analogues have been reported (reviewed in Wu, 2003; Turner and Shieh, 2006), and analogues continue to be optimized in terms of both potency and selectivity for potential utility in neurodegenerative and smooth muscle diseases with erectile dysfunction and urinary incontinence.

In contrast to openers, few non-peptide blockers of BK_{Ca} channels are known at present. Iberiotoxin (IbTx) is a selective, high-affinity blocker that served as a valuable tool to characterize BK_{Ca} channels (Kaczorowski *et al*., 1996). Alkaloids such as paxilline, penitrem A and verruculogen are blockers of smooth muscle maxi-K^+ channels and have been shown to increase spontaneous contractility in smooth muscles including those from the urinary bladder (DeFarias *et al*., 1996; Molinari *et al*., 2000). Although a variety of nonpeptide blockers with range of IK_{Ca}/SK_{Ca} selectivities have been identified at the intermediate/small conductance Ca^{2+}-activated K^+ channels (Campos Rosa *et al*., 2000; Malik-Hall *et al*., 2000), organic small molecule blockers of BK_{Ca} channels yet remain to be identified. This study reports on the identification and characterization of A-272651 (2,4-dimethoxy-*N*-naphthalen-2-yl-benzamide) as a novel blocker of BK_{Ca} channels.

Materials and methods

Animal studies

All studies were carried out in accordance with guidelines outlined by the Animal Welfare Act, the Association for Assessment and Accreditation of Laboratory Animals (AAALAC) and the Institutional Animal Care and Use Committee of Abbott Laboratories.

Radioligand binding

Binding of radiolabelled IbTx – [^{125}I]IbTx-D19Y/Y36F (NEN Life Science Products, Boston, MA, USA) was carried out as described previously (Molinari *et al*., 2000) by incubation in a final volume of 500 μl using about 10–20 μg protein per tube at room temperature. In competition experiments, membranes were preincubated with varying concentrations of compounds for 2 h followed by an additional incubation for 2.5 h in the presence of [^{125}I]IbTx-D19Y/Y36F (8 pM). Incubations were terminated by rapid vacuum filtration over GF/B glass fibre filters presoaked in 0.5% polyethyleneimine and filters washed three times with 1.5 ml of ice-cold 50 mM Tris buffer (pH 7.2). Bound radioactivity was quantitated by γ counting spectroscopy at an efficiency of 80%.

Cation flux assays

Rubidium ($^{86}Rb^+$) flux assays were conducted as described previously (Parihar *et al*., 2003) using HEK-293 cells expressing human BK_{Ca} α-subunits. Briefly, cells were loaded with 1.0–2.0 μCi per well of the radiotracer $^{86}Rb^+$ (NEN Life Science Products, Boston, USA), incubated for 18–24 h in culture media and then washed three times with assay buffer containing (mM) 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 20; NaCl, 120; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; $MgSO_4$, 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 30 min incubation with the test compounds. For the measurements of $^{86}Rb^+$ efflux, supernatants were harvested and saved in 96-well Opti-Plates (Packard Bioscience, Meriden, CT, USA). Cells were subsequently lysed with 1.0 N NaOH and the supernatants again saved in another 96-well Opti-Plate. EcoLume liquid scintillation fluid (ICN, Costa Mesa, CA, USA) was added in both sets of supernatants (efflux and lysate), and the plates were counted on a Packard TopCount (Perkin-Elmer Life Sciences, Downers

Grove, IL, USA). Each test concentration of compounds was tested in duplicate wells.

Electrophysiological recordings

BK_{Ca} currents in HEK-293 cells. The patch-clamp technique was used to measure effects on membrane currents in whole-cell configurations as described previously (Parihar *et al*., 2003). Fire-polished patch electrodes had a resistance of 2–5 MΩ when filled with pipette solution, which contained (mM): KCl, 140; $MgCl_2$, 1; $CaCl_2$, 0.1; ethylene glycol bis (2-aminoethylether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid (EGTA), 1; HEPES, 10; (pH 7.2 with 5 N KOH, 285 mOsm). The bath solution contained (mM): NaCl, 135; KCl, 5; $CaCl_2$, 2.5; $MgCl_2$, 1.2; HEPES, 5 (pH 7.4 with 5 N NaOH, 310 mOsm). HEK-293 cells transfected with BK_{Ca} α and BK_{Ca} αβ1-subunits were voltage-clamped at a holding potential of −80 mV and the ionic current was measured at test potentials from −40 to +100 mV for 400 ms (20 mV each step). The whole-cell currents were amplified using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) 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.

