The smooth muscle-type $\beta_1$ subunit potentiates activation by DiBAC$_4$(3) in recombinant BK channels

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Abbreviations: BK channel, large conductance calcium-activated potassium channel; DiBAC$_4$(3), Bis (1,3-Dibutylbarbituric Acid) Trimethine Oxonol

Large-conductance $\mathrm{Ca}^{2+}$-activated (BK) channels, expressed in a variety of tissues, play a fundamental role in regulating and maintaining arterial tone. We recently demonstrated that the slow voltage indicator DiBAC$_4$(3) does not depend, as initially proposed, on the $\beta_1$ or $\beta_2$ subunits to activate native arterial smooth muscle BK channels. Using recombinant mSLO BK channels, we now show that the $\beta_1$ subunit is not essential to this activation but exerts a large potentiating effect. DiBAC$_4$(3) promotes concentration-dependent activation of BK channels and slows deactivation kinetics, changes that are independent of $\mathrm{Ca}^{2+}$. $K_v$ values for BK channel activation by DiBAC$_4$(3) in 0 mM $\mathrm{Ca}^{2+}$ are approximately 20 $\mu$M ($\alpha$) and 5 $\mu$M ($\alpha+\beta_1$), and G-V curves shift up to ~40mV and ~110 mV, respectively. $\beta_1$ to $\beta_2$ mutations R11A and C18E do not interfere with the potentiating effect of the subunit. Our findings should help refine the role of the $\beta_1$ subunit in cardiovascular pharmacology.

Introduction

BK channels, part of the voltage-activated potassium channel family, can be activated by membrane depolarization and/or intracellular $\mathrm{Ca}^{2+}$ increases. In smooth muscle as well as several other tissues, the opening of BK channels provides a negative feedback mechanism that opposes cell excitation events.1-3 BK channel exist, in most tissues, as heteroctomers formed by 2 types of subunits: $\alpha$ and $\beta$ subunits. The regulatory $\beta$ subunit, first described in smooth muscle, comprises 2 transmembrane domains (TM1-TM2) linked by an extracellular glycosylated loop.4 Four different subtypes of $\beta$ subunits (B1 to B4) with distinctive tissue-specific expression patterns have been identified in mammals; these correspond to the products of 4 different genes, $\text{KCNMB1}$ to $\text{KCNMB4}$.5-7 A recently described type of auxiliary subunit, named $\gamma$, belongs to the family of leucine-reach repeat-containing protein 26.8 We did not find evidence for this subunit in aortic smooth muscle cDNA.9 The $\alpha$ subunit comprises 7 transmembrane regions (S0-S6) and a large cytosolic region.10 This subunit and its multiple alternative splice variants are encoded by one gene, $\text{Slo/KCNMA1}$.11-14

The transmembrane regions of the $\alpha$ subunit contain the pore gate domain (PGD) that expands between S5–S6 segments and the voltage-sensor domain (VSD) that, presumably, includes S0–S4 segments.15,16 The large C-terminal cytoplasmic domain (CTD) comprises approximately two-thirds of the protein. CTD contains a tandem of 2 regions homologous to the regulator of K+ (RCK1 and RCK2) conductance domain; this forms the gating ring of the channel and involves $\mathrm{Ca}^{2+}$ binding sites.17,18 The PGD, VGD, and CTD modules are proposed (Horrigan and Aldrich, HA model)19-21 to interact allosterically to activate the channel, consistent with the modular nature of BK channels.15,22 Specifically, structural rearrangements are transmitted to the PGD upon $\mathrm{Ca}^{2+}$ binding to the

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gating ring of the channel or activation of the voltage sensors, or both, thereby increasing the probability of opening the gate. These allosteric components (Ca²⁺-dependent, voltage-dependent, or intrinsic gating) are potential effectors of BK channel openers.

Intensive research is being devoted to the identification of pharmacological BK channel openers. In particular, a channel-opening compound with subunit specificity is relevant for cardiovascular physiology, where the β subunit is predominant and acts as a molecular tuner critical to vasoregulation. The importance of subunit specificity of channel activators extends to other fields, as well, because of the tissue-specific distribution of β subunits (i.e., β₁ in nervous system). The slow voltage indicator DiBAC₄(3) was found to have BK channel-specific effects. Moreover, these activating effects on recombinant BK channels were described to be specific only for the β₁ and β₄ subunit. We recently demonstrated, however, that DiBAC₄(3) does not depend, as initially proposed, on the β or β₄ subunits to activate native arterial smooth muscle BK channels. In this addendum, we extended our studies to investigate the requirement of the smooth muscle-type β₁ subunit to activate recombinant BK channels by DiBAC₄(3). We report that the β₁ subunit is not essential to this activation but exerts a large potentiating effect. These findings provide a foundation for the development of BK channel-mediated vasoregulators and for the dissection of intricate molecular mechanisms involved in BK channel modulation.

