**RESEARCH PAPER**

**Differential efficacy of GoSlo-SR compounds on BKα and BKαγ1–4 channels**

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**ABSTRACT**

Large conductance voltage and Ca$^{2+}$ activated K$^+$ channels (BK channels) are abundantly expressed throughout the body and are important regulators of smooth muscle tone and neuronal excitability. Their dysfunction is implicated in various diseases including overactive bladder, hypertension and erectile dysfunction. Therefore, BK channel openers bear significant therapeutic potential to treat the above diseases. GoSlo-SR compounds were designed to be potent and efficacious BK channel openers. Although their structural activity relationships, activation in both BKα and BKαβ channels and the hypothetical mode of action of these compounds has been studied in detail in recent years, their effectiveness to open the BKαγ channels still remains unexplored. In this study, we have examined the efficacy of 3 closely related GoSlo-SR openers, GoSlo-SR-5-6 (SR-5-6), GoSlo-SR-5-44 (SR-5-44) and GoSlo-SR-5-130 (SR-5-130) using inside out patches on BKα channels coexpressed with 4 different LRRC (γ1–4) subunits in HEK293 cells. Our data suggests that the activation effects due to SR-5-6 were not significantly affected in the presence of γ1–4 subunits. Interestingly, the effects of more efficacious BK channel opener SR-5-44 were altered by different γ subunits. In cells expressing BKα channels, the shift in $V_{1/2}$ (Δ$V_{1/2}$) induced by SR-5-44 (3 μM) was −76 ± 3 mV, whereas it was significantly reduced by ~70% in BKαγ1 channels (Δ$V_{1/2}$ = −23 ± 3, p < 0.0001, ANOVA). In BKαγ2 channels the Δ$V_{1/2}$ was −36 ± 1 mV, which was less than that observed in BKαγ3 and BKαγ4 channels where the Δ$V_{1/2}$ was −47 ± 5 mV, and −82 ± 5 mV, respectively. Additionally, the excitatory effects of a ‘β specific’ BK channel opener, SR-5-130 were only partially restored in the patches containing BKαγ1–4 channels. Together this data highlights that subtle modifications in GoSlo-SR structures alter their effectiveness on BK channels with accessory γ subunits and this study might provide a scaffold for the development of more tissue specific BK channel openers.

**Introduction**

Large conductance voltage and Ca$^{2+}$ activated K$^+$ channels (BK channels) govern various physiological processes in both neuronal and non-neuronal cells. They act as negative feedback regulators between membrane voltage and intracellular Ca$^{2+}$ to control the membrane excitability. These channels are homo-tetramers that are formed by the pore-forming α subunits, which are encoded by the KCNMA1 gene. In addition to this, 2 different classes of accessory subunits, (β3,4,15,25 and γ28,29) modulate the physiological activity of BKα channels in different cell types. The four members of the β (β1–4) and γ (γ1–4) families influence the Ca$^{2+}$ dependent activation, inactivation, voltage dependence of activation and also the pharmacology of BKα channels. The newly characterized γ subunits are structurally and functionally distinct to the 2 transmembrane containing β subunits. These γ subunits are leucine rich repeat (LRR) containing membrane proteins comprising an N terminal signal peptide, an extracellular LRR domain, a single transmembrane segment (TM) and a short intracellular C-terminus. When γ subunits are coexpressed with BKα channels, their activation $V_{1/2}$ are shifted by ~ −140 mV (γ1, LRRC26), −100 mV (γ2, LRRC52), −50 mV (γ3, LRRC55) and −20 mV (γ4, LRRC38, 28,29).

Recent studies have suggested that the effect of the BK channel opener, mallotoxin (MTX) is reduced in the presence of γ subunits in both native parotid...

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acinar cells and heterologously expressed BKαγ channels.\textsuperscript{1} Thus, it has been postulated that γ subunits can selectively alter the efficacy of BK channel modulators.

The anilinoanthraquinone based GoSlo-SR family of compounds, are potent and efficacious BK channel openers\textsuperscript{22,23} that can relax a variety of smooth muscles including bladder and corpus cavernosum.\textsuperscript{11,16} Although their structure activity relationships\textsuperscript{23,22} have been determined and a number of residues in BKα channels hypothesized to interact with them,\textsuperscript{26} little is known about their effects in the presence of different regulatory subunits. Interestingly, Large et al. (2015) demonstrated that GoSlo-SR-5-6, mediated its full effects in the absence of β1 subunits, whereas its deaminated derivative GoSlo-SR-5-130, was \textasciitilde 4 fold, less effective at shifting $V_{1/2}$ in the absence of β1 or β4 subunits. To date however, no study has yet established the effectiveness of these compounds on the newly characterized γ regulatory subunits. Therefore, the focus of this study was to examine if the effects of 3 different GoSlo-SR openers (SR-5-6, SR-5-44 & SR-5-130) were altered, when applied to BKα channels coexpressing γ1–4 subunits. The structures of these 3 closely related compounds are shown in Figure 1.

