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RESEARCH PAPER

Effects of the novel BK ($K_{Ca}1.1$) channel opener GoSlo-SR-5-130 are dependent on the presence of BK_{β} subunits

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BACKGROUND AND PURPOSE

GoSlo-SR compounds are efficacious BK ($K_{Ca}1.1$) channel openers, but little is known about their mechanism of action or effect on bladder contractility. We examined the effects of two closely related compounds on BK currents and bladder contractions.

EXPERIMENTAL APPROACH

A combination of electrophysiology, molecular biology and synthetic chemistry was used to examine the effects of two novel channel agonists on BK channels from bladder smooth muscle cells and in HEK cells expressing BKα alone or in combination with either β_1 or β_4 subunits.

KEY RESULTS

GoSlo-SR-5-6 shifted the voltage required for half maximal activation ($V_{1/2}$) of BK channels approximately –100 mV, irrespective of the presence of regulatory β subunits. The deaminated derivative, GoSlo-SR-5-130, also shifted the activation $V_{1/2}$ in smooth muscle cells by approximately –100 mV; however, this was reduced by ~80% in HEK cells expressing only BKα subunits. When β_1 or β_4 subunits were co-expressed with BKα, efficacy was restored. GoSlo-SR-5-130 caused a concentration-dependent reduction in spontaneous bladder contraction amplitude and this was abolished by iberiotoxin, consistent with an effect on BK channels.

CONCLUSIONS AND IMPLICATIONS

GoSlo-SR-5-130 required β_1 or β_4 subunits to mediate its full effects, whereas GoSlo-SR-5-6 worked equally well in the absence or presence of β subunits. GoSlo-SR-5-130 inhibited spontaneous bladder contractions by activating BK channels. The novel BK channel opener, GoSlo-SR-5-130, is approximately fivefold more efficacious on BK channels with regulatory β subunits and may be a useful scaffold in the development of drugs to treat diseases such as overactive bladder.

Abbreviations

BK ($K_{Ca}1.1$) channels, large conductance, Ca^{2+} and voltage-activated K^+ channels; CI, confidence intervals; GoSlo-SR-5-130, 9,10-dioxo-4-((3-(trifluoromethyl)phenyl)amino)-9,10-dihydroanthracene-2-sulfonic acid; GoSlo-SR-5-6, sodium 1-amino-4-((3-trifluoromethylphenyl)amino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate); IbTx, iberiotoxin; n.Po, number of channels (n) multiplied by the open probability; RBSM, rabbit bladder smooth muscle; RBSMC, rabbit bladder smooth muscle cells; $V_{1/2}$, voltage required for half maximal activation of BK channels; $\Delta V_{1/2}$, change in $V_{1/2}$ caused by administration of drug

Tables of Links

TARGETS
BK ($K_{Ca}1.1$) channel
L-type Ca^{2+} channels

LIGANDS		
Carbachol	Evans blue dye	Mallotoxin
Docosahexaenoic acid	Iberiotoxin (IbTx)	NS1619
		Tamoxifen

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Large conductance Ca^{2+} and voltage-activated potassium (BK also known as $K_{Ca}1.1$) channels contribute to a variety of cellular functions ranging from neuronal excitability to the regulation of tone in uterine, gastrointestinal, vascular and urinary tract smooth muscle (Anwer *et al.*, 1993; Imaizumi *et al.*, 1996; 1998; Hollywood *et al.*, 2000; Kyle *et al.*, 2013). Functional channels are formed by at least three different subunits: a pore-forming α-subunit and auxiliary β (Knaus *et al.*, 1994) and γ subunits that regulate Ca^{2+} and voltage sensitivity (Knaus *et al.*, 1994; Brenner *et al.*, 2000; Yan and Aldrich, 2010). Increases in intracellular Ca^{2+} and membrane depolarization can activate BK channels allosterically (Horrigan and Aldrich, 2002) and can contribute to the rapid repolarization phase of smooth muscle action potentials. The activation of BK channels can therefore limit the excitability of bladder smooth muscle cells (Heppner *et al.*, 1997; Hashitani and Brading, 2003; Hristov *et al.*, 2011; 2012; 2013; Petkov, 2011). BKα subunit deletion ($Slo^{-/-}$) in mice leads to increased spontaneous and neurogenic bladder contractions *in vitro* (Meredith *et al.*, 2004; Thorneloe *et al.*, 2005) and when bladder function was examined *in vivo*, $Slo^{-/-}$ mice exhibited increased voiding frequency with subthreshold voiding contractions (Meredith *et al.*, 2004; Thorneloe *et al.*, 2005).

In urinary bladder smooth muscle (UBSM), the BKα subunit is expressed along with β_1 (Petkov *et al.*, 2001) and β_4 subunits in some species (Chen and Petkov, 2009) including humans (Hristov *et al.*, 2011; 2013). The β_1 subunit increases the Ca^{2+} sensitivity and both β subunits significantly alter the kinetics of activation and deactivation (Brenner *et al.*, 2000). Deletion of the β_1 subunit also enhanced the phasic activity of UBSM (Petkov *et al.*, 2001) and a number of studies on human bladder suggest that the loss or reduced function of BK channels may contribute to overactive bladder (OAB)/urinary incontinence (Chang *et al.*, 2010; Hristov *et al.*, 2013). It is therefore apparent that BK channel activators could help in the treatment of OAB.

BK channels are therefore attractive targets for novel drugs and over the past two decades a number of small-molecule BK channel modulators have been developed (Argentieri and Butera, 2006; Nardi *et al.*, 2006; Garcia *et al.*, 2007; Nardi and Olesen, 2007; 2008). These include the Neurosearch compounds, NS1608 (Strøbaek *et al.*, 1996), NS1619 (Olesen *et al.*, 1994) and more recently NS19504 (Nausch *et al.*, 2014). However, no BK channel opener has yet progressed to market as a therapeutic treatment. Three activators have advanced into clinical trials for the treatment of stroke (Jensen, 2002), OAB (Tanaka *et al.*, 2003) and asthma (Mushtaq, 2014). Although BMS-204532 showed promising results in rodent models of stroke, it failed to show efficacy when compared with placebo in a phase 3 study (Jensen, 2002). NS-8 advanced to a phase 2 study for the treatment of OAB (Tanaka *et al.*, 2003); however, it was later terminated due to lack of efficacy at a therapeutic dose. Currently, andolast is the only BK channel modulator currently undergoing clinical trials (Mushtaq, 2014).

The observed lack of efficacy of these BK channel openers led us to design and synthesize a novel series of anilinoanthraquinones (Roy *et al.*, 2012; 2014). This group of small molecules, known as the GoSlo-SR family, are potent, efficacious openers of BK channels and can shift the voltage required for half maximal activation of BK channels ($V_{1/2}$) in excess of −100 mV. Although the structure activity of the GoSlo-SR compounds has been comprehensively studied (Roy *et al.*, 2012; 2014), little is known about their effects on different BK subunits or if regulatory β subunits are essential for their BK agonist activity. Furthermore, no study has yet examined the effect of any of these compounds on spontaneous mechanical activity in the bladder. The current study demonstrates that GoSlo-SR-5-130 required either the regulatory β_1 or β_4 subunits to mediate its full effects and inhibited spontaneous mechanical activity in bladder smooth muscle.