**Results**

DiBAC₄(3) can activate α alone BK channels and markedly activate α+β₁ BK channels

In our previous paper, we demonstrated that native single BK channels lacking β₁ (or β₄) subunits were readily activated by DiBAC₄(3). Using the heterologous expression of recombinant BK channels in HEK cells, we analyzed the effect of DiBAC₄(3) on macroscopic BK currents from excised patches in the absence of Ca²⁺. In α alone BK channels, 10 μM DiBAC₄(3) produced an increase in the size of steady-state ionic currents and slowed the deactivation of the current when the channels were stepped back to -120 mV (Fig. 1A, left). For comparison, a steady-state current-voltage relationship was plotted in the absence and in the presence of 10 μM DiBAC₄(3); the dye shifted the threshold for current activation from 80 mV to 50 mV (Fig. 1B, left). The increase in current together with the slowing of current deactivation kinetics appear to be the most salient features for the effect of DiBAC₄(3) on recombinant BK channels. These 2 characteristic effects of DiBAC₄(3) were enhanced in the presence of the β₁ subunit. Currents from α+β₁ BK channels were recorded using solutions identical to those used for α alone and using a similar voltage protocol, except for a longer duration of pulses to allow the channels to reach steady-state. These currents showed typical slower kinetics, a signature of β₁ modulation of BK channels, and were enhanced ~3-fold, at steady-state, by the application of 10 μM DiBAC₄(3) (Fig. 1A, right). Indeed, DiBAC₄(3) displaced the threshold for current activation from 95 mV to 15 mV (Fig. 1B, right). The dramatic effect on deactivation kinetics can be observed from the traces.

DiBAC₄(3) shifts the voltage-dependent activation of BK channels, and slows current deactivation, in a concentration-dependent manner

We performed a series of similar experiments to examine the
concentration-dependent characteristics of DiBAC(3) effects (Fig. 2 and Table 1). Tail currents at -120 mV, normalized to the maximum tail current at 200 μM Ca^2+, were used to obtain conductance vs. voltage (G-V) relationships. Grouped G-V relationships obtained from α alone channels (9 to 16 independent experiments) showed that increasing DiBAC(3) concentrations promoted a leftward shift in the G-V relationship in a concentration-dependent manner (Fig. 2A, left). Further, this effect was more pronounced in the presence of the β subunit (Fig. 2A, right), investigated using α + β channels (5 to 8 experiments).

To accurately estimate the differential effect of DiBAC(3) on α vs. α + β1 BK channels, we fitted each individual experiment to a Boltzmann equation, and plotted the averaged V_{1/2} as a function of DiBAC(3) concentration (Table 1 and Figure 2). As shown in Figure 2B, V_{1/2} decreased with increasing concentrations of DiBAC(3) in α alone channels, and to a greater extent in α + β1 channels. Hill function fitting of the data suggested that the β subunit promotes a 4-fold decrease in the K_d for DiBAC(3) from 22 μM in α channels (with an N of Hill of 0.93), to 5 μM in α + β1 channels (N of Hill 1.27). We also analyzed the concentration dependence of DiBAC(3) in the slowing of channel deactivation kinetics. Time constants obtained from mono-exponential fittings of current relaxation at -120 mV (from -65 to -190 mV) after an activating step to +90 mV (α alone channels) or to +150 mV (α + β1 channels). Currents were recorded in the absence (control) or in the presence of 30 μM DiBAC(3) for both, α alone or α + β1 channels. Continuous lines represent single exponential fittings to the data. (E) Plot of activation time constant vs. DiBAC(3) concentration. Symbols represent averaged activation time constants obtained from single exponential fits of current activation at 200 mV from a holding potential of -120 mV. Data are expressed as mean ± s.e.
function fitting of the data, and obtained a $K_v$ value of 10 μM for $\alpha$ alone and 6.8 μM for $\alpha+\beta$ BK channels; with $N$s of Hill of 1.7 and 1.9, respectively.

We next examined the voltage-dependent characteristics of current relaxation kinetics after a depolarizing step in the presence and absence of 30 μM DiBAC$_{(3)}$. Time constants obtained from these currents are plotted as a function of voltage in Figure 2D. In $\alpha$ alone channels, DiBAC$_{(3)}$ produced an increase in the deactivation time constant with an apparent disruption of its voltage dependence. On the other hand, in $\alpha+\beta$ channels the deactivation time constants were also increased in the presence of DiBAC$_{(3)}$ (approximately by a factor of 50), but the voltage dependence was unaltered. The slope of the exponential fitting remains similar.

We also evaluated activation kinetics at 200 mV. We were not able to detect any noticeable effect of DiBAC$_{(3)}$; time constants remained almost unchanged at increasing concentrations of DiBAC$_{(3)}$ (Fig. 2E).