The overall conclusions of this study are as follows: i) the effects of SR-5-6 were largely unaffected by γ1–4 subunits, ii) the effects of SR-5-44 were reduced in BKαγ1 channels and this reduction was less evident in BKαγ2, BKαγ3 and BKαγ4 channels and lastly, iii) the effects of SR-5-130 were partially enhanced in BKαγ1–4 subunits compared to BKα alone.

**Results**

**The effects of SR-5-6 were unaltered in the presence of γ1–4 subunits**

We first began by examining the effects of the BK channel opener, SR-5-6 on heterologously expressed BKα and BKαγ1–4 channels. Figure 2 shows a typical record of currents recorded from patches containing BKα (Panel A) and BKαγ1 (Panel B) channels evoked by 25 ms voltage pulses, from $-100$ mV to $+100$ mV in 20 mV steps. As shown previously by Yan & Aldrich,\textsuperscript{28,29} the co-expression of γ1 subunits resulted

![Figure 1. Structures of different GoSlo-SR compounds. Panels A, B & C display the structures of SR-5-6, SR-5-44 and SR-5-130, respectively. These anthraquinones are structural analogs to the commerically available dye, Acid Blue 25 (AB25). SR-5-44 is a methylated derivative of SR-5-6 that contains an additional $–CH_3$ group on the D ring at para-position. SR-5-130 is a deaminated derivative of SR-5-6 since it lacks the amino group (NH$_2$) on the C ring.](image-url)
in a leftward shift in the voltage dependent activation of BKα channels such that pronounced K⁺ currents can be readily observed. As the typical record suggests, the BKα currents in control conditions (100 nM Ca²⁺) began to activate at ~ +80 mV, whereas BKαγ₁ currents activated at potentials < −40 mV. Application of 10 µM SR-5-6 (structure shown in Fig. 1A) to BKα channels produced a significant increase in current amplitude, suggesting that its effects were independent of any regulatory subunits as shown before.16,26 Similar to the effects on BKα channels, SR-5-6 induced large inward currents in BKαγ₁ channels.

Figure 2. GoSlo-SR-5-6 excitatory effects are largely unaffected when γ subunits are co-expressed with BKα channels in HEK cells. The typical records in A and B shows the currents recorded from BKα and BKαγ₁ patches in response to 100 nM Ca²⁺ (left) and 10 µM SR-5-6 (middle), when depolarized from −100 mV to +100 mV in 20 mV increments. The summary activation curves obtained from analyzing these currents in low (100 nM) and high (10 µM) Ca²⁺ and SR-5-6 are also shown (right) in the respective panels. The smooth curves are the fits to Boltzmann equation. Panel C displays the mean V₁/₂ values in 3 different Ca²⁺ concentrations (100 nM, 1 µM and 10 µM) and SR-5-6 for BKα and BKαγ₁-4 channels. The orange arrow indicates the shift induced by 10 µM SR-5-6. The mean ΔV₁/₂ produced by SR-5-6 in BKαγ₁-4 channels is compared against BKα channels in Panel D. The dotted line indicates the mean ΔV₁/₂ of BKα channels, which is −103 ± 4 mV. In the presence of γ subunits, this ΔV₁/₂ was retained by SR-5-6 and was not significantly different to that in BKα channels. The numbers of replicates are shown in parentheses.
channels, indicating that γ₁ subunits do not alter its effects. The mean $V_{1/2}$ obtained from the Boltzmann fits to the data in 100 nM Ca²⁺ for BKα channels was 162 ± 2 mV and was shifted to −36 ± 2 mV in 10 μM Ca²⁺. In the presence of SR-5-6, the $V_{1/2}$ was left shifted to 51 ± 2 mV (n = 15). For BKαγ₁ channels, the $V_{1/2}$ in 100 nM and 10 μM Ca²⁺ and SR-5-6 in 100 nM Ca²⁺ was 16 ± 3 mV, −162 ± 9 mV and −66 ± 6 mV (n = 6) respectively.

