Molecular mechanism underlying β1 regulation in voltage- and calcium-activated potassium (BK) channels

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Being activated by depolarizing voltages and increases in cytoplasmic Ca^{2+}, voltage- and calcium-activated potassium (BK) channels and their modulatory β-subunits are able to dampen or stop excitatory stimuli in a wide range of cellular types, including both neuronal and nonneuronal tissues. Minimal alterations in BK channel function may contribute to the pathophysiology of several diseases, including hypertension, asthma, cancer, epilepsy, and diabetes. Several gating processes, allosterically coupled to each other, control BK channel activity and are potential targets for regulation by auxiliary β-subunits that are expressed together with the α (BK)-subunit in almost every tissue type where they are found. By measuring gating currents in BK channels coexpressed with chimeras between β1 and β3 or β2 auxiliary subunits, we were able to identify that the cytoplasmic regions of β1 are responsible for the modulation of the voltage sensors. In addition, we narrowed down the structural determinants to the N terminus of β1, which contains two lysine residues (i.e., K3 and K4), which upon substitution virtually abolished the effects of β1 on charge movement. The mechanism by which K3 and K4 stabilize the voltage sensor is not electrostatic but specific, and the α (BK)-residues involved remain to be identified. This is the first report, to our knowledge, where the regulatory effects of the β1-subunit have been clearly assigned to a particular segment, with two pivotal amino acids being responsible for this modulation.

BK channels | gating currents | voltage sensor | BK beta-subunits

High-conductance voltage- and calcium-activated potassium (BK) channels are homotetrameric proteins of α-subunits encoded by the *slo1* gene (1). These channels are expressed in virtually all mammalian tissues, where they detect and integrate membrane voltage and calcium concentration changes dampening the responsiveness of cells when confronted with excitatory stimuli. They are abundant in the CNS and nonneuronal tissues, such as smooth muscle or hair cells. This wide distribution is associated with an outstandingly large functional diversity, in which BK channel activity appears optimally adapted to the particular physiological demands of each cell type (2). On the other hand, small alterations in BK channel function may contribute to the pathophysiology of hypertension, asthma, cancer, epilepsy, diabetes, and other conditions in humans (3–8). Alternative splicing, posttranslational modifications, and regulation by auxiliary proteins have been proposed to contribute to this functional diversity (1, 2, 9–16).

The BK channel α-subunit is formed by a single polypeptide of about 1,200 amino acids that contains all of the key structural elements for ion permeation, gating, and modulation by ions and other proteins. Tetramers of α-subunits form functional BK channels. Each subunit has seven hydrophobic transmembrane segments (S0–S6), where the voltage-sensor domain (VSD) and pore domain (PD) reside (2). The N terminus faces the extracellular side of the membrane, whereas the C terminus is intracellular. The latter contains four hydrophobic α-helices (S7–S10) and the main Ca^{2+} binding sites (2). VSDs formed by segments S1–S4 harbor a series of charged residues across the membrane that contributes to voltage sensing (2). Upon membrane depolarization, each VSD undergoes a rearrangement (17) that prompts the opening of a highly K^+-selective pore formed by the four PDs that come together at the symmetry center of the tetramer.

Although BK channel expression is ubiquitous, in most physiological scenarios their functioning is provided by their coassembly with auxiliary proteins, such as β-subunits. This coassembly brings channel activity into the proper cell/tissue context (11, 13). Four different β-subunits have been cloned (β1–β4) (18–24), all of which have been observed to modify BK channel function. Albeit to a different extent, all β-subunits modify the Ca^{2+} sensitivity, voltage dependence, and gating properties of BK channels, hence modifying plasma membrane excitability balance. Regarding auxiliary β-subunits, β1- and β2-subunits increase apparent Ca^{2+} sensitivity and decelerate macroscopic current kinetics (14, 20, 21, 25–30); β2 and β3 induce fast inactivation as well as an instantaneous outward rectification (20, 21, 24, 31, 32); and β4 slows down activation and deactivation kinetics (12, 23) and modifies Ca^{2+} sensitivity (12, 33, 34).

It should be kept in mind that β-subunits are potential targets for different molecules that modulate channel function, such as alcohol (35), estrogens (15), hormones (36), and fatty acids (37, 38). Additionally, scorpion toxin affinity in BK channels would tend to increase when β1 is coexpressed with the α-subunit (22).

