Large conductance, Ca$^{2+}$- and voltage-activated K$^+$ (BK) channels are exquisitely regulated to suit their diverse roles in a large variety of physiological processes. BK channels are composed of pore-forming $\alpha$ subunits and a family of tissue-specific accessory $\beta$ subunits. The smooth muscle-specific $\beta$1 subunit has an essential role in regulating smooth muscle contraction and modulates BK channel steady-state open probability and gating kinetics. Effects of $\beta1$ on channel’s gating energetics are not completely understood. One of the difficulties is that it has not yet been possible to measure the effects of $\beta1$ on channel’s intrinsic closed-to-open transition (in the absence of voltage sensor activation and Ca$^{2+}$ binding) due to the very low open probability in the presence of $\beta1$. In this study, we used a mutation of the $\alpha$ subunit (F315Y) that increases channel openings by greater than four orders of magnitude to directly compare channels’ intrinsic open probabilities in the presence and absence of the $\beta1$ subunit. Effects of $\beta1$ on steady-state open probabilities of both wild-type $\alpha$ and the F315Y mutation were analyzed using the dual allosteric HA model. We found that mouse $\beta1$ has two major effects on channel’s gating energetics. $\beta1$ reduces the intrinsic closed-to-open equilibrium that underlies the inhibition of BK channel opening seen in submicromolar Ca$^{2+}$. Further, $P_O$ measurements at limiting slope allow us to infer that $\beta1$ shifts open channel voltage sensor activation to negative membrane potentials, which contributes to enhanced channel opening seen at micromolar Ca$^{2+}$ concentrations. Using the F315Y $\alpha$ subunit with deletion mutants of $\beta1$, we also demonstrate that the small N- and C-terminal intracellular domains of $\beta1$ play important roles in altering channel’s intrinsic opening and voltage sensor activation. In summary, these results demonstrate that $\beta1$ has distinct effects on BK channel intrinsic gating and voltage sensor activation that can be functionally uncoupled by mutations in the intracellular domains.

**INTRODUCTION**

Large conductance Ca$^{2+}$-activated K$^+$ channels (BK-type potassium channel) are activated by intracellular Ca$^{2+}$ and depolarizing voltages. When open, BK channels have a very large outward potassium conductance ($\sim 250$ pS) and are therefore very effective in hyperpolarizing the membrane. The coincident activation of BK channels by Ca$^{2+}$ and voltage makes these channels uniquely tailored to regulates voltage-dependent Ca$^{2+}$ channels in a number of cell types (Kaczorowski et al., 1996; Gribkoff et al., 1997; Calderone, 2002). BK channels in smooth muscle use the accessory $\beta1$ subunit to promote channel opening (Knaus et al., 1994; Tanaka et al., 1997). Previously, the important role of the $\beta$ subunit has been demonstrated by targeted gene knockout of the $\beta1$ locus in mice. Knockout mice have BK channels with reduced openings, increased vascular tone, and hypertension (Brenner et al., 2000b; Plüger et al., 2000).

BK channel open probability is dependent on its intrinsic closed to open equilibrium that is described by the equilibrium constant $L$ (Horrigan and Aldrich, 2002). This is the inherent $P_O$ of the channel without influence of other gating mechanisms. BK channel gating is also allosterically coupled to voltage sensor activation and Ca$^{2+}$ binding (Horrigan and Aldrich, 2002). A prominent effect of $\beta1$ subunits is an increase in BK channel openings. However, it is not well established how, and to what degree $\beta1$ subunit effects on L, voltage sensor activation, or Ca$^{2+}$ binding contribute to enhanced $P_O$.

Historically, because $\beta1$ causes a negative voltage shift of the conductance–voltage (G-V) relationship, in a manner similar to increased Ca$^{2+}$, the effects of the $\beta1$ subunit was first described as an “increase in apparent Ca$^{2+}$ sensitivity” (McManus et al., 1995; Dworetzky et al., 1996; Meera et al., 1996). Later, it was found that this effect may not be due exclusively to changes in Ca$^{2+}$ binding equilibrium (Cox and Aldrich, 2000; Nimigean and Magleby, 2000; Bao and Cox, 2005; Orio and Latorre, 2005). Using gating current measurements, Bao and Cox clearly demonstrated that the bovine $\beta1$ subunit shifts voltage sensor activation to more negative membrane potentials, and this may account for $\beta1$ enhanced openings (Bao and Cox, 2005). Orio and Latorre (2005) also suggested that human $\beta1$ shifts open channel voltage sensor activation to more negative membrane potentials.

**ARTICLE**

An S6 Mutation in BK Channels Reveals $\beta1$ Subunit Effects on Intrinsic and Voltage-dependent Gating

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Effects of β1 on channel’s intrinsic gating are less clear. Whereas Oriol and Latorre proposed that human β1 reduces channel’s intrinsic equilibrium (L) for opening, Bao and Cox suggested otherwise for the bovine β1.

Based on HA model for BK channel gating (Horrigan and Aldrich, 2002), it is advantageous to directly compare α and α+β1 under conditions that isolate the influence of intrinsic gating. This is accomplished by measuring ionic current at 0 Ca2+ (to exclude effects on Ca2+ binding) and very negative membrane potentials (the limiting slope, to exclude effects on voltage sensor activation). Measurement at higher voltages can then indicate the contribution of voltage sensor activation. This approach has proven useful for evaluating BK channel α subunits alone (Horrigan and Aldrich, 2002; Ma et al., 2006). However, under such conditions, β1 channel openings fall below detection levels and this approach has not been feasible (Bao and Cox, 2005; Oriol and Latorre, 2005).