Figure 2C shows a summary of the mean $V_{1/2}$ values (in 100 nM, 1 μM and 10 μM Ca²⁺) in the absence and presence each of the 4 γ subunits. It also shows the effect of SR-5-6 in 100 nM Ca²⁺. It is evident from these data that the left shift in $V_{1/2}$ in control conditions (100 nM Ca²⁺, clear circles) was greatest with $γ₁$ and decreased in the order of $γ₂ > γ₃ > γ₄$. However, it is clear that the effects of SR-5-6 (blue circles) were unaltered by any of the γ subunits. This is reflected in Figure 2D, which shows the summary $ΔV_{1/2}$ produced by SR-5-6. Although a small reduction in the $ΔV_{1/2}$ induced by SR-5-6 (−86 ± 3 mV, n = 6) was observed with the $γ₁$ subunits, this was not significantly different to the $ΔV_{1/2}$ observed with SR-5-6 from BKα channels (−103 ± 4 mV, n = 15). Similarly, the $ΔV_{1/2}$ was −99 ± 8 mV (n = 5) in BKαγ₂, −91 ± 3 mV (n = 3) in BKαγ₃ and −108 ± 7 mV (n = 6) in BKαγ₄ and none of these were significantly different to that observed with the BKα channels.

**SR-5-44 effects were selectively reduced in the presence of different γ subunits**

It is apparent from the data above, that the effects of SR-5-6 were largely unaffected in the presence of any of the γ subunits. To test if this held true for other members of the GoSlo-SR family, we next examined the effects of SR-5-44 (structure shown in Fig. 1B). Note that this compound differs from SR-5-6 in having an extra methyl group in ring D at the para-position. This compound was previously shown to be the most efficacious member of the GoSlo-SR family.²³ We applied SR-5-44 at 3 μM (EC₅₀ = 2.3 μM, ²³) and examined its effects on the BKαγ₁,γ₄ subunits.

Figure 3A-C shows a family of currents evoked by stepping through a range of potentials from −100 mV to +100 mV in BKα, BKαγ₁ and BKαγ₄ channels respectively. The left panels show control currents (in 100 nM Ca²⁺) and the middle panels show currents recorded in the presence of 3 μM SR-5-44. Although, SR-5-44 significantly enhanced current amplitude in both BKα (Fig. 3A) and BKαγ₄ (Fig. 3C) channels, only a small excitatory effect of SR-5-44 was observed in BKαγ₁ channels (Fig. 3B). Summary activation curves are shown in the right panels of Figure 3A-C, where the light blue circles indicate the shift induced by SR-5-44. In BKα channels, the control $V_{1/2}$ was 162 ± 2 mV and this was shifted to 75 ± 2 mV in SR-5-44 ($ΔV_{1/2} = −76 ± 3 mV$, n = 10). However in BKαγ₁ channels, SR-5-44 only shifted $V_{1/2}$ from 3 ± 1 mV to −18 ± 2 mV ($ΔV_{1/2} = −23 ± 3 mV$, n = 8). In contrast, SR-5-44 shifted the $V_{1/2}$ of BKαγ₄ from 133 ± 2 mV in 100 nM Ca²⁺ to 51 ± 3 mV (n = 5). The effects of this drug on the remaining γ subunits are shown as a dot plot in Figure 3D. Figure 3E shows the mean effect of SR-5-44 on $ΔV_{1/2}$ in all of the γ subunits compared with the BKα channels. In the BKα channels, the $ΔV_{1/2}$ of SR-5-44 was −76 ± 3 mV (n = 10) and this was significantly reduced to −23 ± 3 mV (p < 0.001, n = 8) in BKαγ₁ channels. The effects of SR-5-44 on $ΔV_{1/2}$ were also reduced compared to BKα in the BKαγ₂ (−36 ± 1 mV, n = 3, p < 0.01) and BKαγ₃ channels (−47 ± 5 mV, n = 4, p < 0.05). In the least effective γ₄ subunit, the $ΔV_{1/2}$ of SR-5-44 was −82 ± 5 mV (n = 5), which was not significantly different from the effect of SR-5-44 on BKα channels.