Action potential recordings in dorsal root ganglion neurons. DRG from L6 and S1 were harvested from male Sprague–Dawley rats (200–250 g, Charles River, Wilmington, MA, USA) (Zhang *et al*., 2003). Briefly, DRG neurons were dissociated enzymatically with Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Rockville, MD, USA) containing 0.1% collagenase for 20 min. This was followed by incubation at 37°C with 0.1% collagenase/dispase for 10 min and then with 2.5% trypsin for 10 min. Individual DRG neurons were suspended in enzyme-free DMEM, triturated with fire-polished pipettes and plated in polyethyleneimine treated 24 well-plates supplemented with 10% fetal bovine serum, 50 nM nerve growth factor, 2 mM glutamine and 100 U ml^{-1} penicillin–streptomycin. Neurons were cultured at 37°C in an atmosphere of 5% CO_2/95% O_2 and 90% humidity for electrophysiological characterization within 48 h. Whole-cell current clamp was used to record changes in action potential firing (Zhang *et al*., 2003). Current clamp recording was obtained by switching to current clamp mode after a stable whole-cell configuration was established in voltage-clamp mode. Pipette solution contained (mM): KCl 140, $MgCl_2$ 2, EGTA 10, HEPES 10, pH 7.2 adjusted with KOH (osmolarity, 285 mOsm). The external solution contained (mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, HEPES 10, pH 7.4 adjusted with NaOH (osmolarity, 310 mOsm). Action potentials were evoked by 300 ms depolarizing pulses from 0.1 up to 0.6 nA at 0.1 nA steps and were filtered at 2 kHz and sampled at 10 kHz. Only cells with a stable resting membrane potential (more negative than −50 mV) were used in this study.

Isolated bladder smooth muscle studies

Studies on detrusor preparations were carried out as described previously (Buckner *et al*., 2000; Shieh *et al*., 2001). Briefly, urinary bladders from Landrace pigs (9–25 kg, Wilson's PrairieView Farm, Burlington, WI, USA) killed with pentobarbital, 150–200 mg kg^{-1}, Somlethol (JA Webster Inc., Sterling MA, USA) or guinea pigs (250–300 g, Hartley, Charles River Laboratories) were removed and immediately placed in Krebs–Ringer–bicarbonate solution. Muscle strips, 3–5 mm in width and 10 mm in length, dissected free of mucosa were prepared from the bladder tissue by cutting in a circular manner. One end of the strip was fixed to a stationary glass rod and the other was attached to a Grass FT03 transducer at a basal preload of 1.0 g in a 10 ml organ bath chamber. This preload proved to be the best condition for a steady-state baseline. Tissues were allowed to equilibrate for at least 60 min before the assays. In studies with pig detrusor smooth muscle strips, electrical field stimulation (0.05 Hz, 0.5 ms, 20 V) was applied after equilibration via two parallel platinum electrodes included in the stationary rod. In studies with guinea-pig tissues, following equilibration, an additional 15 mM K^+ was added (from stock solution of 3 or 4 M KCl) to maintain a final extracellular K^+ of 20 mM, which sustained regular phasic activity of the smooth muscle (Malysz *et al*., 2004). Cumulative concentration–response curves were generated for each tissue and each tissue was exposed to only one test compound (Buckner *et al*., 2000).

Pharmacological selectivity assays

The activity of A-272651 (10 μM) was evaluated in assays to assess pharmacological selectivity relative to other cell-surface receptors, ion channels, transport sites and enzymes (a total of 75 targets) by use of standardized assay protocols (CEREP, Poitiers, France) as described previously (El Kouhen *et al*., 2005).