Methods

Animal welfare and ethical statement

All experiments were approved by the Dundalk Institute of Technology Animal Care and Use Committee and were in accordance with EU Directive 2010/63/EU. The urinary bladder was removed from both male and female New Zealand white rabbits after they had been killed by injection of pentobarbitone (120 $mg \cdot kg^{-1}$, i.v.). Thirty-three animals were used in this study. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Cell dispersal

The bladder was immediately removed and placed in Krebs' solution, opened up and the urothelium removed by sharp

dissection. Strips of smooth muscle tissue were cut into 1 mm^3 pieces and placed in Ca^{2+}-free Hanks solution for 30 min at 4°C prior to cell dispersal. Rabbit bladder smooth muscle cells (RBSMCs) were isolated while stirring at 35–37°C in a dispersal medium containing 1 mg of protease (Sigma type XXIV; Arklow, Wicklow, Ireland), 10 mg of trypsin inhibitor (Sigma), 10 mg of BSA (Sigma) and 15 mg of collagenase (Sigma) 5 mL^{-1} Ca^{2+}-free Hanks solution. The tissue suspension was centrifuged at 145× *g* for 1 min and the supernatant removed. Tissue pieces were resuspended in Ca^{2+}-free solution and stirred for ~6 min until single relaxed RBSMC were released, plated in Petri dishes containing 100 μM Ca^{2+} and stored at 4°C until use.

Ion channel cloning and cell culture

Total RNA was isolated from rabbit urethral smooth muscle (RUSM) strips using the RNeasy mini kit (Qiagen, Crawley, West Sussex, UK). 5′ and 3′ cDNA fragments of the α subunit of the rabbit BK channel were synthesized using the SMARTer RACE kit (Clontech, Saint-Germain-en-Laye, France). Amplicons were cloned into pcDNA TOPO (Life Technologies, Paisley, UK) and the two fragments were combined through a unique BsrGI site using standard molecular biology procedures. The identified transcript corresponded to the ZERO variant of mouse BKα and to variant 2 (NM_002247.3) of human BKα. cDNA encoding the β_1 subunit was similarly synthesized from RUSM total RNA through a one-step amplification procedure. cDNA encoding the β_4 subunit was likewise synthesized from whole rabbit brain total RNA, again utilizing a one-step amplification procedure. HEK-293 cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 1% penicillin/streptomycin at 37°C in a 5% CO_2 95% air-humidified incubator. cDNAs encoding BKα or the BKα/β complexes were co-transfected into HEK-293 cells along with plasmids encoding GFP using a standard calcium phosphate method. Currents were recorded 48 h post-transfection.

Electrophysiology recordings

All experiments were carried out between 35 and 37°C. Voltage ramps were used to elicit BK channel openings in inside out patches of membrane excised from RBSMC, and voltage steps were used to record currents from HEK cells expressing BKα and BKαβ subunits. Electrodes were pulled from Corning borosilicate glass (1.5 mm O.D. × 0.86 mm I.D. for excised patches; 1.5 mm O.D. × 1.17 mm I.D. for whole cell; Harvard Apparatus, Edenbridge, Kent, UK) using a Sutter P-97 pipette puller (Sutter Instruments, Novato, CA, USA) and were fire polished. Standard single-channel patch clamp recording methods were used in the inside out patch conformation (Hamill *et al.*, 1981). Voltage clamp commands were delivered via Axopatch 1D or Axopatch 200B patch clamp amplifiers (Axon Instruments; Molecular Devices, Wokingham, Berkshire, UK) connected to Digidata 1322A AD/DA converters (Axon Instruments) interfaced to a computer running pClamp software (Axon Instruments). Data were acquired at 10 kHz and filtered at 2 kHz. Patches were held at –60 or –100 mV (as denoted in the text), and the patch potential was ramped from either –100 to +100 or –50 to +150 mV over 2 s before returning to –100 mV. The –50 to +150 mV ramps were normally used in 100 nM Ca^{2+} and in low drug concentrations (<3 μM), whereas the –100 to +100 mV ramps were normally used for higher drug concentrations (3–30 μM). All drugs were applied to the patches in 100 nM Ca^{2+}. Voltage ramps were repeated 15 times and currents were averaged, divided by the single-channel conductance at each voltage and thus corrected for driving force to obtain the number of channels (n) multiplied by the open probability (n.Po). Maximum n.Po was determined by bathing the patch in 1 μM Ca^{2+} and repeating the ramps. Data were fitted with the Boltzmann equation of the form:

$$\text{n.Po} = 1/(1+\exp((V_{1/2} - V\mathit{patch})/K))$$

where $V_{1/2}$ is the membrane potential at which there was half maximal activation, K the slope factor and V*patch* the patch potential (mV). The change in activation $V_{1/2}$ ($\Delta V_{1/2}$) caused by drugs was obtained by subtracting the $V_{1/2}$ in control from that in the presence of the drugs. Voltage ramps were sufficiently slow (100 $mV{\cdot}s^{-1}$) so that the activation curves were not distorted by the time constants of activation or deactivation (Carl and Sanders, 1990).

For experiments with HEK-293 cells, inside out patches were held at either –60 or –100 mV and depolarized in 20 mV increments from –100 to 200 mV for 25 ms. Under our recording conditions, the tail currents from patches expressing BKα or BKαβ_4 subunits deactivated so rapidly in 100 nM Ca^{2+} that we were unable to accurately determine their activation $V_{1/2}$. Consequently, conductance (*G*) was derived from steady-state currents according to Ohm's law:

$$(G = I/(V - E_K))$$

where $E_K = 0$ mV in symmetrical [K^+]. *G* – *V* relationships were fitted with the Boltzmann equation. Data from each patch were normalized to the peak conductance observed in 10 μM Ca^{2+} to obtain G_{max} and all curves were constrained to this value. Series resistance compensation of up to 80% was applied and residual capacitance and leakage currents were subtracted using either a P/4 protocol or offline by manual leak subtraction.

L-type Ca^{2+} currents were recorded in Cs^+-rich pipette solutions with the perforated patch configuration of the whole cell patch clamp technique from RBSMC. The cell membrane was perforated using amphotericin B (600 $\mu g{\cdot}mL^{-1}$). Pipettes were pulled to a tip of diameter approximately 1–1.5 μm and resistance of 2–4 MΩ.

Drugs and solutions

GoSlo-SR-5-6 (sodium 1-amino-4-((3-trifluoromethylphenyl)amino)-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate) and GoSlo-SR-5-130 were synthesized as described previously (Roy *et al.*, 2012; 2014). Iberiotoxin (IbTx) was purchased from Smartox Biotechnology (Saint Marin d'Heres, France) and Sigma.

The following solutions were used. Concentrations in mM are given in parentheses.

Krebs solution. NaCl (120), KCl (5.9), $NaHCO_3$ (25), $NaH_2PO_4.2H_20$ (1.2), glucose (5.5), $MgCl_2$ (1.2), $CaCl_2$ (2.5). pH was adjusted to 7.4 by bubbling the solution with 95% O_2–5% CO_2 continuously.

Ca^{2+}-*free Hanks solution for cell dispersal.* NaCl (125), KCl (5.4), glucose (10), sucrose (2.9), $NaHCO_3$ (4.2), KH_2PO_4 (0.4), NaH_2PO_4 (0.3), HEPES (10). pH was adjusted to 7.4 using NaOH.

Single-channel recording solutions. KCl (140), glucose (10), HEPES (10), EGTA (1) (for Ca^{2+} free and 100 nM Ca^{2+}), H-EDTA (1) (for 1 μM Ca^{2+}), pH adjusted to 7.2 using KOH. Solutions containing 100 nM Ca^{2+} were used as the pipette solution. Chelator software (Schoenmakers *et al.*, 1992) was used to calculate the $[Ca^{2+}]$ required to give the desired free $[Ca^{2+}]_i$ and these were confirmed with a Ca^{2+} electrode.

Cs^+ *pipette solution (perforated patch).* CsCl (133), $MgCl_2$ (1), EGTA (0.5), HEPES (10). pH adjusted to 7.2 using CsOH.