We next repeated the experiments on BKαγ₁ channels using SR-5-6 at a concentration of 3 μM. The representative traces in Figure 4A show tail currents recorded at −80 mV after depolarizing 2 different patches to −20 mV. The top and bottom traces in each record indicate the currents evoked in 100 nM and 10 μM Ca²⁺, respectively. The effect of 3 μM SR-5-6 is shown in Figure 4Ai, (light blue trace) and 3 μM SR-5-44 in Aii (dark blue trace). SR-5-6 had a greater effect on tail current amplitude compared to SR-5-44 and as it increased from 0.6 nA in 100 nM Ca²⁺ to 2.6 nA (~4-fold). However, application of SR-5-44 increased the tail current amplitude ~2-fold (from 0.8 nA to 1.7 nA). These traces show that the activation effects of 3 μM SR-5-44 but not 3 μM SR-5-6 were attenuated in BKαγ₁ channels. Figure 4B shows a comparison of G/Gₘₙ plots for SR-5-6 (Bi, left, light blue symbols) and SR-5-44 (Bii, right, dark blue symbols). SR-5-6 shifted the $V_{1/2}$ in 100 nM Ca²⁺ from 8 ± 1 mV to −32 ± 3 mV, yielding a mean $ΔV_{1/2}$ of −41 ± 2 mV (n = 7). In contrast, SR-5-44...
only shifted the $V_{1/2}$ from $3 \pm 1$ mV to $-18 \pm 3$ mV, resulting in a $\Delta V_{1/2}$ of $-23 \pm 3$ mV ($n = 8$).

Figure 4C compares the mean $\Delta V_{1/2}$ values of SR-5-6 and SR-5-44 on BK$\alpha$ and BK$\alpha$γ1 channels. The $\Delta V_{1/2}$ produced by SR-5-6 in the absence and presence of γ1 subunits was not statistically different whereas, the effects of SR-5-44 were significantly reduced ($p < 0.001$).
SR-5-130 activation effects were enhanced by γ subunits

We finally examined the effect of SR-5-130 on BK\(\alpha\) channels co-expressing \(\gamma_{1-4}\) subunits. SR-5-130 is the deaminated derivative of SR-5-6,\(^{23}\) since it lacks the NH\(_2\) group on C ring (structure shown in Fig. 1C). In agreement with the previous studies by Large et al., (2015), this drug was approximately 4-fold less effective at shifting the \(V_{1/2}\) of BK\(\alpha\) channels compared to BK\(\alpha\)\(\gamma_{1}\) channels. Figure 5A & B compares the effects of 10 \(\mu\)M SR-5-130 on BK\(\alpha\) and BK\(\alpha\)\(\beta_{1}\) channel...
currents recorded from −100 mV to +100 mV. It is evident from the traces in Panel B that the presence of β1 subunits significantly slows the activation and deactivation kinetics of BKα channels as previously reported (Orio and Latorre, 2005, Ahring et al., 1997). In BKα channels, SR-5-130 increased the current amplitude slightly, whereas when β1 subunits were present, it enhanced current amplitude significantly. The efficacy of SR-5-130 (pink circles) in the absence and presence of β1 subunits is also displayed in the summary activation curves of Panels A and B respectively. Interestingly, when this drug was applied to BKαγ1 channels (Fig. 5C), its effects were significantly increased compared to its effects on BKα channels. Similarly, when the effects of SR-5-130 were examined in the presence of the other γ subunits (Fig. 5D & E) a small, but significant increase in ΔV1/2 was observed. Thus, in BKα channels the ΔV1/2 produced by SR-5-130 was −31 ± 3 mV (n = 4) compared to −106 ± 7 mV (n = 5, p < 0.001) in BKαβ1 channels. The ΔV1/2 values of SR-5-130 were −44 ± 3 mV (n = 5) in BKαγ1, −51 ± 3 mV (n = 4) in BKαγ2, −44 ± 3 mV (n = 6) in BKαγ3 and −52 ± 2 mV (n = 4) in BKαγ4 (p < 0.05) channels.

A comparison of free energy changes (ΔΔG0) associated with drug binding on BKα and BKαγ channels

The energetic contribution of these drugs to activate the channels was also estimated to provide a direct indication of any alteration in the drugs’ efficacy. Figure 6A-C compares the free energy (ΔΔG0) provided by SR-5-6, SR-5-44 and SR-5-130 to open the BKα and BKαγ1-4 channels. The ΔΔG0 for these drugs were estimated using the V1/2 and slope (z) values obtained from Boltzmann fits to the GV relationships as described in the methods section. Similar to the unchanged ΔV1/2 effects of SR-5-6 in the presence of any γ subunit, the ΔΔG0 (SR-5-6) in BKαγ1-4 channels was also not significantly different compared to the BKα channels (Fig. 6A). Interestingly, the ΔV1/2 data of SR-5-44 in BKαγ1-4 channels was found to be consistent with its energetic effect (ΔΔG0 SR-5-44) on each of the channel (Fig. 6B). In BKα channels, SR-5-44 reduced the free energy of channel opening by −1.5 ± 0.1 kcal/mol. In each of the BKαγ1-4 constructs, this was significantly reduced in a similar pattern to the ΔV1/2 data, i.e. BKαγ1 (−0.6 ± 0.1 kcal/mol, p < 0.001), BKαγ2 (−0.7 ± 0.01 kcal/mol, p < 0.001) and BKαγ3 (−0.9 ± 0.1 kcal/mol, p < 0.05) channels. However, there was no significant change observed in BKαγ4 channels (−1.3 ± 0.1 kcal/mol). Small changes were observed in ΔΔG0 (SR-5-130) with some of the BKαγ constructs as shown in Figure 6C. It is also interesting to note that SR-5-130 reduced the free energy of BKα channels opening by −0.4 ± 0.1 kcal/mol, compared to −1.7 ± 0.1 kcal/mol in BKαβ (p < 0.001) and −0.8 kcal/mol in BKαγ1-4 channels.