Here, we have used a previously described α subunit mutation (E380Y in human cDNA) (Lippiat et al., 2000) that increases channel openings to investigate β1 subunit effects on channel gating. This allows, for the first time, measurement of α+β1 P0 in the absence of Ca2+ and voltage sensor activation. Analysis of P0-V relationships using the dual allosteric HA gating model revealed that the α1 subunit confers two opposing effects on channel openings: both a negative voltage shift for voltage sensor activation (Vh0) that contributes to increased channel openings seen in micromolar Ca2+, and a reduced closed to open equilibrium (L0) that contributes to reduced channel openings seen in submicromolar Ca2+. Further, deletion analysis demonstrates that interactions at the small intracellular domains mediate intrinsic and voltage-dependent gating effects of β1.

**MATERIALS AND METHODS**

**Patch Clamp Recording β1 Subunit Mutants**

To study channel functional properties, mouse β1 cDNAs (Brenner et al., 2000a) and mouse α cDNAs (GenBank/EMBL/DDBJ accession no. MMU09383) were co-transfected into HEK293 cells. The F380Y mutation, originally described in the human cDNA (Lippiat et al., 2000), was introduced in the mouse α subunit cDNA (site is F315Y in mouse) using the Stratagene Quick-Change Mutagenesis kit.

Mouse β1 mutants were generated by PCR amplification of the β1 cDNAs with amplification primers that delete the N-terminal residues KKIKMVAKRGE (residues 3–13) and C-terminal residues NRSLIILAAQK (residues 181–191) for β1ΔN11 and β1ΔC11, respectively. The double mutant, β1ΔN11ΔC11 differs in that the E13 residue was not deleted. Using a C-terminal epitope-tagged β1ΔN11ΔC11, immunostaining showed expression. However, electrophysiology recordings showed no evidence of functional interactions with BK α subunits using stimulus protocols and a broad range of calcium as in Fig. 1.

Mutant and wild-type mouse β1 subunits were cloned in the mammalian expression vector pIRE2-EGFP (CLONTech Laboratories, Inc.), which fluorescently labels cells with channel expression. The mouse α subunit was cotransfected at a ratio of 1:10 α to β1 to ensure saturation of BK channels with β1 subunits.

Macropatch recordings were made using the excised inside-out patch clamp configuration. To limit series resistance errors, currents 5 nA or less were used for steady-state G-V and analysis of channel kinetics. Experiments were performed at 22°C. Data were sampled at 10–30-μs intervals and low-pass filtered at 8.4 kHz using the HEKA EPCL four-pole bessel filter. Data were analyzed without further filtering. Leak currents were subtracted after the test pulse using P/5 negative pulses from a holding potential of −120 mV. For BK/α+β1, leak subtraction was not performed at 18.5 and 100 μM Ca2+. Patch pipettes (borosilicate glass WVR micropipettes) were coated with Sticky Wax (Kerr Corp.) and fire polished to ~1.5–3 MΩ resistance.

The external recording solution (electrode solution) was composed of 20 mM HEPES, 140 mM KMeSO4, 2 mM KCl, 2 mM MgCl2, pH 7.2. Internal solutions were composed of a pH 7.2 solution of 20 mM HEPES, 140 mM KMeSO4, 2 mM KCl, and buffered with 5 mM HEDTA and CaCl2 to the appropriate concentrations to give 1.7, 7, and 18.5 μM buffered Ca2+ solutions. Higher √Ca2+ solutions were buffered with 5 mM NTA. Low Ca2+ solutions (0.3 μM and 0 Ca2+) were buffered with 5 mM EGTA, and Ba2+ was chelated with 40 μM (+)-18-crown-6-tetracarboxylic acid (Cox et al., 1997b). Free [Ca2+] of buffered solutions were measured using an Orion calcium-sensitive electrode (Orion Research, Inc.).

**Analysis of Macroscopic Currents**

Conductance–voltage (G-V) relationships were obtained using a test pulse to positive potentials followed by a step to a negative voltage (−80 at low Ca2+, −120 at high Ca2+), and then measuring instantaneous tail current 200 μs after the test pulse. In experiments where Gmax were not reached, including BK/α+β1 and BK/α+β1ΔN11 at 0.005 and 0.3 μM [Ca2+]+, BK/α+β1ΔC11 and BK/α+β1ΔN11ΔC11 at 0.005 μM [Ca2+]2, Gmax values at higher [Ca2+] from the same patch were used. G/Gmax-V data were fitted with the Boltzmann function: $G = G_{\text{max}}[1/(1 + e^{-(V - V_{1/2})/\Delta ZF/RT})]$, where V is the test potential, $V_{1/2}$ is the membrane potential at half-maximal conductance, z is the effective gating charge, and F, R, and T are constants.

**Single Channel Analysis**

Single channel opening events were obtained from patches containing one to hundreds of channels. Recordings are of 20 s to hundreds of seconds duration. Analysis was performed using TAC and TACFIT programs (Bruxton Corporations). NP0 was determined using either all-point amplitude histogram or by event detection using a 50% amplitude criteria. The probability (P0) of occupying each open level (k) give rise to $NP_0 = \sum_k \mu_k P_k$.

P0 was then determined by normalizing NP0 values by channel number (N). N was obtained from the instantaneous tail current amplitude during maximal opening at saturating [Ca2+]2, divided by the unitary conductance for each channel at the tail voltage. Combined single channel and macroscopic steady-state data in 0 Ca2+ in the presence of F315Y mutation were fit with the dual allosteric model assuming voltage-dependent transitions only (Horrigan and Aldrich, 2002). Details for fitting parameters are included in figure legends.