To identify the molecular elements that give β1 the ability to modulate the voltage sensor of BK channels, we constructed

Significance

β-Subunits (β1–β4) play a critical role in defining the properties of the voltage- and calcium-activated potassium (BK) channel, which in turn determines the physiological role that this channel can perform in different tissues. In particular, the β1-subunit causes an increase in the apparent BK Ca^{2+} sensitivity due to a stabilization of the voltage sensor in the active configuration. We investigated the molecular details of such voltage-sensor stabilization by mutagenesis and gating current measurements. Mixing regions of β1 and β3 made it possible to identify the N terminus, in particular the third and fourth lysine residues, as the structural element necessary to recover the full effect of β1 on the voltage sensor.

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chimeric proteins of β1/β2- and β1/β3-subunits by swapping their N and C termini, the transmembrane (TM) segments, and the extracellular loops and recorded their gating currents. Two lysine residues that are unique to the N terminus of β1 were identified to be sufficient for BK voltage-sensor modulation.

Results

By aligning all four known β-subunits (Fig. 1*A*), we divided these auxiliary proteins into three main regions: the intracellular N- and C-terminal domains, the transmembrane (TM1 and TM2) domains, and the extracellular loop, all of which had the same predicted secondary structure. Based on these criteria, we designed chimeras combining different β1-segments with β2 or β3 auxiliary

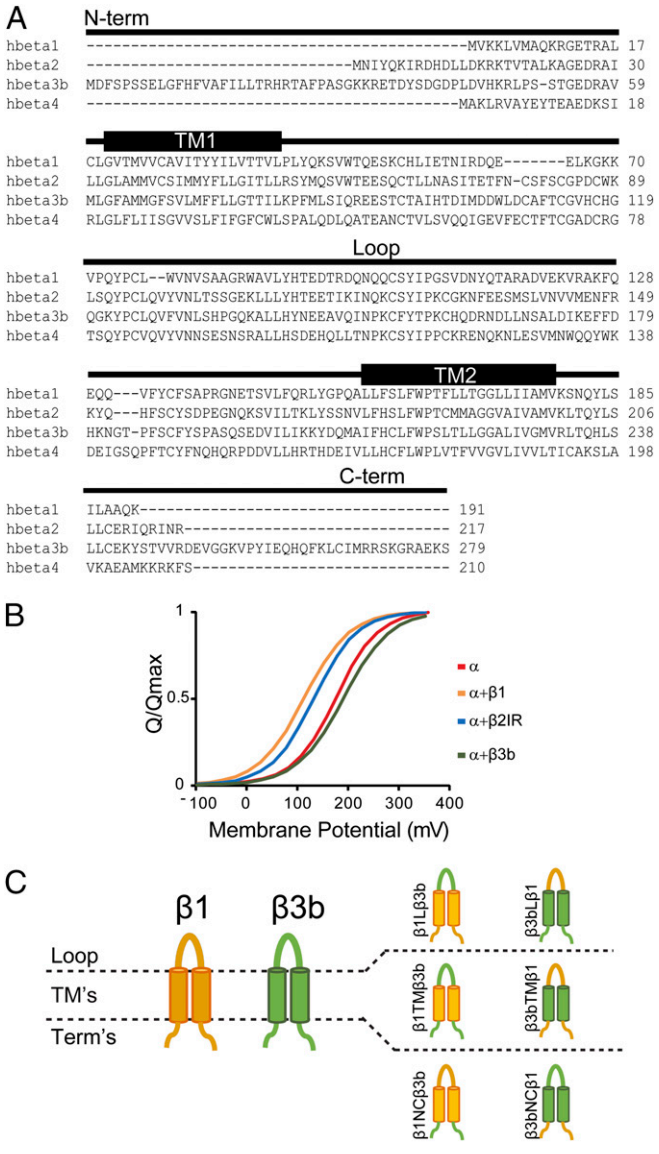

Fig. 1. Predicted topology of chimeric β-subunits. (*A*) Alignment of distinct β-subunits identified to date, highlighting the main domains of their putative structure, intracellular C and N termini, transmembrane domains (TM1 and TM2), and the extracellular loop. (*B*) Gating charge–voltage relationships for BK (red), BK/β1 (orange), BK/β2 (blue), and BK/β3 (green) channels, from ref. 39. (*C*) Topological model of chimeric β1/β3-subunit constructs, in which we exchanged the main regions of the auxiliary protein, namely the extracellular loop as well as the transmembrane domains and N and C termini. The β1-subunit is shown in orange and β3 is in green.