Discussion

The current study investigated the effects of 3 different GoSlo-SR family members (SR-5-6, SR-5-44 and SR-5-130) on each of the 4 regulatory γ subunits. Despite the subtle differences in the overall structures of each compound, we found that each behaved differently in BKαγ1-4 channels. The results demonstrate that the effects of SR-5-6 appear unaltered in the presence of γ1-4 subunits. However, the more hydrophobic compound SR-5-44 appeared to be much less effective at activating BKαγ1 channels. Finally, we found that the effects of SR-5-130 were partially enhanced in BKαγ1-4 expressing patches.

SR-5-6 has previously been characterized as a relatively potent (EC50 = 3.2 μM) and efficacious (shifts V1/2 > −100 mV at 10 μM) activator of BKα channels that did not require BKαβ subunits to mediate its full effects.16,23,26 The current study suggests that its effects were not significantly altered when γ subunits were co-expressed. These results were qualitatively similar to those obtained previously with the Neurosearch compound, NS1619,1 since the ΔV1/2 was −51 mV in BKαγ1 channels,1 which is similar to that observed in BKα channels (−62 mV at 50 μM; Gessner et al., 2012). Our data therefore suggests that SR-5-6 is a rather non-selective BK channel opener, in that it appears equally efficacious in the absence and presence of regulatory subunits. This compound would therefore be expected to lack the tissue selectivity necessary for the development of a therapeutic BK channel opener. Nevertheless, SR-5-6 may prove useful as BK channel opener for research purposes.

In contrast however, the effects of SR-5-44 were altered in BK channels comprising different γ subunits, which was surprising, given the similarity of this compound to SR-5-6. Intriguingly, the ability of SR-5-44 to shift the V1/2 of BKαγ1 channels, was reduced by ∼70%
compared to the BKα channels. These effects are similar to those observed with MTX. In their study, Almassy and Begenisich demonstrated that 0.5 mM MTX caused a \( \Delta V_{1/2} \) of \( \sim -100 \) mV but its efficacy was significantly reduced in the presence of \( \gamma_1 \) subunits (\( \Delta V_{1/2} = -17 \) mV with 5 mM concentration). It is also interesting to note that SR-5-44 was more effective in the remaining \( \gamma \) subunits in ascending order (i.e., \( \gamma_4 > \gamma_3 > \gamma_2 > \gamma_1 \)).

