

Structural Determinants for Functional Coupling Between the β and α Subunits in the Ca^{2+} -activated K^+ (BK) Channel

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High conductance, calcium- and voltage-activated potassium (BK, MaxiK) channels are widely expressed in mammals. In some tissues, the biophysical properties of BK channels are highly affected by coexpression of regulatory (β) subunits. The most remarkable effects of $\beta 1$ and $\beta 2$ subunits are an increase of the calcium sensitivity and the slow down of channel kinetics. However, the detailed characteristics of channels formed by α and $\beta 1$ or $\beta 2$ are dissimilar, the most remarkable difference being a reduction of the voltage sensitivity in the presence of $\beta 1$ but not $\beta 2$. Here we reveal the molecular regions in these β subunits that determine their differential functional coupling with the pore-forming α -subunit. We made chimeric constructs between $\beta 1$ and $\beta 2$ subunits, and BK channels formed by α and chimeric β subunits were expressed in *Xenopus laevis* oocytes. The electrophysiological characteristics of the resulting channels were determined using the patch clamp technique. Chimeric exchange of the different regions of the $\beta 1$ and $\beta 2$ subunits demonstrates that the NH_3 and COOH termini are the most relevant regions in defining the behavior of either subunit. This strongly suggests that the intracellular domains are crucial for the fine tuning of the effects of these β subunits. Moreover, the intracellular domains of $\beta 1$ are responsible for the reduction of the BK channel voltage dependence. This agrees with previous studies that suggested the intracellular regions of the α -subunit to be the target of the modulation by the $\beta 1$ -subunit.

INTRODUCTION

Large conductance Ca^{2+} -activated K^+ (BK, MaxiK) channels are ubiquitously distributed in different cells and tissues. One of their roles is to dampen excitatory signals induced by increases in cytoplasmic Ca^{2+} concentration and/or membrane depolarization (Meech, 1978; McManus and Magleby, 1991; Toro et al., 1998; Vergara et al., 1998). The BK channel is a homotetramer of its pore-forming α -subunit, which is coded by the gene *Slo1* (KNCMA1). This protein contains seven putative transmembrane segments (S0–S6) where the primary sequence of S1–S6 is homologous to the corresponding domains in K_v channels (Shen et al., 1994; Meera et al., 1997). The channel is gated both by membrane depolarization and by increased intracellular Ca^{2+} concentration. Thorough studies of BK channel gating suggest that calcium and voltage act as allosteric modulators

(Cox et al., 1997; Cox and Aldrich, 2000; Horrigan and Aldrich, 2002).

In some mammal tissues, BK channel α subunits are coexpressed with modulatory β subunits, four of which have been cloned (Knaus et al., 1994b; Jiang et al., 1999; Wallner et al., 1999; Xia et al., 1999; Behrens et al., 2000; Brenner et al., 2000; Meera et al., 2000; Uebele et al., 2000; for review see Orio et al., 2002). Purification and cloning of the $\beta 1$ -subunit unveiled the presence of a protein containing two putative transmembrane regions joined together by a large extracellular loop (Knaus et al., 1994a,b). $\beta 1$ increases the apparent Ca^{2+} sensitivity of the channel, slows down the macroscopic activation and deactivation kinetics, and decreases the voltage sensitivity (McManus et al., 1995; Wallner et al., 1995; Dworetzky et al., 1996; Meera et al., 1996; Cox and Aldrich, 2000; Nimigeon and Magleby, 2000; Bao and Cox, 2005; Orio and Latorre, 2005). The presence of the $\beta 1$ -subunit is also a requirement for the binding of channel activators (McManus et al., 1995; Valverde et al., 1999; Dick et al., 2001). The S0 domain and the extracellular NH_2 terminus of the α -subunit are crucial for the adequate functional coupling between α - and

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$\beta 1$ -subunit (Wallner et al., 1996). The $\beta 2$ -subunit has an N-type or fast inactivation motif in its NH_2 terminus, causing BK currents to inactivate when this subunit is present (Wallner et al., 1999; Xia et al., 1999). This motif can be removed ($\beta 2\text{IR}$ -subunit) and the activation properties of the channel can be studied in the presence of $\beta 2\text{IR}$ without the contamination of the inactivation process. Like the $\beta 1$ -subunit, $\beta 2\text{IR}$ also increases the apparent Ca^{2+} sensitivity of the BK channels and slows down its macroscopic kinetics (Wallner et al., 1999; Xia et al., 1999). But unlike the $\beta 1$ -subunit, $\beta 2\text{IR}$ does not modify the voltage dependence and confers low affinity to CTX compared with that exhibited by the α -subunit (Xia et al., 1999; Orio and Latorre, 2005).

Although the region in the α -subunit responsible for $\beta 1$ -subunit regulation has been mapped to the S0 membrane-spanning and NH_2 -terminal regions, very little is known about the structural determinants contained in the β subunits that determine the functional coupling between α and β subunits. We showed in detail that $\beta 1$ and $\beta 2\text{IR}$ subunits conferred different gating properties to the BK channel (Orio and Latorre, 2005). In particular, they impart to BK channels different calcium sensitivities, voltage dependences, and macroscopic gating kinetics. Here we use these differences to map the regions in the β subunits responsible for β -subunit regulation by constructing chimeras between $\beta 1$ and $\beta 2\text{IR}$ subunits. Our results show that intracellular NH_2 and COOH termini are responsible for most of the modulatory effects on channel activity, with some participation of the transmembrane regions. The extracellular regions have no participation in the differences between $\beta 1$ and $\beta 2\text{IR}$ subunits and may play a structural function related to other effects of the β subunits on the channel.

MATERIALS AND METHODS

DNA Clones

cDNAs coding for BK channel α -subunit (KCNMA1) from myometrium (GenBank/EMBL/DBJ accession no. U11058), human $\beta 1$ and $\beta 2$ subunits (KCNMB1 and KCNMB2, GenBank/EMBL/DBJ accession no. U25138 and AF099137), and $\beta 2$ -subunit without inactivation domain ($\beta 2\text{IR}$) (Wallner et al., 1999) were used. The boundaries of the transmembrane domains of $\beta 1$ and $\beta 2\text{IR}$ subunits were defined according to Wallner et al. (1999). Chimeric β subunits were made with the overlapping extension method (Horton et al., 1990) and confirmed by DNA sequencing.

Channel Expression

mMESSAGE mMACHINE (Ambion) *in vitro* transcription kit was used to obtain mRNAs. Channels were expressed in *Xenopus* oocytes using standard techniques (Stühmer and Parekh, 1995). α -Subunit cRNA (0.75 to 2 ng) or a mixture of α -subunit (0.5–1.5 ng) and β -subunit (2–3 ng) cRNAs were injected per oocyte. The approximate molar ratio was at least 6:1 (β : α), ensuring saturation of β -subunit.

Electrophysiological Recordings and Solutions

Currents were recorded 1–5 d after cRNA injection using the inside-out configuration of the patch-clamp technique. All recordings were performed at room temperature (20–22°C). Intracellular

(bath) and extracellular (pipette) solutions contained (in mM) 110 KOH, 10 HEPES, and 2 KCl and were adjusted to pH 7.4 with methanesulfonic acid. Depending on the desired free calcium concentration, 1.8–3.5 mM CaCl_2 and 5 mM EGTA (5–200 nM), HEDTA (0.6–12 μM), or NTA (20–150 μM) was added. Free calcium concentrations were calculated using the WinMaxChelator Software (<http://www.stanford.edu/~cpatton/maxc.html>) and checked with a calcium electrode (World Precision Instruments). The solution designated as “5 nM” is an upper estimation based on contaminating calcium. Depending on the magnitude of currents, and in order to minimize voltage drops due to series resistance, some experiments were done with solutions containing 36 mM KOH and 74 mM NMDG. The pipette solution contained 100 nM or lower free calcium.

Pipettes were pulled in a horizontal pipette puller (Sutter Instruments) from Corning 7740 (Pyrex) or Custom 8250 (Warner Instruments) borosilicate capillary glass. Pipette resistance was 0.8–2 M Ω in 110 mM K^+ , and series resistance error was always <10 mV.

Data were acquired with an Axopatch 200B (Axon Instruments) or an EPC-7 (List Medical) amplifier, filtered with an 8-pole Bessel filter (Frequency Devices) at 1/5 of the acquisition rate, and sampled with a 16-bit A/D converter (NI-6036, National Instruments). Typical acquisition rates ranged from 66.6 to 100 kHz (α -subunit alone) or from 16.6 to 40 kHz ($\alpha + \beta$ subunits). Homemade acquisition software was developed in the LabView programming environment (National Instruments). Primary data analysis was performed with Analysis (provided by F. Bezanilla, University of California Los Angeles, CA) and Clampfit 9 (Axon Instruments) software.

Steady-state Activation Analysis

The Solver function of Microsoft Excel was used to fit a Boltzmann function of the form

$$I = \frac{I_{\max}}{1 + \exp\left(-\frac{zF(V - V_{0.5})}{RT}\right)} \quad (1)$$

to instantaneous tail currents, where I_{\max} is the maximum obtained tail current, z is the voltage dependency of activation, $V_{0.5}$ is the half-activation voltage, T is the absolute temperature (typically 295K), F is the Faraday's constant, and R is the universal gas constant. When $[\text{Ca}^{2+}]$ was <500 nM, only low conductances were achieved and the estimation of I_{\max} became unreliable. In those cases, I_{\max} was fixed using the value obtained with higher calcium concentrations in the same patch. All the analysis that follows was performed using I/I_{\max} values.