Isometric tension recordings

Strips of detrusor (8 × 1 × 1 mm) were mounted in water-jacketed organ baths, perfused with warmed Krebs solution, adjusted to 10 mN tension and allowed to equilibrate for ~60 min. Isometric contractions were measured using a Myobath system and data were acquired using DataTrax 2 software (WPI, Hitchin, Hertfordshire, UK). Mean contraction amplitude (normalized to control) and frequency were measured 5 min before drug addition and 5 min during which drugs had their maximal effect.

Data analysis and statistics

Experiments on RBSM were obtained in three or more animals. *n* refers to the number of cells, patches or tissue strips studied. Summary data are presented as mean ± SEM and statistical comparisons were made with Student's paired *t*-tests, unpaired *t*-tests, ANOVA using Dunnett's *post hoc* test or Wilcoxon signed rank test (for normalized data) as appropriate, taking $P < 0.05$ as significant. In all figures $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Results

Effect of GoSlo-SR-5-6 and GoSlo-SR-5-130 on RBSMC BK channels

Figure 1A and B shows the structure of the anilinoanthraquinone compounds synthesized for this study, GoSlo-SR-5-6, and its deaminated derivative, GoSlo-SR-5-130. The effects of these compounds on BK currents recorded from excised patches of RBSMC are shown in Figure 1C and D respectively. Currents were elicited by voltage ramps in 100 nM Ca^{2+} and activated at potentials positive to +50 mV. GoSlo-SR-5-6 produced a concentration-dependent negative shift in the activation $V_{1/2}$ of the currents. Thus, application of 1, 3 and 10 μM GoSlo-SR-5-6 shifted the activation $V_{1/2}$ by –54, –93 and –121 mV, respectively, in this patch. GoSlo-SR-5-130 produced similar effects, shifting the activation $V_{1/2}$ by –66 and –94 mV in 1 and 3 μM GoSlo-SR-5-130 respectively (Figure 1D). The mean shift in $V_{1/2}$ produced by 1, 3, 10 and 30 μM GoSlo-SR-5-6 was –41 ± 4, –76 ± 10, –107 ± 7 and –137 ± 9 mV (n = 4–7), whereas GoSlo-SR-5-130 shifted the mean $V_{1/2}$ by –42 ± 9, –84 ± 9, –99 ± 4 and –121 ± 10 mV (n = 6–9) at the same concentrations respectively. These data suggest that the amine group present at 1-position of the C-ring (which is absent in GoSlo-SR-5-130) has little effect on the efficacy of these compounds to open BK channels from RBSMC.

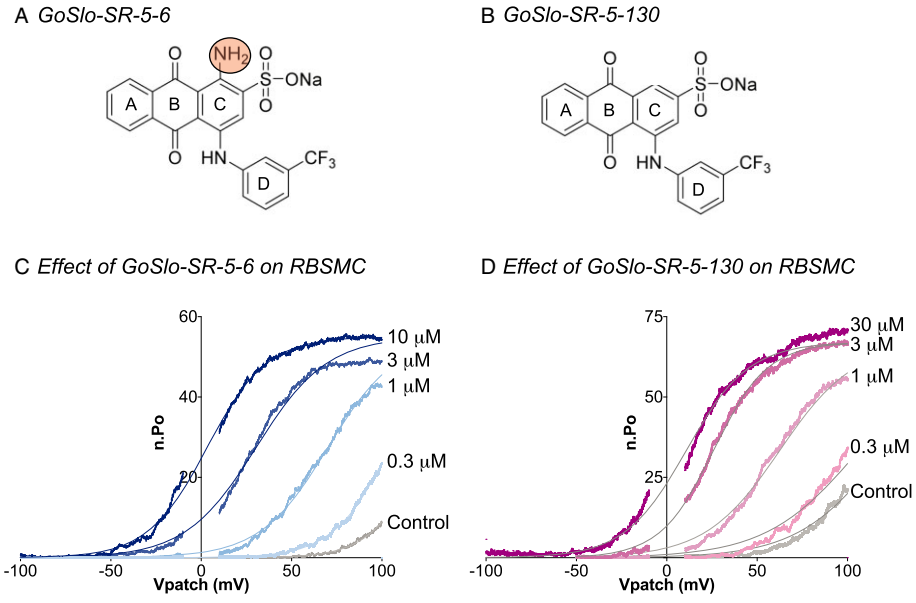

Figure 1

Structures and effects of GoSlo-SR-5-6 and GoSlo-SR-5-130 on rabbit bladder smooth muscle BK channels. (A) Shows the structure of GoSlo-SR-5-6 and (B) shows the deaminated derivative GoSlo-SR-5-130. Voltage ramps were applied to excised inside out patches of membrane from rabbit bladder SMC in the continued presence of 100 nM Ca^{2+}. Both GoSlo-SR-5-6 (C) and GoSlo-SR-5-130 (D) produced concentration-dependent negative shifts in the activation of BK channels.

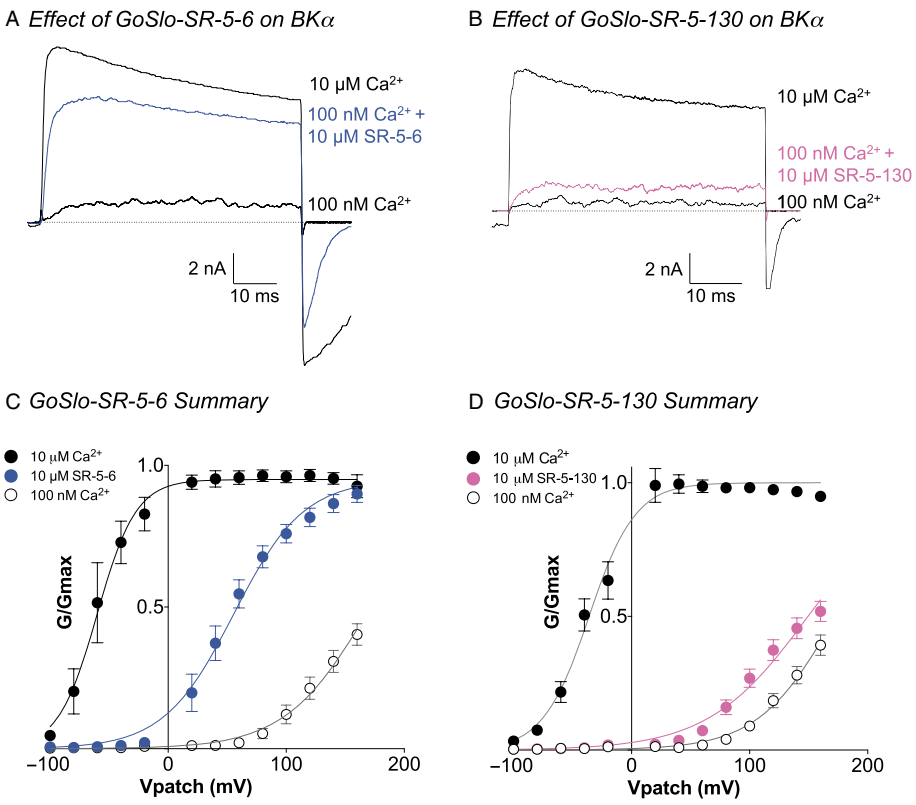

Figure 2

C-ring deamination reduces the efficacy of GoSlo compounds on HEK cells expressing BKα. Excised inside out patches of membrane from HEK293 cells expressing BKα were voltage clamped at –60 mV and stepped in 20 mV increments between –100 and +160 mV. The dotted lines signify zero current. (A) Shows a typical experiment in which application of 10 μM GoSlo-SR-5-6 in the presence of 100 nM Ca^{2+} caused an increase in current amplitude in response to a step to +100 mV. Summary data ($n = 6$) are shown in (C) where GoSlo-SR-5-6 shifted the activation $V_{1/2}$ by –112 ± 4 mV ($P < 0.001$). In contrast, as shown in (B) and (D), 10 μM GoSlo-SR-5-130, in the presence of 100 nM Ca^{2+} only shifted the activation $V_{1/2}$ by –22 ± 6 mV ($P < 0.01$, $n = 10$). The shift in $V_{1/2}$ evoked by GoSlo-SR-5-6 was significantly different ($P < 0.001$) from that observed with GoSlo-SR-5-130.

