Published January 12, 2009

ARTICLE

Measuring the Influence of the BK_{Ca} $\beta 1$ Subunit on Ca^{2+} Binding to the BK_{Ca} Channel

Tara-Beth Sweet and Daniel H. Cox

Molecular Cardiology Research Institute, Tufts Medical Center, and The Department of Neuroscience, Tufts University School of Medicine, Boston, MA 02111

The large-conductance Ca^{2+} -activated potassium (BK_{Ca}) channel of smooth muscle is unusually sensitive to Ca^{2+} as compared with the BK_{Ca} channels of brain and skeletal muscle. This is due to the tissue-specific expression of the BK_{Ca} auxiliary subunit $\beta 1$, whose presence dramatically increases both the potency and efficacy of Ca^{2+} in promoting channel opening. $\beta 1$ contains no Ca^{2+} binding sites of its own, and thus the mechanism by which it increases the BK_{Ca} channel's Ca^{2+} sensitivity has been of some interest. Previously, we demonstrated that $\beta 1$ stabilizes voltage sensor activation, such that activation occurs at more negative voltages with $\beta 1$ present. This decreases the work that Ca^{2+} must do to open the channel and thereby increases the channel's apparent Ca^{2+} affinity without altering the real affinities of the channel's Ca^{2+} binding sites. To explain the full effect of $\beta 1$ on the channel's Ca^{2+} sensitivity, however, we also proposed that there must be effects of $\beta 1$ on Ca^{2+} binding. Here, to test this hypothesis, we have used high-resolution Ca^{2+} dose–response curves together with binding site–specific mutations to measure the effects of $\beta 1$ on Ca^{2+} binding. We find that coexpression of $\beta 1$ alters Ca^{2+} binding at both of the BK_{Ca} channel's two types of high-affinity Ca^{2+} binding sites, primarily increasing the affinity of the RCK1 sites when the channel is open and decreasing the affinity of the Ca^{2+} bowl sites when the channel is closed. Both of these modifications increase the difference in affinity between open and closed, such that Ca^{2+} binding at either site has a larger effect on channel opening when $\beta 1$ is present.

INTRODUCTION

The Journal of General Physiology

Large-conductance Ca²⁺-activated potassium (BK_{Ca}) channels are crucial for the regulation of arterial tone, where they facilitate a negative feedback mechanism that opposes vasoconstriction (Nelson et al., 1995; Nelson and Quayle, 1995; Brenner et al., 2000). Intravascular pressure increases arterial tone by a complex process that includes membrane depolarization and the subsequent elevation of cytoplasmic Ca²⁺ via voltage-dependent Ca²⁺ channels. This global increase in Ca²⁺ leads to vasoconstriction, but it also triggers localized Ca²⁺ release events from ryanodine receptors on the smooth muscle sacroplasmic reticulum. These release events, termed Ca²⁺ sparks, activate nearby BK_{Ca} channels that then create a hyperpolarizing K⁺ current known as a STOC. STOCs then oppose further constriction (Nelson et al., 1995; Perez et al., 1999). The BK_{Ca} channel's accessory β1 subunit has been show to be critically important in this regulatory process, as mice that lack β1 have greatly reduced STOCs in response to sparks as well as hypercontractile smooth muscle and hypertension (Brenner et al., 2000; Pluger et al., 2000). In heterologous expression systems β1 subunits, four of which assemble with a single channel (Shen et al., 1994), make the BK_{Ca} channel

substantially more Ca^{2+} sensitive (McManus et al., 1995; Meera et al., 1996; Cox and Aldrich, 2000a). Thus, in the absence of $\beta 1$ it appears that BK_{Ca} channels lack the Ca^{2+} sensitivity required for BK_{Ca} -mediated feedback regulation of smooth muscle tone.

The mechanism by which β1 enhances the BK_{Ca} channel's Ca²⁺ sensitivity has been the subject of many studies (Wallner et al., 1996; Nimigean and Magleby, 1999b, 2000; Cox and Aldrich, 2000b; Qian et al., 2002; Qian and Magleby, 2003; Bao and Cox, 2005; Orio and Latorre, 2005; Morrow et al., 2006; Orio et al., 2006; Wang and Brenner, 2006; Yang et al., 2008), but it is still unclear. Nimigean and Magleby (1999a,b, 2000) found that β1 increases the length of time that the BK_{Ca} channel spends in bursting states and that this effect persists in the absence of Ca²⁺ (Nimigean and Magleby, 1999a). They suggested that a Ca²⁺-independent effect underlies most of the channel's increased Ca2+ sensitivity. In support of this notion, Bao and Cox (2005) found, when studying gating currents, that β1 stabilizes voltage sensor activation such that activation occurs at more negative voltages with \$1 present. At most voltages this decreases the work that Ca2+ must do to open the channel and thereby increases the channel's apparent Ca²⁺

Correspondence to Daniel H. Cox: dan.cox@tufts.edu

Abbreviations used in this paper: BK_{Ca} , large-conductance Ca^{2+} -activated potassium; G-V, conductance-voltage; HA, Horrigan and Aldrich; *Popen*, open probability.

^{© 2009} Sweet and Cox This article is distributed under the terms of an Attribution–Non-commercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.jgp.org/misc/terms.shtml). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).

affinity. However, to account for the full change in Ca^{2+} sensitivity brought about by $\beta 1$, Bao and Cox (2005) also proposed that $\beta 1$ alters the true affinities of the channel's high-affinity Ca^{2+} binding sites (Bao et al., 2004), a conclusion supported by the earlier study of Cox and Aldrich (2000).

A recent paper by Yang et al. (2008), however, suggests that this may not be the case. Their experiments revealed that mutation of the voltage sensor residue R167 eliminates the ability of $\beta 1$ to enhance the BK_{Ca} channel's Ca^{2+} sensitivity, implying that $\beta 1$ enhances Ca^{2+} sensitivity solely by altering the conformation or movements of the voltage sensor. Here, to clarify this issue, we have used high-resolution Ca^{2+} dose–response curves to determine directly whether or not $\beta 1$ alters the BK_{Ca} channel's affinity for Ca^{2+} at either of its two types of high-affinity Ca^{2+} binding sites. We find effects of $\beta 1$ on the real affinities of both sites.

MATERIALS AND METHODS

Heterologous Expression of BK_{Ca} Channels in TSA 201 Cells

TSA 201 cells (modified human embryonic kidney cells) were transiently transfected with expression vectors (pcDNA 3; Invitrogen) encoding the mouse α subunit (mslo-mbr5) (Butler et al., 1993), the mouse β1 subunit of the BK_{Ca} channel, enhanced green fluorescent protein (eGFP-N1; BD), and the empty pcDNA 3.1+ vector (Invitrogen) to control for the total amount of transfected DNA. Cells were transiently transfected using the Lipofectamine 2000 reagent (Invitrogen). The eGFP was used to monitor successfully transfected cells. For transfection, cells at 80-90% confluence in 35-mm falcon dishes were incubated with a mixture of the plasmids (total of 4 µg DNA) Lipofectamine and Optimem (Invitrogen) according to the manufacturer's instructions. In brief, the mixture was left on the cells 4-8 h after which the cells were replated into recording Falcon 3004 dishes in standard tissue culture media: DMEM with 1% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin solution (all from Invitrogen). The cells were patch clamped 1-3 d after transfection.

The molar ratio of $\beta 1$ to α -expressing plasmids transfected was $1~\beta 1$ plasmid to between 0.6 and $2~\alpha$ plasmids for all experiments. These ratios were determined to be well above that required to maximize the effects of $\beta 1$ on channel gating in experiments in which the relative amount of $\beta 1$ to α plasmid was titrated until no further effect of $\beta 1$ was observed. The minimal saturating ratio was determined to be $1~\beta 1$ plasmid to $6.8~\alpha$ plasmids, as determined by the magnitudes of $\beta 1$ -induced G-V shifts at $100~\mu M$ [Ca²+]. This is likely due to more efficient transcription and or translation of $\beta 1$ relative to the much larger α subunit.

Electrophysiology

All recordings were done in the inside-out patch clamp configuration (Hamill et al., 1981). Patch pipettes were made of borosilicate glass (VWR micropipettes) with 0.8–5-M Ω resistances that were varied for different recording purposes. The tips of the patch pipettes were coated with sticky wax (KerrLab) and fire polished. Data were acquired using an Axopatch 200B patch clamp amplifier and a Macintosh-based computer system equipped with an ITC-16 hardware interface and Pulse acquisition software (HEKA). For macroscopic current recordings, data were sampled at 50 kHz and filtered at 10 kHz. In most macroscopic current recordings, capacity and leak current were

subtracted using a P/5 subtraction protocol with a holding potential of -120~mV and leak pulses in opposite polarity to the test pulse, but with BK_{Ca} currents recorded with >100 μM Ca²+, no leak subtraction was performed. Unitary–current recordings acquired at 0 mV were sampled at 100 kHz and filtered at 2 kHz. All experiments were performed at room temperature, $22\text{-}24^{\circ}\text{C}$.