Statistical Analysis

The τ_{act}/V curves were compared by a two-way ANOVA test, thus assessing the statistical significance of the datasets as a whole. The $V_{0.5}/[\text{Ca}^{2+}]$ curves could not be compared by a two way ANOVA test because not all the chimeras were studied at the same calcium concentrations. Instead, the statistical difference between two datasets was performed by testing whether they can be fit by two (independent fit) or a single (global fit) dose–response curve. Comparison between independent and global fits was done by an extra sum-of-squares F test, using the formula

$$F = \frac{(SS_{\text{glb}} - SS_{\text{ind}}) / (DF_{\text{glb}} - DF_{\text{ind}})}{SS_{\text{ind}} / DF_{\text{ind}}}$$

where SS and DF are the sum-of-squares and the degrees of freedom of the fit, respectively. glb and ind denote the global and the independent fit, respectively. The same procedure was used to test the difference of the exponential factors for the τ_{deact} values in the –50 to 60 mV range. The z values showed the greatest variability

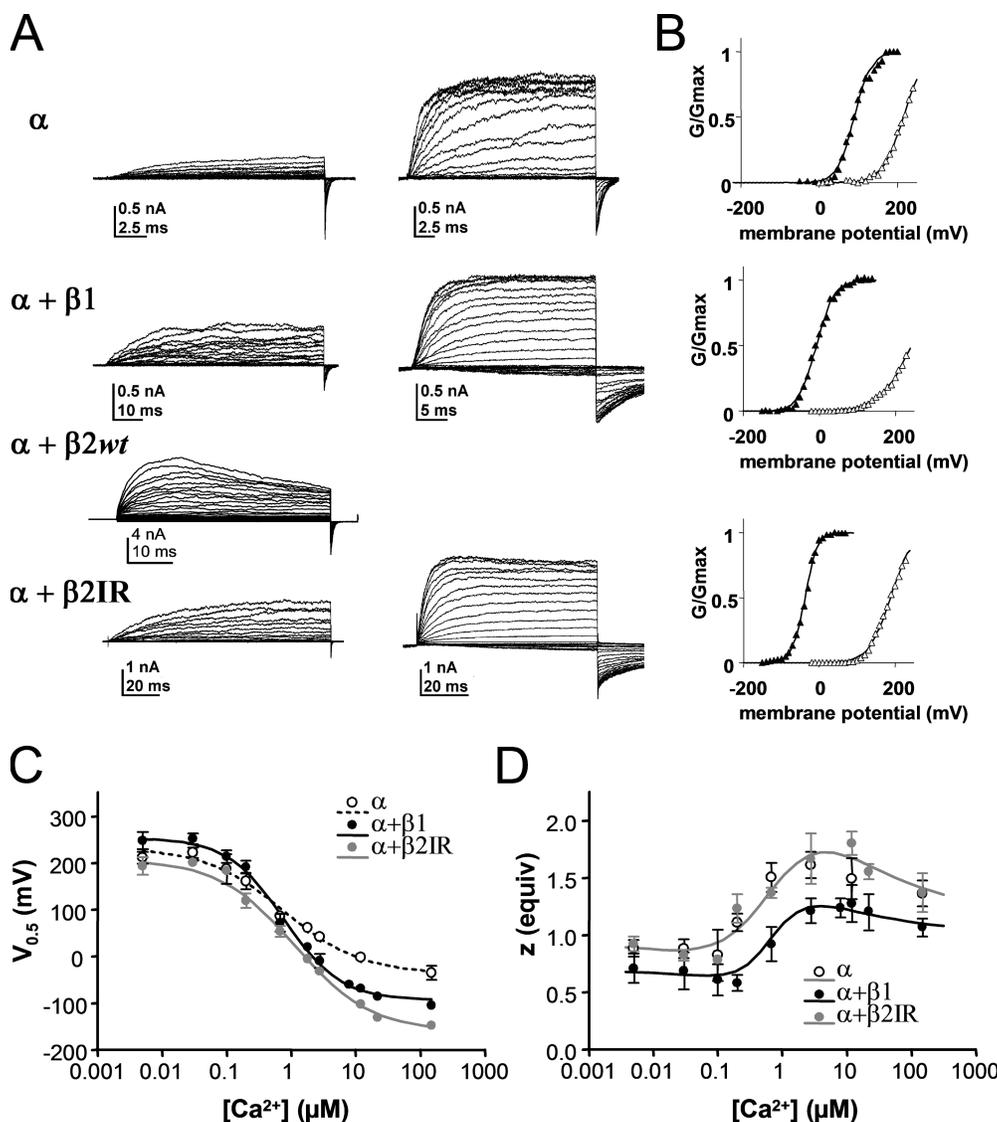


Figure 1. Effects of $\beta 1$ and $\beta 2^{IR}$ subunits on BK channel steady-state activation parameters. (A) Macroscopic currents recorded in the inside-out configuration at 5 nM (left) and 3 μ M (right) intracellular calcium. The voltage protocol for 5 nM was: holding potential (HP), 0 mV; prepulse (PP), -80 mV; test pulse (TP), -10 to 250 mV in 10-mV steps; tail, -60 mV. The voltage protocol for 3 μ M was: HP, 0 mV; PP, -150 mV; TP, -150 to 140 in 10-mV steps; tail, -60 mV. (B) Normalized G/V curves at 3 μ M (closed symbols) and 5 nM Ca²⁺ (open symbols) for the α , $\alpha + \beta 1$ and $\alpha + \beta 2^{IR}$ current traces shown in A. Lines are the best fit of a Boltzmann distribution (Eq. 1). Fit parameters are as follows. α , $V_{0.5} = 215$ mV, $z = 0.95$ (5 nM); $V_{0.5} = 91$ mV, $z = 1.19$ (3 μ M). $\alpha + \beta 1$, $V_{0.5} = 243$ mV, $z = 0.61$ (5 nM); $V_{0.5} = -7$ mV, $z = 1.11$ (3 μ M). $\alpha + \beta 2^{IR}$, $V_{0.5} = 189$ mV, $z = 0.91$ (5 nM); $V_{0.5} = -37$ mV, $z = 1.58$ (3 μ M). (C) Average of the obtained $V_{0.5}$ values plotted against calcium concentration. A best fit sigmoid concentration-effect curve was added as reference for visual comparison in the following figures. (D) Average of the obtained z values plotted against calcium concentration. A best fit dual effect sigmoid curve was added as reference for visual comparison in the following figures. Error bars are SD, $n = 7-8$. When SD bars are not visible, they are smaller than symbol size.

of all the parameters studied, and the statistical comparisons were not reliable. All the analyses were done with the GraphPad Prism software (GraphPad Software Inc.).

RESULTS

Differential Modulation of the BK Channel by its $\beta 1$ and $\beta 2$ Subunits

In a previous report (Orio and Latorre, 2005) we showed clear differences in the way that the $\beta 1$ and $\beta 2$ subunits modulate BK channel's properties, suggesting

a different mechanism of action. The differences in the modulation of steady-state activation of the channel are summarized in Fig. 1. As the $\beta 2$ -subunit induces a fast or N-type inactivation of the current (Fig. 1 A, $\beta 2^{wt}$), in the previous as well as in this work we studied a truncated $\beta 2$ -subunit ($\Delta 2-19\beta 2$, $\beta 2^{IR}$) that lacks the inactivation peptide. In the presence of the $\beta 2^{IR}$ -subunit, BK currents are sustained (Fig. 1 A, $\alpha + \beta 2^{IR}$) and therefore the effects of this subunit in the activation parameters of the BK channel are best studied.

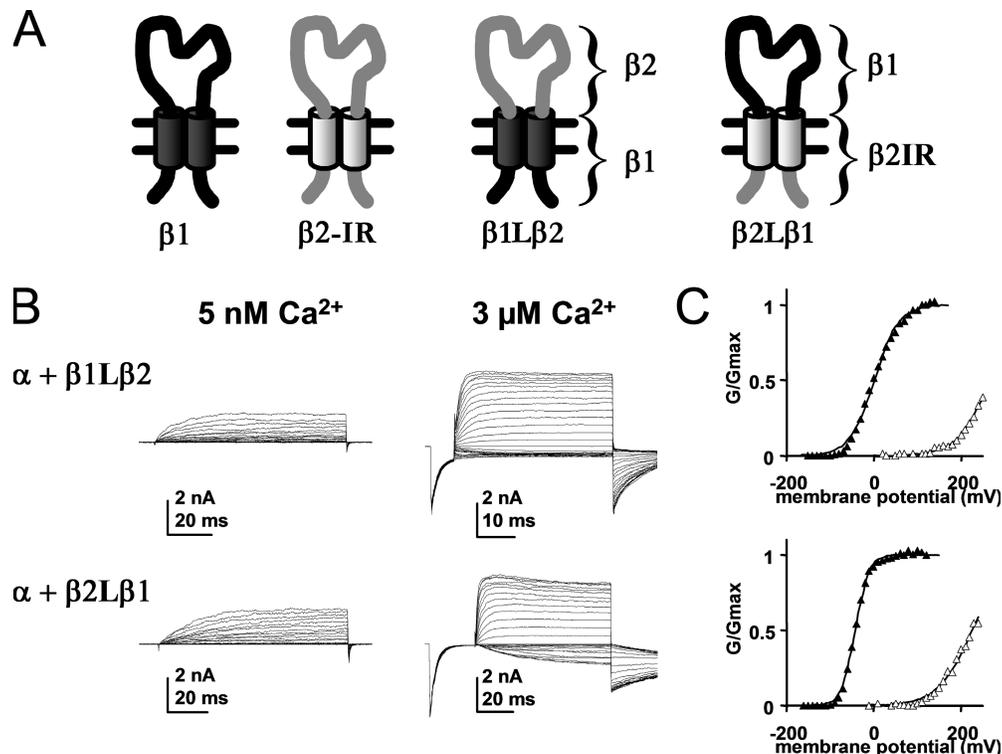


Figure 2. Effects of $\beta 1L\beta 2$ and $\beta 2L\beta 1$ subunits on BK channel steady-state activation parameters. (A) Schematic description of the $\beta 1L\beta 2$ and $\beta 2L\beta 1$ chimeras. (B) Macroscopic currents recorded in the inside-out configuration at 5 nM (left) and 3.3 μM (right) intracellular calcium, for $\alpha + \beta 1L\beta 2$ (top) and $\alpha + \beta 2L\beta 1$ (bottom) channels. The voltage protocols are as described in legend of Fig. 1. (C) Normalized G/V curves at 3.3 μM Ca^{2+} (closed symbols) and 5 nM Ca^{2+} (open symbols) for the current traces shown in B. Lines are the best fit of a Boltzmann distribution (Eq. 1). Fit parameters are as follows. $\alpha + \beta 1L\beta 2$, $V_{0.5} = 267$ mV, $z = 0.7$ (5 nM); $V_{0.5} = 2$ mV, $z = 0.92$ (3.3 μM). $\alpha + \beta 2L\beta 1$, $V_{0.5} = 226$ mV, $z = 0.65$ (5 nM); $V_{0.5} = -42$ mV, $z = 1.7$ (3.3 μM).

A convenient way to represent the voltage and calcium sensitivity of the BK channel is by fitting the G/V curve to a Boltzmann distribution (Eq. 1; Fig. 1 B). The half-activation voltage ($V_{0.5}$) reports the position of the curve along the voltage axis while the z value reports the voltage sensitivity of the channel. Increasing the calcium concentration lowers the half-activation voltage (Fig. 1 C) and in the presence of both the $\beta 1$ and $\beta 2\text{IR}$ subunits the effect of calcium is enhanced, as the $V_{0.5}$ values for $\alpha + \beta 1$ and $\alpha + \beta 2\text{IR}$ are lower than for α at $[\text{Ca}^{2+}] \geq 1 \mu\text{M}$. However, the half potential shift to more hyperpolarized voltages is more pronounced in $\alpha + \beta 2\text{IR}$ channels. At $[\text{Ca}^{2+}] \leq 100$ nM, $\alpha + \beta 1$ channels have higher values than either α or $\alpha + \beta 2\text{IR}$ channels while $\alpha + \beta 2\text{IR}$ is not different from α . Finally, the $\beta 1$ -subunit produces a reduction in the BK voltage dependence, not observed when channels are formed by $\alpha + \beta 2\text{IR}$ subunits (Fig. 1 D).