Effect of GoSlo-SR compounds on BKα subunits expressed in HEK-293 cells

In order to examine if the effects of either compound were altered in the absence of the regulatory β_1 subunit, we cloned the BKα subunit and stably transfected HEK cells with this cDNA. Figure 2A shows a typical example of BKα currents evoked by a voltage step from –60 to +100 mV in the presence of 100 nM Ca^{2+}, 10 μM Ca^{2+} and 100 nM Ca^{2+} plus 10 μM GoSlo-SR-5-6. Application of GoSlo-SR-5-6 to these patches produced three main effects: (i) an increase in the rate of current activation; (ii) a large increase in current amplitude; and (iii) a decrease in the rate of deactivation as evidenced by the slowing of tail currents. In this example, the peak current increased approximately fivefold after application of 10 μM GoSlo-SR-5-6. To assess the effects of GoSlo-SR-5-6 on activation $V_{1/2}$, we constructed activation curves from the *I/V* relationships. The summary activation curves in Figure 2C show that 10 μM GoSlo-SR-5-6 significantly shifted the activation $V_{1/2}$ from 170 ± 6 to 57 ± 7 mV ($\Delta V_{1/2} = -113 \pm 6$ mV, $n = 6$). This value was not significantly different from that observed in native RBSMC and suggests that the efficacy of GoSlo-SR-5-6 is unaltered in the absence of the regulatory $BK\beta_1$ subunit.

However, when we examined the effects of GoSlo-SR-5-130 on HEK cells expressing only BKα subunits, we noticed a significant reduction in its efficacy. Figure 2B shows a typical record where the patch was stepped to +100 mV in the presence of 100 nM Ca^{2+}, 10 μM Ca^{2+} and 100 nM Ca^{2+} plus 10 μM GoSlo-SR-5-130. The deaminated derivative increased current less than twofold. The reduced efficacy of GoSlo-SR-5-130 was apparent when the activation curves shown in Figure 2D were compared with those for GoSlo-SR-5-6 (Figure 2C). Thus, 10 μM GoSlo-SR-5-130 significantly ($P < 0.01$) shifted the activation $V_{1/2}$ from 173 ± 3 to 149 ± 3 mV ($\Delta V_{1/2} = -22 \pm 6$ mV, $n = 10$). This shift was significantly less than its effects on native RBSMC BK channels ($P < 0.001$). The EC_{50} of GoSlo-SR-5-130 on BKα was 14 μM [95% confidence intervals (CI) 5–38 μM, $n = 6$], suggesting that the efficacy and potency of this compound were reduced in the absence of BKβ subunits.

GoSlo-SR-5-130 efficacy is restored in HEK cells expressing BKαβ_1

To test if the effects of either drug were altered in the presence of the regulatory β_1 subunit, BKα and $BK\beta_1$ were co-expressed in HEK cells and the drugs applied. As shown in Figure 3A,

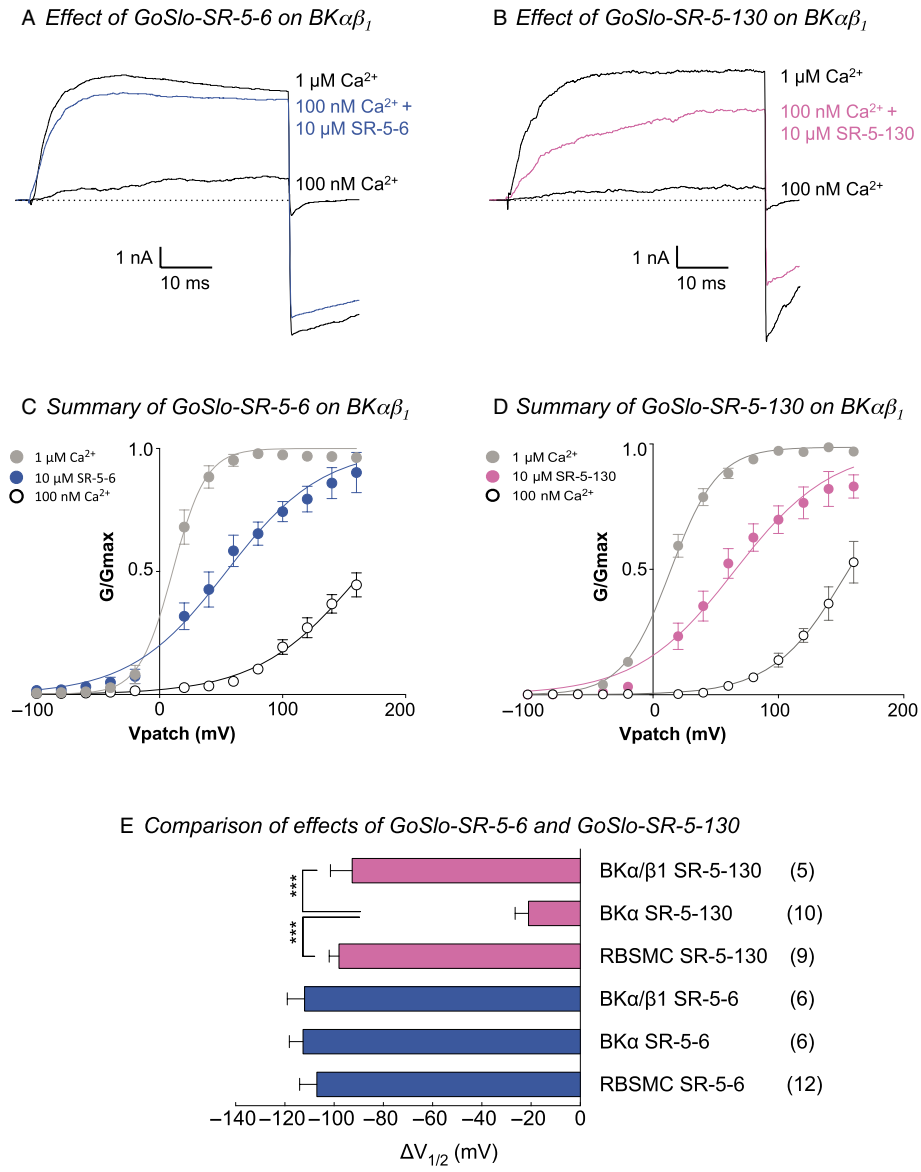

Figure 3

The efficacy of GoSlo-SR-5-130 is restored in HEK cells expressing BKαβ$_1$. Typical experiments in which application of 10 μM GoSlo-SR-5-6 (A) or 10 μM GoSlo-SR-5-130 (B) in the presence of 100 nM Ca^{2+} caused an increase in current amplitude in response to a step to +100 from −60 mV. (C) and (D) Summary activation curves in which GoSlo-SR-5-6 ($n = 6$) or GoSlo-SR-5-130 ($n = 5$) shifted the activation curve negatively compared with 100 nM Ca^{2+}. (E) A comparison of the effects of GoSlo-SR-5-6 and GoSlo-SR-5-130 on RBSMC, BKα and BKαβ$_1$. Numbers of replicates are displayed in parentheses.

the BKαβ$_1$ currents were slower to activate and deactivate compared with the BKα currents (Figure 2) and application of 1 μM Ca^{2+} maximally activated the currents at this potential (+100 mV). As Figure 3A suggests, application of 10 μM GoSlo-SR-5-6 increased the rate of current activation, slowed deactivation and increased peak current amplitude ~4.5-fold. In the six experiments shown in Figure 3C, 10 μM GoSlo-SR-5-6 shifted the $V_{1/2}$ of activation by −112 ± 7 mV (ns compared with RBSMC or BKα).