Solutions

The pipette solution for macroscopic current recordings contained the following (in mM): 118 KMeSO₃, 20 N-methyl-glucamine-MeSO₃, 2 KCl, 2 MgCl₂, and 2 HEPES, pH 7.20. The pipette solution for current recordings at 0 mV contained the following (in mM): 3 KMeSO₃, 135 N-methyl-glucamine-MeSO₄, 2 KCl, 2 MgCl₂, and 2 HEPES, pH 7.20. 10 µM GdCl₃ was added to both pipette solutions to block endogenous stretch-activated channels (Yang and Sachs, 1989; Qian and Magleby, 2003). The bath solution for all recordings contained the following (in mM): 118 KMeSO₃, 20 N-methyl-glucamine-MeSO₃, 2 KCl, and 2 HEPES, pH 7.20. 1 mM EGTA (Fluka) was used as the Ca2+ buffer for solutions containing 3-500 nM free [Ca²⁺], 1 mM HEDTA (Sigma-Aldrich) was used as the Ca²⁺ buffer for solutions containing 0.8–20 μM free [Ca²⁺], and no Ca²⁺ chelator was used as the Ca²⁺ buffer in solutions containing between 20 µM and 2.5 mM free Ca²⁺. 50 μM (+)-18-crown-6-tetracarboxylic acid (18C6TA) was added to all internal solutions to prevent contaminant Ba2+ block at high voltages. Both internal and external solutions were brought to pH 7.20.

The appropriate amount of total Ca²⁺ (100 mM CaCl₂ standard solution; Orion Research, Inc.) to add to the buffered solutions to yield the desired approximate free Ca²⁺ concentrations of 3 nM to 2.5 mM was calculated using the program MaxChelator (see Online supplemental material below), and the solutions were prepared as described previously (Bao et al., 2002). The Ca²⁺ concentrations reported above were determined with an Orion Ca²⁺ sensitive electrode. The solutions bathing the intracellular side of the patch were changed by means of a DAD valve controlled pressurized superfusion system (ALA Scientific Instruments).

Data Analysis

All data analysis was performed with Igor Pro graphing and curve-fitting software (WaveMetrics, Inc.), and the Levenberg-Marquardt algorithm was used to perform nonlinear least-square curve fitting. Values in the text are given \pm the standard error of the mean.

Conductance-Voltage (G-V) Curves

G-V relations were determined from the amplitude of tail currents measured 200 μ s after repolarizations to -80 mV following voltage steps to the test voltage. Each G-V relation was fitted with a Boltzmann function,

$$G = G_{\rm max} \, / \, [1 + e^{\frac{-z F (V - V_{1/2})}{RT}} \,], \label{eq:G}$$

and normalized to the maximum of the fit.

Single-channel Analysis

Under conditions where the open probability (*Popen*) is small ($<10^{-2}$), single-channel openings were observed in patches containing hundreds of channels and I_K was measured from steady-state recordings 30 s in duration. *NPopen* was determined from all-points histograms by measuring the fraction of time spent (P_K) at each open level (k) using a half-amplitude criteria and summing their contributions $NPopen = \Sigma \ kP_K$, where N is the number of channels in the patch.

Popen versus Ca2+ Curves

The effect of Ca²⁺ on *Popen* was determined from the ratio of *NPopen* at a given $[Ca^{2+}]$ to *NPopen* at 0.88 or 5.4 μ M Ca^{2+} for all $[Ca^{2+}]$ tested on a given patch. In the presence of β1, the range of total activity per patch obtained over the range of [Ca²⁺] tested (3 nM to 2.5 mM) was greater than could be covered with a single normalization point. Therefore, in patches with many channels from which we were most interested in making low Popen measurements, the NPopen data at each [Ca²⁺] was normalized by the NPopen value measured at 0.88 μM [Ca²⁺]. Such curves were then averaged. Likewise, in patches with fewer channels, from which we were most interested in making higher *Popen* measurements, the *NPopen* data at each $[Ca^{2+}]$ was normalized by the *NPopen* value measured at $5.4\,\mu\mathrm{M}$ $[Ca^{2+}]$, and these curves were then averaged. The curve normalized to $0.88\,\mu\text{M}$ was then adjusted so as to have the same value at 0.88 µM [Ca²⁺] as the curve normalized to $5.4 \,\mu\text{M} \, [\text{Ca}^{2+}]$. To yield a single curve at the $[\text{Ca}^{2+}]$ at which the two curves overlapped, the two values present were subjected to a weighted average, weighted by the number of measurements in each of the two curves being brought together. This yielded a Ca²⁺ dose–response curve with a value of 1 at 5.4 μM. This curve was then rescaled vertically so that the bottom of the curve equaled 1, or 0 on a log scale. This final curve we plotted as $(NPopen/NPopen_{min}).$

In some cases, *Popen* rather than (*NPopen/NPopen_{min}*) was reported as a function of $[Ca^{2+}]$. This was done by determining *Popen* for each channel type at a single $[Ca^{2+}]$ in separate experiments, and then adjusting the average log (*NPopen/NPopen_{min}*) versus log $[Ca^{2+}]$ curve vertically, such that *Popen* was correct at the $[Ca^{2+}]$ at which *Popen* was known. This *Popen*, used for calibration, was determined at 2.5 mM $[Ca^{2+}]$ from patches whose channel content was apparent (n = 1-4).

Online Supplemental Material

The amount of Ca²⁺ to add to internal solutions to yield the desired free Ca²⁺ concentrations was calculated using the program Max-Chelator, which was downloaded from http://www.stanford.edu/~cpatton/maxc.html and is included as executable files available at http://www.jgp.org/cgi/content/full/jgp.200810094/DC1.

RESULTS

Steady-state Effects of $\beta1$

The BK_{Ca} channel is both Ca²⁺ and voltage sensitive, and the effects of these stimuli are often displayed as a series of G-V relations determined over a series of Ca²⁺ concentrations. Such a series, determined from current families recorded from BK_{Ca} channels expressed in TSA 201 cells, is shown in Fig. 1 C. Representative data used to construct such a series are shown in Fig. 1 A. The data are from an excised inside-out macropatch expressing the mouse BK_{Ca} α subunit (mSlo1). Increasing intracellular Ca²⁺ shifts the channels' G-V curve leftward, an effect that is generally known to be due to three types of Ca²⁺ binding sites, two of high affinity and one of low affinity (Schreiber and Salkoff, 1997; Bian et al., 2001; Bao et al., 2002, 2004; Shi et al., 2002; Xia et al., 2002). The channels in these patches, however, contained the mutation E399N, which eliminates low-affinity Ca²⁺ sensing (Shi et al., 2002; Xia et al., 2002). So here only the effects of Ca²⁺ binding at the channel's high-affinity

Ca²⁺ binding sites are evident. We refer to the mSlo1 channel carrying this mutation as ΔE . Increasing Ca²⁺ from 3 nM to 2.5 mM shifts the ΔE G-V relation \sim 200 mV leftward.

When the mouse BK_{Ca} $\beta 1$ subunit is expressed with the α subunit (see currents in Fig. 1 B), the Ca²⁺-induced leftward shifting evident in Fig. 1 C becomes more pronounced (Fig. 1 D), and thus it may be said that β1 increases the Ca2+ sensitivity of the BKCa channel in that it increases its G-V shift in response to a given change in [Ca²⁺] (McManus et al., 1995). On a plot of half-maximal activation voltage $(V_{1/2})$ versus $[Ca^{2+}]$, this effect is seen as an increase in slope (Fig. 1 E). At a single membrane voltage, 0 mV for example, it appears as an increase in both the efficacy and the apparent affinity of the channel for Ca²⁺ (Fig. 1 F). These data reveal that Ca²⁺ binding to the BK_{Ca} channel's low-affinity Ca²⁺ binding sites (eliminated by the E399N mutation) is not required for β1's effects on Ca²⁺ sensing. β1 has similar effects on the ΔE channel as it does on wild-type mSlo1 (McManus et al., 1995; Cox and Aldrich, 2000a; Bao and Cox, 2005).