In the case of the kinetic parameters, both β subunits slow down the activation kinetics. However, $\alpha + \beta 2\text{IR}$ channels show a slower activation and the voltage dependence of the deactivation kinetics is modified by the presence of the $\beta 1$ - but not the $\beta 2$ -subunit (Orío and Latorre, 2005; see also Fig. 4, C and D).

The External Loop of β Subunits Is Not Involved in Differential Coupling

The rationale of the experiments that follow is based upon the electrophysiological differences shown by channels formed by $\alpha + \beta 1$ and $\alpha + \beta 2\text{IR}$ subunits. We, therefore, predicted that regions essential for β -subunit modulation of the BK channel may be identified by exchanging regions between $\beta 1$ and $\beta 2$ subunits. The first set of chimeras tested was one in which the external loops were exchanged: $\beta 2$ containing the loop of $\beta 1$ ($\beta 2L\beta 1$) and $\beta 1$ containing the loop of $\beta 2$ ($\beta 1L\beta 2$) (Fig. 2 A). Fig. 2 B shows that coexpression of these chimeras with the α -subunit produces robust currents in excised patches of oocyte membrane. In particular, it is apparent from the current records that both chimeras are able to slow down the activation and deactivation kinetics with respect to currents generated by α -subunit alone (see also Fig. 4). Fig. 2 C shows that the conductance is voltage and Ca^{2+} dependent. A more detailed inspection indicates that the G/V curves for the $\beta 2L\beta 1$ chimera are steeper and that at 3 μM Ca^{2+} it is shifted ~ 40 mV to the left compared with the $\beta 1L\beta 2$ chimera. To quantify the effects of each chimera and to have an adequate comparison of their characteristics with those

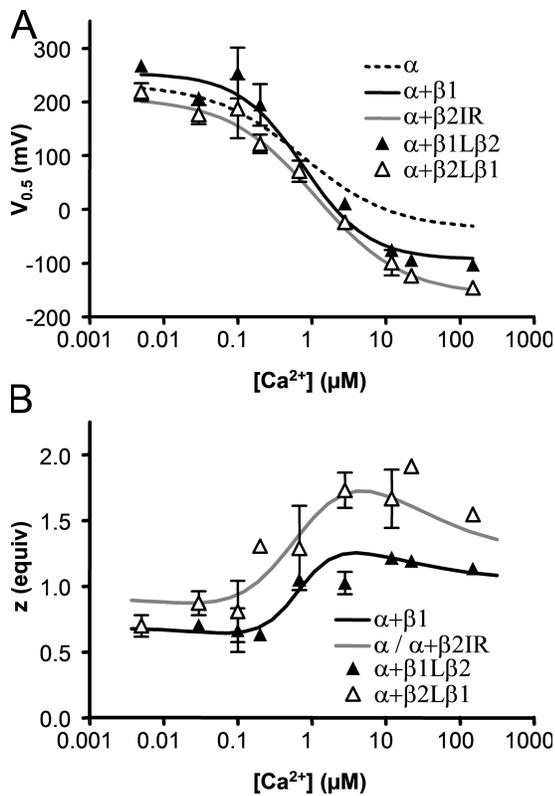


Figure 3. Steady-state activation parameters for $\alpha + \beta 1L\beta 2$ and $\alpha + \beta 2L\beta 1$ channels. (A) Average of the obtained $V_{0.5}$ values plotted against calcium concentration. For α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$ only the best fit sigmoid concentration–effect curve (see Fig. 1 C) is depicted. (B) Average of the obtained z values plotted against calcium concentration. For α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$ only the best fit curve (see Fig. 1 D) is depicted. Error bars are SD, $n = 5$ –6. When SD bars are not visible, they are smaller than symbol size.

of the parent β subunits we performed experiments over a wide range of Ca^{2+} concentrations. Fig. 3 (A and B) summarizes the results obtained from the fitting of the G/V curves to Eq. 1. Fig. 3 A shows a plot of $V_{0.5}$ against $[Ca^{2+}]$ for $\beta 1L\beta 2$ (black triangles) and $\beta 2L\beta 1$ (open triangles). Fig. 3 B shows the z values against $[Ca^{2+}]$ for $\beta 1L\beta 2$ (black triangles) and $\beta 2L\beta 1$ (open triangles). For visual comparison purposes, the data for α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$ channels are shown only as the best fit curves (see figure legends for details). The results demonstrate that the behavior of channels formed by $\alpha + \beta 1L\beta 2$ resembles that of channels produced by the coexpression of $\alpha + \beta 1$ ($P = 0.6$ and 0.002 compared with $\alpha + \beta 1$ and $\alpha + \beta 2IR$, respectively, in an extra sum-of-squares F test; see MATERIALS AND METHODS) and that the activity of channels formed by $\alpha + \beta 2L\beta 1$ is similar to channels formed by $\alpha + \beta 2IR$ ($P = 0.002$ and 0.72 compared with $\alpha + \beta 1$ and $\alpha + \beta 2IR$, respectively). This result suggests that the loop region is not responsible for the functional differences between $\beta 1$ and $\beta 2$ subunits.

As mentioned before, both $\beta 1$ and $\beta 2IR$ subunits slow down the activation process of the channel but the time

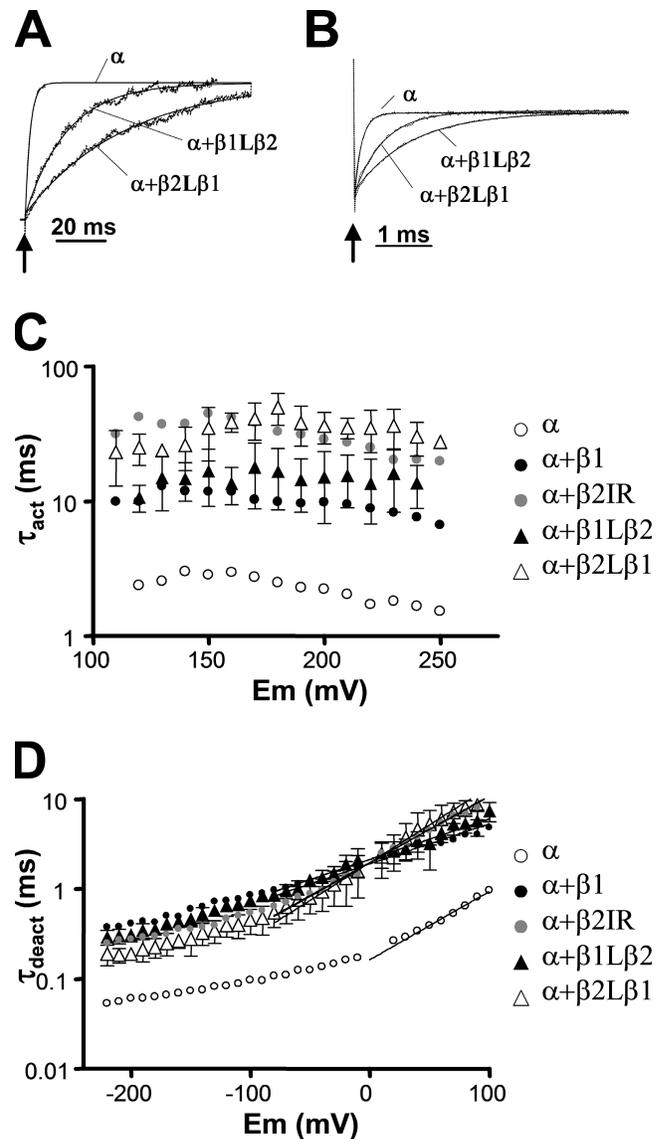


Figure 4. Effect of the $\beta 1L\beta 2$ and $\beta 2L\beta 1$ subunits on BK channel macroscopic kinetics in the absence of calcium. (A) Current traces evoked by a 200 mV pulse after a -80 mV prepulse. Over the traces, the best fit to an exponential rise is shown. Current magnitude of each trace was normalized by the steady-state current predicted by the fit. For α , the fit was extrapolated to show that the traces are effectively normalized to the same maximum. (B) Tail current traces evoked by a -60 mV pulse after opening the channels with a $+200$ mV activation pulse. Current magnitude of each trace was normalized by the peak of the tail current. Over the traces, the best fit to an exponential decay is shown. (C) Activation time constant (τ_{act}) plotted against activation voltage. Symbols represent mean \pm SD. $n = 7$ –10. (D) Deactivation time constant (τ_{deact}) plotted against voltage. Symbols represent mean \pm SD. $n = 3$ –5. In the case of α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the mean is shown. Lines represent the best fit of a single exponential function to the data between -50 and $+60$ mV extrapolated from -80 to $+100$ mV.

course of the current induced by $\alpha + \beta 2IR$ channels is slower than that promoted by $\alpha + \beta 1$ channels (Orio and Latorre, 2005). Fig. 4 A shows representative

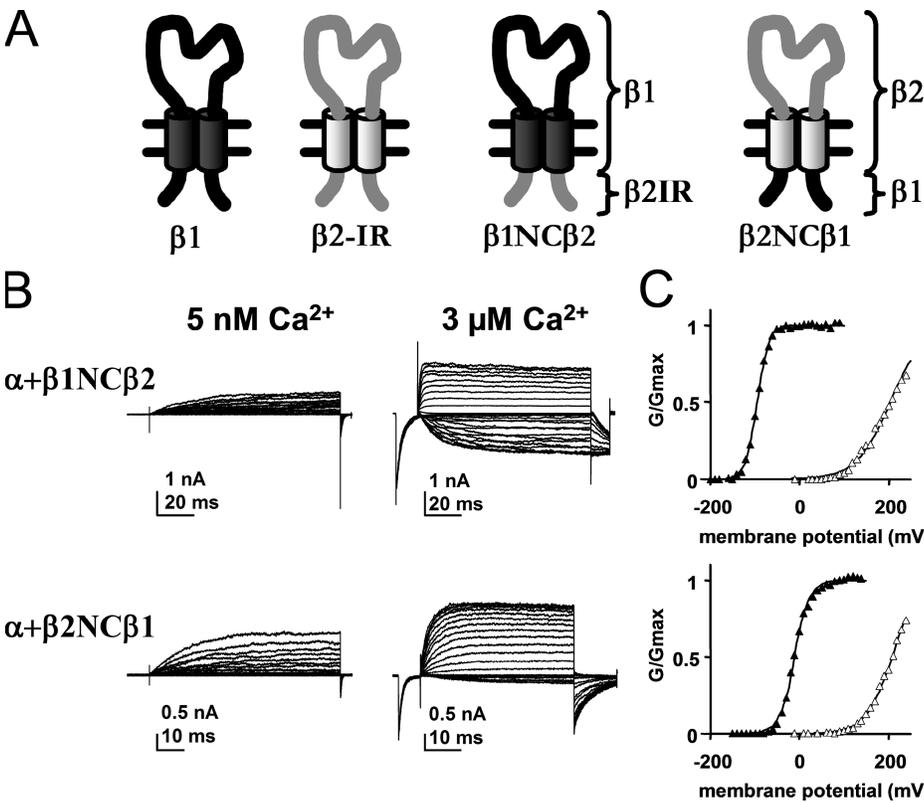


Figure 5. Effects of $\beta 1\text{NC}\beta 2$ and $\beta 2\text{NC}\beta 1$ subunits on BK channel steady-state activation parameters. (A) Schematic description of the $\beta 1\text{NC}\beta 2$ and $\beta 2\text{NC}\beta 1$ chimeras. (B) Macroscopic currents recorded in the inside-out configuration at 5 nM (left) and 3.3 μM (right) intracellular calcium, for $\alpha + \beta 1\text{NC}\beta 2$ (top) and $\alpha + \beta 2\text{NC}\beta 1$ (bottom) channels. The voltage protocols are as described in legend of Fig. 1. (C) Normalized G/V curves at 3.3 μM (closed symbols) and 5 nM Ca^{2+} (open symbols) for the current traces shown in B. Lines are the best fit of a Boltzmann distribution (Eq. 1). Fit parameters are as follows. $\alpha + \beta 1\text{NC}\beta 2$, $V_{0.5} = 204$ mV, $z = 0.7$ (5 nM); $V_{0.5} = -95$ mV, $z = 2.0$ (3.3 μM). $\alpha + \beta 2\text{NC}\beta 1$, $V_{0.5} = 208$ mV, $z = 0.9$ (5 nM); $V_{0.5} = -10$ mV, $z = 1.5$ (3.3 μM).