In contrast, the effects of GoSlo-SR-5-130 were increased in cells expressing BKαβ$_1$. In the lower trace of Figure 3B, the patch was exposed to 100 nM Ca^{2+} and this elicited a peak current of 688 pA. Application of 10 μM GoSlo-SR-5-130 (middle trace) increased the amplitude of the current sixfold, increased the rate of current activation and slowed deactivation. As the activation curves in Figure 3D suggest, 10 μM GoSlo-SR-5-130 shifted the activation $V_{1/2}$ in 100 nM Ca^{2+} from 156 ± 3 to 64 ± 5 mV ($\Delta V_{1/2} = -92 \pm 9$ mV, $n = 5$), which was not significantly different to that recorded in RBSMC BK channels. Similarly, when we constructed concentration–response curves with GoSlo-SR-5-130 in these cells, the EC_{50} was 2.8 μM (95% CI 1.5–5.4 μM, $n = 5$) compared with 14 and 1.8 μM (Roy *et al.*, 2014) in BKα and RBSMC BK channels respectively.

Figure 3E shows a comparison of the efficacy of 10 μM GoSlo-SR-5-6 and 10 μM GoSlo-SR-5-130 on BK channels from RBSMC as well as BKα and BKαβ$_1$ expressed in HEK cells. In this

summary, the mean $\Delta V_{1/2}$ was plotted for each cell type for both compounds. It was clear from these data that the effects of GoSlo-SR-5-6 depended little on the presence of the regulatory β_1 subunit. However, the effects of GoSlo-SR-5-130 were reduced by ~80% in HEK cells expressing only BKα subunits and this was restored when the β_1 subunit was co-expressed.

GoSlo-SR-5-130 effects are restored in HEK cells expressing BKαβ4

Given that co-expression of the β_1 subunit restored the effect of GoSlo-SR-5-130, we also assessed if the efficacy of GoSlo-SR-5-130 was altered in the presence of the β_4 subunit, which has been shown to be present in the bladder of some species (Chen and Petkov, 2009). Figure 4B shows a typical record of currents evoked by a step to +100 mV from a patch expressing BKαβ_4 channels in 100 nM Ca^{2+}, 10 μM Ca^{2+} and 100 nM Ca^{2+} plus 10 μM GoSlo-SR-5-130. Consistent with other reports (Brenner *et al.*, 2000), the BKαβ_4 was slowly activating and its activation $V_{1/2}$ in 100 nM Ca^{2+} was more depolarized compared with BKα channels. As shown in Figure 4B application of GoSlo-SR-5-130 (pink trace) increased peak current from ~10-fold. In the seven experiments summarized in Figure 4D, the shift in activation $V_{1/2}$ was –84 ± 4 mV, suggesting that its efficacy was largely restored when the β_4 was co-expressed with the α subunit. In contrast, the effects of GoSlo-SR-5-6 were not altered in patches co-expressing BKαβ_4 channels

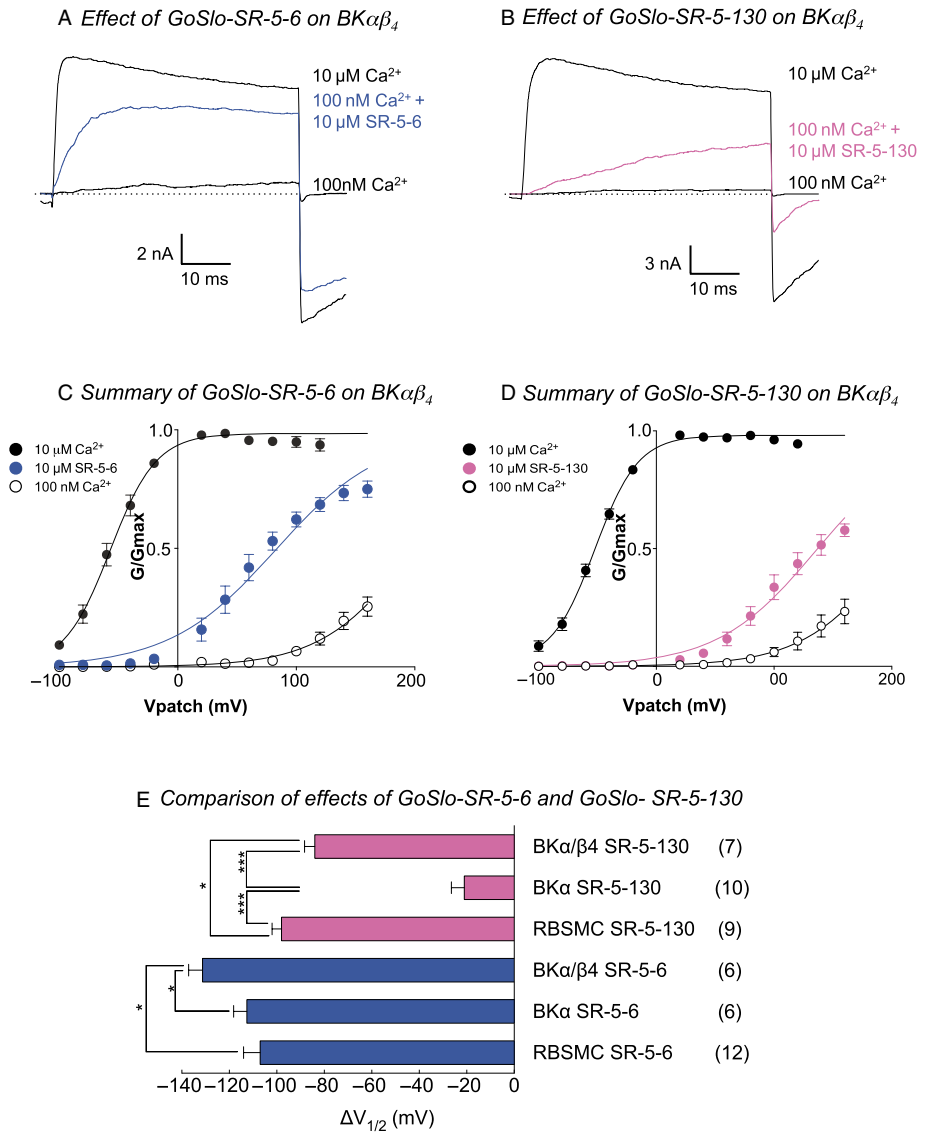

Figure 4

The efficacy of GoSlo-SR-5-130 is restored in HEK cells expressing BKαβ_4. Upper panels show typical experiments in which application of 10 μM GoSlo-SR-5-6 (A) or GoSlo-SR-5-130 (B), both in 100 nM Ca^{2+}, caused an increase in current amplitude in response to a step to +100 mV. (C) and (D) Summary activation curves in which GoSlo-SR-5-6 (n = 6) and GoSlo-SR-5-130 (n = 7) shifted the activation negatively in BKαβ_4 compared with 100 nM Ca^{2+}. (E) A comparison of the effects of GoSlo-SR-5-6 and GoSlo-SR-5-130 on RBSMC, BKα and BKαβ_4. Numbers of replicates are displayed in parentheses.