Data analysis

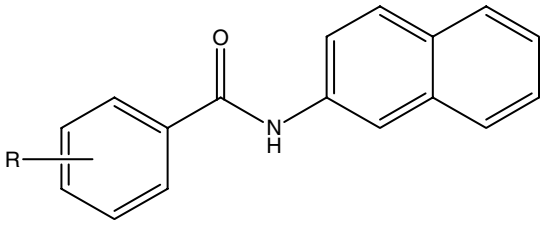
The percent Rb release was defined by measuring the amount of $^{86}Rb^+$ released into the well after stimulation divided by the total amount of $^{86}Rb^+$ loaded per well (% $^{86}Rb^+$ release = ($^{86}Rb^+$ in buffer of stimulated cells/$^{86}Rb^+$ in buffer from lysed cells) × 100). The concentration dependence of changes in $^{86}Rb^+$ efflux or tension responses was fitted by nonlinear regression analysis (GraphPad Prism, San Diego, CA, USA) to obtain IC_{50} values. Guinea-pig spontaneous myogenic activity was analysed for changes in the area under the contractile response curve during a 15 min interval. In electrical field-stimulated tissues of pig, the concentration-dependent change in the peak amplitude of the response (measured in grams) was used for calculating potencies. Results are expressed as means ± s.e.m. Significant differences between groups of means were assessed by the unpaired Student's *t*-test.

Materials

2,4-Dimethoxy-*N*-naphthalen-2-yl-benzamide (A-272651), related analogues (see Table 1) and NS-1608 were synthesized at Abbott Laboratories (Abbott Park, IL, USA). All other chemicals and reagents were purchased from Sigma

Chemical Co. (St Louis, MO, USA) unless specified otherwise. Compounds were prepared in DMSO (Sigma) as 10 mM stock solutions unless indicated otherwise, kept protected from light and serial dilutions were prepared in appropriate assay buffer just before use.

Table 1 Electrophysiological assignment of BK_{Ca} opening activity in HEK-293 cells stably transfected with BK_{Ca} α-subunits



Compound	*Name*	*R*	*Effect on outward current in the presence of compound (10 μM) data expressed as % over control*
1	A-272651	2, 4-OCH_3	$-69.0 \pm 2.4\%$
2		2-OCH_3, 4-Cl	$-64.6 \pm 5.3\%$
3		2-OCH_3, 5-$C(CH_3)_3$	$481 \pm 204\%$
4	A-411873	—	$107.8 \pm 24.5\%$[a]
5	NS-8	—	$21.7 \pm 7.0\%$[a]
6	NS-1608	—	$503.8 \pm 18.42\%$

Abbreviation: Bk_{ca}, large-conductance Ca^{2+}-activated $K+$ channel.
[a]Previously reported in Turner *et al*. (2003). Minus sign indicates inhibition of current responses.

Results

A-272651 enhances [^{125}I]IbTx binding

In initial high-throughput screening efforts, A-272651 (Figure 1) was identified as a compound that enhanced the binding of the radioligand, [^{125}I]IbTx-D19Y/Y36F. Unlike unlabelled IbTx that displaced radioligand binding, A-272651 increased specific [^{125}I]IbTx-D19Y/Y36F binding by about 3.5-fold at 1 μM ($n=4$; $EC_{50}=0.29$ μM). This increase in binding is akin to that observed previously with indole alkaloids such as paxilline and verruculogen under similar assay conditions (Molinari *et al*., 2000).

Inhibition of BK_{Ca} channel-mediated $^{86}Rb^+$ efflux

As reported previously (Parihar *et al*., 2003), various structurally diverse BK_{Ca} openers belonging to the aryl pyrrole, benzimidazolone, diphenylurea and aryl hydroxy oxindole classes have been shown to stimulate IbTx-sensitive cation flux in HEK-293 cells expressing BK_{Ca} α-subunit. Consistent with earlier observations from our laboratory, NS-1608, a diphenylurea BK_{Ca} opener, evoked concentration-dependent $^{86}Rb^+$ efflux from transfected HEK-293 cells with an EC_{50} value of 2.71 ± 1.36 μM ($n=6$). As shown in Figure 2, preincubation with 10 μM A-272651, before addition of various concentrations of NS-1608, completely abolished $^{86}Rb^+$ efflux responses.

Inhibition of BK_{Ca} channel-mediated current responses

HEK-293 cells expressing BK_{Ca} α-subunit were voltage-clamped from a holding potential of −80 mV and ionic currents were measured from test membrane potential of