current traces for channel activation at 200 mV after a -80 mV hyperpolarizing pulse in the virtual absence of calcium (5 nM). The time course followed by the currents induced by $\alpha + \beta 2\text{L}\beta 1$ channels is slower than that of $\alpha + \beta 1\text{L}\beta 2$ channels. Fig. 4 C plots the time constant of an exponential fit of the current time course as a function of the activation voltage. The tendency ($\alpha + \beta 2\text{L}\beta 1$ slower than $\alpha + \beta 1\text{L}\beta 2$ slower than α) is maintained at all the activation voltages explored, resembling the behavior of the $\beta 1$ and $\beta 2\text{IR}$ subunits. As in the $V_{0.5}/[\text{Ca}^{2+}]$ and $z/[\text{Ca}^{2+}]$ plots, the data obtained for $\alpha + \beta 1\text{L}\beta 2$ channels superimposes to that obtained for $\alpha + \beta 1$ channels ($P = 0.13$ and $P < 0.0001$ when compared with $\alpha + \beta 1$ and $\alpha + \beta 2\text{IR}$, respectively, in a two-way ANOVA test), and the data obtained for $\alpha + \beta 2\text{L}\beta 1$ channels is not different from that obtained for $\alpha + \beta 2$ channels ($P < 0.0001$ and $P = 0.94$ when compared with $\alpha + \beta 1$ and $\alpha + \beta 2\text{IR}$, respectively).

The deactivation process at -60 mV after a 200-mV activation pulse is also slower when the channel is coexpressed with the chimeric subunits $\beta 1\text{L}\beta 2$ and $\beta 2\text{L}\beta 1$ (Fig. 4 B). Moreover $\alpha + \beta 1\text{L}\beta 2$ is slower than $\alpha + \beta 2\text{L}\beta 1$, confirming that $\beta 1\text{L}\beta 2$ behaves like $\beta 1$ while $\beta 2\text{L}\beta 1$ is like $\beta 2\text{IR}$. However, the deactivation kinetics must be studied in a wide voltage range because the $\beta 1$ -subunit also affects the voltage dependence of the deactivation process. Fig. 4 D plots the deactivation time constant (τ_{deact}) in the -220 to $+100$ mV voltage range. The semilogarithmic τ_{deact}/V plot shows two apparent

slopes for all α , $\alpha + \beta 1$, and $\alpha + \beta 2\text{IR}$ channels. While the first slope (limiting slope for the voltage dependence of channel deactivation) is not affected by the type of β -subunit, the second slope ($z = 0.44$ electronic equivalents for the α -subunit alone) is severely affected by the $\beta 1$ ($z = 0.25$) but not the $\beta 2\text{IR}$ ($z = 0.44$) subunit. This is due to the reduction of the voltage sensor-associated voltage dependence of the BK channel that occurs in the presence of the $\beta 1$ -subunit (Orio and Latorre, 2005; but see Bao and Cox, 2005). The chimeric $\beta 1\text{L}\beta 2$ -subunit modifies the voltage dependence of the deactivation process of the channel in the same way as the $\beta 1$ -subunit, with a z value of 0.28 ($P = 0.8$ and $P < 0.0001$ compared with $\alpha + \beta 1$ and $\alpha + \beta 2\text{IR}$, respectively, in an extra sum-of-squares F test). The $\beta 2\text{L}\beta 1$ -subunit shows a voltage sensor-related slope a little higher than that observed for $\alpha + \beta 2\text{IR}$ channels ($z = 0.48$, $P < 0.0001$ and $P = 0.003$ compared with $\alpha + \beta 1$ and $\alpha + \beta 2\text{IR}$, respectively).

These results strongly suggest that the differential effects of the β subunits on the BK channel properties are related to the transmembrane and/or the intracellular domains of these regulatory subunits but not to the extracellular domain.

Functional Coupling Is Mainly Determined by Intracellular Domains

To circumscribe the structure(s) in the β subunits determining the differential coupling with the α -subunit,

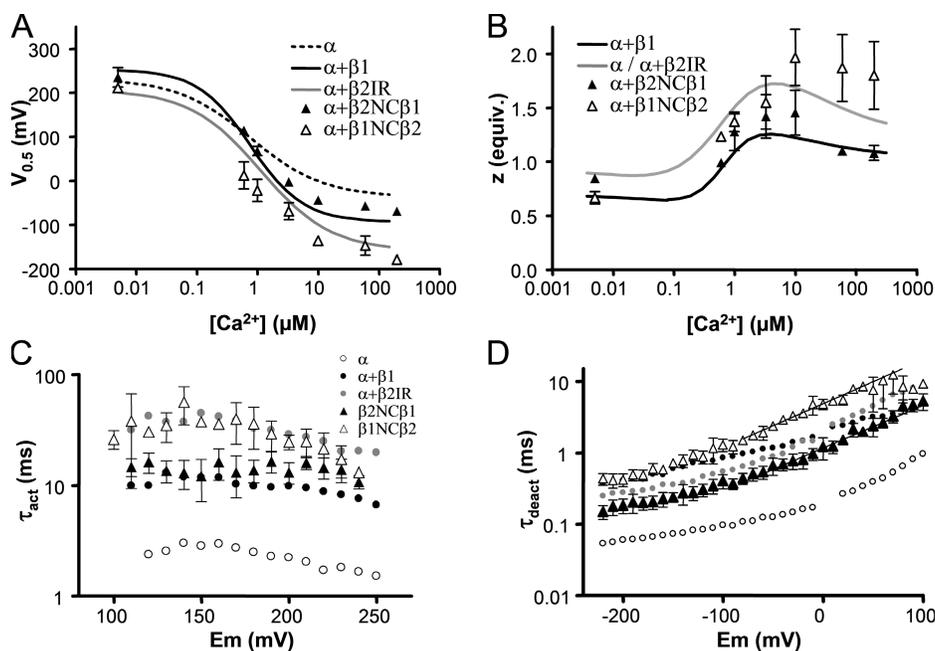


Figure 6. Activation parameters for $\alpha + \beta 1NC\beta 2$ and $\alpha + \beta 2NC\beta 1$ channels. (A) Average of the obtained $V_{0.5}$ values plotted against calcium concentration. For α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the best fit sigmoid concentration–effect curve (see Fig. 1 C) is depicted. (B) Average of the obtained z values plotted against calcium concentration. For α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$ only the best fit curve (see Fig. 1 D) is depicted. Error bars are SD, $n = 4–7$. When SD bars are not visible, they are smaller than symbol size. (C) Activation time constant (τ_{act}) at 5 nM intracellular $[Ca^{2+}]$ plotted against activation voltage. Symbols represent mean \pm SD, $n = 3–5$. (D) Deactivation time constant (τ_{deact}) at 5 nM intracellular $[Ca^{2+}]$ plotted against voltage. Symbols represent mean \pm SD, $n = 3–7$. In the case of α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the mean is shown. Lines are defined as in Fig. 4.

we exchanged the NH_2 and $COOH$ terminus between $\beta 1$ and $\beta 2IR$. The two chimeras generated in this way were denominated $\beta 1NC\beta 2$ ($\beta 1$ with NH_2 and $COOH$ termini of $\beta 2$) and $\beta 2NC\beta 1$ ($\beta 2$ with NH_2 and $COOH$ termini of $\beta 1$) (Fig. 5 A). Fig. 5 B shows that the coexpression of both chimeras with the BK channel produces robust, calcium- and voltage-dependent currents. From the G/V plots (Fig. 5 C), it is evident that in the presence of the $\beta 1NC\beta 2$ chimera, the channel has higher calcium sensitivity than in the presence of $\beta 2NC\beta 1$ (the curve for 3 μM is more displaced to the left). Also, $\alpha + \beta 1NC\beta 2$ channels have a higher voltage dependency. The analysis of the calcium and voltage dependence (Fig. 6, A and B) in the presence of these chimeras shows that the $\beta 2NC\beta 1$ chimera (filled triangles) imparts to the BK channel similar properties than those obtained when the α -subunit is coexpressed with the $\beta 1$ -subunit (black solid line), both in terms of the $V_{0.5}/[Ca^{2+}]$ ($P = 0.03$ and $P < 0.0001$ compared with $\alpha + \beta 1$ and $\alpha + \beta 2IR$, respectively) as well as the $z/[Ca^{2+}]$ relationships. The $\beta 1NC\beta 2$ chimera (empty triangles) on its hand resembles the behavior of the $\beta 2IR$ (gray solid lines) subunit, though the $V_{0.5}$ values are displaced 40–50 mV toward more negative voltages ($P < 0.0001$ and $P = 0.003$ compared with $\alpha + \beta 1$ and $\alpha + \beta 2IR$, respectively).

$\beta 1NC\beta 2$ and $\beta 2NC\beta 1$ chimeras also show an effect on channel kinetics, slowing down both the activation and

deactivation processes. Fig. 6 C plots the time constant of the macroscopic activation kinetics (τ_{act}) at several voltages and in the absence of calcium. $\beta 1NC\beta 2$ resembles the behavior of the $\beta 2IR$ -subunit ($P < 0.0001$ and $P = 0.08$, respectively) and though the $\beta 2NC\beta 1$ is statistically different from both ($P = 0.0002$ and $P < 0.0001$, respectively), it is closer to the $\beta 1$ -subunit. This suggests that the intracellular portions of the β subunits are responsible for the modulation of the activation kinetics.