**RESULTS**

Effects of mβ1 on BK Channel Steady-State G-V Relationships

Fig. 1 demonstrates effects of mβ1 on BK channel steady-state gating between 0 and 100 μM Ca2+. BK channels
composed of α subunit alone (BK/α) or α with saturating mβ1 expression (BK/α+β1) were transiently expressed in HEK293 cells, and macroscopic BK currents were recorded in the inside-out patch clamp configuration. BK currents were evoked by step depolarization at controlled intracellular Ca2+ (Fig. 1, A and B, left panels) to obtain normalized steady-state tail conductance versus voltage (G-V) relationships (Fig. 1, A and B, right panels). Averaged V1/2-Ca2+ and Q-Ca2+ relationships obtained from Boltzmann fits of the G-V relationship (Fig. 1, C and D) show that mβ1 subunit alters V1/2 and Q in a Ca2+-dependent fashion. In the presence of mβ1, there is a steeper V1/2-Ca2+ relationship (Fig. 1 C) that indicates an increase in apparent Ca2+ sensitivity. Below 1.7 μM Ca2+, mβ1 subunit shifts the G-V relationships to positive potentials. This is most dramatic at nominal 0 Ca2+, where G/Gmax for BK/α+β1 channels only reaches ~0.23 at 300 mV. Extrapolation of the V1/2 from the Boltzmann fit predicts that mβ1 confers an ~150-mV positive shift in V1/2. Above 1.7 μM Ca2+, however, mβ1 causes a negative shift in the V1/2 (~50 mV shift at 100 μM Ca2+). In addition, the mβ1 subunit reduces the apparent equivalent gating charge (Q) at low Ca2+ (Fig. 1 D).

**Understanding Effects of mβ1 on Channel Gating Energetics in the Context of the HA Gating Model**

What are the mechanisms underlying mβ1 modulation of BK channel gating? The current view of BK channel gating is described by a dual allosteric (HA) model (Rothberg and Magleby, 1999; Horrigan and Aldrich, 2002). In this model, channel opening is governed by three equilibrium constants, L (closed-to-open transition), J (voltage sensor activation), K (Ca2+ binding), and D, C, and E, the allosteric couplings between L and J, L and K, and J and K, respectively. Open probability is described by Eq. 1, referring to the HA model (Horrigan and Aldrich, 2002):

$$P_O = \frac{1}{1 + \frac{(1 + J + K + JE)^4}{L(1 + KC + JD + JKCD)^4}}.$$  

(1)

In the absence of Ca2+, the occupied states are reduced to 10 (Fig. 2 A, left),

$$P_O = \frac{1}{1 + \frac{(1 + J)^4}{L(1 + JD)^4}}.$$  

(2)

In the absence of Ca2+ and at extremely negative membrane potentials (limiting slope), virtually all voltage sensors reside in the resting state and the occupied states are further reduced to 2 (C0 and O0) (Fig. 2 A, dashed box). Because J is small (J << 1/D), Eq. 2 reduces to

$$P_O = \frac{L}{1 + L}.$$  

(3)

When P0 is small (P0 << 0.01), L << 1,

$$P_O = L = I_o \exp\left(\frac{zFV}{RT}\right).$$  

(4)
In this equation, $z_L$ is the voltage dependence of the closed-to-open transition and $L_0$ is channel’s closed-to-open equilibrium in the absence of $Ca^{2+}$ and voltage sensor activation at 0 mV (Fig. 2 B). Therefore a direct approach to evaluate effects of $\beta_1$ on channel’s intrinsic closed to open equilibrium is to compare logP0-V at 0 $Ca^{2+}$ and limiting slope. As predicted by Eq. 4, the position of the limiting slope of logP0 along the Y axis is determined entirely by $L_0$ and $z_L$ (Fig. 2 C).

Another advantage of P0 measurement near the limiting slope is that it allows one to infer effects on open-channel voltage sensor activation ($Vh_O$, see Table I for definitions). As shown in Fig. 2 D, the HA model predicts that membrane potentials where P0 transitions from weakly voltage dependent to “steep” voltage dependence is critically dependent on $Vh_O$ and relatively unaffected by other voltage-dependent parameters, including closed channel voltage sensor activation ($Vh_C$) or the charge associated with voltage sensor activation ($z_J$).

### m$\beta_1$ Increases Channel’s Intrinsic Energetic Barrier for Opening and Shifts Voltage Sensor Activation to Negative Membrane Potentials

To determine effects of m$\beta_1$ on intrinsic and voltage dependent gating, 0 $Ca^{2+}$ P0 was measured over a wide range of voltages in the presence and absence of m$\beta_1$. Examples of single channel recordings are displayed in Fig. 3 A. Previously, others have measured bovine $\beta_1$ effects on dwell time and P0 at positive voltages (0 $Ca^{2+}$ and +30 mV) and found that bovine $\beta_1$ increased both burst duration (~20-fold) and gap duration (2-3-fold) for a net sevenfold increase in P0 (Nimigean and Magleby, 2000). At a similar voltage (+40 mV), we also found that m$\beta_1$ increases both mean burst duration (sixfold) and mean gap duration (fourfold) (Fig. 3, B and C). This resulted in a P0 that is similar for $\alpha$ vs. $\alpha$ channels ($6.3 \pm 5 \pm 2 \times 10^{-5} \alpha/m\beta_1$ vs. $6.3 \pm 5 \pm 0.8 \times 10^{-5} \alpha$; Fig. 3, D and E). However, at negative voltages, although the fold change in mean burst duration is somewhat reduced (3.6-fold at ~60 mV), m$\beta_1$ causes much longer gaps between open events (3 $e^4 \pm 0.9 \times 10^4$ s BK/\alpha+$\beta_1$ vs. 4.4 $e^4 \pm 9 \times 10^3$ s BK/\alpha, ~60 mV; see Fig. 3 C). The larger increase in gap duration (~70-fold) at negative voltages likely underlies a 15-fold reduction in P0 of BK/\alpha+$\beta_1$ channels over BK/\alpha channels (7.4 $e^3 \pm 5 \times 10^{-8}$ vs. 1.1 $e^3 \pm 2.7 \times 10^{-5}$, respectively).