(Figure 4A) and as the results shown in Figure 4C suggest, the activation $V_{1/2}$ was shifted more than –100 mV.

Figure 4E shows summary data comparing the effect of GoSlo-SR-5-6 and GoSlo-SR-5-130 on $V_{1/2}$ in native RBSMC BK channels and HEK cells expressing either BKα or BKαβ_4. The efficacy of 10 μM GoSlo-SR-5-6 was significantly enhanced ($\Delta V_{1/2}$ = –131 ± 6 mV, $P < 0.05$) in cells expressing BKαβ_4 compared with RBSMC ($\Delta V_{1/2}$ = –113 ± 6 mV), whereas the response to 10 μM GoSlo-SR-5-130 was slightly, but significantly ($P < 0.05$), less than that in RBSMC.

Effect on phasic spontaneous bladder contractions

We next studied the effect of both GoSlo-SR-5-6 and GoSlo-SR-5-130 on RBSM using isometric tension recording. As shown in Figure 5A, GoSlo-SR-5-6 had little effect on the amplitude or frequency of contractions when applied at concentrations up to 30 μM. As the summary for 11 experiments shown in Figure 5B suggests, under control conditions the contraction amplitude was 4.7 ± 1.5 mN (normalized value = 1) and the frequency was 7.6 ± 1 min^{-1}. Application of 10 μM GoSlo-SR-5-6 had no significant effect on either contraction amplitude or frequency (8.9 ± 1.4 min^{-1}). Although 30 μM GoSlo-SR-5-6 reduced contraction amplitude in 3 of the 11 preparations, it had no effect on the frequency of contractions. However, as shown in Figure 5B and C, 30 μM GoSlo-SR-5-6 had no significant effect on contraction amplitude or frequency.

In contrast, GoSlo-SR-5-130 consistently reduced contraction amplitude but had little effect on frequency (Figure 6A). Thus, 10 μM GoSlo-SR-5-130 reduced the contraction amplitude by ~60% and reduced basal tone. When 30 μM GoSlo-SR-5-130 was applied, only small amplitude contractions remained and these returned towards control levels upon washout. Figure 6B shows summary data where under control conditions, the mean contraction amplitude was little affected by concentrations of GoSlo-SR-5-130 below 10 μM. However, the mean contraction amplitude was reduced from a normalized value of 1 to 0.52 ± 0.07 and 0.14 ± 0.03 ($P < 0.001$, $n = 15$) in the presence of 10 and 30 μM GoSlo-SR-5-130, respectively, whereas mean contraction frequency decreased from 8.1 ± 1 to 5.4 ± 1.4 min^{-1} (Figure 6C).

Effects of GoSlo-SR-5-130 on L-type Ca^{2+} channels in RBSMC

The inhibition of contractions by GoSlo-SR-5-130 is consistent with the effects of opening BK channels but could also be explained by non-selective inhibition of Ca^{2+} influx through L-type Ca^{2+} channels. To examine this possibility, we recorded L-type Ca^{2+} channels in RBSMC as shown in Figure 7A. Depolarizing steps from –60 to 0 mV evoked peak L-type Ca^{2+} currents with mean amplitudes of –359 ± 97 pA (n = 7). Application of 30 μM GoSlo-SR-5-130 decreased current amplitude to –288 ± 88 pA (ns). We also performed concentration–response experiments (results shown in Figure 7B; n = 7) and found no significant effect of GoSlo-SR-

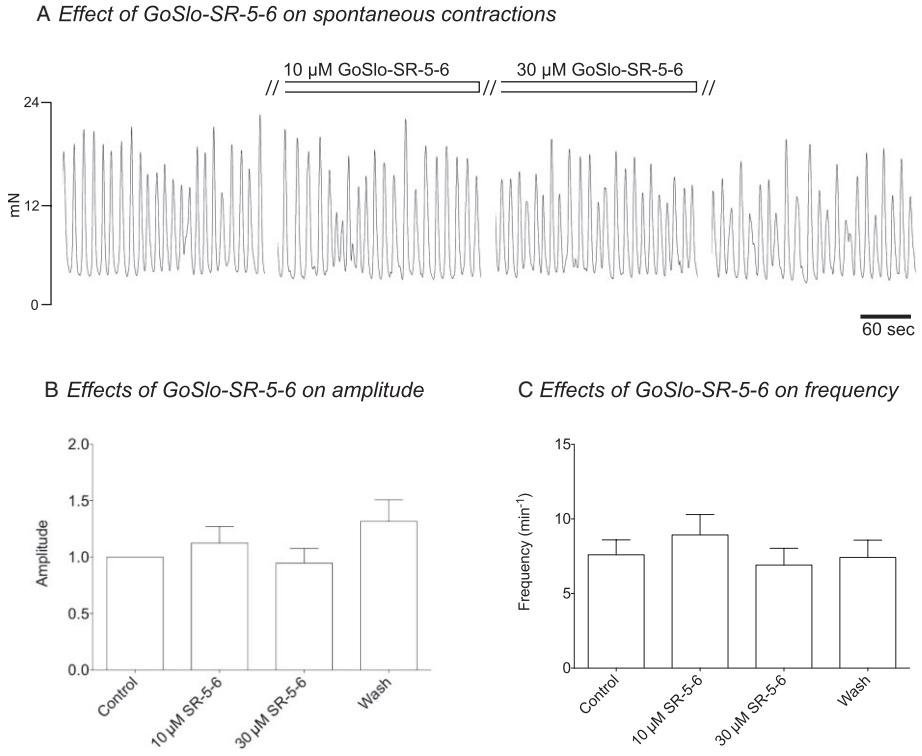

Figure 5

GoSlo-SR-5-6 has little effect on myogenic bladder contractions. The left-hand part of (A) shows a recording of control activity in rabbit bladder smooth muscle strips. Application of 10 or 30 μM GoSlo-SR-5-6 failed to reduce contraction amplitude, frequency or basal tone. Summary data for both normalized contraction amplitude (B) and contraction frequency (C) from 11 similar experiments are also shown.