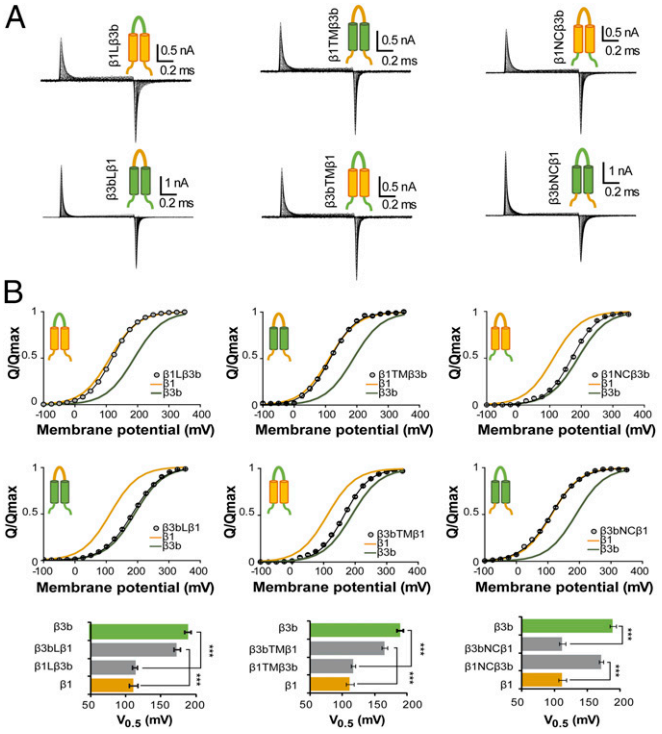

Fig. 2. Gating currents of BK channels coexpressed with chimeric β-subunits. (*A*) Representative gating current recordings from BK channels coexpressed with β1/β3 chimeric constructs, as indicated. (*B*) Gating charge–voltage relationships for the indicated BK+β1/β3 chimeric complexes. For comparison, all Q–V plots include the curve from channels formed by BK/β1 (orange) and BK/β3 (green). (*Lower*) For all chimeras, quantification of $V_{0.5}$ obtained from the Q–V curves is presented (mean ± SEM). The nonparametric *t* test was used to compare statistical significance between the native and corresponding chimeric auxiliary protein. ***$P < 0.001$.

subunits. To characterize the effect of these chimeras on the voltage sensor, we measured gating currents, in permeant ion-free solutions, of BK channels coexpressed with these constructs in *Xenopus laevis* oocytes by using macropatches. β1- and β2-subunits shift the gating charge (Q) vs. voltage (V) relationship for BK channels to more negative potentials compared with those of the α-subunit alone (30, 39). This stabilizes the voltage sensor in its active configuration. In contrast, the β3-subunit displaces the $V_{0.5}$ of the Q–V curve slightly to the right (Fig. 1*B*) (39). We constructed chimeras between the β1- and β3-subunits to identify the molecular determinants that enable β1 to modulate the voltage sensor of BK channels. To establish molecular identity and the mechanism for the voltage-sensor modulation phenotype, we swapped the intracellular N and C termini as well as the transmembrane segments and the extracellular loops between β1 and the neutral β3-isoform. The following chimeric β-proteins were generated: β1Loopβ3, in which the β1-loop was replaced by the β3-homolog, and β3Loopβ1, which is the converse chimera; β1TMβ3 and β3TMβ1, in which transmembrane segments were swapped; and β1NCβ3 and β3NCβ1, which are chimeras in which the N and C termini were exchanged (Fig. 1*C*).

Functional Coupling Between α- and β1-Subunits Is Determined by the Cytoplasmic Region of the β1-Subunit. Robust gating currents (I_g) were recorded from oocyte macropatches 3–4 d after RNA injection for each of the six β1/β3 chimeric β-proteins coexpressed with BK (Fig. 2*A*). Gating currents were elicited by 1-ms pulses between −90 and 350 mV. Visual inspection of I_g did not reveal any major effect on the kinetics of voltage-sensor activation. Q–V curves were obtained by integrating I_g at the end of the depolarizing

pulse and were compared with Q–V curves that were recorded with the parental subunits (Fig. 2*B*). Exchanging the extracellular loop (β1Loopβ3 and β3Loopβ1) preserved the Q–V phenotype of the primary subunit (Fig. 2*B*, *Left*). A similar situation is observed when the transmembrane segments (β1TMβ3 and β3TMβ1; Fig. 2*B*, *Middle*) were swapped. Taken together, these results rule out the loops and TM domain structures as molecular determinants for the modulation of the voltage sensors. In contrast, exchanging the intracellular N and C termini of β1 and β3 shifted Q–V relationships close to those of the primary β intracellular harboring segment. In other words, the β1NCβ3-chimera presented a β3 Q–V phenotype, whereas the β3NCβ1 construct exhibited I_g currents versus membrane potential that resemble β1-currents (Fig. 2*B*, *Right*).