In the case of the macroscopic deactivation kinetics (Fig. 6 D), none of the chimeras behaved exactly as either the $\beta 1$ - or the $\beta 2IR$ -subunit. The voltage dependence of the deactivation kinetics turned out to be different to both parent β subunits ($z = 0.35 \pm 0.02$ for $\alpha + \beta 2NC\beta 1$ and 0.36 ± 0.02 for $\alpha + \beta 1NC\beta 2$ channels; $P \leq 0.01$ for all comparisons with $\alpha + \beta 1$ and $\alpha + \beta 2IR$), suggesting a partial effect on the voltage sensor–associated voltage dependence. $\alpha + \beta 1NC\beta 2$ channels have τ_{deact} limiting values (at the most negative potentials) close to $\alpha + \beta 1$ channels ($P = 0.03$), indicating that the kinetic rate for channel deactivation when all voltage sensors are in the resting position is affected by this chimera in the same way as in the presence of the $\beta 1$ -subunit.

In summary, the study of the “NC” chimeras suggests that the intracellular regions of the $\beta 1$ and $\beta 2IR$ subunits determine the modulation of a great part of the properties studied here, with the exception of the

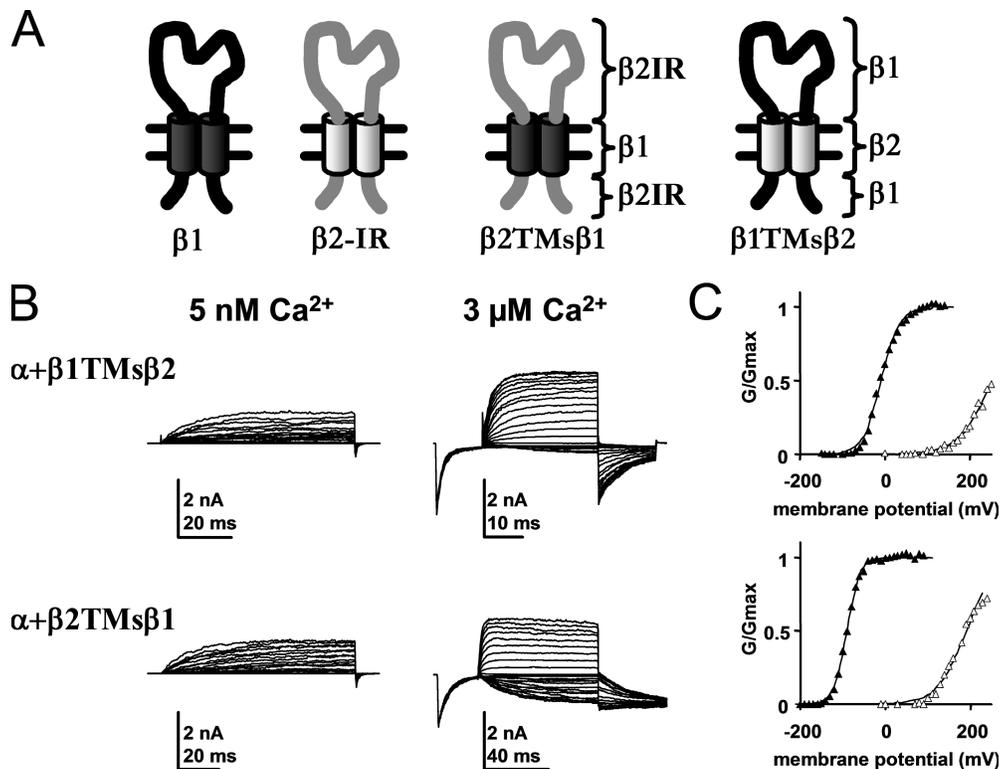


Figure 7. Effects of $\beta 1\text{TMs}\beta 2$ and $\beta 2\text{TMs}\beta 1$ subunits on BK channel steady-state activation parameters. (A) Schematic description of the $\beta 1\text{TMs}\beta 2$ and $\beta 2\text{TMs}\beta 1$ chimeras. (B) Macroscopic currents recorded in the inside-out configuration at 5 nM (left) and 3.3 μM (right) intracellular calcium, for $\alpha + \beta 1\text{TMs}\beta 2$ (top) and $\alpha + \beta 2\text{TMs}\beta 1$ (bottom) channels. The voltage protocols are as described in legend of Fig. 1. (C) Normalized G/V curves at 3.3 μM (closed symbols) and 5 nM Ca^{2+} (open symbols) for the current traces shown in B. Lines are the best fit of a Boltzmann distribution (Eq. 1). Fit parameters are as follows. $\alpha + \beta 1\text{TMs}\beta 2$, $V_{0.5} = 251$ mV, $z = 0.7$ (5 nM); $V_{0.5} = -9$ mV, $z = 1.2$ (3.3 μM). $\alpha + \beta 2\text{TMs}\beta 1$, $V_{0.5} = 191$ mV, $z = 0.7$ (5 nM); $V_{0.5} = -90$ mV, $z = 1.7$ (3.3 μM).

changes induced in the deactivation kinetics and its voltage dependence. Essentially the same conclusion can be obtained from the analysis of the $\beta 1\text{TMs}\beta 2$ and $\beta 2\text{TMs}\beta 1$ chimeras, in which the transmembrane domains were exchanged between $\beta 1$ and $\beta 2\text{IR}$ (Figs. 7 and 8). $\beta 2\text{TMs}\beta 1$ chimera, which contains the same intracellular domains as $\beta 2$ and $\beta 1\text{NC}\beta 2$ subunits, behaves very much like the $\beta 1\text{NC}\beta 2$ subunits. On the other hand, the $\beta 2\text{TMs}\beta 1$ chimera, which contains the same intracellular domains as the $\beta 1$ and $\beta 2\text{NC}\beta 1$ subunits, behaves as the $\beta 2\text{NC}\beta 1$ chimera. This can be seen from another point of view: the $\beta 1\text{NC}\beta 2$ and $\beta 2\text{TMs}\beta 1$ chimeras modulate BK channel properties in a very similar way and they share the transmembrane and intracellular domains with a completely different extracellular loop. The same is true for the $\beta 2\text{NC}\beta 1$ and $\beta 1\text{TMs}\beta 2$ chimeras. This is in complete agreement with the results of the $\beta 1\text{L}\beta 2$ and $\beta 2\text{L}\beta 1$ chimeras, as the extracellular loop does not determine the differential behavior of the β subunits.

The NH_2 Terminus Is Crucial for Defining the Behavior of either β -subunit

To define further the functional coupling structures, we next created chimeras in which the NH_2 terminus

($\beta 1\text{N}\beta 2$, $\beta 2\text{N}\beta 1$) or the COOH terminus ($\beta 1\text{C}\beta 2$, $\beta 2\text{C}\beta 1$) between $\beta 1$ and $\beta 2\text{IR}$ was exchanged (Fig. 9 A and Fig. 10 A).

When the NH_2 terminus of $\beta 2\text{IR}$ is transplanted into the $\beta 1$ -subunit, the resulting chimera ($\beta 1\text{N}\beta 2$) confers to the BK channel many of the properties of the $\beta 2$ -subunit: the $\alpha + \beta 1\text{N}\beta 2$ channels are very similar to $\alpha + \beta 2\text{IR}$ in terms of the $V_{0.5}$ (Fig. 9 B, $P = 0.02$ and $P = 0.1$ compared with $\alpha + \beta 1$ and $\alpha + \beta 2\text{IR}$, respectively), z (Fig. 9 C), and τ_{act} (Fig. 9 D, $P < 0.0001$ and $P = 0.6$, respectively) values. The only reminiscences of the $\beta 1$ -subunit are found in the τ_{deact}/V plot, where the voltage sensor-associated voltage dependence ($z = 0.28$, $P = 0.02$ and $P < 0.0001$, respectively) and the limiting τ_{deact} value are similar to $\alpha + \beta 1$. Something similar occurs with the opposite chimera, $\beta 2\text{N}\beta 1$, with some minor differences. $\alpha + \beta 2\text{N}\beta 1$ channels behave like $\alpha + \beta 1$ in terms of the z (Fig. 9 C) and τ_{act} (Fig. 9 D, $P = 0.5$ and $P < 0.0001$, respectively) values; however, the $V_{0.5}$ values fall between the curves for α alone and $\alpha + \beta 1$ channels (Fig. 9 B; $P = 0.001$ when compared with $\alpha + \beta 1$ and 0.07 when compared with α). The latter may be an indication of a disruption of the effect of the β subunits on the apparent calcium sensitivity. Regarding the

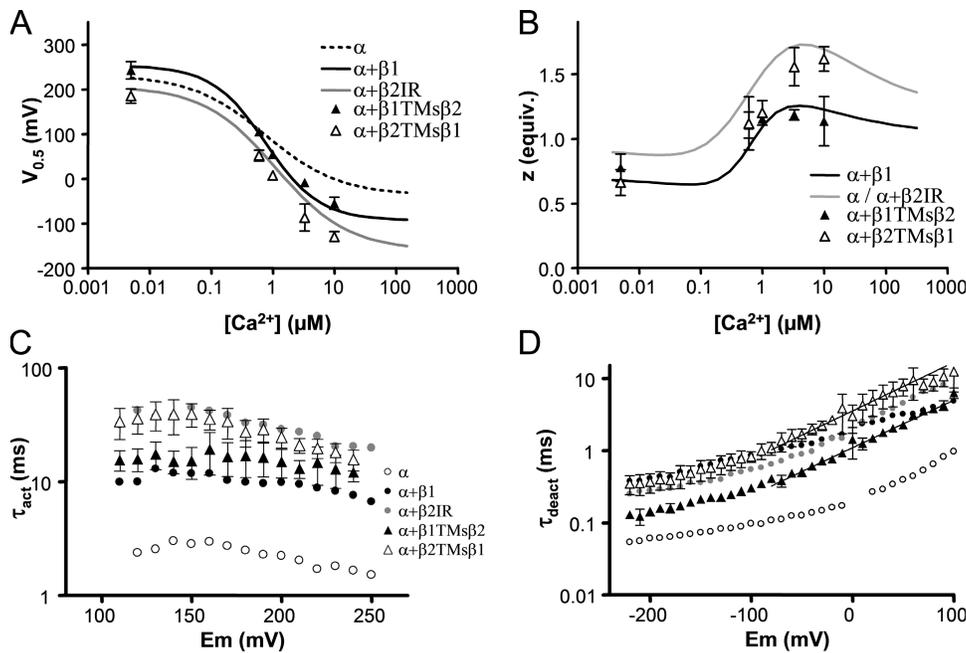


Figure 8. Activation parameters for $\alpha + \beta 1TMs\beta 2$ and $\alpha + \beta 2TMs\beta 1$ channels. (A) Average of the obtained $V_{0.5}$ values plotted against calcium concentration. For α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the best fit sigmoid concentration–effect curve (see Fig. 1 C) is depicted. (B) Average of the obtained z values plotted against calcium concentration. For α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the best fit curve (see Fig. 1 D) is depicted. Error bars are SD, $n = 5-6$. Missing error bars are smaller than symbol size. (C) Activation time constant (τ_{act}) at 5 nM intracellular $[Ca^{2+}]$ plotted against activation voltage. Symbols represent mean \pm SD. $n = 6-8$. (D) Deactivation time constant (τ_{deact}) at 5 nM intracellular $[Ca^{2+}]$ plotted against voltage. Symbols represent mean \pm SD. $n = 4-5$. In the case of α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the mean is shown. Lines are defined as in Fig. 4. The exponent factors expressed as electronic charges ($z = \text{slope} \times RT/F$) are $\alpha + \beta 1TMs\beta 2$, 0.39 ± 0.01 ; $\alpha + \beta 2TMs\beta 1$, 0.39 ± 0.02 .

deactivation kinetics, the behavior is intermediate between α and $\alpha + \beta 2IR$ channels, with a lower limiting τ_{deact} than $\alpha + \beta 2IR$ and a slope for the -50 to $+50$ voltage range of 0.34 ($P < 0.01$ when compared with both $\alpha + \beta 1$ and $\alpha + \beta 2IR$).