Comparison of logP$\alpha$V curves for BK/\alpha (Fig. 3 D) and BK/\alpha+$\beta_1$ (Fig. 3 E) indicates that there are two differences between these channels’ steady-state properties. First, whereas the logP$\alpha$V curve of BK/\alpha displays a clear transition in voltage dependence (approached limiting slope at approximately +40 mV), logP$\alpha$V curve of BK/\alpha+$\beta_1$ does not. Based on the HA model, this result suggests that m$\beta_1$ shifts $Vh_O$ to more hyperpolarized

### Table I: Definitions of Gating Parameters (Horrigan and Aldrich, 2002)

| Parameter | Definition |
|-----------|------------|
| $L_0$ | C-O equilibrium constant (unliganded channel, resting voltage sensors). $L_0 = L_0 \exp(\frac{z_L(Vh_C)}{kT})$ |
| $z_L$ | $z_L$ and $z_J$ are the zero voltage value of $L$ and its partial charge, respectively. $z_L = z_L \exp(\frac{z_L(Vh_O)}{kT})$ |
| $J_0$ | RA equilibrium constant (closed, unliganded channel). $J_0 = J_0 \exp(\frac{z_J(Vh_O)}{kT})$ |
| $J$ | $J_0$ and $z_J$ are the zero voltage value of $J$ and its partial charge, respectively. $J = J_0 \exp(\frac{z_J(Vh_O)}{kT})$ |
| D | Allosteric factor describing interaction between channel opening and voltage sensor activation. $D = \exp[-z_J(Vh_O - Vh_C)]/kT$ |

$m\beta_1$ increases the intrinsic energetic barrier for opening and shifts voltage sensor activation to negative membrane potentials.
membrane potentials (Fig. 2 D). In addition, log$P_O$ at negative voltages for BK/α is substantially greater than BK/α+β1, indicating a decreased closed to open equilibrium ($L_0$) in the presence of mβ1. To quantify mβ1-mediated changes in $L_0$, $V_{hO}$, and $V_{hC}$, data in Fig. 3 (D and E) were fitted using Eq. 2, where $z_L$ and $z_J$ were held at 0.30 e0 and 0.58 e0, respectively, based on previous estimates (Horrigan and Aldrich, 2002; Bao and Cox, 2005; Wang et al., 2006). For BK/α, estimated $L_0$, $V_{hO}$, and $V_{hC}$ were 1 e$^{-6}$, +46 mV, and +202 mV, respectively (Table II). These values are reasonably close to previous estimates (Horrigan and Aldrich, 2002; Bao and Cox, 2005; Wang et al., 2006). For BK/α+β1, because $P_O$ drops so dramatically ($P_O < 10^{-8}$), it was not technically feasible to obtain $P_O$ at the limiting slope. Therefore, existing data only provides estimates for the upper and lower limits for $L_0$ (between 1 e$^{-10}$ and 1 e$^{-8}$) and $V_{hO}$ (between −70 and −20 mV), whereas $V_{hC}$ is estimated to be ≈+130 mV. We also attempted to improve the fitting for BK/α+β1 by setting equivalent gating charge for voltage sensor activation ($z_J$) to a lower value. Previously, others had found that some β1 effects on BK channels could be explained by reducing $z_J$ to 0.37 e0, (Orio and Latorre, 2005). However, we found that holding $z_J$ to 0.37 e0 produces a poor fit of BK/α+β1 data (Fig. 3 F), which suggests that mβ1 does not lower $z_J$.

Effects of mβ1 on $V_{hC}$ and $V_{hO}$ that are estimated by fitting $P_O$ data using the HA model are similar to those of β1 obtained by gating current measurements (Bao and Cox, 2005; Fig. 3 E and Table II). In both cases, β1 shifts $V_{hC}$ to hyperpolarized membrane potentials by ≈70 mV. Gating current measurements found that β1...
shifts $V_{hO}$ by $\sim 60$ mV (Bao and Cox, 2005) and our fits estimate that $m_{\beta 1}$ causes a $V_{hO}$ shift between $-20$ and $-70$ mV. Effects of $m_{\beta 1}$ on $L_0$, however, differ from that proposed for $b_{\beta 1}$. Whereas a $>100$-fold decrease in $L_0$ was estimated for $m_{\beta 1}$, it was proposed that $b_{\beta 1}$ slightly increases $L_0$. Because $P_O$ measurement also did not reach the limiting slope in the study performed by Bao and Cox (2005), it is not clear whether $b_{\beta 1}$ indeed increases $L_0$. Effects of $h_{\beta 1}$ on channel gating was also investigated using ionic currents in the context of the HA model (Orio and Latorre, 2005). The authors proposed that $h_{\beta 1}$ significantly decreases $L_0$, $V_{hO}$, and $z_J$ with little effects on $V_{hC}$.

F315Y Mutation Dramatically Increases Channel's Closed-to-Open Transition

The above analysis indicates that $m_{\beta 1}$ subunits have effects on BK channels that should be apparent at limiting slope. However, the greatly reduced $P_O$ combined with the negative voltage shift of $V_{hO}$ make $P_O$ measurements at limiting slope not feasible. Previously it had been shown that F380Y, a point mutation in the S6 transmembrane domain of hslo, significantly increases $P_O$ even at 0 Ca$^{2+}$ (Lippiat et al., 2000). The F380 residue lies in a position within the C-terminal domain of S6 that may serve as the gate for Kv channels (Swartz, 2005). An mslo equivalent of the F380Y mutation was generated (F315Y in mouse) and characterized at 0 Ca$^{2+}$ using macroscopic and single channel recordings (Fig. 4A). Similar to previous findings, F315Y shows extremely long open dwell times, (Fig. 4A, left panel vs. Fig. 4B). For example, open burst durations are $11 \pm 2$ ms for F315Y vs. $0.36 \pm 0.02$ ms for WT $\alpha$ at $-60$ mV.