We also investigated six equivalent chimeras between β1 and β2 that also modulate voltage-sensor activity (Fig. S1*A*). The differences in Q–V relationships between β1 and β3 are larger than those between β1 and β2 (~60 mV vs. ~20 mV), thus allowing to cleanly discriminate the effects of the different β-segments on VSD stabilization. As in the case for β1/β3-chimeras, the β1/β2 chimeric proteins also showed robust gating currents and, although to a lesser extent, exhibited a similar trend of Q–V relationships when coexpressed with BK (Fig. S1 *B* and *C* and Table S1).

All of this evidence strongly indicates that the cytoplasmic segments (N or/and C termini) of β1-subunits contain the molecular determinants that enable the auxiliary protein to modulate the voltage sensor in BK channels.

The β1-Subunit Phenotype Is Largely Determined by Its Intracellular N-Terminal Domain. To shed more light on the molecular determinants for voltage-sensor modulation, we designed chimeras in which only the N or C terminus of β1 and β3 was exchanged separately to produce the following: β1Nβ3 (β1 with the N-terminal region of β3), β3Cβ1 (β3 containing the C terminus of β1), and β3Nβ1 (β3 expressing the N terminus of β1). After coexpression with BK, we recorded I_g and generated Q–V curves for each of them (Fig. 3 *A* and *B*). Chimeras β1Nβ3 and β3Cβ1 exhibited a phenotype closely related to that of β3, whereas β3Nβ1 showed a Q–V relationship closer to that observed for β1 (Fig. 3*B* and Table S2). All of this evidence together strongly indicates that the molecular elements required for modulating the voltage sensors in BK channels are harbored in the N terminus of β1. Note that the β1Cβ3-chimera did not express in our experimental conditions.

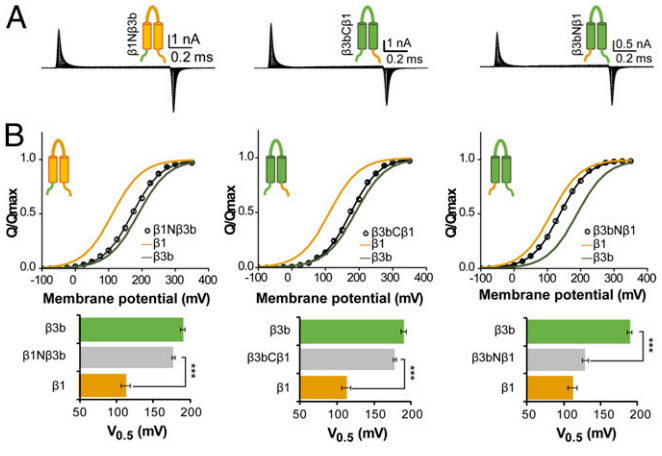

Fig. 3. Gating current recordings of BK channels coexpressed with chimeric β-subunits of the N or C terminus. (*A*) Representative recordings of BK channels with β1/β3 chimeric constructs, as indicated. (*B*) Gating charge–voltage relationships for the indicated BK+β1/β3 chimeric complexes. For comparison, all Q–V plots include the curve from channels formed by BK/β1 (orange) and BK/β3 (green). (*Lower*) Quantification of $V_{0.5}$ obtained from the Q–V curves is presented (mean ± SEM). The nonparametric *t* test was used to compare statistical significance between the native and corresponding chimeric auxiliary protein. ***$P < 0.001$.

We estimated the free energy (ΔG) for β1 modulation of the BK voltage sensor and compared it with the chimera constructs. By calculating the differences between the energy of activation ($\Delta\Delta G$) (40) in the chimeric proteins, we estimated the energetic contribution of different β-regions, hence allowing us to compare their net energetic effects in VSD modulation (Table S3). $\Delta\Delta G$ values of chimeric β-proteins containing intracellular regions of β1 were small, whereas those lacking these segments were significantly different compared with wild-type β1. This confirms the importance of this β1-region for the modulation of the voltage sensor in the BK channel.

A Pair of Basic Amino Acids in β1 Modulates BK Channel Voltage Sensors. The N terminus of β1 is only 18 amino acids long and contains six charged residues that appear to be interesting candidates, because they may have possible electrostatic effects on the voltage sensor. Two pairs of candidates, namely the K3K4 and K10R11 residues, were selected as targets so as to investigate their role in VSD modulation (Fig. 1*A*, hbeta1 line). By using site-directed mutagenesis, we neutralized both pairs of residues by substituting them to alanine, and thus generating $\beta1^{N\_K3AK4A}$ and $\beta1^{N\_K10AR11A}$ mutants for the β1 N terminus. Gating current measurements obtained by coexpressing these mutants with BK revealed that the K3AK4A mutation completely abolished the effect of β1 on the voltage sensor, whereas the K10AR11A mutation did not have any effect (Fig. 4*A*). Consequently, K3 and K4 lysines are required for the complete effect of the β1 N terminus to be exerted on VSD modulation (Table S4).