When the COOH termini of the $\beta 1$ and $\beta 2IR$ subunits are transplanted between each other, the resulting chimeras have almost all the properties of the parent β -subunit. The $\alpha + \beta 1C\beta 2$ channels have $V_{0.5}$ values ($P = 0.05$ and $P < 0.0001$), z values, τ_{act} values ($P = 0.3$ and $P < 0.0001$), and τ_{deact} voltage dependence of $\alpha + \beta 1$ (Fig. 10, B–E); only the limiting τ_{deact} resembles that of $\alpha + \beta 2IR$ (Fig. 10 E; $P = 0.2$). $\alpha + \beta 2C\beta 1$ channels, on the other hand, behave like $\alpha + \beta 2IR$ in terms of the z (Fig. 10 C), τ_{act} (Fig. 10 D; $P < 0.0001$ and $P = 0.07$, respectively), and τ_{deact} values (Fig. 10 E; $P < 0.0001$ and $P = 0.3$, respectively, for the -50 to 50 mV slope). Interestingly, the $V_{0.5}$ values are like $\alpha + \beta 1$ ($P = 0.7$ and $P = 0.0001$, respectively), suggesting again a partial disruption of the normal effect of the $\beta 2IR$ -subunit on the apparent calcium sensitivity.

In summary, a gross analysis of the results with the “N” and “C” chimeras indicates that most of the differences between $\beta 1$ and $\beta 2IR$ subunits reside in the corresponding NH_2 terminus, and that for some of the effects (changes in τ_{deact} for example) both intracellular domains are required.

DISCUSSION

Structural Determinants in α - β Subunit Coupling

The extracellular NH_2 terminus of the α -subunit together with the first transmembrane domain S0 have been proposed to contribute to the functional interaction between β and α subunits (Wallner et al., 1996). This conclusion was put forward based on the results obtained using different chimeras between *hSlo* and the *Drosophila* homologue of *hSlo*, *dSlo*, that does not functionally couple to the $\beta 1$ -subunit. A chimera containing only the extracellular NH_2 terminus together with the first transmembrane domain S0 of *hSlo* is able to successfully couple with the $\beta 1$ -subunit. Here we attempt to draw similar conclusions from the side of the $\beta 1$ and $\beta 2$ subunits, characterizing a set of chimerical β subunits. A general view of our results shows that the intracellular regions of $\beta 1$ and $\beta 2$ are responsible for most of the differences between the modulatory effects that each of them confer to the activity of BK channel.

The $\beta 1$ and $\beta 2$ subunits are the most closely related within the BK channel’s β -subunit family, with a 43% overall sequence identity and a 39% identity in the loop region. Leaving aside the inactivation promoted by the $\beta 2$ -subunit, they have qualitatively similar effects on BK channel properties. Our conclusions, therefore, should be drawn strictly on the differences between these

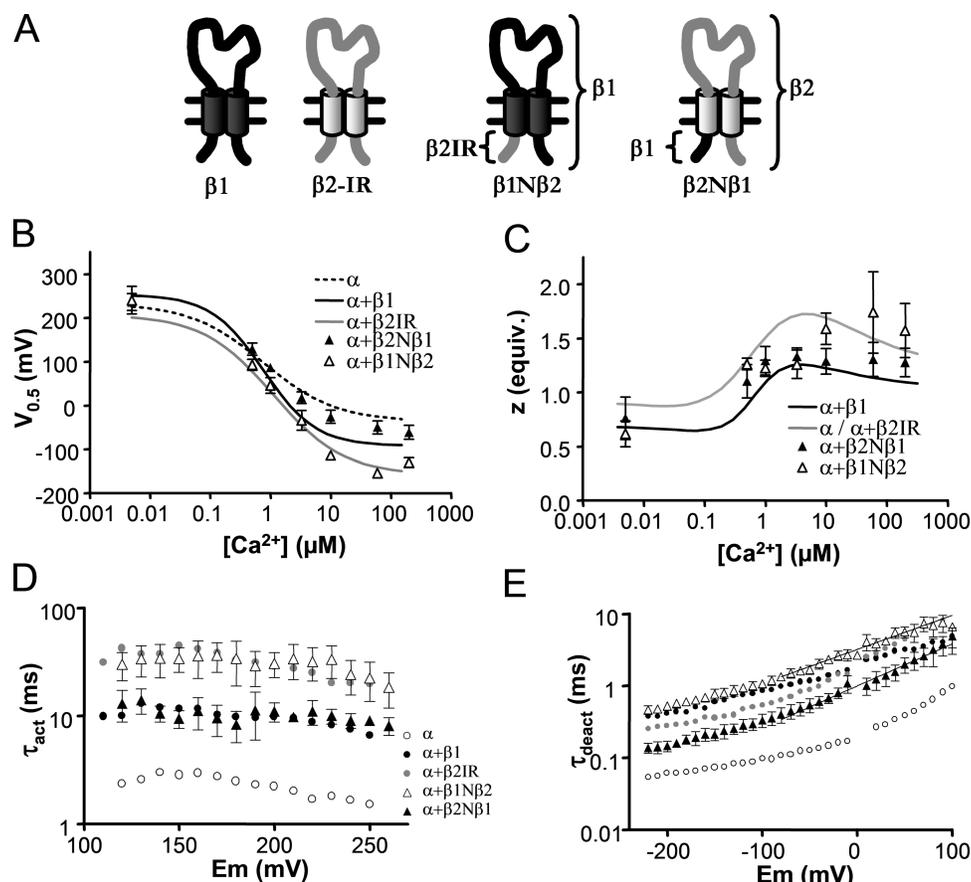


Figure 9. Activation parameters for $\alpha + \beta 1N\beta 2$ and $\alpha + \beta 2N\beta 1$ channels. (A) Schematic description of the $\beta 1N\beta 2$ and $\beta 2N\beta 1$ chimeras. (B) Average of the obtained $V_{0.5}$ values plotted against calcium concentration. For α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the best fit sigmoid concentration–effect curve (see Fig. 1 C) is depicted. (C) Average of the obtained z values plotted against calcium concentration. For α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the best fit curve (see Fig. 1 D) is depicted. Error bars are SD, $n = 4–6$. Missing error bars are smaller than symbol size. (D) Activation time constant (τ_{act}) at 5 nM intracellular $[Ca^{2+}]$ plotted against activation voltage. Symbols represent mean \pm SD. $n = 4–11$. (E) Deactivation time constant (τ_{deact}) at 5 nM intracellular $[Ca^{2+}]$ plotted against voltage. Symbols represent mean \pm SD. $n = 4–6$. In the case of α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the mean is shown. Lines represent the best fit of a simple exponential function to the data between -50 and $+60$ mV, extrapolated from -80 to $+100$ mV. The exponential factors expressed as electronic charges ($z = slope \times RT/F$) are $\alpha + \beta 1N\beta 2$, 0.28 ± 0.01 ; $\alpha + \beta 2N\beta 1$, 0.34 ± 0.01 .

subunits. It is possible that most of the effects of both subunits reside in a common structural feature of the extracellular loop, while the intracellular and transmembrane domains would be responsible for the fine tuning of these effects giving to each subunit its own identity. Though this is an issue that will need experiments with the other β subunits to be categorically addressed, several facts make us think that these domains do much more than subtle changes to the activation properties of the BK channel. First, one of the differences between $\beta 1$ and $\beta 2$ subunits is not quantitative but qualitative: the voltage dependence of the channel is greatly impaired in the presence of the $\beta 1$ -subunit while it remains unaffected by the $\beta 2$ -subunit. At least, our present results map regions of the $\beta 1$ -subunit that must interact with either the voltage sensors of the channel or the domains that couple the voltage sensor activation with channel opening. Second, a thorough analysis of the effects of

these subunits suggested the possibility that both β subunits enhance the apparent calcium sensitivity by different mechanisms (Orio and Latorre, 2005). In this case, the regions that we have mapped would be responsible for a different regulatory mechanism in each β -subunit. Finally, all the voltage and calcium sensitivity in the BK channel has been so far mapped to transmembrane and intracellular domains: the voltage sensor is in the S4 membrane–spanning helix (Diaz et al., 1998), all the postulated calcium binding sites are proposed to be intracellular (Schreiber and Salkoff, 1997; Schreiber et al., 1999; Bian et al., 2001; Bao et al., 2002, 2004; Xia et al., 2002, 2004), and the coupling mechanisms for both voltage and calcium activation have been mapped to intracellular regions as well (see below, Functional Coupling). Therefore we think unlikely that an important contribution to the modulation of calcium- and voltage-dependent activation mechanisms of the BK