Estimating the Affinities of Each Binding Site with and without $\beta 1$

In a previous study we found that much of β 1's effects on Ca²⁺ sensing are due to its effects on voltage sensor movement, but to completely account for our data, we suggested that β1 also has effects on Ca²⁺ binding (Bao and Cox, 2005). Here, to test this hypothesis we sought to estimate the channel's Ca²⁺ dissociation constants at each high-affinity Ca²⁺ binding site in the presence and absence of β1. To do this we used high-resolution Ca²⁺ dose-response curves (Horrigan and Aldrich, 2002; Sweet and Cox, 2008). The channel's Popen was measured at a single voltage over a large range of [Ca²⁺]. Fig. 2 (A and B) shows unitary currents recorded from membrane patches expressing either ΔE or $\Delta E + \beta 1$. Both patches were held at 0 mV and exposed to a range of $[Ca^{2+}]$. Although each patch contained many channels, Popen was low at 3 nM [Ca²⁺], such that activity was observed as the infrequent and brief openings of single channels. Increasing intracellular Ca²⁺ then caused an increase in *Popen.* From data like these we derived the ΔE and ΔE + β1 channels' *Popen* versus [Ca²⁺] relations (Fig. 2 C). So that all parts of each curve could be well determined, Popen was measured over seven orders of magnitude with 21 Ca²⁺ concentrations. To do this, many patches were used and normalized by their values of *NPopen* at 5.3 µM, where N is the number of channels in a given patch. The resulting curve was then either normalized to its minimum to yield curves like those in Fig. 3 C or adjusted so as to have the proper *Popen* at 2.5 mM [Ca²⁺] (determined in separate single-channel experiments; see Materials and methods). This yielded curves like those in Fig. 2 D.

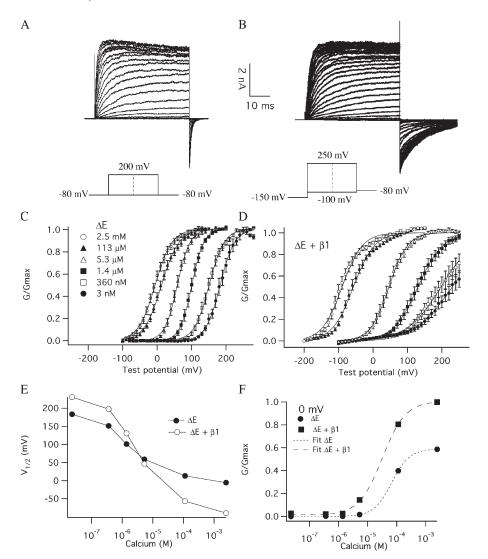


Figure 1. β 1 increases the Ca²⁺ sensitivity of BK_{Ca}-channel activation. (A and B) Macroscopic currents recorded from ΔE channels in the absence and presence of \$1. Currents are from inside-out macropatches from TSA 201 cells exposed to 9.7 µM Ca2+. (C and D) G-V relations determined at the following Ca2+ concentrations: 0.003, 0.360, 1.4, 5.3, 113, and 2,500 µM for the mutant channel ΔE (C) and ΔE + β1 (D). Each curve represents the average of between 4 and 22 individual curves. Error bars indicate SEM. The solid curves are Boltzmann fits with the following parameters: ΔE , 3 nM Ca²⁺: Q= 1.21 *e*, $V_{1/2} = 183 \text{ mV}$; 360 nM Ca^{2+} : Q = 1.18 e, $V_{1/2}$ = 151 mV; 1.4 μ M Ca²⁺: Q = 1.47 e, $V_{1/2}$ = 101 mV; 5.4 μ M Ca²⁺: Q = 1.38 e, $V_{1/2}$ = 59 mV; 113 μ M Ca²⁺: \widetilde{Q} = 1.00 e, $V_{1/2}$ = 13 mV; 2.5 mM Ca²⁺: Q = 1.04 e, $V_{1/2}$ = -5.7 mV. Δ E + β 1, 3 nM Ca^{2+} : Q = 0.46 e, $V_{1/2}$ = 230 mV; 360 nM Ca²⁺: Q = 0.51 e, $V_{1/2} = 197$ mV; 1.4 μM Ca²⁺: $Q = 0.70 \ e$, $V_{1/2} = 131 \ mV$; 5.4 μM Ca²⁺: Q = 0.94 *e*, $V_{1/2}$ = 46 mV; 113 μ M Ca^{2+} : $\widetilde{Q} = 0.83 \ e$, $V_{1/2} = -57 \ mV$; 2.5 mM Ca^{2+} : $\widetilde{Q} = 0.87 \ e$, $V_{1/2} = -90 \ mV$. (E) Plots of half-maximal activation voltage $(V_{1/2})$ versus $[Ca^{2+}]$ for traces shown in C and D. (F) Ca²⁺ dose-response curves determined for ΔE in the presence and absence of $\beta 1$ at 0 mV. The curves are fitted with the Hill equation:

$$\frac{G}{G_{\text{max}}} = \frac{1}{1 + (Kd/[Ca])^n}.$$

Fit parameters are as follows: ΔE (Kd = 68 μ M, n = 1.4); ΔE + β 1 (Kd = 32 μ M, n = 1.1).

Fig. 2 C shows a comparison of the $Popen/Popen_{min}$ versus $[Ca^{2+}]$ relations at 0 mV of the ΔE channel in the presence (filled circles) and absence (open circles) of $\beta 1$. The magnitude of the change in Popen induced by Ca^{2+} (the range the data spans on the ordinate) is ~ 100 -fold larger for the $\Delta E + \beta 1$ channel than it is for the ΔE channel. This magnitude is determined by the energy Ca^{2+} binding imparts to the channel's central closed-to-open conformational change, which in turn is determined by the open- and closed-state Ca^{2+} binding affinities of each binding site. Thus, that this magnitude changes with $\beta 1$ coexpression indicates that $\beta 1$ alters Ca^{2+} binding.

To analyze this effect more rigorously, *Popen* versus $[Ca^{2+}]$ relations were determined for the ΔE (open circles) and $\Delta E + \beta 1$ (filled circles) channels (Fig. 2 D), and these data were then analyzed as follows. If one assumes that there are four of each type of Ca^{2+} binding site and that each site influences channel opening by altering the equilibrium constant of a single conformational change between closed and open—as much evidence suggests (McManus and Magleby, 1991; Cox

et al., 1997; Cui et al., 1997; Horrigan et al., 1999; Horrigan and Aldrich, 1999, 2002; Rothberg and Magleby, 1999, 2000; Cox and Aldrich, 2000a)—and that there are no interactions between binding sites and no interactions between binding sites and voltage sensors (not rigorously true [Sweet and Cox, 2008], but see below), then at constant voltage the channel's *Popen* as a function of voltage can be written as

$$Popen = \frac{M(1 + [Ca]/K_{o1})^4(1 + [Ca]/K_{o2})^4}{(1 + [Ca]/K_{C1})^4(1 + [Ca]/K_{C2})^4 + M(1 + [Ca]/K_{O1})^4(1 + [Ca]/K_{O2})^4},$$

where K_{Cl} and K_{C2} represent the dissociation constants of binding sites 1 and 2 in the closed conformation, K_{Ol} and K_{O2} represent the dissociation constants of binding sites 1 and 2 in the open conformation, and M represents the closed-to-open equilibrium constant when no Ca^{2+} are bound. As relates to the BK_{Ca} channel, M is voltage dependent and incorporates all effects of voltage on opening.

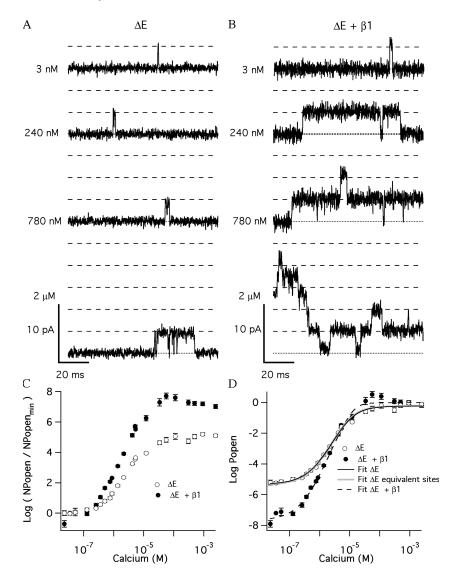


Figure 2. β 1 increases the Ca²⁺ dependence of Popen at a constant voltage. (A and B) Outward K+ currents recorded at 0 mV and filtered at 2 kHz from macropatches containing ΔE channels (A) or $\Delta E + \beta 1$ channels (B) exposed to the indicated [Ca²⁺]. These currents demonstrate that Popen increases in a Ca2+dependent manner when voltage is constant. A comparison of the dose-response relations for the effect of Ca²⁺ on *Popen* at 0 mV in the absence or presence of $\beta 1$ is show. Each point represents the average of between 7 and 30 patches at each [Ca2+] tested. Error bars represent SEM. The Popen curves are fitted with Eq. 1 yielding values of: ΔE , $K_{CI} = 4.3 \,\mu\text{M}$, $K_{OI} =$ 1.1 μ M, $K_{C2} = 7.9 \mu$ M, $K_{O2} = 1.3 \mu$ M, $M = 4.7 \times$ 10^{-6} ; $\Delta E + \beta 1$, $K_{CI} = 2.2 \mu M$, $K_{OI} = 0.5 \mu M$, $K_{C2} = 32 \,\mu\text{M}, K_{O2} = 0.6 \,\mu\text{M}, M = 2.0 \times 10^{-8}. \text{Ad}$ ditionally, ΔE was fitted assuming that the two types of binding sites are equivalent. The fit yield values of $K_C = 5.9 \mu M$, $K_O = 1.2 \mu M$, and $M = 4.2 \times 10^{-6}$.