To explore whether the effect of these two lysine residues is through electrostatic modulation, we measured I_g currents in solutions of increased ionic strength and found that $V_{0.5}$ differences between wild-type and mutant β1 were essentially the same at all ionic strengths evaluated (Fig. 4*B*). This suggests the existence of a site-specific interaction between β1 and a partner in BK, which remains to be determined.

A recent report indicated that conserved residues in the external loop of β1 are key elements for interactions between the external segments of the channel, enabling gate and voltage-sensor modulation of BK channels (41). In fact, mutating conserved residues in the extracellular loop of β1 alters the G–V relationships (41, 42), thus affecting both the equivalent charge as well as intrinsic channel activation (41). By replacing β1 external loop residues Y74 and Y105 by alanine and recording I_g from BK channels coexpressed with these mutated β1s, we determined that these mutations did not alter the ability of β1 to shift charge-movement voltage dependence (Fig. S2*B* and Tables S4 and S5). This evidence strengthens the results showing that the N terminus encompasses the structural determinant for the modulation of BK channel voltage sensors. It should be noted that the $\beta1^{N\_K3AK4A}$ mutant retains the ability to modulate the G–V relationships when coexpressed with BK channels (Fig. S2 *A* and *C*), suggesting that the N terminus of β1 is not participating in the modulation of channel opening. This observation suggests that modulation of channel opening and voltage sensors could depend on different regions of the auxiliary protein.

The role of K3K4 residues in voltage-sensor modulation is also revealed by free energy calculations, comparing mutants with wild-type β1. The change in free energy produced by the K3AK4A mutant of β1 is significantly higher than the wild-type subunit (Table S5). However, for all of the other mutants tested, the $\Delta\Delta G$ values were not significantly different compared with those of wild-type β1, which were taken as reference.

Another possible mechanism by which β1 could modulate the voltage sensors through K3K4 residues is that these residues may interact with an Mg^{2+} binding site in the BK channel, which is thought to interact with the VSD (43). This possibility is suggested by the observation that when BK is coexpressed with β1, Mg^{2+} can

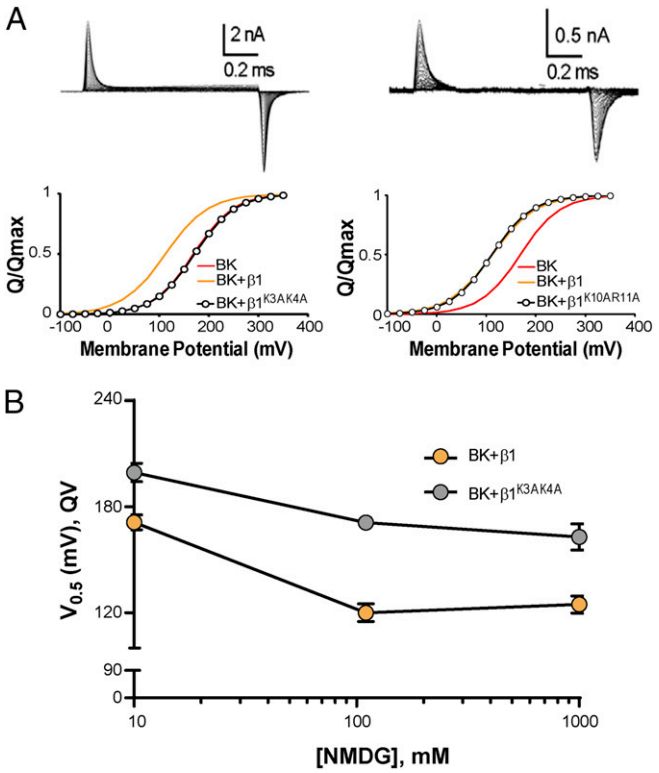

Fig. 4. Gating current recordings of BK channels coexpressed with mutant β1-subunits. (*A*, *Upper*) Representative gating current recordings for the mutants K3AK4A (*Left*) and K10AR11A. (*A*, *Lower*) Gating charge–voltage relationship for the indicated β1-mutants. For comparison, Q–V plots include the curve from channels formed by BK (red) and BK/β1 (orange). (*B*) $V_{0.5}$ values derived from Q–V curves, plotted against NMDG concentration obtained by superfusing different NMDG concentrations to the patch containing the channels. The values corresponding to BK+β1 wild-type proteins are shown in orange circles, whereas data for the $\beta1^{N\_K3K4A}$ mutant are in gray circles. Mean ± SEM is presented. The nonparametric *t* test was used to compare statistical significance between wild-type β1 and the mutant protein.

no longer shift the G–V curve (44). Cross-linking experiments have suggested that β1 could interact with the S0 segment of the BK channel (45–47), and it has been demonstrated that S0 could be a functional and structural part of the VSD (28, 48). Because our results indicate that voltage-sensor modulation is intracellular, we targeted residue D99, which is the closest Mg^{2+}-binding residue that is located in S0. The I_g from BK^{S0_D99A} mutants coexpressed with β1-subunits yields a Q–V curve with shifted $V_{0.5}$, which increases from 112 to 130 mV (Fig. S3 and Table S4). Taken together, our results suggest that the effect of β1 on voltage-sensor equilibrium is driven at least in part by specific interactions with residues in the BK α-subunit, in which magnesium-activating mechanisms could be participating.