![Figure 4](image)

**Figure 4.** F315Y mutation greatly increases $P_O$ at 0 Ca$^{2+}$ by increasing $L_0$. (A) Representative macroscopic (left) and single channel (right) recordings of BK/F315Y at 0 Ca$^{2+}$. (B) Representative single channel currents of BK/$\alpha$ at 0 Ca$^{2+}$ show opening to be much briefer than the F315Y mutant. (C) G-V relations (mean $\pm$ SEM) for BK/$\alpha$ ($n = 12$) and BK/F315Y ($n = 13$). F315Y mutation left shifts G-V and decreases the apparent voltage dependence. (D) Log$P_O$-V relations (mean $\pm$ SEM) for BK/$\alpha$ ($n = 3$–12) and BK/F315Y ($n = 4$–7). (E) Representative log$P_O$-V relations of BK/F315Y where the limiting slope was fitted to Eq. 4 to estimate $z_L$ and $L_0$ values (mean $\pm$ SEM) are indicated in the figure ($n = 6$). (F) Best fits to the HA model (held $z_J = 0.26 e_0$, $z_L = 0.58 e_0$, $V_{hC} = +92$ mV, and $V_{hO} = +35$ mV). (G) Best fits to the HA model assuming F315Y does not alter $V_{hC}$ and $V_{hO}$ (held $z_J = 0.26 e_0$, $V_{hC} = +202$ mV, and $V_{hO} = +46$ mV, yielded $L_0 = 4 e^{-2}$, $z_J = 0.36 e_0$).
leftward shift in the G-V relationship and a decrease in the apparent voltage dependence (Fig. 4 C) (Lippiat et al., 2000). Fitting individual logPO data at limiting slope using Eq. 4 estimated a slight reduction in zL (0.30 wild type α, 0.26 ± 0.04 e0 for F315Y, n = 6). Fitting both logPO and PO data using Eq. 2, gating parameters L_{0}, z_{J}, V_{hC}, and V_{hO} are estimated to be 9 e^{-2}, 0.58 e_{0}, +92 mV, and +35 mV, respectively (Fig. 4 F and Table II). The large decrease in V_{hC} and little change in V_{hO} decreases D from 35.2 to 3.7, which explains the shallower G-V slope (apparent voltage dependence) for F315Y. To rule out the possibility that the reduced G-V slope can be explained by a reduction in z_{J} alone, we also fit the F315Y data by holding V_{hC} and V_{hO} at wild-type values, and z_{J} at 0.26 e_{0} (estimates from limiting slope measurements) (Fig. 4 G). This yielded a poorer fit. In summary, these results indicate that the F315Y has two effects. These are a negative voltage shift of V_{hC} and a greater than 10^4 increase in L_{0} relative to wild-type α subunits.

We next used the large increase in L_{0} in F315Y to investigate mechanisms underlying BK channel modulation by the β subunits at limiting slope.

Investigating Effects of β1 on BK Channel Intrinsic and Voltage-dependent Gating Using F315Y

Steady-state gating properties of BK/F315Y+β1 channels were characterized at 0 Ca^{2+}, combining single channel recordings (Fig. 5, A and D, right panels) and macroscopic recordings (Fig. 5 B). Fig. 5 A shows currents from an excised patch containing a single BK/F315Y+β1 channel. Unlike BK/α+β1, BK/F315Y+β1 maximal PO (11601) (+20 to +40 mV) and maximal conductance at 0 Ca^{2+} can be easily observed (Fig. 5, B and C). Averaged G-V relationships (Fig. 5 C) suggest that mβ1 shifts the V_{1/2} to more depolarized membrane potentials, with a slight increase in the slope of the G-V relation. LogPO\_V curves of individual patches were fitted using Eq. 4. Similar to wild-type BK channels, mβ1 does not significantly alter z_{J} of F315Y (Fig. 5 E and Table II; BK/F315Y 0.26 ± 0.04 e_{0}, BK/F315Y+β1 0.27 ± 0.04 e_{0}, P = 0.64). We estimated V_{hC}, V_{hO}, and L_{0} by fitting both PO\_V and logPO\_V data using Eq. 2. V_{hC} and V_{hO} were estimated to be 72 and −26 mV, respectively (Fig. 5 F and Table II). This is a −20-mV shift of V_{hC} and −61-mV shift of V_{hO} over BK/F315Y channels.
sensor activation estimated by our fits are qualitatively similar to bβ1 measured directly using gating current measurements (Bao and Cox, 2005). However, although shifts of VhO are similar, the −20-mV shift of VhC in the F315Y background is smaller than the −71-mV shift measured by Bao and Cox (2005). Consistent with mβ1 effects on WT α subunit (Fig. 3 E), mβ1 also caused a dramatic (50-fold) reduction of intrinsic gating in the BK/F315Y subunit (Fig. 5 F). L0 for BK/F315Y is 9 e−² versus 1.8 e−³ for BK/F315Y+bβ1. In summary, the F315Y mutation allowed us to measure effects on intrinsic gating by mβ1 despite the dramatic reduction in P0. Further, extending P0 measurement to the limiting slope provides an assay to measure effects of voltage sensor activation on PO and thereby constrain estimates of VhO using the HA model.

For wild-type α subunits, mβ1 causes a positive G-V shift in low Ca²⁺ and a negative G-V shift in high Ca²⁺, with a crossover of the V_{1/2} around 1.7 μM Ca²⁺ (Fig. 1 C).

This creates a steeper V_{1/2}-Ca²⁺ relationship. How does β1 modulation of L0 and VhO contribute to these properties? We simulated wild-type α subunit PO (HA model, Eq. 1) across a range of Ca²⁺ by varying L0, VhO, or both VhO and VhC, either individually or in combination, to understand their effect on the V_{1/2}-Ca²⁺ and Q-Ca²⁺ relations (Fig. 6). As shown in Fig. 6 A, reducing L0 by mβ1 causes a positive shift of the G-V to a lesser extent at high Ca²⁺ than at low Ca²⁺, causing the V_{1/2}-Ca²⁺ relationship to be more steep. In addition, reducing L0 also reduces Q at low Ca²⁺. This is because the decrease of L0 causes significant channel openings to occur at much more positive potentials than VhC where voltage-dependent gating rely on the weak voltage dependence of the closed-to-open transition, zL (Wang et al., 2006). Thus, the reduced intrinsic gating creates a double hit to inhibit channel openings: a greater energetic barrier due to L0 and a much weaker voltage dependence (Q) as significant channel openings occur more much positive
than Vh_C (Fig. 6 A, right). Therefore the V_{1/2} is shifted to far positive values. With the contribution of higher Ca^{2+} (>1.6 μM), channel openings fall within the range of voltage sensor activation (between Vh_O and Vh_C) and the effect of decreased L_0 on V_{1/2} is greatly reduced and fairly uniform across 1.7–100 μM Ca^{2+}. We can see that the HA model predicts that shifting Vh_C to more positive potentials (Fig. 6 B, e.g., +400 mV) places channel openings within the effective range of voltage sensor activation despite the decrease of L_0. In that case, effect of L_0 on V_{1/2} is uniform across both low and high Ca^{2+} concentrations. Thus, the HA model predicts that mβ1 effects on L_0 contribute to a much larger positive shift of the V_{1/2} and reduced voltage dependence (Q) at low Ca^{2+} than high calcium, which would steepen the V_{1/2}-[Ca^{2+}] relations.