BK channels at saturating Ca$^{2+}$ concentrations can be further activated by DiBAC$_{(3)}$

As DiBAC$_{(3)}$ activation appears to be independent of Ca$^{2+}$-dependent activation of the channels, we examined whether DiBAC$_{(3)}$ can activate BK channels at saturating concentrations of Ca$^{2+}$: when all high-affinity Ca$^{2+}$ binding sites of the channel are likely to be occupied. Even under conditions of maximal activation by Ca$^{2+}$ (200 μM), DiBAC$_{(3)}$ produced a further hyperpolarizing shift in the I-V relationship (Fig. 3, left). This effect was more pronounced in channels formed by $\alpha+\beta$, subunits (Fig. 3, right).

$\beta$, to $\beta$ mutations do not prevent the potentiating effect of the $\beta$ subunit on DiBAC$_{(3)}$ activation of BK channels

DiBAC$_{(3)}$ was initially described as a BK channel activator acting only when $\beta_1$ or $\beta_4$ subunits were part of the channel, with the $\beta_2$ subunit having no role—or even an inhibitory one—in the presence of DiBAC$_{(3)}$. Additionally, specific $\beta$ to $\beta$ mutations were recently reported to dampen the differential effect of omega-3 fatty acids on channels containing $\beta_1$ vs. $\beta_3$ subunits. Our observations of the consistent enhancing influence of the $\beta$ subunit on the effects of DiBAC$_{(3)}$ prompted us to introduce these mutations into our experiments. We mutated $\beta_1$ subunits in the residues Arg11 or Cys18 to obtain $\beta_{11}$ and $\beta_{18}$ subunits. We co-expressed $\alpha$ subunits with either $\beta_{11}$ or $\beta_{18}$. Differential effects were investigated by exposing the mutant channels to a DiBAC$_{(3)}$ concentration (1.1 μM) that is not high enough to activate $\alpha$ alone channels. DiBAC$_{(3)}$ activated BK currents in patches with both $\alpha+\beta_{11}$ and $\alpha+\beta_{18}$ subunits (Fig. 4A and B). Further, application of 1.1 μM DiBAC$_{(3)}$ promoted a leftward shift in the current-voltage relationship and slowed the deactivation kinetics in both cases. The $V_{1/2}$ shifted from 178 ± 7 mV to 144 ± 10 mV for $\alpha+\beta_{11}$ channels (n = 6; $P < 0.005$) and from 162 ± 5 mV to 128 ± 4.8 mV for $\alpha+\beta_{18}$ channels (n = 6; $P < 0.005$) (Fig. 4C).

This suggests that $\beta$ residues Arg11 and/or Cys18 are unlikely contributing to the differential effect of DiBAC$_{(3)}$ on $\alpha$ alone vs. $\alpha+\beta$, channels.

Discussion

In our recent single-channel study recording arterial smooth muscle BK channels we showed that DiBAC$_{(3)}$ is able to activate native BK channels in the absence of $\beta_1$ or $\beta_4$ subunits. We now show that recombinant BK channels can also be activated by DiBAC$_{(3)}$ in the absence of auxiliary $\beta$ subunits, suggesting a binding site for the dye is located in the $\alpha$ subunit and the $\beta$ subunit plays a modulatory role. This is not the first time an activator was originally described as $\beta$-specific but was later shown to activate $\alpha$ alone channels as well: DHS-I (dehydrosoyasaponin I) and tamoxifen also fit this description. Importantly, the substantial $\beta$ modulatory effects obtained with common recombinant BK channel variants may mislead the interpretation of activating effects as being produced by genuine $\beta$-specific ligands. Careful inspection of a wide range of concentration-dependent effects is advised for determining the stringency for $\beta$ subunit requirements. The differential effect of DiBAC$_{(3)}$ in $\alpha$ vs. $\alpha+\beta$, channels presented here might be at the root of the initially observed $\beta_1$/ $\beta_3$ specificity. A detailed inspection of the effect of DiBAC$_{(3)}$ on BK channels highlights a mechanism that involves a shift in the voltage-dependent activation of the channel together with a deceleration of the deactivation kinetics.

DiBAC$_{(3)}$ shifts voltage-dependent activation

In the virtual absence of Ca$^{2+}$ we observed a concentration-dependent shift in the voltage-dependent gating of Ca$^{2+}$ channels.}