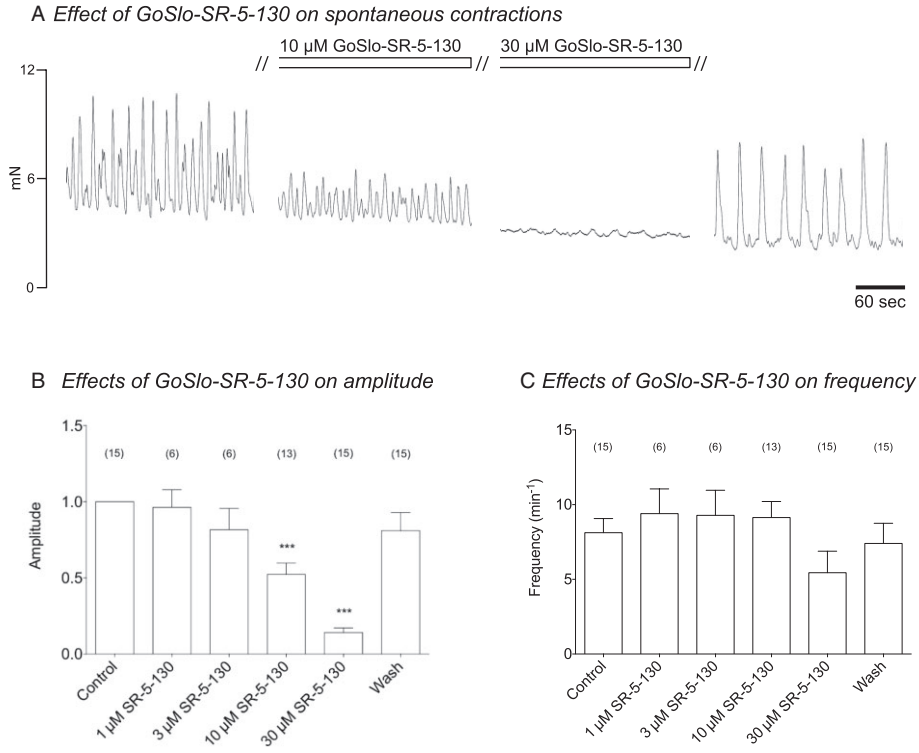

Figure 6

GoSlo-SR-5-130 inhibits myogenic bladder contractions. (A) A recording of control activity in rabbit bladder smooth muscle strips. Application of 10 µM GoSlo-SR-5-130 caused a significant reduction in contraction amplitude and basal tone. Application of 30 µM GoSlo-SR-5-130 in this example practically abolished the spontaneous contractions, with a further decrease in basal tone. Summary data for both normalized contraction amplitude (B) and contraction frequency (C) are also shown. The numbers in parentheses indicate the number of replicates.

5-130 on L-current at any concentration (ANOVA). Because of solubility issues at concentrations greater than 100 µM, we were unable to complete full concentration- effect curves. However, the data shown in Figure 7B suggest that the IC_{50} for GoSlo-SR-5-130 is likely to be in excess of 250 µM. These results suggest that the effect of GoSlo-SR-5-130 on tension was unlikely to be due to inhibition of L-type Ca^{2+} channels.

GoSlo-SR-5-6 (1–30 µM) had no significant effect on RBSMC L-type Ca^{2+} current. In six experiments the peak current, using the above protocol, was −532 ± 145 pA compared with −383 ± 121 pA in 30 µM GoSlo-SR-5-6, which was not significantly different (ANOVA).

GoSlo-SR-5-130 is ineffective in the presence of IbTx

If the effects of GoSlo-SR-5-130 were mediated selectively via BK channels, we might expect that its inhibitory effects would be abolished when BK channels were blocked with the selective toxin IbTx. As shown in the left-hand panels of Figure 8A, 30 µM GoSlo-SR-5-130 caused a large reduction in the amplitude of spontaneous contractions when applied for 30 min and this returned towards control during wash. When 100 nM IbTx was applied for 30 min, it increased both the amplitude and frequency of contraction and re-application of GoSlo-SR-5-130 had little effect. Upon removal of both drugs, the frequency and amplitude of contractions returned towards control. Figure 8B shows summary data in which contraction amplitude was measured before, during and after drug application in the absence (left bars) and presence (right bars) of IbTx and normalized to control. Contraction amplitude was significantly reduced to 0.27 ± 0.06 in the presence of 30 µM GoSlo-SR-5-130 ($P < 0.05$, $n = 6$) and returned towards control levels upon washout. When BK channels were blocked with 100 nM IbTx, mean contraction amplitude was significantly increased to 2.2 ± 0.4 and this was not altered by 30 µM GoSlo-SR-5-130 in the continued presence of IbTx. Figure 8C shows the effect of GoSlo-SR-5-130 on contraction frequency in the absence and presence of IbTx.

Effect of GoSlo-SR-5-130 on neurogenic and carbachol-mediated contractions

To assess the effects of GoSlo-SR-5-130 on neurogenic contractions, bladder strips were stimulated at 4 Hz for 60 s (0.3 ms pulse width) to induce nerve-mediated contractions. Under control conditions, electrical field stimulation elicited mean peak contractions of 16.5 ± 3.7 mN ($n = 7$). After a 30 min application of 10 or 30 µM GoSlo-SR-5-130, there was no significant effect on neurogenic contractions, which were 18.1 ± 3.9 mN ($n = 6$) and 16.8 ± 3.7 mN in amplitude respectively ($n = 7$).

To assess if the effects of carbachol were altered by GoSlo-SR-5-130, we measured contractions induced by 1 µM

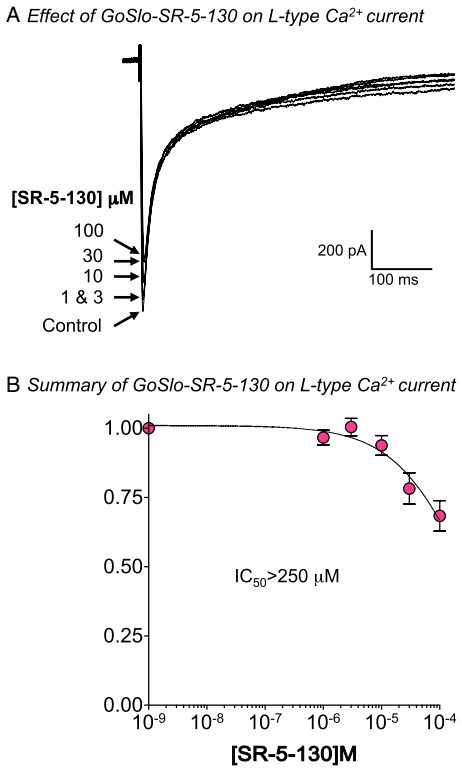

Figure 7

GoSlo-SR-5-130 has little effect on L-type Ca^{2+} currents. (A) Typical currents evoked in response to a depolarizing step to 0 mV from a holding potential of –60 mV. Increasing concentrations of GoSlo-SR-5-130 only reduced thes peak current modestly. (B) Summary of concentration-effect curves from seven experiments and suggests that the IC_{50} is in excess of 250 μM.

carbachol in the absence and presence of the BK channel opener. The mean amplitude of these contractions was 34.5 ± 5.2 mN under control conditions and was not significantly altered (36.4 ± 5.6 mN, n = 4) following re-application of carbachol in the continued presence 30 μM GoSlo-SR-5-130.

Discussion

The results demonstrate that two novel related anilinoanthraquinone compounds have similar effects on native BK channels in RBSMC, but differ significantly in their effect when applied to HEK cells expressing only the pore-forming α subunit. Furthermore, only GoSlo-SR-5-130 inhibited spontaneous contractions of rabbit bladder. These results suggest that subtle modifications of these compounds can alter both their efficacy on BK channels comprised of either BKα or BKαβ subunits and their effects on tissue strips.

We recently demonstrated (Roy *et al.*, 2012; 2014) that the GoSlo-SR family are potent, efficacious BK channel openers. Roy *et al.* (2014) demonstrated that deamination of the C-ring had little effect on either the efficacy or potency of GoSlo-SR-5-130 compared with GoSlo-SR-5-6. Our results, shown in Figure 1, are in agreement with these earlier findings and support the idea that when $BK\alpha\beta_1$ subunits are expressed together, the effects of GoSlo-SR-5-130 and Go-Slo-SR-5-6 are similar. However, our results suggest that the efficacy of these differed when they were applied to BK channels comprised of only the pore-forming α subunit. Thus, GoSlo-SR-5-130 shifted the activation $V_{1/2}$ by approximately –20 mV compared with the approximately –100 mV shift observed with GoSlo-SR-5-6 in the same cells. Interestingly, the effectiveness of GoSlo-SR-5-130 was largely restored when the regulatory β1 or β4 subunits were co-expressed with the α subunit, suggesting that the absence of a regulatory β subunit subtly changes either (i) the binding site for GoSlo-SR-5-130 or (ii) the transduction pathway for GoSlo-SR-5-130. In contrast, the effects of GoSlo-SR-5-6, which differ from GoSlo-SR-5-130 by the presence of an NH_2 group on the C-ring, were effective on patches containing BKα, $BK\alpha\beta_1$ or $BK\alpha\beta_4$ channels.