Countering effects on L_0, negative shift of Vh_O alone or both Vh_O and Vh_C decreases V_{1/2} to a similar extent across [Ca^{2+}] (Fig. 6 C). Depending on quantitative changes in L_0 combined with Vh_O, the V_{1/2}-Ca^{2+} curve may or may not crossover (Fig. 6 D, left and right). In summary, these analyses suggest that mβ1 effects on Vh_O contribute to the negative G-V shift, and L_0 contributes to a steeper V_{1/2} vs. Ca^{2+} relationship. However, our analysis does not rule out the possibility that β1 may also have effects on Ca^{2+} binding or coupling between Ca^{2+} binding and gating that contribute to changes in Ca^{2+} sensitivity.

### Intracellular Domains of β1 Are Required for β1-mediated Modulation of Voltage-dependent Gating

The β1 subunit is composed of a large extracellular domain and small N- (15 amino acids) and C-terminal (12 amino acids) domains. Given that the intracellular domains of the α subunit are required for β1 subunit-mediated G-V shift (Qian et al., 2002), the β1 intracellular domains were deleted to evaluate their role in modulating gating. 11 amino acids that follow the N-terminal
initiating methionine and glycine were deleted in \( \beta^1\Delta N_{11} \) (Fig. 7 A). In addition, the C-terminal 11 residues were deleted in \( \beta^1\Delta C_{11} \) (Fig. 7 E). Effects of \( \beta^1\Delta N_{11} \) and \( \beta^1\Delta C_{11} \) on steady-state gating of wild-type \( \alpha \) subunit were examined over a wide range of \( \text{Ca}^{2+} \) (Fig. 7, B, C, F, and G). These data are summarized in \( V_{1/2} \text{Ca}^{2+} \) and \( Q_{\text{Ca}^{2+}} \) plots (Fig. 7, D and H). Surprisingly, deletion of either intracellular domain has similar effects on the G-V relationship. Both mutants eliminate the negative voltage shift of the G-V relationship in high \( \text{Ca}^{2+} \), but maintain the positive G-V shift to varying extents in low \( \text{Ca}^{2+} \) (Fig. 7, D and H).

\( \beta^1\Delta N_{11} \) and \( \beta^1\Delta C_{11} \) were coexpressed with BK/F315Y to examine whether the mutations affect \( \beta^1 \)'s ability to reduce \( L_0 \) and \( V_{hO} \). Macroscopic and single channel recordings (Fig. 8) were used to obtain the \( P_{o-V} \) relationship. Fitting the log\( P_{o-V} \) relationship (Fig. 8, B and E) at limiting slope using Eq. 4 estimated that \( z_L \) for both \( \beta^1\Delta N_{11} \) (0.24 \( \pm \) 0.05 e\( \theta \)) and \( \beta^1\Delta C_{11} \) (0.24 \( \pm \) 0.05 e\( \theta \)) is not significantly different from wild-type \( \beta^1 \) (0.27 \( \pm \) 0.04 e\( \theta \), \( P = 0.46 \) and \( P = 0.52 \) for \( \beta^1\Delta N_{11} \) and \( \beta^1\Delta C_{11} \) vs. WT m\( \beta^1 \), respectively). Fitting both \( P_{o-V} \) and log\( P_{o-V} \) using Eq. 2 (Fig. 8, C and F; Table II), it was found that the major effect of the m\( \beta^1 \) mutations is a reduced leftward shift of \( V_{hO} \). This is from \(-61\) mV shift for wild-type m\( \beta^1 \) to a \(-20\) mV shift for \( \beta^1\Delta N_{11} \), and complete elimination in \( \beta^1\Delta C_{11} \). \( \beta^1\Delta N_{11} \) and \( \beta^1\Delta C_{11} \) reduced \( L_0 \) compared with \( \alpha \) alone (Fig. 8, B and E; 5.5 \( e^{-3} \) and 8 \( e^{-3} \), respectively, relative to \( 9 e^{-2} \) for \( \alpha \), but to a somewhat lesser extent compared with wild-type
β1 (1.8 e⁻⁻⁻). In summary, these results suggest that the intracellular domains are required for β1 subunit effects on voltage sensor activation and explains why β1ΔN11 and β1ΔC11 do not negatively shift the G-V relationship (Fig. 7, D and H). In contrast, mutation of the intracellular domains has a much weaker effect on L₀.