| Table 1. DiBAC$_{(3)}$ concentration-dependent effects on BK channels |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| DiBAC$_{(3)}$ (μM) | $V_{1/2}$ (mV) | $Q$ (pC/pF) | $\tau_{\text{Activation}}$ (ms) | $\tau_{\text{Deactivation}}$ (ms) | $V_{1/2}$ (mV) | $Q$ (pC/pF) | $\tau_{\text{Activation}}$ (ms) | $\tau_{\text{Deactivation}}$ (ms) |
| 0 | 167 ± 2 | 1.16 ± 0.04 | 2.53 ± 0.33 | 0.15 ± 0.01 | 180 ± 5 | 0.87 ± 0.14 | 21.80 ± 3.92 | 0.55 ± 0.09 |
| 0.12 | 166 ± 1 | 1.09 ± 0.04 | 2.37 ± 0.34 | 0.17 ± 0.01 | 163 ± 8 | 0.82 ± 0.16 | 29.31 ± 7.38 | 0.76 ± 0.05 |
| 0.37 | 163 ± 2 | 1.16 ± 0.08 | 3.08 ± 0.94 | 0.17 ± 0.01 | 158 ± 4 | 0.72 ± 0.10 | 25.76 ± 3.02 | 0.97 ± 0.16 |
| 1.1 | 160 ± 3 | 1.13 ± 0.08 | 2.69 ± 0.51 | 0.18 ± 0.01 | 151 ± 8 | 0.68 ± 0.11 | 25.14 ± 4.30 | 1.26 ± 0.14 |
| 3.33 | 156 ± 3 | 1.11 ± 0.07 | 3.08 ± 0.94 | 0.25 ± 0.02 | 124 ± 8 | 0.71 ± 0.07 | 23.70 ± 5.41 | 2.03 ± 0.06 |
| 10 | 141 ± 4 | 1.01 ± 0.03 | 2.64 ± 0.64 | 0.45 ± 0.08 | 90 ± 11 | 0.83 ± 0.28 | 22.50 ± 5.33 | 5.02 ± 0.71 |
| 30 | 123 ± 8 | 0.95 ± 0.03 | 2.81 ± 0.70 | 0.67 ± 0.10 | 69 ± 6 | 0.87 ± 0.17 | 17.42 ± 2.77 | 6.61 ± 0.92 |

$V_{1/2}$ is expressed in mV. Q is the number of elementary charges associated to channel activation. $\tau_{\text{Activation}}$ and $\tau_{\text{Deactivation}}$ are expressed in ms. $n \geq 5$ independent experiments.

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BK channels upon DiBAC$_4$(3) application. For α alone channels this shift is as much as 40 mV, with a small change in the associated charge observed at high concentrations of DiBAC$_4$(3) (> 10 μM). For α+β1 channels, DiBAC$_4$(3) can produce a shift larger than 110 mV, without significant changes in slope values. These values are smaller than what we have observed in native channels. This disparity probably stems from the molecular heterogeneity of native BK channel α subunits, and/or differences in post-translational modifications. Since DiBAC$_4$(3) also activates BK channels in saturating Ca$^{2+}$ concentrations, the Ca$^{2+}$-sensing domain of the channel appears not to be involved in the activating mechanism. Thus, DiBAC$_4$(3) is not acting as a Ca$^{2+}$ surrogate for BK channels and can activate BK channels beyond the maximal effect of Ca$^{2+}$. The G-V slopes were not greatly affected by DiBAC$_4$(3), suggesting that the voltage-sensing machinery of the channels might not participate; gating current measurements would be more appropriate to resolve this question.

DiBAC$_4$(3) slows deactivation kinetics

In the presence of DiBAC$_4$(3) we observed a consistent slowing of deactivation kinetics that was concentration-dependent. Indeed, (30 μM) DiBAC$_4$(3) prolongs the deactivation time constants more than 4-fold in both α and α+β1 channels, but does not affect activation kinetics. According to the HA model for BK channel gating, the changes induced by DiBAC$_4$(3) may result from a mechanism affecting the intrinsic closed-to-open (C-O) transitions of the channels. This process differs from the voltage-sensor movement but is still (weakly) voltage-dependent. Changes in relaxation kinetics are expected if DiBAC$_4$(3) interferes with the C-O conformational changes. The deceleration of deactivation kinetics suggests that DiBAC$_4$(3) may promote a destabilization of the closed state of the channel. If macroscopic deactivation kinetics are dictated by single-channel bursting behavior, the observed prolongation of deactivation time constants with DiBAC$_4$(3) supports our previous observation in native single channels where DiBAC$_4$(3) shortened the channel’s interburst time in a concentration-dependent fashion (Figure 3C from Scornik et al.). Nevertheless, an intermediate Ca$^{2+}$ concentration (3 μM) was used in that study, yielding a more complex gating scheme.

We also analyzed the voltage dependence of deactivation time constants at a single DiBAC$_4$(3) concentration. The intrinsic C-O transition is slightly voltage-dependent. Under control conditions, the voltage dependence of deactivation time constants can be described by an exponential function that has very similar slopes for both α and α+β1 channels. The application of DiBAC$_4$(3) produces a prolongation of time constants. However, our analysis reveals a differential effect of DiBAC$_4$(3) for α vs. α+β1 channels. At −190 to −70 mV, DiBAC$_4$(3) prolongs deactivation time constants of α alone channels more than 20-fold but disrupts the voltage dependence of channel closing by producing a shallower response to voltage. On the other hand, in the presence of the β1 subunit, DiBAC$_4$(3) prolongs the deactivation time constants more than 50-fold, while preserving the voltage dependence associated to the process. The physical interpretation of this remains unclear; a wider voltage range is needed to firmly establish the differential effect.