In the absence of Ca²⁺, Eq. 1 reduces to:

$$Popen = \frac{M}{1+M},\tag{2}$$

which can be rearranged to

$$M = \frac{Popen}{1 - Popen}. (3)$$

Further, at voltages where Popen is much less than 1 ($\sim 10^{-2}$ or lower, such as 0 mV, which we have used here), Eq. 3 may be simplified to

$$M \approx Popen.$$
 (4)

Thus, in the absence of Ca^{2+} , M can be determined directly from *Popen*.

Conversely, at saturating Ca²⁺, Eq. 1 reduces to

$$Popen = \frac{1}{1 + \frac{1}{M} \left(\frac{1}{C1}\right)^4 \left(\frac{1}{C2}\right)^4}$$
 (5),

where

$$C1 = \left(\frac{K_{C1}}{K_{C1}}\right) \tag{6},$$

and

$$C2 = \left(\frac{K_{C2}}{K_{O2}}\right) \tag{7}$$

and, therefore, the top of the *Popen* versus $[Ca^{2+}]$ curve is determined by M and the ratios (C1 and C2) of the open- and closed-state Ca^{2+} dissociation constants at the two types of sites.

The ΔE channel's *Popen* versus [Ca²⁺] curve at 0 mV (Fig. 2 D, open circles) was fitted with Eq. 1 (solid line). The fit yielded the following values:

Site 1:
$$K_{CI} = 4.3 \pm 21 \,\mu\text{M}$$
, $K_{OI} = 1.1 \pm 19 \,\mu\text{M}$, $C = 3.9$
Site 2: $K_{C2} = 7.9 \pm 20 \,\mu\text{M}$, $K_{O2} = 1.3 \pm 24 \,\mu\text{M}$, $C = 6.1$
 $M = 4.7 \times 10^{-6} \pm 10^{-6}$

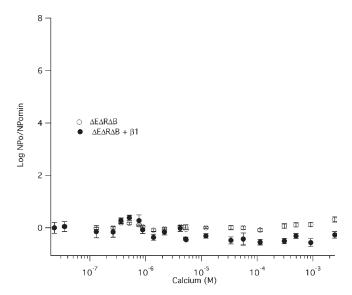


Figure 3. Mutating all three types of Ca^{2+} binding sites eliminates the Ca^{2+} dependence of *Popen*. Dose–response relationships for the effect of Ca^{2+} on *Popen* at 0 mV were obtained by plotting the mean log ratio of NPopen in the presence and absence of Ca^{2+} for $\Delta E \Delta R \Delta B$ (open circles) and $\Delta E \Delta R \Delta B + \beta 1$ (filled circles) channels. Each point represents the average between 7 and 11 patches at each $[Ca^{2+}]$ tested. Error bars represent SEM.

Note the large standard errors of the dissociation constants. This suggests that the fit is not unique and that the RCK1 and Ca²⁺ bowl sites likely have similar affinities at 0 mV, such that changes in the parameters governing one site can be compensated for by changes in the parameters governing the other site. Indeed, the fit was almost as good when we supposed that the two types of sites had identical binding properties (gray curve).

Also shown in Fig. 2 D is the *Popen* versus $[Ca^{2+}]$ curve we determined for the ΔE channel in the presence of $\beta 1$. $\beta 1$ reduces M by 235-fold, which moves the 0 $[Ca^{2+}]$ data point well down the ordinate. This indicates that some of $\beta 1$'s effects are on channel properties that are unrelated to Ca^{2+} binding, as has been demonstrated (Nimigean and Magleby, 1999b, 2000; Bao and Cox, 2005; Orio and Latorre, 2005; Wang and Brenner, 2006). Fitting this curve with Eq. 1 yielded:

Site 1:
$$K_{CI} = 2.2 \pm 0.3 \, \mu\text{M}$$
, $K_{OI} = 0.50 \pm 2.1 \, \mu\text{M}$, $C = 4.4$
Site 2: $K_{C2} = 32 \pm 0.0 \, \mu\text{M}$, $K_{O2} = 0.56 \pm 1.9 \, \mu\text{M}$, $C = 57.1$
 $M = 2.0 \times 10^{-8} \pm 21 \times 10^{-6}$

The fit is better determined than the fit to the ΔE (α only) curve, and it suggests that in addition to decreasing $M \sim 200$ -fold, $\beta 1$ increases the affinity of both binding sites when the channel is open, and it reduces the affinity of one site when the channel is closed. This increases C at both sites and thereby the effect of Ca^{2+} binding on channel opening.

$\beta 1$ Does not Restore Ca²⁺ Sensing to Triple Mutant Channels

To test more directly whether β1 affects Ca²⁺ binding, and if so, at which sites and to what extent, we examined β1's effects on each type of binding site individually using mutations that selectively eliminate the effect of Ca²⁺ at each type of site. D367A eliminates Ca²⁺ sensing via RCK1 sites (Xia et al., 2002), and D898A/D900A eliminates Ca²⁺ sensing via Ca²⁺ bowls (Bao et al., 2004). Before using these mutations, however, it was important to confirm that in conjunction with E399N, they completely eliminate the effect of Ca²⁺ on *Popen*. Shown in Fig. 3 are Ca²⁺ dose-response relations at 0 mV determined from a patch expressing the triple mutant (E399N)(D367A)(D898A/ D900A), which we refer to as $\Delta E \Delta R \Delta B$, in the presence and absence of β1. As is evident, the triple mutant shows no response to Ca²⁺, which demonstrates that the three sites targeted by these mutations can together account for all Ca^{2+} sensing. Importantly, $\Delta E \Delta R \Delta B + \beta 1$ also shows no response to Ca²⁺. Thus, in our hands, β1 does not restore the Ca²⁺ sensitivity of one or more of the sites, as has been suggested (Qian and Magleby, 2003), nor does \(\beta\)1 contain Ca²⁺ binding sites of its own that are coupled to channel opening.

β 1 Alters the Affinity of the Ca²⁺ Bowl Site

We then used the mutant (E399N) (D367A), which we refer to as $\Delta E \Delta R$, to examine $\beta 1$'s effect on Ca^{2+} sensing at the Ca²⁺ bowl. Fig. 4 displays BK_{Ca} currents from individual patches expressing $\Delta E \Delta R$ in the absence (A) or presence (B) of β1. Each patch was held at 0 mV and exposed to various [Ca²⁺]. For the $\Delta E\Delta R$ channels, application of Ca²⁺ caused an increase in *Popen*, but the increase was not as great (\sim 10²-fold) as it was with the Δ E channel ($\sim 10^5$ -fold), likely because the ΔR mutation eliminates half of the channels high-affinity Ca²⁺ binding sites. But more importantly, expression of \$1 increased the Ca^{2+} dependence of *Popen* for the $\Delta E\Delta R$ channels. Fig. 4 C shows a comparison of the *Popen/Popen*_{min} versus [Ca²⁺] relations for the $\Delta E\Delta R$ channel in the presence (filled circles) and absence (open circles) of β1 at 0 mV. Remarkably, for the $\Delta E \Delta R$ channel, the change in *Popen* produced by saturating Ca^{2+} is ~ 50 times larger in the presence of $\beta 1$ than it is in its absence. This indicates that β 1 increases C at the Ca²⁺ bowl site and therefore that it affects Ca²⁺ binding at this site.

To determine the extent to which $\beta 1$ affects Ca^{2^+} binding, in Fig. 4 D, the *Popen* versus $[Ca^{2^+}]$ relations for the $\Delta E \Delta R$ channels (open circles) and $\Delta E \Delta R + \beta 1$ channels (filled circles) are plotted. These data are the same as those in Fig. 4 C, except they have been plotted as absolute *Popen*. The affinities of the intact Ca^{2^+} bowl site in the presence and absence of $\beta 1$ were then determined from fits with Eq. 8 below, which is analogous to Eq. 1, but represents the case where there is only one type of Ca^{2^+} binding site.