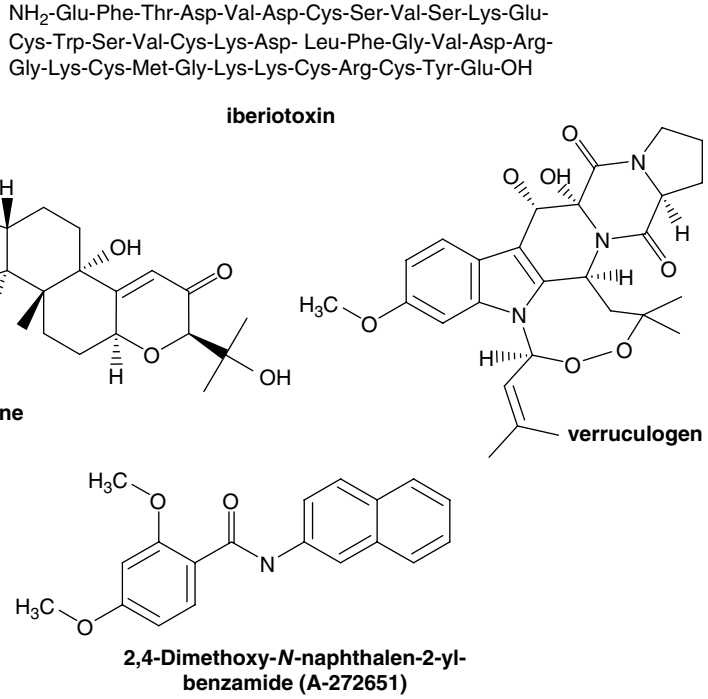


Figure 1 Structure of A-272651 and other representative large-conductance calcium-activated K^+ (BK_{Ca}) channel blockers.

−40 to +100 mV for 400 ms by whole-cell patch clamp. Steep voltage-dependent increases in ionic currents were recorded when test potentials were above −20 mV (Figure 3). Addition of A-272651 resulted in a concentration-dependent inhibition of BK_{Ca} currents in HEK-293 cells expressing BK_{Ca} α-subunit alone or both α- and β1-subunits (Figure 3). The IC_{50} values for A-272651 to inhibit BK_{Ca} α and BK_{Ca} αβ1 currents were 4.59±0.07 μM (Hill coefficient = 1.04, measured at +40 mV, $n = 4$) and 2.82±0.09 μM (Hill coefficient = 0.89, $n = 6$), respectively (Figure 3c). These results suggest that coexpression of the β1-subunit exerts no substantial effects on the inhibitory effects of A-272651.

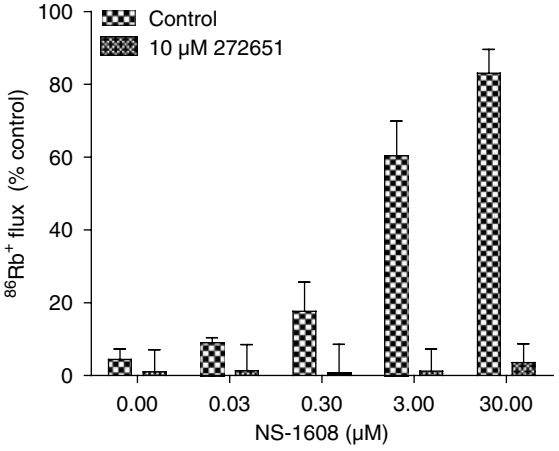


Figure 2 A-272651 suppressed $^{86}Rb^+$ efflux evoked by NS-1608. Activation of BK_{Ca} evoked $^{86}Rb^+$ efflux by NS-1608 in HEK-293 cells expressing BK_{Ca}-α-subunit in the absence and presence of 10 μM A-272651 is shown. Data are from means of 3–4 experiments. Cells were loaded with $^{86}Rb^+$, as described under Materials and methods and the effects of A-272651 were assessed during 30 min incubation.

As shown in Table 1, the 4-chloro analogue of A-272651 (compound 2 in Table 1) also showed comparable inhibition of BK_{Ca} current (see Table 1). Interestingly, when a t-butyl substituent was introduced (compound 3, Table 1), the resulting analogue was found to significantly increase BK_{Ca} currents (481% at 10 μM). The observed enhancement in current responses is comparable to that observed with NS-1608 (504±18%, $n = 5$), and substantially higher than that observed with the previously reported amino-azaindole A-411873 (108%) (compound 28 in Turner *et al*., 2003; Turner and Shieh, 2006), and with NS-8 (22%) under similar experimental conditions. Within this structural series, it is thus possible to produce both strong inhibitors and enhancers of BK_{Ca} currents by judicious selection of aryl substituents.