**Figure 5.** The effects of SR-5-130 are partially enhanced in BKα channels co-expressed with \( \gamma_{1-4} \) subunits. The currents evoked by voltage pulses from \(-100\) mV to \(+100\) mV in 100 nM Ca\(^{2+}\) (left) and 10 \( \mu \)M SR-5-130 (middle) in BKα, BKαβ₁, and BKαγ₁ channels are shown in Panels A, B, and C, respectively. SR-5-130 is the deaminated derivative of SR-5-6, whose structure is depicted in Figure 1C. It is evident from the traces that SR-5-130 increased the current amplitude slightly in BKα channels, whereas when \( \beta_1 \) subunits were present, it enhanced current amplitude much more. Interestingly, in BKαγ₁ channels, SR-5-130 increased the channel activation that is slightly greater than that of BKα but significantly lower to that of BKαβ₁ channels. The summary GV curves are also shown in the respective panels where a pronounced leftward shift of SR-5-130 (pink circles) is seen in BKαβ₁ channels. Panel D demonstrates the mean \( V_{1/2} \) values in 3 different Ca\(^{2+}\) concentrations and SR-5-130 for BKα, BKαβ₁, and BKαγ₁-4 channels. The mean \( \Delta V_{1/2} \) of 10 \( \mu \)M SR-5-130 in BKα channels is compared against BKαβ₁ and BKαγ₁-4 channels in Panel E. The dotted line indicates the mean \( \Delta V_{1/2} \) obtained in BKα channels, which was \( -31 \pm 2 \) mV (\( n = 4 \)). In the presence of \( \gamma \) subunits this shift was increased by only \( \sim -10 \) mV to \( -20 \) mV (p < 0.05, ANOVA) compared to that of BKα channels. However, in BKαβ₁ channels, SR-5-130 produced a shift that is \( \sim -70 \) mV (p < 0.001) greater than that of BKα channels. The numbers in parentheses indicate the number of replicates.
of their effect on V1/2 of BKα channels. It might be possible that the extra methyl group of this drug prevents it from binding effectively when the γ1 subunits (and other efficacious subunits) are present. To strengthen this proposition, future work should be examining various substitutions of this methyl group to more hydrophobic groups such as octyl-, butyl- or phenyl- groups. If the above hypothesis holds true, these compounds will be much less effective than SR-5-44 in BKαγ1 channels. Li et al., (2015, 2016) have reported recently that the γ subunits ability to shift the voltage dependence of activation depends primarily on the γ TM segments and the adjacent cluster of charged residues in the C-tail. The effectiveness of the different γ subunits may rely upon how tightly their TM segment associate with the α subunit to enhance allosteric coupling between voltage sensors and the pore. Such a tight interaction may hinder access or alter binding of the bulkier D ring of SR-5-44. Thus, when the γ1 subunits are present, drug interactions with the channel could be reduced, accounting for its reduced efficacy. In the BKαγ4 constructs, the γ4 TM may not be as strongly associated with the α subunit and the bulkier SR-5-44 conceivably has full access to its binding site. Li et al., (2016) have recently identified a residue, phenylalanine (F273) in the middle of γ1 TM segment to play an essential role in the γ1 subunits modulation and association with BKα channels. This residue is also present in the γ2 subunit (F256), when mutated to serine led to a complete loss of γ2 modulatory function on the BKα channels. Thus it is also possible that this aromatic hydrophobic amino acid in γ1 and 2 subunits might have induced a steric hindrance effect such that the drug’s interaction with its binding sites on BKα channels is altered. This residue is not conserved in γ4 subunits (S259) suggesting that the drug can have its full binding effects on BKα channels. Another alternative is that the number of charged residues in the C-tail of the γ subunits could alter SR-5-44 binding. Previous studies have suggested that the arginines play a significant role in the modulatory effect of γ subunits on BKα.17,18 It is conceivable that the cluster of 6 arginine residues in the tail region of γ1 could alter the hydrophilicity of the local environment and perhaps alter interactions with the more lipophilic compound, SR-5-44.

The β subunit selective opener, SR-5-13016 did not show its maximal effects on BKαγ channels. The enhanced efficacy of SR-5-130 in the presence of β subunits suggests that either the drug’s binding site was

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**Figure 6.** The γ subunits reduce the mean free energy (ΔΔG₀) imparted by SR-5-44 but not SR-5-6. The mean free energies contributed by the 3 drugs to open the BKαγγ1-4 channels are compared against BKα channels in Panels A (SR-5-6), B (SR-5-44), and C (SR-5-130). The ΔΔG₀ by SR-5-6 in BKα channels was not significantly different to that in BKαγγ1-4 channels. Consistent with the reduced efficacy of SR-5-44 in different γ subunits, the ΔΔG₀ (SR-5-44) was significantly reduced in BKαγγ1, 2 (p < 0.001) and BKαγγ3 (p < 0.05) but not in BKαγγ4 channels. The application of SR-5-130 induced a small increase of ΔΔG₀ in all of the 4 γ subunits (γ1, and γ4, p < 0.05) compared to the BKα alone. However, this increase was not as marked as that of in BKαβ1 channels (p < 0.001). The numbers of replicates are shown in parentheses.
subtly changed such that it is more accessible to promote greater channel opening or, the transduction pathway of drug is altered. The inability of the γ subunits to fully restore the effects of SR-5-130 was not surprising, since the structure and mechanism of action of β subunits is quite distinct to that of γ subunits.