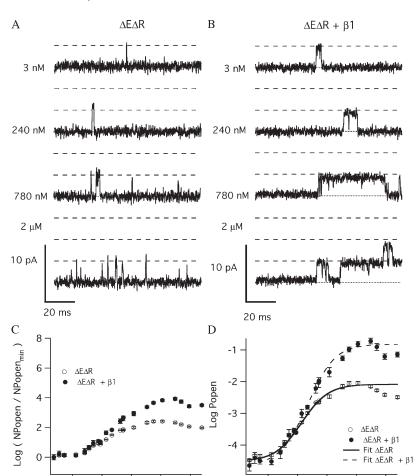


Figure 4. β1 alters the affinity of the Ca^{2+} bowl at constant voltage. (A and B) outward K⁺ currents recorded at 0 mV and filtered at 2 kHz from macropatches containing $\Delta E\Delta R$ channels (A) or $\Delta E\Delta R + \beta 1$ channels (B) exposed to the indicated $[Ca^{2+}]$. These currents demonstrate that *Popen* increases in a Ca^{2+} -dependent manner when voltage is constant. A comparison of the dose–response relation for the effect of Ca^{2+} on *Popen* at 0 mV in the absence or presence of β1 is plotted in C as the ratio of N*Popen* to N*Popen*_{min} versus $[Ca^{2+}]$ and in D as *Popen* versus $[Ca^{2+}]$. Each point represents the average of between 8 and 16 patches at each $[Ca^{2+}]$ tested. Error bars represent SEM.

$$Popen = \frac{M(1 + [Ca]/K_O)^4}{(1 + [Ca]/K_C)^4 + M(1 + [Ca]/K_O)^4}$$
(8)

10⁻³

10⁻⁵ Calcium (M) 10⁻³

10⁻⁷

Importantly, the bottom of the curve is still determined by M, but once M is specified, the top of the curve is determined only by C. That is, at saturating Ca^{2+} ,

10⁻⁵ Calcium (M)

10⁻⁷

$$Popen = \frac{1}{1 + \frac{1}{M} \left(\frac{1}{C}\right)^4} \qquad (9), \quad \text{where} \quad C = \left(\frac{K_C}{K_O}\right) \qquad (10).$$

Thus, when there is a single type of binding site, determining the top and bottom of the channel's *Popen* versus [Ca²⁺] curve greatly constrains the fitting. In fact, in leaves only one parameter free, either of the two dissociation constants, to determine the shape of the curve.

Fitting the $\Delta E \Delta R$ channel's *Popen* versus [Ca²⁺] curve yielded the fit shown in Fig. 4 D (solid line). Gratifyingly, even with these constraints the fit closely approximated the data and yielded the following parameters (see also Table I):

$$K_C = 2.1 \pm 0.28 \ \mu\text{M}, \ K_O = 0.55 \pm 0.08 \ \mu\text{M}, \ C = 3.82$$

$$M = 2.8 \times 10^{-6} \pm 5.3 \times 10^{-6}$$

Next, we fitted the $\Delta E \Delta R + \beta 1$ curve (Fig. 4 D, dashed line), and again the fit appeared to well approximate the data. This suggests that $\beta 1$ does not likely alter the assumptions underlying Eq. 8: a single conformational change between opened and closed; four binding sites; and binding at one site does not affect binding at the other sites, except via promoting opening. The fit yielded the following parameter values:

$$K_C = 5.9 \pm 1.1 \ \mu\text{M}, \ K_O = 0.7 \pm 0.13 \ \mu\text{M}, \ C = 8.4$$

 $M = 2.2 \times 10^{-6} \pm 8.8 \times 10^{-6}$

Note that the fit is well constrained and suggests that $\beta 1$ reduces the affinity of the Ca²⁺ bowl site for Ca²⁺ both in the opened and the closed channel. The effect on K_O however is smaller than on K_C and not clearly significant. The effect on K_C , however, is large (2.8-fold), and it is predominately this change that increases C for this site and accounts for the expanded Popen range spanned by the $\Delta E\Delta R$ channel's Ca²⁺ dose–response relation in the presence of $\beta 1$.

Thus, $\beta 1$ does alter Ca^{2+} binding at the Ca^{2+} bowl site. It lowers the Ca^{2+} bowl's affinity when the channel is closed. Unexpectedly, however, the loss of the channel's

TABLE |
Ca²⁺ Binding Parameters

Binding site	Membrane potential (mV)	$K_C (\mu M)$	$K_{O} \; (\mu M)$	$M\times 10^{-6}$	$C (K_C/K_O)$
Ca^{2+} bowl $(\Delta E \Delta R)$					
α	0	2.1 ± 0.28	0.55 ± 0.08	2.8 ± 5.3	3.8
α+β1	0	5.9 ± 1.1	0.70 ± 0.13	2.2 ± 8.8	8.4
RCK1 (ΔΕΔΒ)					
α	0	15.8 ± 3.1	2.10 ± 0.40	18 ± 4.5	7.5
α+β1	0	18.5 ± 4.4	0.52 ± 0.07	0.15 ± 0.05	36

RCK1 sites also had a dramatic effect on β 1's influence on the Ca²⁺-independent properties of channel gating. The β 1-induced change in M, which was 235-fold for the Δ E channel (Fig. 2 D), is absent in Δ E Δ R channel. Indeed, with the D367A mutation present, it appears β 1 no longer effects M. The bottoms of the two curves in Fig. 2 D are at the same place on the log (*Popen*) axis. This result argues that functional RCK1 sites are required for β 1's effect on the intrinsic equilibrium constant between open and closed, usually referred to as L (for an analysis of mouse β 1's effects on L see Wang and Brenner 2006).

β1 also Alters the Affinities of the RCK1 Sites

To examine β1's effect on Ca²⁺ sensing at the RCK1 sites we used the mutant (E399N) (D898A/D900A), which we refer to as $\Delta E \Delta B$. In Fig. 5, BK_{Ca} currents from individual patches expressing $\Delta E \Delta B$ channels in the absence (A) or presence of $\beta 1$ (B) are displayed. Patches were held at 0 mV and exposed to various [Ca²⁺]. For $\Delta E \Delta B$ channels application of Ca²⁺ caused an increase in *Popen*, but again the increase is not as great ($\sim 10^4$ -fold) as it is with the ΔE channel ($\sim 10^5$ -fold), presumably because the $\Delta E \Delta B$ channel had lost half of its high-affinity Ca²⁺ binding sites. More importantly, however, coexpression of \$1 increased the maximal effect of Ca²⁺ on *Popen*. Fig. 5 C shows a comparison of the *Popen/Popen*_{min} versus [Ca²⁺] relations for the $\Delta E \Delta B$ channel in the presence (filled circles) and absence (open circles) of β 1. The effect of β 1 on Ca²⁺ sensing via the RCK1 sites is substantial. For the $\Delta E \Delta B$ channel, the change in *Popen* produced by saturating Ca^{2+} is ~ 400 times larger in the presence of the β 1. This again requires that β 1 increase $C(K_{CI}/K_{OI})$ at the RCK1 site and therefore that it affects Ca²⁺ binding at this site as well.

To determine how much each dissociation constant is affected, we plotted (Fig. 5 D) the *Popen* versus [Ca²+] relations for the $\Delta E \Delta B$ (open circles) and $\Delta E \Delta B + \beta I$ (filled circles) channels and fit these relations with Eq. 8. The affinities of the intact RCK1 sites in the presence and absence of βI were then determined from the fits. The fit to the $\Delta E \Delta B$ data (solid line) yielded the following values (see also Table I):

$$K_C = 15.8 \pm 3.1 \ \mu\text{M}, \ K_O = 2.1 \pm 0.43 \ \mu\text{M}, \ C = 7.5$$

$$M = 1.8 \times 10^{-5} \pm 0.45 \times 10^{-5}$$

And the fit to the $\Delta E \Delta B + \beta 1$ data (dashed curve) yielded the following values (see also Table I):

$$K_C = 18.5 \pm 4.4 \,\mu\text{M}, K_O = 0.52 \pm 0.07 \,\mu\text{M}, C = 36$$

 $M = 1.5 \times 10^{-7} \pm 0.5 \times 10^{-7}$

Thus, there is a small $\beta1$ -induced change in the affinity of the RCK1 sites when the channel is closed, and a much larger relative change when the channel is open, the opposite of what we observed at the Ca²+ bowl. Most important, however, the changes are diametric and consequently the change in C is large (7.5 \square 36), and it is this change that drives the expansion of the channel's Ca²+ dose–response curve along the ordinate in Fig. 5 C upon $\beta1$ coexpression. Interestingly, the $\beta1$ -induced change in M is \sim 200-fold for the Δ E Δ B channel, similar to what is observed with the Δ E channel. Thus, unlike functional RCK1 sites, functional Ca²+ bowls are not required for $\beta1$'s effects on the Ca²+-independent gating properties of the BK_{Ca} channel.