A caveat to interpreting these results is the possibility that the single deletions are dominant-negative mutants rather than loss of function. It is possible that the intracellular domains of β1 normally do not have a role in stabilizing voltage sensor activation. Deletion of either intracellular domain may expose residues of the other domain for novel interaction with the α subunit that perturbs β1 effects on intrinsic opening and voltage sensor activation. This scenario predicts that deleting both intracellular domains should reconstitute β1 subunit properties. We tested this possibility by generating β1 mutations lacking both N- and C-terminal domains (β1ΔNαβC11 and β1ΔNβαC11). Coexpression of β1ΔNαβC11 with wild-type α demonstrates that the double mutant, like the β1ΔN11 and β1ΔC11 mutants, eliminates the negative voltage shift of the G-V in high Ca²⁺ (Fig. 7 L). In addition, the β1ΔN10C11 mutant also perturbs the positive G-V shift in low Ca²⁺ (Fig. 7 L). These results suggest that the double deletion may also affect β1’s ability in modulating L₀ and Vh₀. To directly examine effects of the double deletions on intrinsic and voltage-dependent gating, logPₒV relationship was obtained for BK/F315Y+β1ΔN10C11 using single channel recordings (Fig. 8 G). Fitting logPₒV relationship at limiting slope showed that unlike the single deletions, the β1ΔN10C11 significantly reduces voltage dependence of the closed to open equilibrium (z₁ is 0.13 ± 0.02 e₀; Fig. 8 H). Analysis using Eq. 2 indicates that β1ΔN10C11 dramatically decreases β1’s reduction of L₀ and eliminates β1’s ability to left shift Vh₀ (Fig. 8 I; Table II). The above findings suggest that it is unlikely that β1ΔN11 and β1ΔC11 are dominant-negative mutations, and provides additional evidence that intracellular domains are required for stabilizing voltage sensor activation. Coexpression of wild-type α and β1ΔN11ΔC11 produced currents indistinguishable from BK/α alone (unpublished data). Although the protein was expressed (as assayed by immunohistochemistry; unpublished data), it appears that the conserved E11 residue is critical for coupling between α and β1 when the 10 and 11 residues of the N and the C terminus are deleted.

The β1 subunit has the additional property of reducing the apparent voltage dependence (Q) of the conductance–voltage relationship. Intracellular domain chimeras (BK α chimeras with related slo₃ channels) that eliminate the negative shift of the G-V relationship do not affect the apparent voltage dependence (Qian et al., 2002). Similarly, we find that deletion of either intracellular domains and the double deletion, to an extent, still decrease Q (Fig. 7, D, H, and L). In combination with the double deletion effect on the V₁/₂ at low Ca²⁺ (Fig. 7 L), these results indicate that some effects by mβ1 are retained by interactions in the transmembrane and/or extracellular domains.

**DISCUSSION**

**Properties of mβ1**

Similar to previous analysis of β1 subunits, our results demonstrate that mβ1 reduces the channel’s apparent voltage dependence (Q) and increases its apparent Ca²⁺ sensitivity. The increase in apparent Ca²⁺ sensitivity is manifested in two ways: a negative shift of the G-V relationship at micromolar Ca²⁺, and a steeper V₁/₂–Ca²⁺ curve. These effects have been previously observed for human β1 (hβ1) (Meera et al., 1996; Nimigean and Magleby, 1999; Lippiat et al., 2003; Orio and Latorre, 2005) and bovine β1 (bβ1) (Cox and Aldrich, 2000; Bao and Cox, 2005). Several of these studies also observed that below ∼1 μM Ca²⁺, β1 either becomes less “effective” in shifting G-V relations (Meera et al., 1996; Cox and Aldrich, 2000; Nimigean and Magleby, 2000; Bao and Cox, 2005) or produces a positive shift in the G-V relationship (Orio and Latorre, 2005). Our studies with mβ1 concur with the later, and indeed show a very large positive shift at submicromolar Ca²⁺.

How do β1 subunits confer an increase in apparent Ca²⁺ sensitivity, and an increased slope for the V₁/₂–Ca²⁺ curve? By combining the F315Y limiting slope analysis with mutagenesis of the intracellular domain, we were able to uncover mechanisms that contribute to these properties. Utilization of the F315Y mutation with β1 allowed us to directly measure the effect on Pₒ by the negative shift of voltage sensor activation, as predicted by previous gating current measurements (Bao and Cox, 2005). The decrease in Vh₀ and the negative shift of the G-V relationship are correlated in our mutations, indicating that effects on voltage sensor equilibrium by β1 may be causal for the negative G-V shift, as predicted by Bao and Cox (2005). However, our simulations indicate that the negative G-V shift occurs equally across Ca²⁺ concentrations. This indicates that the increased slope of the V₁/₂–Ca²⁺ curve is not accounted for by effects on voltage sensor equilibrium. Rather, we found that β1 decrease of intrinsic gating (L₀) contributes to the increased slope of the V₁/₂–Ca²⁺ curve. Unlike Vh₀, the effect of L₀ on V₁/₂ appears to be Ca²⁺ dependent where there is a greater positive shift of the G-V curve at low Ca²⁺ than high Ca²⁺. Surprisingly, it is this β1 effect that reduces Pₒ more so at low Ca²⁺ than at high calcium that gives a steeper Ca²⁺ response.

Previous studies had also inferred that human β1 decreased BK channel’s closed-to-open equilibrium (Orio and Latorre, 2005). However, this is somewhat controversial given that Bao et al. did not require a decreased
closed to open equilibrium to explain bovine β1 subunit effects (Bao and Cox, 2005). In part, this discrepancy may also be due to species differences. At 0 Ca²⁺, \( V_{1/2} \) for oocyte-expressed BK channels composed of mouse α (mso-mbr5; Butler et al., 1993) and bovine β1 (Knaus et al., 1994) is \( \sim 200 \) mV (Bao and Cox, 2005), and for β1 channels composed of human α and human β1 expressed in oocytes is \( \sim 250 \) mV (Orio and Latorre, 2005). In our study, mouse α (Pallanck and Ganetzky, 1994) and mouse β1 expressed in HEK293 cells resulted in an estimated \( V_{1/2} \) to be >300 mV. Thus, mouse (this study) and human β1 subunits (Orio and Latorre, 2005) may have a greater effect on \( L_0 \) than the bovine β1 subunit (Bao and Cox, 2005). An additional variable is the expression system. Functional interaction between BK channel α and β subunits has been shown to be phosphorylation dependent (Erxleben et al., 2002; Jin et al., 2002). It is possible that similar to KCNQ channels (Nakajo and Kubo, 2005), BK channel phosphorylation status differs between oocytes (used in the previous studies) and HEK293 cells (used in this study).