The role of the β1 subunit

The dye concentrations needed to half-maximally activate the channels are reduced by a factor of 4 in the presence of the β1 subunit. This parameter (K_L) changes from around 20 μM to 5 μM when the β1 subunit is present. The latter is very similar to the value obtained using native single channels in our previous work (3.4 μM in canine coronary BK channels), suggesting a similar affinity for the dye. The concentration-dependent effects of the dye on deactivation kinetics showed an expanded range of change (Table 1) in the presence of the β1 subunit, although the K_L for DiBAC$_4$(3) in this series falls within a similar range (10 μM vs. 6.8 μM; α vs. α+β1 channels, respectively). Collectively, these results support a potentiating role of the β1 subunit in modifying G-V relationships and deactivation kinetics with DiBAC$_4$(3).

Point mutation analysis of β1 molecular determinants

The β1 subunit was recently reported to potentiate the activating effect of the omega-3 fatty acid DHA (docosahexaenoic acid) on BK channels. Two key residues, Arg11 in the N terminus and Cys18 in the TM1 region, are critical to produce this potentiation. We mutated those residues to understand the molecular mechanism by which the β1 subunit enhances DiBAC$_4$(3) effects, DiBAC$_4$(3) and DHA share, in principle, β1 and β2 subunit potentiating effects and both lack a role for β3 subunits. Our point mutation study, however, indicates that neither R11A nor C18E mutations reduce the β1 potentiation of DiBAC$_4$(3) effects. In contrast, those mutations in β1 reduce DHA potentiation of the BK channel current. The 2 β1-subunit residues are...
proposed to be close enough to each other to establish a hydrogen bond or an electrostatic interaction. The 2 interacting residues would then interact directly with the pore helix S6 region or the S6-RCK1 linker of the α subunit to destabilize the closed state of the channel.31 Our analysis of DiBAC₄(3) argues for a mechanism that also involves destabilization of the closed state of the channel. However, β₂ residues, suspected to contribute to the destabilization of the closed state of the channel, do not appear to play a role during DiBAC₄(3) activation.

Notably, while DiBAC₄(3) conspicuously prolongs deactivation kinetics, DHA has only a slight effect on this process. This suggests that DiBAC₄(3) and DHA target different molecular mechanisms to activate the channel. Gessner et al.38 recently suggested a classification for BK channel activators based on the HA model. They proposed activators could be grouped based on their capacity to modify, for example, the C-O equilibrium constant, and/or the resting-activated equilibrium constant of the voltage sensor (i.e., HA model parameters L, and J, respectively). It would be interesting to know if a unifying picture can emerge from this classification in relation to activators potentiated by β subunits, reconciling a classification based on mechanistic considerations with one based on auxiliary subunit (tissue) specificity. We did not perform an extended study to obtain HA model parameters. However, based on the effects presented here, DiBAC₄(3) may belong to L⁺ class activators, potentiated by the β₁ subunit, despite that specific β₂ residues do not influence its effects. Whether this feature constitutes a class of openers that can eventually be mapped to specific structural components of BK channels remains to be elucidated. Our findings shed light on specific molecular mechanisms of BK channel pharmacology that are key for the rational design of compounds of both research and therapeutic interest.

Materials and Methods

Heterologous expression of recombinant mouse (mslo) BK channels

Human Embryonic Kidney (HEK) ts201 cells (Health Protection Agency Culture Collections) were grown in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum (GIBCO, Invitrogen) under standard cell culture conditions (5% CO₂ incubator at 37 °C). Mouse BK channels, mslo α cDNA (kcnma1, GenBank: MMU09383) subcloned into the mammalian expression vector pcDNA3 (Invitrogen, www.lifetechnologies.com), and mslo β₁ cDNA (kcnmb1, GenBank: EDL23733.1) subcloned into the mammalian expression vector pIRE32-EGFP (CLONTECH) were used for transfection. HEK cells were plated in 35 mm dishes and transfected...
24 h later at 60–70% confluence, using the transfection reagent GenCellin (BioCellChallenge). In experiments assessing the α subunit alone, msls was co-transfected with green fluorescent protein as a marker. In co-transfection experiments assessing msls α+β channel expression, the β subunit (WT or mutated) was co-transfected at a ratio of 1:10, α to β, to ensure saturation of BK channels with β subunits. The mouse β subunit construct fluorescently labels cells with channel subunit expression. Four to six h after transfection, cells were trypsinized and re-plated on glass coverslips and maintained in culture for their use between 24–48 h post-transfection.

Site-directed mutagenesis

The construct pRES2-EGFP-kcnmb1 was used as a template to engineer mutations p.R11A and p.C18E by site-directed mutagenesis using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene) and the following primer pairs (mutation underlined): p.R11A forward, 5′GGTGATGGCC CAGAAGGCCG GAGAG 3′; reverse 5′GCTCGTGCTCT CTCGGCGCTT CTGCGGCCC 3′; p.C18E forward, 5′GGAGAGACAC GAGCCTCTCTA GCTGCGG 3′; reverse 5′CCATTGGCAC TCCACACCTC AGGGGCT 3′. The resultant constructs (kcnmb1_R11A and kcnmb1_C18E) were sequenced and prepared with Qiagen Maxi Prep kit (QIAGEN).