A-272651 increases bladder smooth muscle contractility

To assess the functional effects of A-272651 on smooth muscle contractility, effects on myogenic phasic activity and on electrical field stimulated bladder smooth muscle were assessed. In the presence of 20 mM extracellular K^+, the guinea-pig detrusor smooth muscle segments exhibit spontaneous phasic contractile activity, which can be augmented by IbTx and attenuated by compounds such as NS-8 and NS-1619 suggesting the participation of BK_{Ca} channels (Buckner *et al*., 2002). As illustrated by Figure 4a, A-272651

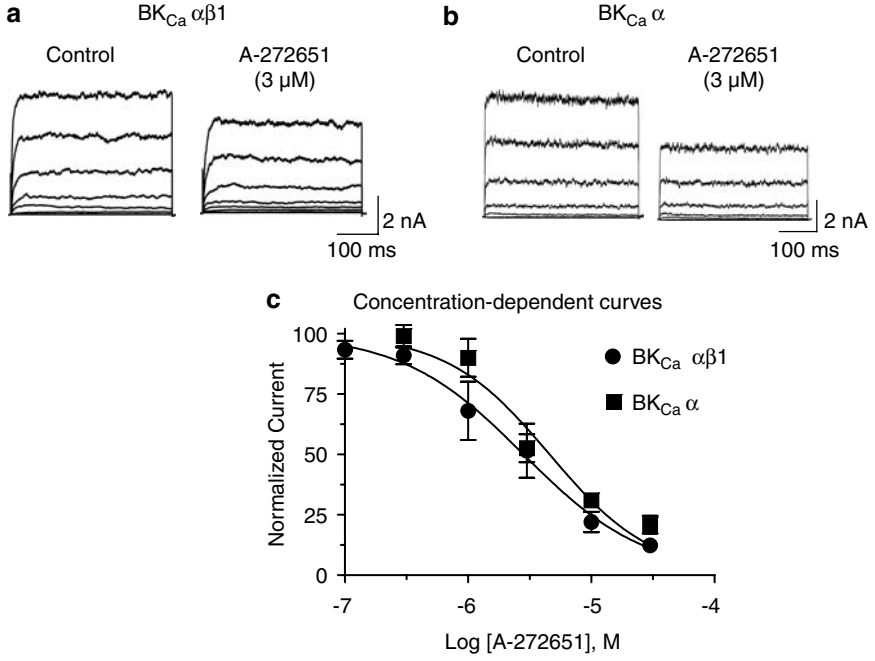


Figure 3 Inhibition of BK_{Ca} currents by A-272651. Membrane currents, mediated by BK_{Ca} αβ1-subunits (**a**) and BK_{Ca} α-subunit stably transfected in HEK-293 cells (**b**), were evoked by testing potentials from −40 to +100 mV for 400 ms (20 mV each step) from a holding potential of 80 mV in control and in the presence of 3 μM A-272651. (**c**) Shows the concentration-dependent inhibition of currents, with IC_{50} values of 4.59±0.07 μM (Hill coefficient = 1.04, measured at +40 mV) for BK_{Ca} α and 2.82±0.09 μM (Hill coefficient = 0.89) for BK_{Ca} αβ1, respectively.