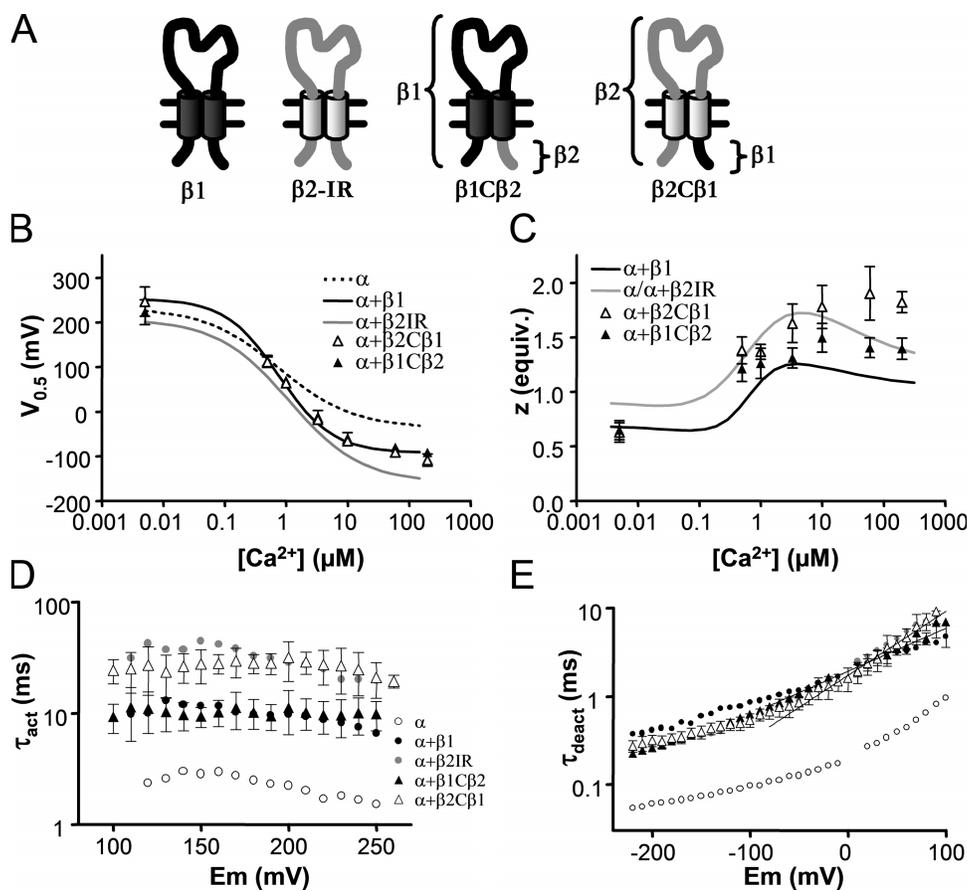


Figure 10. Activation parameters for $\alpha + \beta 1C\beta 2$ and $\alpha + \beta 2C\beta 1$ channels. (A) Schematic description of the $\beta 1C\beta 2$ and $\beta 2C\beta 1$ chimeras. (B) Average of the obtained $V_{0.5}$ values plotted against calcium concentration. For α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the best fit sigmoid concentration–effect curve (see Fig. 1 C) is depicted. (C) Average of the obtained z values plotted against calcium concentration. For α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the best fit curve (see Fig. 1 D) is depicted. Error bars are SD, $n = 9–11$. Missing error bars are smaller than symbol size. (D) Activation time constant (τ_{act}) at 5 nM intracellular $[Ca^{2+}]$ plotted against activation voltage. Symbols represent mean \pm SD. $n = 8–12$. (E) Deactivation time constant (τ_{deact}) at 5 nM intracellular $[Ca^{2+}]$ plotted against voltage. Symbols represent mean \pm SD. $n = 4–8$. In the case of α , $\alpha + \beta 1$, and $\alpha + \beta 2IR$, only the mean is shown. Lines represent the best fit of a simple exponential function to the data between -50 and $+60$ mV, extrapolated from -80 to $+100$ mV. The exponential factors expressed as electronic charges ($z = \text{slope} \times RT/F$) are $\alpha + \beta 1C\beta 2$, 0.29 ± 0.01 ; $\alpha + \beta 2C\beta 1$, 0.43 ± 0.01 .

channel may arise from an interaction of extracellular domains of β and α subunits.

Function of the Extracellular Region

Several roles have been attributed to the interaction between extracellular regions of the α and β subunits of the BK channel, all of them unrelated to changes in the voltage or calcium sensitivity of the channel. The external loop of β subunits plays an important role in determining ion permeation and the characteristics of toxin binding. The $\beta 4$ -subunit, for instance, induces a resistance of BK channels to CTX and IbTX block that is reproduced only by a chimera containing the loop of $\beta 4$ (Meera et al., 2000). The $\beta 3$ -subunit produces an open channel rectification (Xia et al., 2000; Lingle et al., 2001; Zeng et al., 2001) that is dependent on the disulfide bridges in its extracellular region. Again, this effect is only reproduced by a chimera containing the extra-

cellular loop of $\beta 3$ (Zeng et al., 2003). In the case of the bovine $\beta 1$ -subunit, the adequate loop conformation appears to be a requisite for BK channel high affinity binding of CTX, as any of the four cysteines contained in the external $\beta 1$ linker are critical in defining the ability of $\beta 1$ in enhancing CTX binding (Hanner et al., 1998). Regarding the functional coupling between α and $\beta 1$ subunits, the results that propose the extracellular domains as part of the interaction domain (Wallner et al., 1996) may be only of structural meaning, these domains being needed for the assembly of the heteromultimer rather than for functional coupling.

The present results add to this list of findings that the β -subunit external region may play only a minor role in the modulation of the calcium- and voltage-dependent activation mechanisms of the channel. This minor contribution cannot be excluded, though. We have recently shown that a point mutation in the extracellular loop of

the human $\beta 1$ -subunit modifies its effect on BK calcium sensitivity (Fernandez-Fernandez et al., 2004). Despite the physiological relevance of this effect, it is less than the differences between $\beta 1$ and $\beta 2$ subunits (e.g., at 500 nM calcium the E65K mutation induces a shift of 10 mV in $V_{0.5}$ while the difference between $\beta 1$ and $\beta 2$ subunits at 680 nM calcium is ~ 20 mV), and this mutant still changes the channel kinetics the way the wild-type $\beta 1$ -subunit does. Thus, the extracellular loop may play a partial role in the modulation of apparent calcium sensitivity, possibly via some interaction with the external side of the voltage sensor (see Bao and Cox, 2005; Orio and Latorre, 2005).

Role of the Intracellular Termini

When only the intracellular and transmembrane regions are dissociated (NC and TMs chimeras) the resulting chimeras induce changes in channel properties that resemble the donor of the intracellular regions, suggesting that the intracellular ends are responsible for most of the functional coupling between α and β subunits. However in this case the results were not as clean as with the L chimeras, suggesting a shared role with the transmembrane regions in the modulation of channel properties. Interestingly, the $\beta 1\text{NC}\beta 2$ and $\beta 2\text{TMs}\beta 1$ chimeras (both having the NH_2 and COOH termini of $\beta 2$) showed an enhanced effect on BK channel's calcium sensitivity, promoting a $V_{0.5}$ shift higher than that promoted by the $\beta 2\text{IR}$ -subunit.

Chimeras with only the NH_2 terminus exchanged ($\beta 1\text{N}\beta 2$ and $\beta 2\text{N}\beta 1$) showed a mixed behavior; however, there were important characteristics similar to the β -subunit contributing to the NH_2 terminus. In particular, the NH_2 terminus suffices to determine the BK channel activation kinetics and the behavior of z as a function of the Ca^{2+} concentration. A different result was obtained with the chimeras with only the COOH terminus exchanged ($\beta 1\text{C}\beta 2$ and $\beta 2\text{C}\beta 1$); in which there were minor differences in behavior compared with the parental β subunits. Thus, the NH_2 terminus of the β subunits is the most important structural determinant in the differences of modulation of the α -subunit by its auxiliary $\beta 1$ and $\beta 2$ subunits.

This finding is not surprising when other evidences are considered. In the case of $\beta 4$ -subunit, phosphorylation of its NH_2 and COOH termini is crucial for the functional coupling with the α -subunit (Jin et al., 2002). The addition of the phosphatase inhibitor okadaic acid (OA) eliminates the effect of coexpression of $\beta 4$ subunits, indicating that phosphorylation of $\beta 4$ eliminates its modulatory effects. This shows that the relevance of the intracellular ends of the β subunits in the α - β subunit coupling may be a characteristic of all β subunits. The NH_2 terminus of $\beta 2$ -subunit has a conserved cAMP, cGMP-dependent phosphorylation site (sequence KRKT), and its relevance for modulation of channel properties remains unknown.

Functional Coupling

It has been proposed that the intracellular domains of each BK α -subunit contributes two RCK (regulatory of K^+ conductance) domains and that these eight RCK domains form a gating ring similar to the gating ring of MthK channels (Jiang et al., 2001, 2002). The free energy provided by Ca^{2+} binding to the gating ring (chemical energy) is transformed in mechanical energy used to open the pore. The linker between the sixth (S6) transmembrane domain and the first RCK domain of the BK channel has also been shown to play a role in this molecular coupling, acting as a passive spring (Niu et al., 2004). In the case of the molecular coupling between voltage sensor activation and channel opening, the S4-S5 linker as well as the COOH -terminal half of the S6 segment have been postulated to play this role in other voltage-gated channels (Chen et al., 2001; Lu et al., 2002; Tristani-Firouzi et al., 2002).

It appears, therefore, inescapable that the intracellular domains of the BK channel are the most probable site of interaction for the modulation of the calcium and voltage sensitivity. Indeed, Qian et al. (2002) showed that the $\beta 1$ -subunit cannot enhance the calcium sensitivity of BK channels when the intracellular "tail" domain is replaced with the tail from the highly related, pH-dependent, Slo3 channel. The only effect of the $\beta 1$ -subunit that can be detected on this channel is a reduction of the voltage dependence as it does for the wt channel in the absence of calcium. This and other observations were interpreted as the $\beta 1$ -subunit interacting with the intracellular calcium-sensing machinery of the BK channel. We show here that the intracellular domains of the $\beta 1$ and $\beta 2$ subunits are indeed responsible for most of the channel activity modulation. An attractive hypothesis would be that the cytoplasmic regions of the β subunits interacts directly with the linker-gating ring complex, modifying the allosteric coupling factors involved in channel opening.

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REFERENCES

- Bao, L., and D.H. Cox. 2005. Gating and ionic currents reveal how the BKCa channel's Ca^{2+} sensitivity is enhanced by its $\beta 1$ subunit. *J. Gen. Physiol.* 126:393–412.