A large number of BK channel modulators have been synthesized over the last 20 years (reviewed in Nardi and Olesen, 2008), but the molecular mechanisms underlying some of their effects have only recently been examined in detail (Gessner *et al.*, 2012; Hoshi *et al.*, 2013). It is clear from the literature that BK channel activators can be broadly divided into two groups, which either do or do not require the presence of a regulatory β subunit to mediate their full effects. Early studies on the dehydrosaponin group of compounds (McManus *et al.*, 1995) demonstrated that the regulatory β_1 subunit was essential for their action. A similar requirement for the β_1 subunit has been demonstrated for a range of unrelated compounds including tamoxifen (Dick *et al.*, 2001), Evans blue dye (Yamada *et al.*, 2001) and omega-3 docosahexaenoic acid (Hoshi *et al.*, 2013). In contrast, the NS series of compounds, as well as pimaric acid (Imaizumi *et al.*, 2002), mallotoxin (Zakharov *et al.*, 2005) and Cym-04 (Gessner *et al.*, 2012), appear to activate BK channels equally well in the absence of the β_1 subunit. This suggests the presence of multiple regulatory sites on BK channels, which can be targeted to modify their function.

An unexpected finding of this study was the inability of GoSlo-SR-5-6 to significantly alter the spontaneous contractions of RBSM. This was surprising given that GoSlo-SR-5-6 and GoSlo-SR-5-130 produced almost identical effects ($\Delta V_{1/2}$ approximately –100 mV) when applied to RBSMC BK channels and to $BK\alpha\beta_1$ expressed in HEK cells. One possible explanation for the differential effect of these two compounds on mechanical activity is that GoSlo-SR-5-6 may be less tissue or cell permeant than its deaminated derivative, GoSlo-SR-5-130. Alternatively, GoSlo-SR-5-6 may be selectively metabolized by bladder smooth muscle cells or excreted via multidrug resistant transporters known to be present in bladder smooth muscle cells (Rius *et al.*, 2005).

The observed effects of GoSlo-SR-5-130 could also be explained by non-selective actions of this compound on other ion channels such as blockade of L-type Ca^{2+} channels. Although we cannot fully exclude an inhibitory effect of GoSlo-SR-5-130 on Ca^{2+} influx, a number of lines of evidence suggest that the effects of this compound on tension are due to activation of BK channels. Firstly, GoSlo-SR-5-130, at the concentrations used in the tension experiments, only produced a small decrease in the amplitude of L-type Ca^{2+} currents recorded from RBSMC. Indeed, the estimated IC_{50}

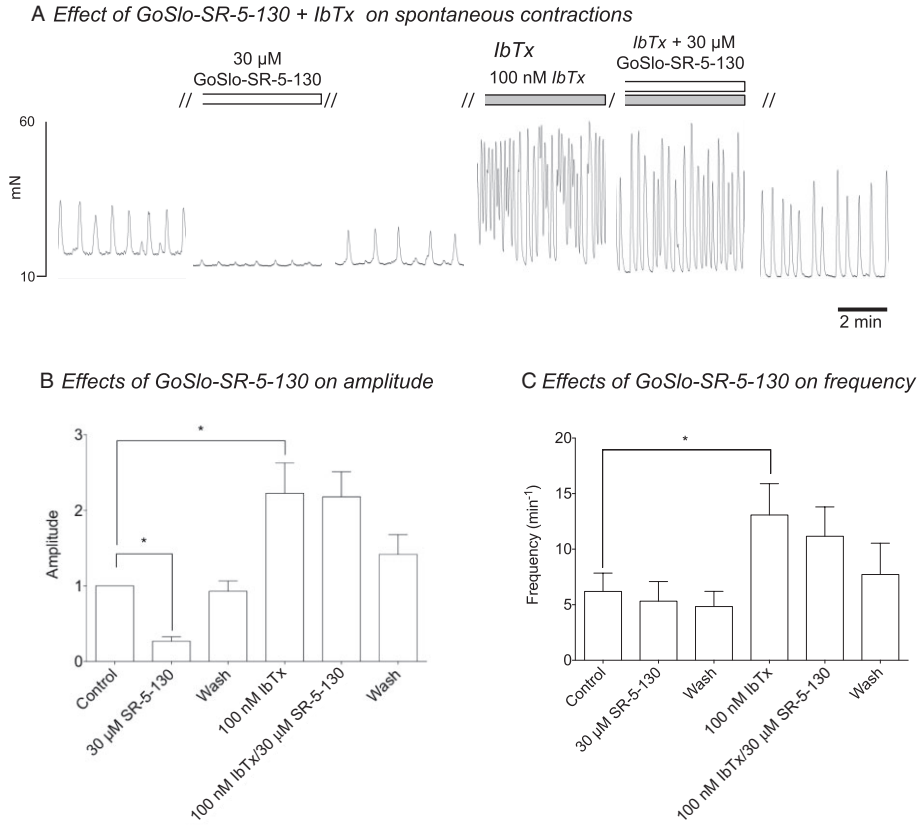

Figure 8

The effects of GoSlo-SR-5-130 are abolished when BK channels are blocked with IbTx. The left-hand side of (A) shows spontaneous contractions before, during and after application of 30 μM SR-5-130, which reversibly inhibited contractions. The right-hand side of (A) suggests IbTx application increased the amplitude and frequency of contractions and co-application of GoSlo-SR-5-130 had little effect on these contractions when BK channels were blocked. (B) A summary of results from six experiments in which normalized mean amplitude was measured before, during and after GoSlo-SR-5-130 in the absence and presence of IbTx. (C) A summary of the results from the same six experiments showing the mean frequency in the presence of IbTx. When BK channels were blocked, GoSlo-SR-5-130 failed to inhibit the spontaneous contractions.

(>250 μM) of GoSlo-SR-5-130 on L-current was some 130-fold higher than the EC_{50} on BK channels (Roy *et al.*, 2014). Secondly, when we pharmacologically ablated BK channels with IbTx, re-application of GoSlo-SR-5-130 failed to significantly alter contractile activity. Taken together, the results of these experiments suggest that GoSlo-SR-5-130 can inhibit contractile activity in RBSM and these effects appear to be due to selective activation of BK channels.

It is also important to note that although GoSlo-SR-5-130 significantly reduced the amplitude of spontaneous contractions, it had no effect on neurogenic contractions elicited in response to EFS. These data suggest that GoSlo-SR-5-130 has little effect on the release of excitatory neurotransmitters or on post-junctional muscarinic receptors. This is further supported by the observation that the response to exogenous application of carbachol was not significantly altered in the presence of GoSlo-SR-5-130. Consequently, we would expect that application of this compound *in vivo* would not lead to urinary retention as it is unlikely to have an effect on micturition contractions.

In summary, the results of this study suggest that GoSlo-SR-5-130 is a novel BK channel modulator that requires regulatory β subunits to mediate its full excitatory effects on BK channels and may serve as a useful scaffold for the development of more potent BK channel openers.

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Author contributions

R. J. L., E. B., A. K., S. R., T. I. W. and A. K. performed the research. G. P. S., K. D. T., N. G. M. and M. A. H. designed the study. S. R. contributed essential reagents. R. J. L., A. K., T. I. W., S. R., A. A. and M. A. H. analysed the data. R. J. L., G. P. S. and M. A. H. wrote the paper.

Conflicts of interest

K. D. T., N. M. H., G. P. S., S. R. and M. A. H. have submitted a Patent application (IPN WO 2012/035122 A11) on this family of molecules.

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