### β1 and β4 Subunits Share Similar Mechanisms

Interestingly, the major effects of β4 are similar to the mouse β1 subunit. Both cause a decrease in intrinsic opening and leftward voltage shifts for voltage sensor activation (Wang et al., 2006). The distinction is that the β1 subunit has a crossover between inhibition and activation at low micromolar Ca²⁺ concentrations and is therefore generally regarded to promote channel activation. The β4 subunit, in contrast, has a crossover at tens of micromolar Ca²⁺ concentration and is generally regarded to be a down-regulator for BK channels (Weiger et al., 2000; Brenner et al., 2005). It is indeed possible that quantitative differences in these two opposing effects, intrinsic gating or voltage sensor activation, underlie the distinction between β1 and β4 subunits.

#### β1 Functional Domains

Finally, these studies contribute to our understanding of β1 subunit domains that mediate interaction with BK channels. Previous studies using chimeras between β1 and β2 indicate that differences between these subunits can be ascribed to differences in the intracellular domains of the β subunits (Orio and Latorre, 2005). Consistent with these studies, we find that most, but not all, of the effects of β1 (effects on \( V_{hO} \) and \( L_0 \)) are mediated by the intracellular domains. Predominant effects of the extracellular and transmembrane domain appear to be its influence on the equivalent gating charge conferred by β1 subunits, and also a small effect on \( L_0 \). An intriguing possibility may be that the intracellular domains of the β1 subunit directly interact with the voltage sensor domain to modulate channel activation. Indeed, the recent finding that residues in S2 and S3, in addition to the S4 transmembrane domains, contribute to voltage sensor equilibrium (Horrigan and Aldrich, 2002; Ma et al., 2006) present the possibilities that β1 intracellular domains may be tugging on any of the respective intracellular loops for S2–S4 to mediate effects on \( V_{hO} \).

However, other studies have found that perturbing the α subunit N-terminal extracellular domain and the first transmembrane (S0) domains also has a profound effect on the negative shift of the G-V relationship conferred by β1 (Waller et al., 1996; Morrow et al., 2006). We cannot rule out the possibility that intracellular domains and transmembrane/extracellular domains of β1 are allosterically coupled so that mutations in either domain perturb β1 subunit effects. Alternatively, mutations in the extracellular domain of α and intracellular domains of β1 affect different aspects of BK channel gating that appear qualitatively similar if measured by the net effect of the G-V relationship. In this regard, future studies using the F315Y limiting slope analysis should provide a more accurate mapping of α and β1 subunit functional domains.

#### F315Y Provides a Useful Reagent for Measuring BK Channel Properties at the Limiting Slope

Historically, a number of other ion channel mutations have served to uncover mechanisms that would otherwise be difficult or not possible to resolve. One example is the ILT Shaker mutation. By separating the final open transitions from charge movement steps (Smith-Maxwell et al., 1998), the ILT mutation allowed biophysical studies to probe channel gating mechanisms (del Camino et al., 2005; Pathak et al., 2005). As well, the W434F mutation of Shaker channel blocks potassium conductance and facilitates gating current measurements (Perozo et al., 1993). Yet, as useful as these mutations are, they have their own caveats with regard to how they affect other channel gating properties. For example, W434F, in addition to blocking channel conductance, it also retains channels in a c-type inactivated state (Yang et al., 1997). This begs the question of how the F315Y mutation affects our ability to infer β1 modulation of gating.

The F315Y mutation is located in the C-terminal residues of the S6 domain, a region that is ascribed to serving as the gate for Kv channels (Swartz, 2005). Our observations were that the F315Y had two effects. Most dramatic was an increase in intrinsic gating that is apparent as a large (30-fold) increase in open channel dwell times (Fig. 4; 11 ± 2 ms F315Y vs. 0.36 ± 0.02 ms WT α at −60 mV, 5 nM Ca²⁺) and ~10,000-fold increase in limiting slope \( P_0 \) (Fig. 4 D). As well, fitting to the HA model indicates a negative shift of voltage sensor activation of closed channels (\( V_{hO} \), Table II), perhaps indicating a change in channel conformation in the closed state. Taken together, a simplistic hypothesis is that the F315Y mutation destabilizes the closed gate. Thus,
although F315Y may not be useful in reporting effects on VhC, several lines of evidence suggest that other F315Y and β1 properties are qualitatively additive, indicating that their mechanisms are independent and not masked. Compared with wild-type BK/α channels, BK/β1 and BK/F315Y both display increased mean burst duration (Fig. 5 B; Nimigean and Magleby, 1999). Despite the dramatically increased burst durations of F315Y, this property of β1 is conserved in the F315Y background (Fig. 5 D; F315Y+β1 is 334 ± 12 ms vs. 11 ± 2 ms F315Y alone at −60 mV, 5 nM Ca2+). In addition, β1 subunits confer a reduction in I0 in the F315Y background despite the large increase in intrinsic gating (L0) by the α mutation. Other properties of β1 also appear to be qualitatively retained, including the negative shift of open channel voltage sensor activation previously reported by Bao and Cox (2005). Thus, in many aspects, F315Y has effectively uncovered β1-mediated modulation of BK channels.

With regard to estimating VhC, it is not clear if the F315Y mutation reports β1 effects. Bao and Cox saw that ββ1 conferred similar shifts of both VhC (−61 mV) and VhC (−71 mV). Our estimates of mβ1 were an unequal shift of VhC (−61 mV) and VhC (−20 mV) in the F315Y background. The fact that F315Y alone has a VhC (+110 mV, Table II) that is quite different than wild-type α subunits (+202 mV) creates the possibility that the F315Y mutation perturbs β1 effects on VhC.

In conclusion, the increase in P0 by the F315Y mutation has uncovered properties that were predicted by gating current measurements, and novel properties such as effects on intrinsic gating that were previously difficult to measure. One can predict that the mutation should continue to provide a valuable tool to identify critical residues that bridge functional interactions between the BK channel α and β1 subunits.

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