Although β 1 has very large effects on the Δ E Δ B channel's Ca²⁺ dose-response relation, there is a complicating factor that makes our conclusions about the effects of β1 on this site less than definitive. Our analysis assumes that there are no direct interactions between Ca2+ binding sites and voltage sensors. We have however shown previously (Sweet and Cox, 2008) that Ca2+ binding at the RCK1 site is voltage dependent, an effect that is most reasonably attributed to a direct intrasubunit interaction between voltage sensor and RCK1 Ca²⁺ binding site. Further, we have estimated the allosteric factor by which voltage sensor activation alters the equilibrium constant for Ca²⁺ binding at the RCK1 site (E) to be \sim 2.8, and Horrigan and Aldrich (2002) arrived at a similar value, 2.4, based on the effect of Ca²⁺ on voltage sensor movement measured with gating currents. This means that Eq. 8 is not strictly correct for the $\Delta E \Delta B$ channel because as Ca^{2+} binds and the channels open, more voltage sensors will become active, even if the voltage is held constant, and this could lead to enhanced binding not accounted for by Eq. 8. The magnitude of this effect will depend on E and on the properties of the channel's voltage sensors. In terms of the Horrigan and Aldrich (HA) model, it will depend on L and V_{hc} , and V_{ho} , z_L , and z_L (Horrigan and Aldrich, 2002). Furthermore, if \(\beta \)1 changes any of these

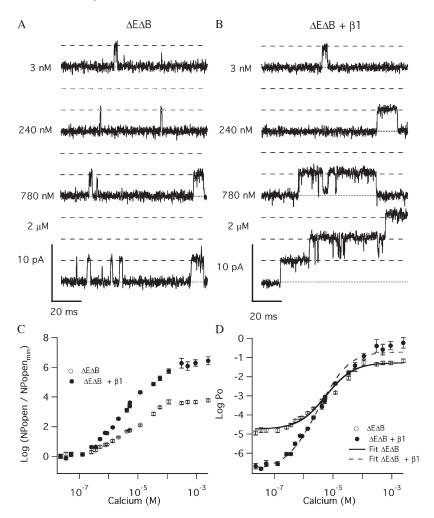


Figure 5. β1 alters the affinity of the RCK1 site at constant voltage. (A and B) Outward K^+ currents recorded at 0 mV and filtered at 2 kHz from macropatches containing $\Delta E \Delta B$ channels (A) or $\Delta E \Delta B + \beta 1$ channels (B) exposed to the indicated $[Ca^{2+}]$. These currents demonstrate that *Popen* increases in a Ca^{2+} -dependent manner when voltage is constant. A comparison of the dose–response relation for the effect of Ca^{2+} on *Popen* at 0 mV in the absence or presence of $\beta 1$ is shown in C as the ratio N*Popen* to N*Popen*_{min} versus $[Ca^{2+}]$ and in D as *Popen* versus $[Ca^{2+}]$. Each point represents the average of between 5 and 22 patches at each $[Ca^{2+}]$ tested. Error bars represent SEM.

parameters this could alter the channel's Ca^{2+} dose–response curve in a way that looks like an increase in C, when no real change in C has occurred.

Ideally, our experiments would have been performed at a negative voltage where the channel's voltage sensors are very rarely active; however, in the presence of the $\beta 1$ subunit voltage sensors become active at far negative voltages where Popen at 3 nM [Ca2+] is too low to measure well, making this approach impractical. However, we examined whether the effects of β1 on the BK_{Ca} channel's voltage sensing parameters could account for its effects on the $\Delta E \Delta B$ channel's *Popen* versus $[Ca^{2+}]$ curve as follows. We simulated $\Delta E \Delta B$ *Popen* versus [Ca²⁺] curves using the HA model and voltage-sensing parameters that have been well established for the wild-type mSlo1 channel (V_{ho} = 27 mV, V_{hc} = 151 mV, z_I = 0.58, z_L = 0.41, $L\theta$ = 2 × 10⁻⁶, and E = 2.8) and Ca²⁺ dissociation constants we determined for the RCK1 site (K_C = 23.2 μ M and K_O = 4.9 μ M). This led to the *Popen* versus [Ca²⁺] curve shown in Fig. 6 B (dark curve). We then simulated the effects of $\beta 1$ on the voltage-sensing parameters of the channel by lowering L from 2×10^{-6} to 2×10^{-9} to account for the large decline in M (*Popen* at 3 nM Ca²⁺), and we altered V_{hc} to +80 mV and V_{ho} to -34 mV, parameters we have determined previously

for α+β1 channels (Bao and Cox, 2005). The resulting curve is also shown in Fig. 6 B (gray curve), and as anticipated, altering these voltage-sensing parameters did increase the maximum effect of Ca²⁺ on *Popen*. The change in *Popen* brought about by saturating [Ca²⁺] increased 1.55-fold. Thus, some of the changes we have observed in the $\Delta E \Delta B$ channel's *Popen* versus [Ca²⁺] curve upon $\beta 1$ expression likely arise from β1's effects on voltage sensing rather than Ca²⁺ binding. However, as seen in Fig. 6 A, β1 increases the maximum effect of Ca²⁺ on Popen 708-fold far greater than the effect we anticipate due to the linkage between Ca²⁺ binding and voltage sensing (1.55-fold). In fact the anticipated effect is <1% of the true effect of β1. Thus, this complication not withstanding, it does appear that β1 alters the true Ca²⁺ affinities of the RCK1 sites as well as the Ca²⁺ bowl sites.

DISCUSSION

Here, we have examined the mechanism by which the BK_{Ca} channel's $\beta 1$ subunit increases the Ca^{2+} sensitivity of channel activation. To be as accurate as possible, we used unitary–current recordings from patches containing from a few hundred to just a few channels. This

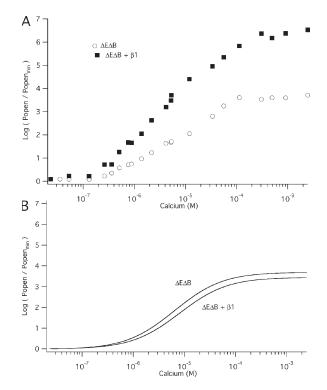


Figure 6. β1's effects on the BK_{Ca} channel's voltage-sensing parameters cannot account for the β1-induced changes in the $\Delta E \Delta B$ channel's Ca^{2+} dose–response relation. (A) $\Delta E \Delta B$ (open circles) and $\Delta E \Delta B + \beta 1$ (filled squares) Ca^{2+} dose–response relations normalized to their minima. (B) Simulated Ca^{2+} dose–response curves for the $\Delta E \Delta B$ (black curve) and $\Delta E \Delta B + \beta 1$ (gray curve) channels assuming that $\beta 1$ only affects the voltage-sensing properties of the channel. The curves were simulated with an HA model using parameters we have determined previously (Bao and Cox, 2005; Sweet and Cox, 2008). For the $\Delta E \Delta B$ (α-only channel): L = 2e-6, Vhc =151 mV, Vho = 27 mV, E = 2.8, and E = 0.41, and E = 0.58, E = 2.8, and E = 0.41, and E = 0.58, E = 0.58

allowed us to determine Popen over seven orders of magnitude. To be as model independent as possible, we made measurements at constant voltage, and where possible low Popen, such that the amplitudes and shapes of the resulting Ca²⁺ dose-response curves were dependent primarily, if not exclusively, on the channel's Ca²⁺ binding parameters. The essential assumptions we made in fitting our data were as follows: (1) that there is a single conformational change between open and closed that can occur with any number of Ca²⁺ bound, an idea that is consistent with a great many single-channel and macroscopic BK_{Ca} channel studies and all current models (McManus and Magleby, 1991; Cox et al., 1997; Cui et al., 1997; Horrigan et al., 1999; Horrigan and Aldrich, 1999, 2002; Rothberg and Magleby, 1999, 2000; Cox and Aldrich, 2000a); (2) that there are four of each type of high-affinity site. This has been established for the Ca²⁺ bowl (Niu and Magleby, 2002), and given the fourfold symmetry of the channel, this seems likely to be the case for the RCK1 site as well; and (3) that there are no interactions between binding sites of the same type or between Ca²⁺ binding and voltage sensor movement.

We found that the 0 mV Ca^{2+} dose–response curve of BK_{Ca} channels containing only functional RCK1 sites could be well fitted by supposing that each site independently influences opening, and that each site has a dissociation constant of 15.8 μM when the channel is closed and 2.1 μM when the channel is open. These values produce a C value of 7.5, which allows us to calculate that each Ca^{2+} bound to a Ca^{2+} bowl site decreases the energy difference between open and closed by 4.9 KJ/mol.