Electrophysiological recordings

Macroscopic BK currents were recorded from excised inside-out patches of transfected HEK cells. Pipette and initial bath (200 μM Ca²⁺) solution contained 140 mM KCl, 10 mM HEPES, 1 mM MgCl₂, and 0.2 mM CaCl₂ (pH 7.2). After patch excision an initial voltage protocol was routinely applied to establish the macroscopic BK channel current. After patch excision an initial voltage protocol was then extensively washed with a 0 mM Ca²⁺ bath solution. The external recording solution was then extensively washed with a 0 mM Ca²⁺ solution containing 140 mM KCl, 10 mM HEPES, and 11 mM EGTA. Increasing concentrations of DiBAC₄(3), dissolved in the same 0 mM Ca²⁺ solution, were applied with a gravity driven perfusion system (Cell MicroControls). After each solution exchange, excisions were equilibrated for 2 min before recordings. Pipette resistance was between 1 and 3 MΩ. Data were acquired on an Axopatch 200B amplifier (Molecular Devices), filtered at 5 kHz, and sampled at 100 kHz by using a Digidata 1322A (Molecular Devices). All recordings were performed at room temperature. Leak correction was achieved using P/N leak subtraction protocols. DiBAC₄(3) was obtained from Invitrogen. DiBAC₄(3) was dissolved in dimethyl sulfoxide (DMSO) to a 20 mM stock solution, aliquoted, and stored at −20 °C until use. Unless stated otherwise, all chemicals were from Sigma-Aldrich.

Data analysis

To obtain G-V curves, we measured instantaneous tail currents at 200 μs after the end of the preceding activating pulse using Clampfit 10.2 (Molecular Devices). Instantaneous tail current values were then normalized to the maximum tail current amplitude obtained in 200 μM Ca²⁺. Data were imported into Origin, and each curve was individually fitted by using a built-in Boltzmann equation as a data descriptor. Individual V_{1/2} and slope values were obtained from the fit. Charge (Q) associated with channel activation was estimated from the slope using RT/f = 25.3 mV. Activation and deactivation time course measurements were fit with a single exponential function using Clampfit and visually inspected for proper fit. For deactivation kinetics, a family of tail currents were elicited by step pulses at different voltages, preceded by an initial 250 ms activating step to 90 mV for α alone channels, and a 150 ms activating step to 150 mV for α+β channels. Concentration-response curves were fitted using Origin built-in Hill function. Apparent dissociation constant (K₁) and N of Hill values were obtained from the fit.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Nelson MT, Cheng H, Rubart M, Santana LF, Bonev AD, Knot HJ, Lederer WJ. Relaxation of arterial smooth muscle cells by calcium sparks. Science 1995; 270:633-7; PMID:7570021; http://dx.doi.org/10.1126/science.270.5236.633
2. Pérez GJ, Bonev AD, Nelson MT. Micromolar Ca(2+) from sparks activates Ca(2+)-sensitive K(+) channels in rat coronary artery smooth muscle. Am J Physiol Cell Physiol 2001; 281:C1769-75; PMID:11698234
3. Pérez GJ, Bonev AD, Patlak JB, Nelson MT. Functional coupling of ryanodine receptors to KCa channels in smooth muscle cells from rat cerebral arteries. J Gen Physiol 1999; 113:229-38; PMID:9925821; http://dx.doi.org/10.1085/jgp.113.2.229
4. Knaus HG, Folander K, Garcia-Calvo M, Garcia ML, Kaczorowski GJ, Smith M, Swanson R. Primary sequence and immunological characterization of beta-subunit of high conductance Ca(2+)-activated K+ channel from smooth muscle. J Biol Chem 1994; 269:17274-8; PMID:8006036
5. Wallner M, Meera P, Toro L. Molecular basis of fast inactivation in voltage- and Ca(2+)-activated K+ channels: a transmembrane beta-subunit homolog. Proc Natl Acad Sci U S A 1999; 96:4137-42; PMID:10097176; http://dx.doi.org/10.1073/pnas.96.7.4137
6. Jiang Z, Wallner M, Meera P, Toro L. Human and rodent MaxiK channel beta-subunit genes: cloning and characterization. Genomics 1999; 55:57-67; PMID:9888999; http://dx.doi.org/10.1006/geno.1998.5627
7. Brenner R, Jegla TJ, Wickenden A, Liu Y, Aldrich RW. Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4. J Biol Chem 2000; 275:6453-61; PMID:10692449; http://dx.doi.org/10.1074/jbc.275.9.6453
8. Yan J, Aldrich RW. LRRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium. Nature 2010; 466:513-6; PMID:20613726; http://dx.doi.org/10.1038/nature09162
9. Scornik FS, Bucicor RS, Wu Y, Selga E, Bosch Calero C, Brugada R, Pérez GJ. DiBAC₄(3) hits a “sweet spot” for the activation of arterial large-conductance Ca(2+)-activated potassium channels independently of the β-subunit. Am J Physiol Heart Circ Physiol 2013; 304:H1471-82; PMID:2342916; http://dx.doi.org/10.1152/ajpheart.00939.2012
10. Meera P, Wallner M, Song M, Toro L. Large conductance voltage- and calcium-dependent K+ channel, a distinct member of voltage-dependent ion channels with seven N-terminal transmembrane segments (S0-S6), an extracellular N terminus, and an intracellular (S9-S10) C terminus. Proc Natl Acad Sci U S A 1997; 94:14066-71; PMID:9391153; http://dx.doi.org/10.1073/pnas.94.25.14066
11. Arkinson NS, Robertson GA, Ganetzky B. A component of calcium-activated potassium channels encoded by the Drosophila slo locus. Science 1991; 253:551-5; PMID:1857984; http://dx.doi.org/10.1126/science.1857984
12. Pallannec L, Ganetzky B. Cloning and characterization of human and mouse homologs of the Drosophila calcium-activated potassium channel gene, slowpoke. Hum Mol Genet 1994; 3:1239-43; PMID:7987297; http://dx.doi.org/10.1093/hmg/3/8.1239
13. Tseng Cram J, Foster CD, Krause JD, Mertz R, Godinot N, DiChiara TJ, Reinhart PH. Cloning, expression, and distribution of functionality distinct Ca(2+)-activated K+ channel isoforms from human brain. Neuron 1994; 13:1335-30; PMID:7993625; http://dx.doi.org/10.1016/0896-6273(94)90418-9