- Bao, L., A.M. Rapin, E.C. Holmstrand, and D.H. Cox. 2002. Elimination of the BK(Ca) channel's high-affinity Ca^{2+} sensitivity. *J. Gen. Physiol.* 120:173–189.
- Bao, L., C. Kaldany, E.C. Holmstrand, and D.H. Cox. 2004. Mapping the BKCa channel's " Ca^{2+} bowl": side-chains essential for Ca^{2+} sensing. *J. Gen. Physiol.* 123:475–489.
- Behrens, R., A. Nolting, F. Reimann, M. Schwarz, R. Waldschutz, and O. Pongs. 2000. hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel β subunit family. *FEBS Lett.* 474:99–106.
- Bian, S., I. Favre, and E. Moczydowski. 2001. Ca^{2+} -binding activity of a COOH-terminal fragment of the *Drosophila* BK channel involved in Ca^{2+} -dependent activation. *Proc. Natl. Acad. Sci. USA.* 98:4776–4781.
- Brenner, R., T.J. Jegla, A. Wickenden, Y. Liu, and R.W. Aldrich. 2000. Cloning and functional characterization of novel large conductance calcium-activated potassium channel β subunits, hKCNMB3 and hKCNMB4. *J. Biol. Chem.* 275:6453–6461.
- Chen, J., J.S. Mitcheson, M. Tristani-Firouzi, M. Lin, and M.C. Sanguinetti. 2001. The S4-S5 linker couples voltage sensing and activation of pacemaker channels. *Proc. Natl. Acad. Sci. USA.* 98:11277–11282.
- Cox, D.H., and R.W. Aldrich. 2000. Role of the β 1 subunit in large-conductance Ca^{2+} -activated K^+ channel gating energetics. Mechanisms of enhanced Ca^{2+} sensitivity. *J. Gen. Physiol.* 116:411–432.
- Cox, D.H., J. Cui, and R.W. Aldrich. 1997. Allosteric gating of a large conductance Ca-activated K^+ channel. *J. Gen. Physiol.* 110:257–281.
- Diaz, L., P. Meera, J. Amigo, E. Stefani, O. Alvarez, L. Toro, and R. Latorre. 1998. Role of the S4 segment in a voltage-dependent calcium-sensitive potassium (hSlo) channel. *J. Biol. Chem.* 273:32430–32436.
- Dick, G.M., C.F. Rossow, S. Smirnov, B. Horowitz, and K.M. Sanders. 2001. Tamoxifen activates smooth muscle BK channels through the regulatory β 1 subunit. *J. Biol. Chem.* 276:34594–34599.
- Dworetzky, S.I., C.G. Boissard, J.T. Lum-Ragan, M.C. McKay, D.J. Post-Munson, J.T. Trojnecki, C.P. Chang, and V.K. Gribkoff. 1996. Phenotypic alteration of a human BK (hSlo) channel by hSlo β subunit coexpression: changes in blocker sensitivity, activation/relaxation and inactivation kinetics, and protein kinase A modulation. *J. Neurosci.* 16:4543–4550.
- Fernandez-Fernandez, J.M., M. Tomas, E. Vazquez, P. Orio, R. Latorre, M. Senti, J. Marrugat, and M.A. Valverde. 2004. Gain-of-function mutation in the KCNMB1 potassium channel subunit is associated with low prevalence of diastolic hypertension. *J. Clin. Invest.* 113:1032–1039.
- Hanner, M., R. Vianna-Jorge, A. Kamassah, W.A. Schmalhofer, H.G. Knaus, G.J. Kaczorowski, and M.L. Garcia. 1998. The β subunit of the high conductance calcium-activated potassium channel. Identification of residues involved in charybdotoxin binding. *J. Biol. Chem.* 273:16289–16296.
- Horrigan, F.T., and R.W. Aldrich. 2002. Coupling between voltage sensor activation, Ca^{2+} binding and channel opening in large conductance (BK) potassium channels. *J. Gen. Physiol.* 120:267–305.
- Horton, R.M., Z.L. Cai, S.N. Ho, and L.R. Pease. 1990. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. *Biotechniques.* 8:528–535.
- Jiang, Y., A. Pico, M. Cadene, B.T. Chait, and R. MacKinnon. 2001. Structure of the RCK domain from the *E. coli* K^+ channel and demonstration of its presence in the human BK channel. *Neuron.* 29:593–601.
- Jiang, Y., A. Lee, J. Chen, M. Cadene, B.T. Chait, and R. MacKinnon. 2002. Crystal structure and mechanism of a calcium-gated potassium channel. *Nature.* 417:515–522.
- Jiang, Z., M. Wallner, P. Meera, and L. Toro. 1999. Human and rodent MaxiK channel β -subunit genes: cloning and characterization. *Genomics.* 55:57–67.
- Jin, P., T.M. Weiger, Y. Wu, and I.B. Levitan. 2002. Phosphorylation-dependent functional coupling of hSlo calcium-dependent potassium channel and its h β 4 subunit. *J. Biol. Chem.* 277:10014–10020.
- Knaus, H.G., A. Eberhart, G.J. Kaczorowski, and M.L. Garcia. 1994a. Covalent attachment of charybdotoxin to the β -subunit of the high conductance Ca^{2+} -activated K^+ channel. Identification of the site of incorporation and implications for channel topology. *J. Biol. Chem.* 269:23336–23341.
- Knaus, H.G., K. Folander, M. Garcia-Calvo, M.L. Garcia, G.J. Kaczorowski, M. Smith, and R. Swanson. 1994b. Primary sequence and immunological characterization of β -subunit of high conductance Ca^{2+} -activated K^+ channel from smooth muscle. *J. Biol. Chem.* 269:17274–17278.
- Lingle, C.J., X.H. Zeng, J.P. Ding, and X.M. Xia. 2001. Inactivation of BK channels mediated by the NH_2 terminus of the β 3b auxiliary subunit involves a two-step mechanism: possible separation of binding and blockade. *J. Gen. Physiol.* 117:583–606.
- Lu, Z., A.M. Klem, and Y. Ramu. 2002. Coupling between voltage sensors and activation gate in voltage-gated K^+ channels. *J. Gen. Physiol.* 120:663–676.
- McManus, O.B., and K.L. Magleby. 1991. Accounting for the Ca^{2+} -dependent kinetics of single large-conductance Ca^{2+} -activated K^+ channels in rat skeletal muscle. *J. Physiol.* 443:739–777.
- McManus, O.B., L.M. Helms, L. Pallanck, B. Ganetzky, R. Swanson, and R.J. Leonard. 1995. Functional role of the β subunit of high conductance calcium-activated potassium channels. *Neuron.* 14:645–650.
- Meech, R.W. 1978. Calcium-dependent potassium activation in nervous tissues. *Annu. Rev. Biophys. Bioeng.* 7:1–18.
- Meera, P., M. Wallner, Z. Jiang, and L. Toro. 1996. A calcium switch for the functional coupling between α (hSlo) and β subunits (Kv,ca β) of maxi K channels. *FEBS Lett.* 385:127–128.
- Meera, P., M. Wallner, M. Song, and L. Toro. 1997. 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. USA.* 94:14066–14071.
- Meera, P., M. Wallner, and L. Toro. 2000. A neuronal β subunit (KCNMB4) makes the large conductance, voltage- and Ca^{2+} -activated K^+ channel resistant to charybdotoxin and iberiotoxin. *Proc. Natl. Acad. Sci. USA.* 97:5562–5567.
- Nimigeon, C.M., and K.L. Magleby. 2000. Functional coupling of the β 1 subunit to the large conductance Ca^{2+} -activated K^+ channel in the absence of Ca^{2+} . Increased Ca^{2+} sensitivity from a Ca^{2+} -independent mechanism. *J. Gen. Physiol.* 115:719–736.
- Niu, X., X. Qian, and K.L. Magleby. 2004. Linker-gating ring complex as passive spring and Ca^{2+} -dependent machine for a voltage- and Ca^{2+} -activated potassium channel. *Neuron.* 42:745–756.
- Orio, P., and R. Latorre. 2005. Differential Effects of β 1 and β 2 subunits on BK channel activity. *J. Gen. Physiol.* 125:395–411.
- Orio, P., P. Rojas, G. Ferreira, and R. Latorre. 2002. New disguises for an old channel: maxiK channel β -subunits. *News Physiol. Sci.* 17:156–161.
- Qian, X., C.M. Nimigeon, X. Niu, B.L. Moss, and K.L. Magleby. 2002. Slo1 tail domains, but not the Ca^{2+} bowl, are required for the β 1 subunit to increase the apparent Ca^{2+} sensitivity of BK channels. *J. Gen. Physiol.* 120:829–843.
- Schreiber, M., and L. Salkoff. 1997. A novel calcium-sensing domain in the BK channel. *Biophys. J.* 73:1355–1363.
- Schreiber, M., A. Yuan, and L. Salkoff. 1999. Transplantable sites confer calcium sensitivity to BK channels. *Nat. Neurosci.* 2:416–421.

- Shen, K.Z., A. Lagrutta, N.W. Davies, N.B. Standen, J.P. Adelman, and R.A. North. 1994. Tetraethylammonium block of Slowpoke calcium-activated potassium channels expressed in *Xenopus* oocytes: evidence for tetrameric channel formation. *Pflügers Arch.* 426:440–445.
- Stühmer, W., and A.B. Parekh. 1995. Electrophysiological recordings from *Xenopus* oocytes. In *Single Channel Recording*. B. Sakmann and E. Neher, editors. Plenum Press, New York. 341–356.
- Toro, L., M. Wallner, P. Meera, and Y. Tanaka. 1998. Maxi-K(Ca), a unique member of the voltage-gated K channel superfamily. *News Physiol. Sci.* 13:112–117.
- Tristani-Firouzi, M., J. Chen, and M.C. Sanguinetti. 2002. Interactions between S4-S5 linker and S6 transmembrane domain modulate gating of HERG K⁺ channels. *J. Biol. Chem.* 277:18994–19000.
- Uebele, V.N., A. Lagrutta, T. Wade, D.J. Figueroa, Y. Liu, E. McKenna, C.P. Austin, P.B. Bennett, and R. Swanson. 2000. Cloning and functional expression of two families of β -subunits of the large conductance calcium-activated K⁺ channel. *J. Biol. Chem.* 275:23211–23218.
- Valverde, M.A., P. Rojas, J. Amigo, D. Cosmelli, P. Orío, M.I. Bahamonde, G.E. Mann, C. Vergara, and R. Latorre. 1999. Acute activation of Maxi-K channels (hSlo) by estradiol binding to the β subunit. *Science.* 285:1929–1931.
- Vergara, C., R. Latorre, N.V. Marrion, and J.P. Adelman. 1998. Calcium-activated potassium channels. *Curr. Opin. Neurobiol.* 8:321–329.
- Wallner, M., P. Meera, M. Ottolia, G.J. Kaczorowski, R. Latorre, M.L. Garcia, E. Stefani, and L. Toro. 1995. Characterization of and modulation by a β -subunit of a human maxi KCa channel cloned from myometrium. *Receptors Channels.* 3:185–199.
- Wallner, M., P. Meera, and L. Toro. 1996. Determinant for β -subunit regulation in high-conductance voltage-activated and Ca²⁺-sensitive K⁺ channels: an additional transmembrane region at the N terminus. *Proc. Natl. Acad. Sci. USA.* 93:14922–14927.
- Wallner, M., P. Meera, and L. Toro. 1999. Molecular basis of fast inactivation in voltage and Ca²⁺-activated K⁺ channels: a transmembrane β -subunit homolog. *Proc. Natl. Acad. Sci. USA.* 96:4137–4142.
- Xia, X.M., J.P. Ding, and C.J. Lingle. 1999. Molecular basis for the inactivation of Ca²⁺- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells. *J. Neurosci.* 19:5255–5264.
- Xia, X.M., J.P. Ding, X.H. Zeng, K.L. Duan, and C.J. Lingle. 2000. Rectification and rapid activation at low Ca²⁺ of Ca²⁺-activated, voltage-dependent BK currents: consequences of rapid inactivation by a novel β subunit. *J. Neurosci.* 20:4890–4903.
- Xia, X.M., X. Zeng, and C.J. Lingle. 2002. Multiple regulatory sites in large-conductance calcium-activated potassium channels. *Nature.* 418:880–884.
- Xia, X.M., X. Zhang, and C.J. Lingle. 2004. Ligand-dependent activation of Slo family channels is defined by interchangeable cytosolic domains. *J. Neurosci.* 24:5585–5591.
- Zeng, X.H., J.P. Ding, X.M. Xia, and C.J. Lingle. 2001. Gating properties conferred on BK channels by the β 3b auxiliary subunit in the absence of its NH₂ and COOH termini. *J. Gen. Physiol.* 117:607–628.
- Zeng, X.H., X.M. Xia, and C.J. Lingle. 2003. Redox-sensitive extracellular gates formed by auxiliary β subunits of calcium-activated potassium channels. *Nat. Struct. Biol.* 10:448–454.