In the presence of $\beta 1$, the RCK1 site's dose–response curve at 0 mV could also be well fitted with a simple model and yielded values for K_C and K_O of 18.5 and 0.52 μ M, respectively. These values produce a substantial C value of 36 (8.8 KJ/mol per binding event), which we think is in large part responsible for the dramatic increase in the influence of Ca^{2+} on *Popen* brought about by $\beta 1$ in the $\Delta E\Delta B$ channels. As discussed above, however, our estimates of these values are complicated by an interaction between Ca^{2+} binding and voltage at the RCK1 sites. But we have simulated the influence that this complication will likely have on Ca^{2+} binding in the presence of $\beta 1$, and it turns out to very small compared with the effect of $\beta 1$ observed. Thus, we do not think this linkage has greatly distorted our measurement of K_C and K_O .

Ca²⁺ binding to the Ca²⁺ bowl is not voltage sensitive (Sweet and Cox, 2008); thus, our conclusions drawn from data for the Ca²⁺ bowl sites are more clear. We found that the Ca²⁺ bowl's dose-response curve at 0 mV could be well fitted by supposing that each Ca²⁺ bowl independently influences opening, and that each site has a dissociation constant for Ca²⁺ of 2.1 µM when the channel is closed and 0.56 µM when the channel is open. These values produce a C of 3.75, which allows us to calculate that each Ca²⁺ bound at a Ca²⁺ bowl site decreases the energy difference between open and closed by 3.2 KJ/mol. These numbers may be compared with previous estimates of K_C and K_0 for this site. Xia et al. (2002) estimated $K_0 = 4.5 \pm$ $1.7 \,\mu\text{M}$ and $K_O = 2.0 \pm 0.7 \,\mu\text{M}$ (C = 2.25), and Bao et al. (2002) estimated $K_C = 3.8 \pm 0.2$ and $K_O = 0.94 \pm 0.06 \,\mu\text{M}$ (C=4.0). And in Sweet and Cox (2008), we estimated $K_C=$ $3.13 \pm 0.28 \,\mu\text{M}$ and $K_0 = 0.88 \pm 0.06 \,\mu\text{M}$ (C = 3.55). Thus, our current estimates are close to our previous estimates, especially in terms of C.

More interestingly, we found that coexpression of $\beta 1$ changed the $\Delta E \Delta R$ channel's *Popen* versus [Ca²+] relation in a manner that indicated that the affinity of the Ca²+ bowl site for Ca²+ is changed in the presence of $\beta 1$. In the presence of $\beta 1$, the $\Delta E \Delta R$ channel's dose–response curve at 0 mV could be well fitted by supposing that each Ca²+ bowl independently influences opening, and that each site has an affinity of 5.9 μ M when the channel is closed and 0.67 μ M when the channel is open. These values produce a C value of 8.8, which allows us to calculate that with $\beta 1$

present, each Ca²⁺ bound at a Ca²⁺ bowl decreases the energy difference between open and closed by 5.3 KI/mol.

These numbers are difficult to compare with previous estimates of the affinities of the BK_{Ca} channel in the presence of \$1. Bao and Cox (2005) estimated the affinities of the channel in the presence of $\beta 1$ to be $K_C =$ 3.71 μ M and $K_O = 0.88 \mu$ M (C = 4.2) for site 1 and $K_C =$ 5.78 μ M and $K_0 = 0.73 \,\mu$ M (C = 7.9) for site 2. These estimates, however, were based on the assumption that $\beta 1$ affected the affinity of only one of the two binding sites (site 2), which we now think is not the case. But interestingly, and of relevance here, Cox and Aldrich (2000) observed that the affinity of the channel for Ca²⁺ in the open conformation must not change upon expression of β 1, as β 1 does not alter the critical [Ca²⁺] required to begin to see a leftward shift in the channel's G-V relation (Cox and Aldrich, 2000a). This is in agreement with our data that show that expression of β1 does not appreciably alter K_0 for the higher-affinity site (Ca²⁺ bowl: $Ko_{\alpha} = 0.56 \, \mu\text{M}$, $K_{O\alpha + \beta I} = 0.67 \, \mu\text{M}$).

In considering the effects of $\beta 1$ on Ca^{2+} binding, it is interesting to note that the BK_{Ca} $\beta 1$ subunit has very little intracellular sequence, just its N and C termini—15 and 13 amino acids long, respectively. The rest of the protein is comprised of two transmembrane domains and a large extracellular loop—118 amino acids. Thus, if $\beta 1$ subunits alter the channel's affinity for Ca^{2+} by interacting directly with Ca^{2+} binding sites, generally accepted to be contained within the cytoplasmic C terminus of the channel, they seemingly must do so through these short intracellular termini. Supporting this idea, chimera experiments with $\beta 1$ and $\beta 2$ (Orio and Latorre, 2005) and $\beta 1$ N- and C-terminal deletion experiments (Wang and Brenner, 2006) have shown that these termini play a critical role in transmitting $\beta 1$'s effects to the channel proper.

In addition to β 1's effects on Ca²⁺ binding, and in agreement with the previous findings of Wang and Brenner (2006), we found that mouse β 1 reduces *Popen* at 0 mV in the absence of Ca²⁺, presumably by lowering *L*. Strikingly, our results show that a mutation at a Ca²⁺ binding site (D367A) not only eliminates Ca²⁺ binding at the RCK1 sites, but it also eliminates β 1's ability to reduce *Popen* at 0 mV in the absence of Ca²⁺. That is, a single Ca²⁺ binding site mutation affects both the mechanism by which the channel senses Ca²⁺ and also the mechanism by which β 1 influences the intrinsic energetics of opening.

We also found that when we combine mutations at all three types of Ca^{2+} binding sites—E399N (low-affinity site), D367A (RCK1 site), and D898/D900 (Ca^{2+} bowl)—we see no effect of Ca^{2+} on channel gating in the α -only channel, and we see no restoration of an effect of Ca^{2+} on channel gating by β 1. This in contrast to the work of Qian and Magleby (2003), who found that coexpression of β 1 restored some Ca^{2+} sensitivity to a similar triple mutant. We do not know why we did not see the restorative effect of β 1 observed by Qian and Magleby. The most straightfor-

ward explanation is that the Ca^{2^+} binding sites were disabled by the mutations and could not be fixed by $\beta 1$. However, the main differences between the two studies were as follows: our recordings were made at $0\,\text{mV}$, whereas theirs were conducted at $+50\,\text{mV}$. The mutations used at each binding site were not exactly the same between the two studies, and Qian and Magleby recorded from single-channel patches, whereas the patches we used typically contained many channels. Furthermore, Qian and Magleby did not see the restorative effect of $\beta 1$ in every experiment, but rather they reported a large variability from patch to patch. This would seem to suggest that perhaps there is a subtle environmental factor required for the effect they observed that was absent from our experiments.

Our measurements of the effects of $\beta 1$ on Ca^{2+} binding also stand in some contrast to the recent paper of Yang et al. (2008). They found that the mutation R167A in the voltage-sensing domain of the mSlo1 channel eliminates $\beta 1$'s ability to alter the channel's G-V curve at all $[Ca^{2+}]$. This result suggests that by altering just voltage sensor movement one can disrupt the $\beta 1$ -induced enhancement of Ca^{2+} sensitivity altogether. It seems hard to imagine how such a mutation could simultaneously eliminate $\beta 1$'s effects on voltage sensor movement and Ca^{2+} binding at both binding sites, unless these processes are physically more intertwined than previously thought.

Perhaps rather than affecting Ca^{2+} binding directly, the $\beta 1$ subunit might be affecting a linkage between Ca^{2+} binding and voltage sensing, which in turn affects Ca^{2+} binding. Indeed, we have shown recently (Sweet and Cox, 2008) that such a linkage exists between the RCK1 sites and the voltage sensors, so it is possible that an enhancement of the allosteric factor at this linkage, E, could create enhanced binding affinity. It would be interesting to explore this linkage in $\alpha+\beta 1$ channels. However, we found no such linkage between Ca^{2+} bowl sites and the channel's voltage sensors (Sweet and Cox, 2008); thus, we do not think that such an effect is involved in the $\beta 1$ -induced changes in the binding affinity we observed at the Ca^{2+} bowl sites.

In conclusion, we have shown that the BK $\beta 1$ subunit has effects on the affinities of the BK_{Ca} channel's high-affinity Ca²⁺ binding sites. It primarily increases the affinity of the RCK1 sites when the channel is open, and it primarily decreases the affinity of the Ca²⁺ bowl sites when the channel is closed. Both of these modifications increase the difference in affinity between open and closed, such that Ca²⁺ binding at either site has a larger effect on channel opening when $\beta 1$ is present.