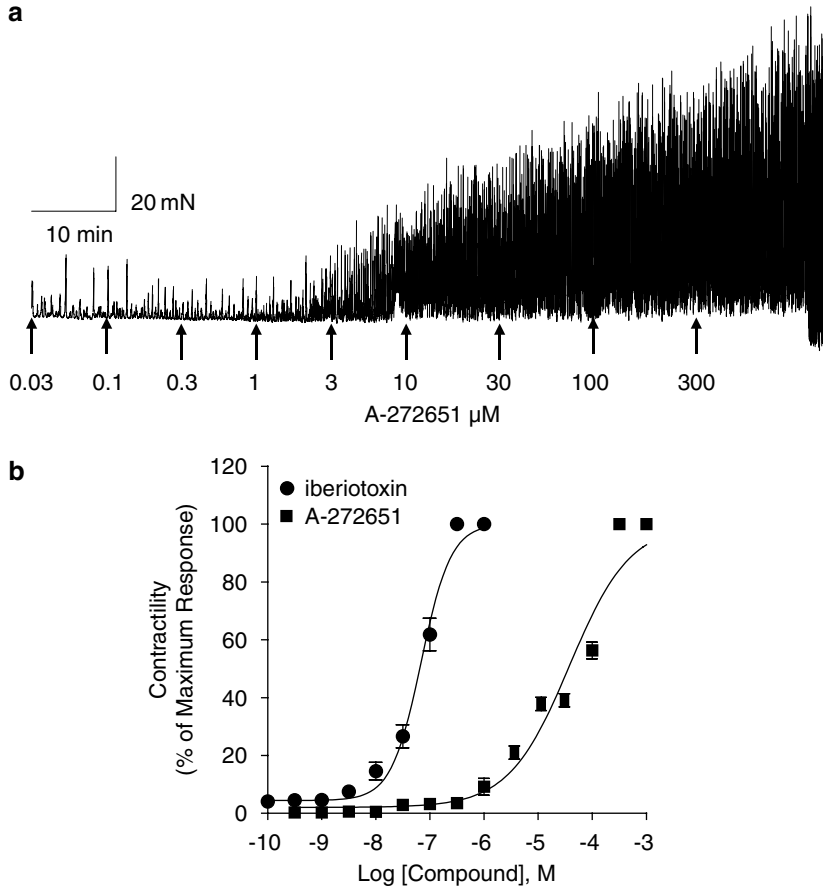


Figure 4 The effect of A-272651 and IbTx on the spontaneous phasic activity of guinea-pig detrusor. (**a**) A-272651 evoked concentration-dependent increases in spontaneous phasic contractions starting at concentration of 1 μM and attaining maximal levels comparable to IbTx. (**b**) The EC_{50} value for A-272651 to enhance spontaneous phasic contraction is 37.95 ± 0.12 μM, which is about 500-fold higher than the EC_{50} of IbTx (0.068 ± 0.04 μM). The data are mean values from four experiments.

increased spontaneous bladder contractions in a concentration-dependent manner. The maximal increase in contractility was comparable to that observed with IbTx, whereas the EC_{50} value was about 500-fold higher than IbTx itself. As reported previously from our laboratory, low-frequency stimulus (0.05 Hz) produced a continuous transient twitch response in the pig detrusor that can be effectively abolished by potassium channel openers such as the potent K_{ATP} channel-opener P1075 (Buckner *et al*., 2000) and BK_{Ca} openers such as NS-1619 (Malysz *et al*., 2004). Under similar conditions, A-272651 increased twitch responses in a concentration-dependent manner with an EC_{50} value of 4.66 ± 0.06 μM (Figure 5).

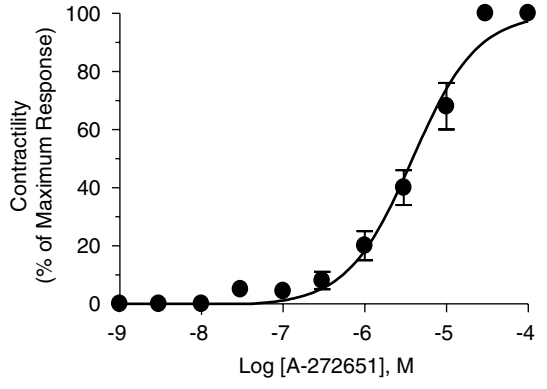


Figure 5 The effect of A-272651 on electrical field-stimulated contractility of the pig detrusor. A-272651 evoked concentration-dependent increases in contractility evoked by low frequency of field stimulation in pig detrusor with an EC_{50} value of 4.05 ± 0.05 μM (Hill coefficient $= 1.11 \pm 0.11$, $n = 4$).

A-272651 increases action potential firing in DRG neurons

We also examined the effects of A-272651 in DRG neurons. In capsaicin-sensitive small-diameter DRG neurons, action potential responses were evoked by injecting threshold current (200–300 pA). Application of 3 μM A-272651 increased the firing frequency and prolonged action potential duration of DRG neurons (Figure 6). As shown in Figure 6c, the effects were readily reversed upon washout.

Pharmacological selectivity

To determine the specificity of A-272651, the compound was profiled across a panel of *in vitro* binding assays (CEREP, Poitiers, France). These assays included G protein-coupled receptors, enzymes, transporters and ion channels. A-272651