Discussion

The β1-subunit greatly enhances the BK channel's Ca^{2+} sensitivity mainly by stabilizing the active configuration of the voltage sensor, but also Ca^{2+} binding at the regulating domain for K^+ conductance (RCK1) and Ca^{2+} bowl sites has a larger effect on channel opening when β1 is present (30, 39, 49). The gating current studies have shown that β1, β2, and β4 stabilize the voltage sensors of α-subunits in their active conformation, in contrast with β3, which does not impact voltage-sensor equilibrium (14, 25, 30, 39). By measuring the activity of the voltage sensor based on gating current recordings, we aimed at demonstrating here that the N-terminal segment of β1 encompasses the main structural determinant for the stabilization of the BK voltage sensor. Furthermore, we identified lysine residues K3 and K4 in the N terminus as sufficient to account for the whole effect on VSD charge stabilization.

All β-subunits are composed of two transmembrane segments (i.e., TM1 and TM2) linked by an extracellular loop with their NH_2 and COOH termini facing the cytoplasm. Functional interaction between α- and β-subunits appears to depend on the presence of the S0 segment of α (28). Actually, disulfide–cross-linking experiments showed that the TM2 segment is next to S0 whereas TM1 is in the neighborhood of S1 and S2, suggesting that β1 can interact with the VSD of two adjacent α-subunits (46, 47). All β-subunits are localized in the same position when interacting with the α-subunit of BK channels (50–52). Cross-linking experiments also showed that β1 increases the efficiency of cross-linking between amino acids located in the extracellular ends of S0 and S4, suggesting that this subunit is altering the VSD architecture (45). We note here that the BK tail domains are required for the β1-subunit to increase the apparent Ca^{2+} sensitivity of the channel (53). Therefore, the β1-subunit maybe also be interacting with the BK C terminus (see below and ref. 54). The proximity between the TMs of β1 and the VSD of BK channels makes it tempting to suggest that the TMs of the β1-subunit are involved in the modulation of the voltage-sensor equilibrium between resting and active configurations. This hypothesis is supported by the results of Kuntamallappanavar et al. (55), who, by making chimeras between β1 and β4, proposed that both transmembrane segments of β1 are needed to confer the phenotype of α/β1 BK channels. However, the high degree of homology among β-subunit TMs and the differences in the modulation exerted by the different β-subunits (39) cast doubt on the thought that TM segments of β-subunits are the only molecular determinants involved in VSD modulation. This idea is already supported by experiments using chimeric constructs between β1- and β2-subunits and β1 N- and C-terminal deletion experiments (25, 56), which revealed that the N and C termini are the relevant β1-subunit regions in defining the behavior of either subunit. Our functional results also argue against the possibility that TMs play an important role in VSD modulation by β-subunits. This is in agreement with the sequence homology between β-subunits that shows a high degree of concordance, contrasting with the phenotypic divergence observed in BK channels when coexpressed with the different auxiliary β-proteins. It is now reasonable to think that the TM segments of β likely have primarily a structural role in maintaining interactions with α-subunits.

Our findings together with free energy calculations (Tables S3 and S5) suggest that only when the N-terminal and K3K4 residues of β1 are missing does a significant destabilization of the active conformation of the voltage sensor occur. A simplified view of VSD modulation by β1 is provided in the cartoons in Fig. 5. Because lysines are involved in the modulatory effect of the BK channel by the β1-subunit, we hypothesized that these residues could be stabilizing the active configuration of the voltage sensor by electrostatic interaction with the positive charges contained in the voltage-sensor domain (43). However, the effects of ionic strength on the half-voltage of the Q–V curves suggest that voltage-sensor stabilization in its active configuration by the β1-subunit is not mediated by long-range electrostatic interactions between both proteins. A specific interaction could have prevalence here, or a combination of different types of protein–protein interactions. Although the residues from the BK α-pore–forming subunit involved in these interactions remain to be determined, the work of Sun et al. (54) indicates that β1 alters the interface between the VSD and the cytosolic domain by disrupting a disulfide-bond formation. This observation may explain the effect of the mutation D99A, which perturbs Mg^{2+} binding and at the same time shifts the Q–V curve of the α/β1 BK channel toward the right along the voltage axis. Our results suggest that