We have also calculated the free energy changes using Boltzmann fits to the the GV curves to quantify the effects of these drugs on different constructs. In agreement with their effects on $\Delta V_{1/2}$, a similar pattern in their free energy of channel opening was also observed. The free energy of channel opening was only moderately reduced ($-0.4$ kcal/mol) by SR-5-130 in BKα and this was reduced slightly more in the BKαγ1-4 channels (Fig. 6C). However in BKαβ1 channels, SR-5-130 reduced the energy of channel opening ~4 fold, perhaps suggesting that the presence of β1 subunit alters the binding affinity of the drug. As shown in Figure 6A, the reduction in the energy required for BKα channel opening was reduced by $\sim -1.7$ kcal/mol with SR-5-6 ($\Delta G_0$ SR-5-6), consistent with the data that it was a more efficacious opener of BKα than SR-5-130. 16 The effects of SR-5-6 on the $\Delta G_0$ were not significantly altered in any of the BKαγ constructs, perhaps suggesting that neither SR-5-6 binding, nor its transduction pathway were altered by γ subunits. Intriguingly, SR-5-44 was much less effective at reducing the energy required for channel opening in BKαγ1-3 channels, suggesting that its efficacy was reduced when γ subunits are present. To precisely distinguish between either SR-5-44 binding or its transduction pathways are perturbed by γ subunits, more detailed biophysical and biochemical binding studies are needed. It will also be of interest to determine which regions of the γ1 subunit are responsible for reducing the effects of SR-5-44. Utilizing γ1 and γ4 chimera to allow the swapping of the TM segments or C-tails would facilitate in the identification of potential residues (such as F273) that may participate in modifying the binding or transduction pathway of SR-5-44.

This study might be advantageous in the future classification of BK channel openers. A large number of BK channel openers have already been classified based on their requirement for regulatory β subunits to mediate their maximal effects e.g. tamoxifen, 8 Evans blue, 27 docosahexaenoic acid (DHA), 13 lithocholic acid 5 and SR-5-130. 16 The present study suggests that BK openers can also be classified in terms of their effectiveness on γ subunits. Thus, BK channel openers such as MTX 1 and SR-5-44 (Roy et al., 2012) can be placed into a γ1 ineffective sub-category, whereas NS1619 1 and SR-5-626 appear to fit into γ1 effective category.

In conclusion, this study suggests that subtle modifications of the GoSlo-SR compounds can alter their efficacy when applied to BKαγ constructs. Some members of the GoSlo-SR family may serve as useful starting structures for the development of more tissue specific BK channel openers.

**Materials and methods**

*Ion channel cloning and heterologous expression in HEK293 cells*

The BKα and β1 subunit were isolated from rabbit urethra smooth muscle (RUSM) and cloned into pcDNA3.3 TOPO vector (Life Technologies) using standard molecular biology procedures. The identified rabbit BKα transcript (rBKα) corresponded to the ZERO variant of murine BKα and to variant 2 (NM_002247.3) of the human BKα (hBKα) subunit. The rBKα channel was “humanized” by mutating A60T and S657T residues. The GoSlo-SR-BKα-130 experiments were performed on rBKα and rBKαβ1 constructs and all other electrophysiology experiments were performed using the hBKα construct.

Since initial transient transfection of HEK cells with separate BKα and γ plasmids (1:5 ratio) failed to produce reliable expression of γ subunits, we utilized a bicistronic plasmid expression system to permit simultaneous expression of 2 genes (BKα and BKγ1-4). These BKαγ1-4 fusion constructs reliably produced BK currents that activated at more negative potentials, as shown previously. 26 HEK cells were grown in DMEM/MEM medium supplemented with 10% heat-inactivated FBS (fetal bovine serum) and 1% Penicillin/streptomycin antibiotic at 37°C in a humidified incubator with 5% CO2. The BKα and BKαγ1-4 encoding plasmid DNAs were introduced into HEK cells using the calcium phosphate transfection method. 9 HEK cells were used for electrophysiological recordings 24 to 48 hours after transfection.

**Electrophysiology**

Electrodes were pulled from Corning borosilicate glass (1.5 mm O.D. × 0.86 mm I.D.) using a Sutter P-97 pipette puller and were fire polished using a Narashige MF 83 microforge. Pipettes had a resistance of 2-5 MΩ when filled with recording solutions and series resistance
was compensated by up to 80%. Standard single-channel patch clamp recording methods were used in the inside-out patch conformation in whole cell mode. 