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14. Adelman JP, Shen KZ, Kavanaugh MP, Warren RA, Wu YN, Lagrutta A, Bond CT, North RA. Calcium-activated potassium channels expressed from cloned complementary DNAs. Neuron 1992; 9:209-16; PMID:1497896; http://dx.doi.org/10.1016/0896-6273(92)90160-F

15. Hoshi T, Pantazis A, Olcese R. Transduction of voltage and Ca2+ signals by Slo BK channels. Physiology (Bethesda) 2013; 28:172-89; PMID:23636263; http://dx.doi.org/10.1152/physiol0955.2012

16. Liu G, Zakharov SI, Yang L, Deng SX, Landry DW, Karlin A, Marx SO. Position and role of the BK channel alpha subunit S0 helix inferred from disulfide crosslinking. J Gen Physiol 2008; 131:537-48; PMID:18474637; http://dx.doi.org/10.1085/jgp.200809968

17. Yusifov T, Javaherian AD, Pantazis A, Gandhi CS, Olcese R. The RCK1 domain of the human BKCa channel transduces Ca2+ binding into structural rearrangements. J Gen Physiol 2010; 136:189-202; PMID:20624858; http://dx.doi.org/10.1085/jgp.200910374

18. Yusifov T, Savalli N, Gandhi CS, Otness M, Olcese R. The RCK2 domain of the human BKCa channel is a calcium sensor. Proc Natl Acad Sci U S A 2008; 105:576-81; PMID:18162557; http://dx.doi.org/10.1073/pnas.0705261105

19. Horrigan FT, Cui J, Aldrich RW. Allosteric voltage-gating of potassium channels I. Miso ionic currents in the absence of Ca2+. J Gen Physiol 1999; 114:277-304; PMID:10436003; http://dx.doi.org/10.1085/jgp.114.2.277

20. Horrigan FT, Aldrich RW. Allosteric voltage gating of potassium channels II. Miso channel gating charge movement in the absence of Ca2+. J Gen Physiol 1999; 114:305-36; PMID:10436004; http://dx.doi.org/10.1085/jgp.114.2.305

21. Horrigan FT, Aldrich RW. Coupling between voltage sensor activation, Ca2+ binding and channel opening in large conductance (BK) potassium channels. J Gen Physiol 2002; 120:267-305; PMID:12198087; http://dx.doi.org/10.1085/jgp.20026605

22. Latore R, Moreira FJ, Zaelzer C. Allosteric interactions and the modular nature of the voltage- and Ca2+-activated (BK) channel. J Physiol 2010; 588:3141-8; PMID:20603535; http://dx.doi.org/10.1113/jphysiol.2010.199999

23. Cui YM, Yasunome E, Otani Y, Ido K, Yoshinaga T, Sawada K, Ohwada T. Design, synthesis, and characterization of BK channel openers based on oximation of abietane diterpene derivatives. Bioorg Med Chem 2010; 18:8642-59; PMID:20607932; http://dx.doi.org/10.1016/j.bmc.2010.09.072