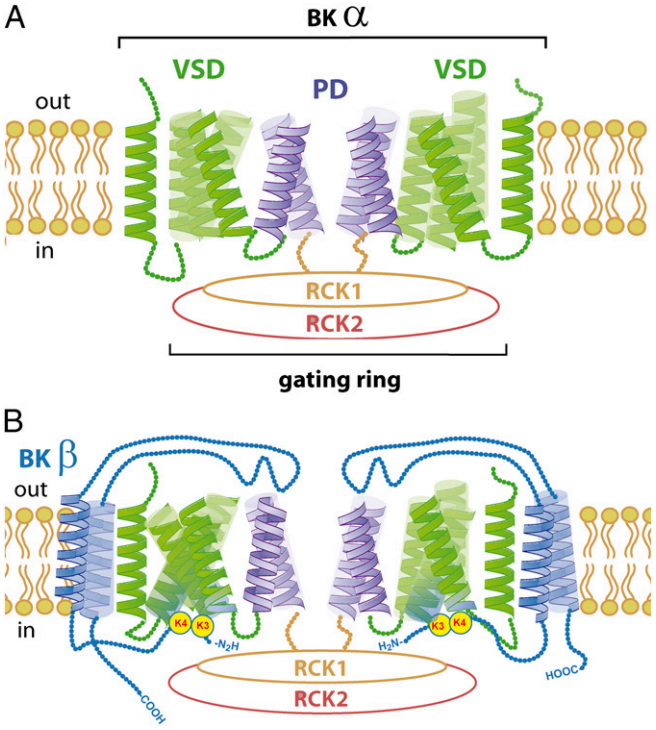

Fig. 5. Molecular model of BK and β1 interaction. *(A)* The illustration shows the α-subunit of the BK channel with the voltage-sensor domain in green and the pore domain in violet. RCK domains in the C terminus of BK are also shown. *(B)* The amino terminus of the β1-subunit (blue) is involved in the modulation of gating through the intracellular N terminus, specifically by K3K4 residues, shown as yellow balls.

a disruption between the VSD and cytosolic domain may be mediated by the β1 N terminus.

Recently, a role in the modulation of the open probability dependence in the BK channel was attributed to the extracellular loop of the β1-subunit. By substituting a series of conserved residues in the loop, changes in the G–V curve were observed both in the equivalent charge as well as in the intrinsic constant of activation, L_0 (41). In addition, mutations in the loop E65K and R140W (related to hypertension and asthma, respectively) also modified the G–V relationships (42, 57). By contrast, our results do not support a role for the β-subunit loops in the modulation of VSDs, and clearly establish that the intracellular N terminus is responsible for VSD stabilization in BK, in the absence of Ca^{2+}. Although our results appear to disagree with previous reports establishing a role for residues in the extracellular loop of β1 in VSD modulation (41, 58), it is important to recall here the studies of the group of Cox (30, 49), which reported that to fully account for the increase in apparent Ca^{2+} sensitivity induced by the β1-subunit it was necessary that β1 also decrease the true Ca^{2+} affinity to the closed channel. Moreover, Sweet and Cox (49) further studied this problem, and combining high-resolution Ca^{2+} dose–response curves together with mutagenesis of the two high-affinity Ca^{2+} binding sites led to the conclusion that indeed the β1-subunit modifies Ca^{2+} binding to the BK channel. β1 increases the affinity of Ca^{2+} to the RCK1 high-affinity Ca^{2+} binding site when the BK channel is open and decreases the affinity of the Ca^{2+} bowl sites when the channel is closed. These modifications in Ca^{2+} induced by the β1-subunit binding offer us a reasonable explanation for the findings of Gruslova et al. (41) and Fernández-Mariño et al. (58), because it is possible that the mutations described by these authors are inducing a further decrease of the Ca^{2+} affinity when the channel is closed and/or an increase in the Ca^{2+} affinity of the open channel.

In conclusion, we have identified the molecular determinants in β1 responsible for the stabilization of the active configuration of the BK channel voltage sensor. We argue that those results involving the transmembrane domains or the loops of β1 in determining the BK phenotype are a possible consequence of modification of Ca^{2+} binding.

Materials and Methods

Use of *X. laevis* was approved by the Ethics Committee of the University of Valparaiso. We used *X. laevis* oocytes as a heterologous system to express BK channels. All recordings were made by patch clamp in excised patches in the inside-out configuration.