This work was supported by program project grant P01-HL077378 and predoctoral fellowship F31 NS047823 from the National Institutes of Health.

Edward N. Pugh Jr. served as editor.

Submitted: 1 October 2008 Accepted: 18 December 2008

REFERENCES

- Bao, L., and D.H. Cox. 2005. Gating and ionic currents reveal how the BK_{Ca} 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 Ca²⁺ sensitivity. *J. Gen. Physiol.* 120:173–189.
- Bao, L., C. Kaldany, E.C. Holmstrand, and D.H. Cox. 2004. Mapping the BK_{Ca} channel's "Ca²⁺ bowl": side-chains essential for Ca²⁺ sensing. *J. Gen. Physiol.* 123:475–489.
- Bian, S., I. Favre, and E. Moczydlowski. 2001. Ca2+-binding activity of a COOH-terminal fragment of the Drosophila BK channel involved in Ca2+-dependent activation. *Proc. Natl. Acad. Sci. USA*. 98:4776–4781.
- Brenner, R., G.J. Perez, A.D. Bonev, D.M. Eckman, J.C. Kosek, S.W. Wiler, A.J. Patterson, M.T. Nelson, and R.W. Aldrich. 2000. Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature*. 407:870–876.
- Butler, A., S. Tsunoda, D.P. McCobb, A. Wei, and L. Salkoff. 1993. mSlo, a complex mouse gene encoding "maxi" calcium-activated potassium channels. *Science*. 261:221–224.
- Cox, D.H., and R.W. Aldrich. 2000. Role of the $\beta 1$ subunit in large-conductance Ca²⁺-activated K⁺ channel gating energetics. Mechanisms of enhanced Ca²⁺ 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.
- Cui, J., D.H. Cox, and R.W. Aldrich. 1997. Intrinsic voltage dependence and Ca²⁺ regulation of *mslo* large conductance Ca-activated K⁺ channels. *J. Gen. Physiol.* 109:647–673.
- Hamill, O.P., A. Marty, E. Neher, B. Sakmann, and F.J. Sigworth. 1981.
 Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391:85–100.
- Horrigan, F.T., and R.W. Aldrich. 1999. Allosteric voltage gating of potassium channels II; mSlo channel gating charge movement in the absence of Ca²⁺. *J. Gen. Physiol.* 114:305–336.
- Horrigan, F.T., and R.W. Aldrich. 2002. Coupling between voltage sensor activation, Ca²⁺ binding and channel opening in large conductance (BK) potassium channels. *J. Gen. Physiol.* 120:267–305.
- Horrigan, F.T., J. Cui, and R.W. Aldrich. 1999. Allosteric voltage gating of potassium channels I: mSlo ionic currents in the absence of Ca²⁺. J. Gen. Physiol. 114:277–304.
- 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 beta subunit of high conductance calcium-activated potassium channels. *Neuron*. 14:645–650.
- Meera, P., M. Wallner, Z. Jiang, and L. Toro. 1996. A calcium switch for the functional coupling between alpha (hslo) and beta subunits (KV,Ca beta) of maxi K channels. *FEBS Lett.* 382:84–88.
- Morrow, J.P., S.I. Zakharov, G. Liu, L. Yang, A.J. Sok, and S.O. Marx. 2006. Defining the BK channel domains required for beta1-subunit modulation. *Proc. Natl. Acad. Sci. USA*. 103:5096–5101.
- Nelson, M.T., and J.M. Quayle. 1995. Physiological roles and properties of potassium channels in arterial smooth muscle. Am. J. Physiol. 268:C799–C822.
- Nelson, M.T., H. Cheng, M. Rubart, L.F. Santana, A.D. Bonev, H.J. Knot, and W.J. Lederer. 1995. Relaxation of arterial smooth muscle by calcium sparks. *Science*. 270:633–637.
- Nimigean, C.M., and K.L. Magleby. 1999a. β subunits increase the calcium sensitivity of mSlo by stabilizing bursting kinetics. *Biophys. I.* 76:A328.
- Nimigean, C.M., and K.L. Magleby. 1999b. The β subunit increases the Ca²⁺ sensitivity of large conductance Ca²⁺-activated potassium

- channels by retaining the gating in the bursting states. J. Gen. Physiol. 113:425–440.
- Nimigean, 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., and K.L. Magleby. 2002. Stepwise contribution of each subunit to the cooperative activation of BK channels by Ca2+. Proc. Natl. Acad. Sci. USA. 99:11441–11446.
- Orio, P., and R. Latorre. 2005. Differential effects of β1 and β2 subunits on BK channel activity. *J. Gen. Physiol.* 125:395–411.
- Orio, P., Y. Torres, P. Rojas, I. Carvacho, M.L. Garcia, L. Toro, M.A. Valverde, and R. Latorre. 2006. Structural determinants for functional coupling between the β and α subunits in the Ca²⁺-activated K⁺ (BK) channel. *J. Gen. Physiol.* 127:191–204.
- Perez, G.J., A.D. Bonev, J.B. Patlak, and M.T. Nelson. 1999. Functional coupling of ryanodine receptors to KCa channels in smooth muscle cells from rat cerebral arteries. J. Gen. Physiol. 113:229–238.
- Pluger, S., J. Faulhaber, M. Furstenau, M. Lohn, R. Waldschutz, M. Gollasch, H. Haller, F.C. Luft, H. Ehmke, and O. Pongs. 2000. Mice with disrupted BK channel beta1 subunit gene feature abnormal Ca(2+) spark/STOC coupling and elevated blood pressure. Circ. Res. 87:E53–E60.
- Qian, X., and K.L. Magleby. 2003. Beta1 subunits facilitate gating of BK channels by acting through the Ca2+, but not the Mg2+, activating mechanisms. Proc. Natl. Acad. Sci. USA. 100:10061–10066.
- Qian, X., C.M. Nimigean, X. Niu, B.L. Moss, and K.L. Magleby. 2002. Slo1 tail domains, but not the Ca²⁺ bowl, are required for the β1 subunit to increase the apparent Ca²⁺ sensitivity of BK channels. *J. Gen. Physiol.* 120:829–843.
- Rothberg, B.S., and K.L. Magleby. 1999. Gating kinetics of single large-conductance Ca²⁺-activated K⁺ channels in high Ca²⁺ suggest a two-tiered allosteric gating mechanism. *J. Gen. Physiol.* 114: 93–124 (published erratum in 114:337).
- Rothberg, B.S., and K.L. Magleby. 2000. Voltage and Ca²⁺ activation of single large-conductance Ca²⁺-activated K⁺ channels described by a two-tiered allosteric gating mechanism. *J. Gen. Physiol.* 116:75–99.
- Schreiber, M., and L. Salkoff. 1997. A novel calcium-sensing domain in the BK channel. *Biophys. J.* 73:1355–1363.
- Shen, K.Z., A. Lagrutta, N.W. Davies, N.B. Standen, J.P. Adelman, and R.A. North. 1994. Tetraethylammonium block of Slowpoke calciumactivated potassium channels expressed in Xenopus oocytes: evidence for tetrameric channel formation. *Pflugers Arch.* 426:440–445.
- Shi, J., G. Krishnamoorthy, Y. Yang, L. Hu, N. Chaturvedi, D. Harilal, J. Qin, and J. Cui. 2002. Mechanism of magnesium activation of calcium-activated potassium channels. *Nature*. 418:876–880.
- Sweet, T.B., and D.H. Cox. 2008. Measurements of the BK_{Ca} channel's high-affinity Ca²⁺ binding constants: effects of membrane voltage. *J. Gen. Physiol.* 132:491–505.
- Wallner, M., P. Meera, and L. Toro. 1996. Determinant for beta-subunit regulation in high-conductance voltage-activated and Ca(2+)sensitive K+ channels: an additional transmembrane region at the N terminus. *Proc. Natl. Acad. Sci. USA*. 93:14922–14927.
- Wang, B., and R. Brenner. 2006. An S6 mutation in BK channels reveals β1 subunit effects on intrinsic and voltage-dependent gating. *J. Gen. Physiol.* 128:731–744.
- Xia, X.M., X. Zeng, and C.J. Lingle. 2002. Multiple regulatory sites in large-conductance calcium-activated potassium channels. *Nature*. 418:880–884.
- Yang, H., G. Zhang, J. Shi, U.S. Lee, K. Delaloye, and J. Cui. 2008. Subunit-specific effect of the voltage sensor domain on Ca2+ sensitivity of BK channels. *Biophys. J.* 94:4678–4687.
- Yang, X.C., and F. Sachs. 1989. Block of stretch-activated ion channels in Xenopus oocytes by gadolinium and calcium ions. *Science*. 243:1068–1071.