24. Cui YM, Yasunome E, Otani Y, Yoshinaga T, Ido K, Sawada K, Kawahara M, Yamaguchi K, Ohwada T. Novel oxime and oxime ether derivatives of 12,14-dichlorohydroxyacid: design, synthesis, and BK channel-opening activity. Bioorg Med Chem Lett 2008; 18:6366-9; PMID:18999579; http://dx.doi.org/10.1016/j.bmcl.2008.10.078

25. Cui YM, Yasunome E, Otani Y, Yoshinaga T, Ido K, Sawada K, Ohwada T. Novel BK channel openers containing dehydroacetic acid skeleton: structure-activity relationship for peripheral substrates on ring C. Bioorg Med Chem Lett 2008; 18:5201-5; PMID:18789660; http://dx.doi.org/10.1016/j.bml.2008.08.078

26. Cui YM, Yasunome E, Otani Y, Yoshinaga T, Ido K, Sawada K, Ohwada T. Design, synthesis and characterization of podocarpate derivatives as openers of BK channels. Bioorg Med Chem Lett 2008; 18:5197-200; PMID:18789663; http://dx.doi.org/10.1016/j.bml.2008.08.081

27. Deng PF, Roisman ZZ, Blundon JA, Cho Y, Cui J, Cavalli V, Zakharenko SS, Klyachko VA. FMRF regulates neurotransmitter release and synaptic information transmission by modulating action potential duration via BK channels. Neuron 2013; 77:696-711; PMID:23493122; http://dx.doi.org/10.1016/j.neuron.2012.12.018

28. Nardi A, Olesen SP. BK channel modulators: a comprehensive overview. Curr Med Chem 2008; 15:3126-46; PMID:18473808; http://dx.doi.org/10.2174/092986708784221412

29. Brenner R, Perès GJ, Bonev AD, Eckman DM, Kosek JC, Wiler SW, Patterson AJ, Nelson MT, Aldrich RW. Vasoregulation by the beta subunit of the calcium-activated potassium channel. Nature 2000; 407:870-6; PMID:11057658; http://dx.doi.org/10.1038/35038011

30. Morimoto T, Sakamoto K, Sade H, Ohya S, Muraki K, Imaizumi Y. Voltage-sensitive oxonol dyes are novel large-conductance Ca2+-activated K+ channel activators selective for beta1 and beta2 but not for beta3 subunits. Mol Pharmacol 2007; 71:1075-88; PMID:17209121; http://dx.doi.org/10.1124/mol.106.03146

31. Hoshi T, Tian Y, Xu R, Heinemann SH. Novel mechanism of the modulation of BK potassium channel complexes with different auxiliary subunit compositions by the omega-3 fatty acid DHA. Proc Natl Acad Sci U S A 2013; 110:4822-7; PMID:23487786; http://dx.doi.org/10.1073/pnas.1222003110

32. Pérez GJ. Dual effect of tansyroxin on arterial KCa channels does not depend on the presence of the beta1 subunit. J Biol Chem 2005; 280:21739-47; PMID:15826942; http://dx.doi.org/10.1074/jbc.M413953200

33. Chen L, Shipston MJ. Cloning of potassium channel splice variants from tissues and cells. Methods Mol Biol 2008; 491:35-60; PMID:18989082; http://dx.doi.org/10.1007/978-1-59745-526-8_3

34. Selga E, Pérez-Serra A, Moreno-Asso A, Anderson S, Thomas K, Desai M, Brugada R, Pérez GJ, Scornici FS. Molecular heterogeneity of large-conductance calcium-activated potassium channels in canine intracardiac ganglia. Channels (Austin) 2013; 7:7; PMID:23807990; http://dx.doi.org/10.4161/chan.25945

35. Poulsen AN, Wolf H, Hay-Schmidt A, Jansen-Olesen I, Olesen J, Klaerke DA. Differential expression of BK channel isoforms and beta-subunits in rat neuro-vascular tissues. Biochim Biophys Acta 2009; 1788:380-9; PMID:18927929; http://dx.doi.org/10.1016/j.bbamem.2008.10.001

36. Chen L, Tian L, MacDonald SH, McClafferty H, Hammond MS, Huibant JM, Ruth P, Knaus HG, Shipston MJ. Functionally diverse complement of large conductance calcium- and voltage-activated potassium channel (BK) alpha-subunits generated from a single site of splicing. J Biol Chem 2005; 280:33599-609; PMID:16081418; http://dx.doi.org/10.1074/jbc.M505838200

37. Nimisean CM, Magleby KL. The beta subunit increases the Ca2+ sensitivity of large conductance Ca2+-activated potassium channels by retaining the gating in the bursting states. J Gen Physiol 1999; 113:425-40; PMID:10051518; http://dx.doi.org/10.1085/jgp.113.3.425

38. Gesser G, Cui YM, Otani Y, Ohwada T, Soom M, Hoshi T, Heinemann SH. Molecular mechanism of pharmacological activation of BK channels. Proc Natl Acad Sci U S A 2012; 109:3552-7; PMID:22351907; http://dx.doi.org/10.1073/pnas.1114332109

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