Chimera Construction. Chimeric β-subunits were made by using the overlap extension technique (59). Briefly, three pairs of primers were used for each chimeric subunit (P1–P6). PCR was used to generate three fragments using the primers P1 and P2; P3 and P4; and P5 and P6, where P2 and P4 share a region of homology with P3 and P5, respectively. Later, the three fragments were mixed and amplified by using the primers P1 and P6, which can be extended to form the recombinant product. Finally, the construct was digested with restriction enzymes, ligated to the vector, and confirmed by DNA sequencing.

Channel Expression. The BK subunit [GenBank/European Molecular Biology Laboratory (EMBL)/DNA Data Bank of Japan (DDBJ) accession no. U11058] and the human β1-subunit (KCNMB1, GenBank/EMBL/DDBJ accession no. U25138) were provided by L. Toro (University of California, Los Angeles). The β3b-subunit (GenBank/EMBL/DDBJ accession no. AF160968) and the chimera β3Lβ1 were provided by Christopher Lingle (Washington University School of Medicine, St. Louis). The D99A mutant from BK was kindly provided by Teresa Giraldez (Research Division, University Hospital of Nuestra Señora de Candelaria, Tenerife, Spain). mMESSAGE mMACHINE (Ambion) was used for in vitro transcription. *X. laevis* oocytes were injected in a proportion of 1:5 of α:β with 50 nL of 1 μg/μL cRNA 4–8 d before recording.

Solutions.
Gating currents. Internal solution: 110 mM *N*-methyl-D-glucamine (NMDG)-$MeSO_3$, 10 mM Hepes, 5 mM EGTA. External (pipette) solution: 110 mM tetraethylammonium (TEA)-$MeSO_3$, 10 mM Hepes, 2 mM $MgCl_2$. pH was adjusted to 7. Internal Ca^{2+} concentration was assumed to be zero in 5 mM EGTA.
Macroscopic currents. Internal solution: 1 mM KOH, 110 mM NMDG, 10 mM Hepes, 5 mM EGTA. External (pipette) solution: 1 mM KOH, 110 mM NMDG, 10 mM Hepes, 2 mM $MgCl_2$.

Recordings. Gating currents were elicited by 1-ms steps to increasing voltages from −90 to 350 mV in increments of 10 mV. Voltage command and current output were filtered at 20 kHz with an eight-pole Bessel filter. Acquisition rate was 500 kHz. Experiments were performed at room temperature (20–22 °C). To measure gating currents in different ionic conditions in the same oocyte, the patch was excised and washed with internal solution at least 10–20 times the volume of the chamber.

Pipettes of borosilicate capillary glass (World Precision Instruments; 1B150F-4) were pulled in a horizontal pipette puller (Sutter Instruments). Pipette resistance was 400–900 kΩ (20–60 μm) after being fire-polished. Data were acquired with an Axopatch 200B (Axon Instruments) amplifier. Both the voltage command and current output were filtered at 20 kHz with an eight-pole Bessel low-pass filter (Frequency Devices). Current signals were sampled with a 16-bit A/D converter (Digidata 1322A; Axon Instruments), using a sampling rate of 500 kHz. Experiments were performed using Clampex 8 (Axon Instruments) acquisition software. Leak subtraction was performed based on a P/4 protocol (60).

Data Analysis. All data analyses were performed with Clampfit 10 (Axon Instruments) and Excel 2007 (Microsoft). Gating currents were integrated between 0 and 400 μs after the voltage step to obtain the net charge movement. Q–V relationships were fitted with a Boltzmann function: $Q = Q_{max}/(1 - \exp(-zF(V - V_{0.5})/RT))$, where Q_{max} is the maximum charge, z is the voltage dependency of activation, $V_{0.5}$ is the half-activation voltage, T is the absolute temperature (typically 295 K), F is Faraday's constant, and R is the universal gas constant. Q_{max}, $V_{0.5}$, and z were determined by using the solver complement of Microsoft Excel.

For wild-type and chimeric constructs, a mean $V_{0.5}$ ($<V_{0.5}>$) value was obtained. All corresponding Q–V curves were displaced in the voltage axis by

$V_{0.5} - \langle V_{0.5} \rangle$, allowing us to construct an average Q–V curve that preserved the shape of the individual Q–V curves.

The amount of free energy required to activate the sensors was calculated as $\Delta G = zFV_{0.5}$, with the z and $V_{0.5}$ values determined from Q–V curves as was previously done (61). All $\Delta\Delta G$ values were calculated as $\Delta\Delta G = F(z_i V_{0.5\ i} - z_0 V_{0.5})$, where z_0 and $V_{0.5}$ correspond to the values for BK + β1, which are taken as reference, and z_i and $V_{0.5\ i}$ are the values for the other BK + β combinations (chimeras and mutants).

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