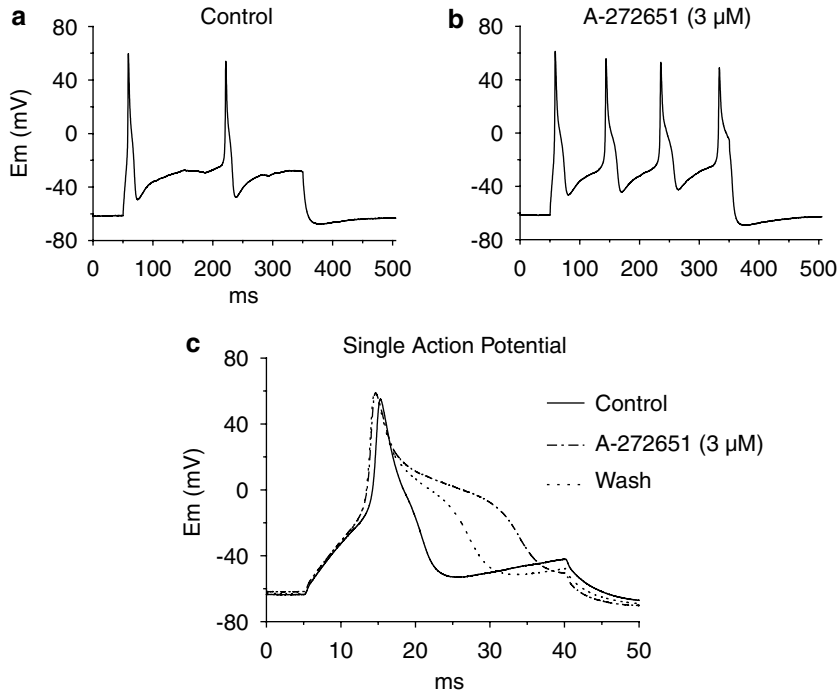


Figure 6 The effect of A-272651 on firing activity of sensory neurons from DRG. Action potentials were evoked in control (**a**) or in the presence of 3 μM A-272651 (**b**), in capsaicin-sensitive small-diameter DRG neurons. A-272651 significantly enhanced firing frequency in sensory DRG neurons. Single action potential analysis revealed that A-272651 induced a reversible prolongation of action potential duration (**c**). The recordings shown are representative of multiple recordings ($n = 4$).

(10 μM) was found to be inactive at most of the tested targets including voltage-gated K^+ channels, small-conductance Ca^{2+}-activated K^+ channels, L-type voltage-gated Ca^{2+}-channels and voltage-gated Na^+ channels. Some degree of interaction was observed at the concentration tested (10 μM) with adenosine receptor type 3 (94%; percent inhibition of control specific binding), peripheral (benzodiazepine receptor (86%), human CB_2 (cannabinoid receptor, 87%) and human $5HT_{1A}$ (5-hydroxytryptamine receptor, 81%).

Discussion

Apart from peptide toxins such as IbTx, some indole alkaloids such as paxilline and verruculogen (Figure 1), are thus far the best characterized blockers of BK_{Ca} channels (Turner and Shieh, 2006). In contrast to previously reported blockers of the BK_{Ca} channel, A-272651 is a low-molecular-weight biaryl amide. A-272651 and related analogues are prepared by single-step amide coupling reactions from the corresponding naphthyl amine and substituted benzoic acid starting materials. In this study, interactions of A-272651 with BK_{Ca} channels were demonstrated across a number of assays including cation flux, whole-cell patch clamp in BK_{Ca} expressing cells and primary DRG neurons and tissue reactivity studies in bladder smooth muscle.

The IC_{50} values of A-272651 to inhibit BK_{Ca} α- and $\alpha\beta$1-mediated currents (4.59 and 2.82 μM respectively) are $\sim$1000-fold higher than that of IbTx (1.7 nM) and paxilline (1.9 nM) (Kaczorowski *et al*, 1996; Sanchez and McManus, 1996). In addition to modifying channel-gating properties and calcium sensitivity, the β-subunits can influence BK_{Ca} pharmacology. For example, it has been shown that β-subunits can influence interactions of toxins such as charybdotoxin (Hanner *et al*., 1997). Likewise, changes in pharmacological properties of BK_{Ca} channels in the presence of the β1-subunit have also been revealed for the peptide toxin IbTx and the alkaloid blocker tetrandrine (Dworetzky *et al*., 1996). Although A-272651 is 1.6-fold more potent in blocking BK_{Ca} $\alpha\beta$1-mediated currents compared with BK_{Ca} α-mediated currents, the difference was not statistically significant, suggesting that the β1-subunit has no substantial effects on interaction of A-272651 with the channel.