Voltage clamp commands were delivered via either Axopatch 1D or Axopatch 200B patch clamp amplifiers (Axon Instruments) connected to Digidata 1322A AD/DA converters (Axon Instruments) interfaced to computers running pClamp software (Axon Instruments). Data was acquired at 100 kHz and filtered at 2 kHz. Patches were held at either −60 mV or −100 mV and depolarized in 20 mV increments to 200 mV. In the BKαγ1 and BKαγ2 experiments with 10 μM Ca2+ the patches were stepped from −200 mV to +200 mV. Residual capacitance and leakage currents were subtracted using either a P/4 protocol, or offline by manual leak subtraction. However, in BKαγ1 patches, the channel open probability in 10 μM Ca2+ was ~0.75 even at holding potentials of −100 mV. We initially tried to hold these patches at potentials more negative to −100 mV but the patch stability was affected at these potentials, resulting in lost seals. Given that the seal resistance of these patches was very stable over time, we assumed that the leakage current remained the same in low and high Ca2+. Consequently, we utilized the leak current obtained in higher Ca2+ to estimate the leakage current recorded in higher Ca2+. The current records were copied as metafiles and pasted into PowerPoint for figure preparations.

Data analysis

The currents recorded in the presence of BKαγ1 displayed prominent voltage dependent block of steady-state currents at very positive potentials consistent with the previous studies. As a result, activation curves (G/Gmax) were constructed from the tail current obtained in 100 nM Ca2+.

G/Gmax were constructed from the tail current obtained in 100 nM Ca2+

The currents recorded in the presence of BK currents were recorded at 37°C and BKαβ1/γ1.4 channels was calculated using.

\[
\Delta G_0 = 0.2389zFV_{1/2}
\]

Where F is the Faraday constant. ΔG0 has the units of energy (kcal/mol). The change in ΔG0 produced in the presence of a drug in BKα and BKαβ1/γ1.4 channels was calculated using.

\[
\Delta \Delta G_0(\text{drug}) = \Delta G_0(\text{drug}) - \Delta G_0(100 \text{ nM Ca}^{2+})
\]

Synthesis of GoSlo-SR family

The GoSlo-SR compounds were synthesized by a microwave assisted Ullmann coupling reaction as described previously in Roy et al., (2012, 2014). The structures of these compounds were confirmed using 1H NMR and high-resolution mass spectra and their purity was determined by HPLC analysis. Stocks of 10 mM of these drugs were made up in DMSO/water and stored at 4°C until use. All drugs were applied to the cytosolic face of the patches in 100 nM Ca2+.

Recording solutions

BK currents were recorded at 37°C in 140 mM symmetrical K+ solutions containing (in mM) 140 KCl, 10 Glucose, 10 HEPES and 1 EGTA (for free [Ca2+] 100 nM to 300 nM) or 1 HEDTA (for free [Ca2+] 1 μM to 10 μM). Pipette solutions had a free [Ca2+] of 100 nM. We used this low divalent cation pipette solutions to allow us to directly compare our results with those published previously on rabbit bladder smooth muscle cells. The concentration of total Ca2+ required to yield the
desired free Ca\(^{2+}\) was calculated using Schoenmaker’s Chelator program (http://www.organphy.science.ru.nl/chelator/Chelmain.html). The Ca\(^{2+}\) was added as CaCl\(_2\) and pH of all the solutions was adjusted to 7.2 with KOH. All solutions were made with double distilled, deionized, filtered water from a MilliQ water purification system. The cytosolic side of the membrane was perfused continuously and the solutions were interchanged using a gravity-fed perfusion system. In all experiments, GoSlo-SR compounds were applied to the cytosolic face of patches in the presence of 100nM Ca\(^{2+}\). The EC\(_{50}\) of these compounds range from 2-4 \(\mu\)M and \(^{16,22,23}\) and were applied to patches at concentrations of either 3 \(\mu\)M or 10 \(\mu\)M.

**Abbreviations**

| Abbreviation          | Description                                                                 |
|-----------------------|----------------------------------------------------------------------------|
| BK channels           | Large conductance voltage and Ca\(^{2+}\)-activated K\(^{+}\) channels     |
| GoSlo-SR-5-6          | (Sodium 1-Amino-4-((3-trifluoromethylphenyl)amino)-9,10-dioxo-9,10-dihydroantracene-2-sulfonate) |
| GoSlo-SR-5-44         | Sodium 1-Amino-4-(4-methyl-3-(trifluoromethyl)phenylamino)-9,10-dioxo-9,10-dihydroantracene-2-sulfonate |
| GoSlo-SR-5-130        | (9,10-dioxo-4-((3-(trifluoromethyl)phenyl)amino)-9,10-dihydroantracene-2-sulfonic acid) |
| LRRC proteins         | Leucine rich repeat containing proteins                                    |
| \(V_{1/2}\)            | Voltage required to reach half maximal activation of channels in a patch    |
| \(\Delta V_{1/2}\)     | Change in \(V_{1/2}\); G-V, Conductance-Voltage                            |

**Disclosure of potential conflicts of interest**

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

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