It is known that membrane-bound Ca^{2+} can influence the binding interactions of [^{125}I]IbTx-D19Y/Y36F with BK_{Ca} channels (Knaus *et al*., 1994; Molinari *et al*., 2000). In our previous studies, paxilline and verruculogen both increased [^{125}I]IbTx-D19Y/Y36F binding to some 400% above control values when assays were performed in the absence of ethylenediaminetetraacetic acid (EDTA). Interestingly, in the presence of EDTA, the modulatory effect of these alkaloids was not observed. The binding interactions of A-272651 reported in this study appears to be similar to that reported for the indole alkaloids with significant potentiation of [^{125}I]IbTx-D19Y/Y36F in the presence of EDTA. The fact that removal of membrane-bound Ca^{2+} by EDTA abolishes the effect of indole alkaloids as reported previously, suggests that Ca^{2+} is required for the observed potentiatory effect on IbTx binding. While chelating agents (EDTA, EGTA) at micromolar concentrations increase the binding affinity by removal of bound Ca^{2+}, indole alkaloids (paxilline,

verruculogen) at nanomolar concentrations may increase the binding affinity by an allosteric interaction that increases the toxin affinity or indirectly by decreasing membrane-bound Ca^{2+} interactions with the BK_{Ca} channel.

While we have not examined functionally for effects on other Ca^{2+}-activated K^+ channels, A-272651 was evaluated in a panel of receptor binding assays for more than 70 diverse neurotransmitter receptor and ion channel sites (CEREP, France). No significant displacement of L-type voltage-gated Ca^{2+}-channels (verapamil site) and small conductance Ca^{2+}-activated ligand (apamin) binding sites was noted at 10 μM. In addition, the effect of A-272651 on BK_{Ca} channels appears selective as no significant effect on basal $^{86}Rb^+$ flux, baseline bladder twitch or action potential responses were noted at the concentration ranges examined. It remains to be elucidated whether A-272651 inhibits Ca^{2+}-influx, which could possibly diminish opening probabilities of BK_{Ca} channels in an indirect manner. However, A-272651 evoked spontaneous contraction (Figure 4) and prolonged action potential duration (Figure 6), suggesting that A-272651 appears not to alter Ca^{2+}-regulation, which could indirectly modulate BK_{Ca} channels.

Although A-272651 increased contractility of smooth muscle under both neurogenic and myogenic conditions, differences in potencies are apparent. A-272651 was about 10-fold more potent in increasing twitch responses in electrically driven bladder smooth muscles ($EC_{50} = 4\,\mu$M) compared to increasing contractility under myogenic conditions ($EC_{50} = 38\,\mu$M). Apart from species differences, the nature of two separate models is likely to account for these observations. In guinea-pig detrusor, the elevation of extracellular K^+ is thought to increase contractility by a direct depolarization of the smooth muscle and activation of L-type Ca^{2+}-channels. In contrast, electrical field stimulation excites the intramural nerve to release excitatory neurotransmitters, the action of which elicits characteristic twitches (Buckner *et al*., 2002). Thus, the contractions evoked by K^+-induced depolarization involve only a myogenic component, whereas electrically evoked contractions involve both a neurogenic component and a myogenic component. A likely basis for the differential effect between the two models is the possibility that A-272651 also inhibits neuronal BK_{Ca} channels, in addition to those present at the level of the bladder smooth muscle. This is supported by the observations that A-272651 increased firing frequency in DRG neurons (*vide infra*).

Neuronal BK_{Ca} channels, especially those present in CNS and DRG neurons, are a target for the action of BK_{Ca} openers such as aryloxindolone and benzimidazole analogues. For example, the benzimidazolone NS-1619 increased opening activity of a IbTx-sensitive Ca^{2+}-dependent channel and reversibly suppressed action potential firing, attributable to increases in threshold for evoking action potentials, reductions in action potential amplitude and increases in amplitude of after-hyperpolarization (Zhang *et al*., 2003). These effects potentially underlie the reported reduction in transmitter release and neuroprotective effects with BK_{Ca} openers (Gribkoff *et al*., 2001; Hewawasam *et al*., 2002). In contrast to the observations with BK_{Ca} openers, A-272651 was found to increase firing frequency in DRG neurons, which was readily reversible upon washout, unlike IbTx. Collectively, our studies show that A-272651 can modulate BK_{Ca} channels in both bladder smooth muscle and neurons, and this compound represents one of the first small-molecule BK_{Ca} channel blockers that could serve as useful tools for investigation of BK_{Ca} channels in physiological and pathological states.

Conflict of interest

The authors state no conflict of interest.

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