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Measuring the Influence of the BK$_{Ca}$ β1 Subunit on Ca$^{2+}$ Binding to the BK$_{Ca}$ Channel

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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 β1, whose presence dramatically increases both the potency and efficacy of Ca$^{2+}$ in promoting channel opening. β1 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 β1 stabilizes voltage sensor activation, such that activation occurs at more negative voltages with β1 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 β1 on the channel's Ca$^{2+}$ sensitivity, however, we also proposed that there must be effects of β1 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 β1 on Ca$^{2+}$ binding. We find that coexpression of β1 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 β1 is present.

INTRODUCTION

Large-conductance Ca$^{2+}$-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 Ca$^{2+}$ via voltage-dependent Ca$^{2+}$ channels. This global increase in Ca$^{2+}$ leads to vasoconstriction, but it also triggers localized Ca$^{2+}$ release events from ryanodine receptors on the smooth muscle sarcoplasmic reticulum. These release events, termed Ca$^{2+}$ 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 shown to be critically important in this regulatory process, as mice that lack β1 have greatly reduced STOCs in response to sparks as well as hyper-contraction 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 β1 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 Ca$^{2+}$ 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 Ca$^{2+}$ (Nimigean and Magleby, 1999a). They suggested that a Ca$^{2+}$-independent effect underlies most of the channel's increased Ca$^{2+}$ 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 β1 present. At most voltages this decreases the work that Ca$^{2+}$ must do to open the channel and thereby increases the channel's apparent Ca$^{2+}$ sensitivity.
affinity. However, to account for the full change in Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} sensitivity, implying that \(\beta_1\) enhances Ca\textsuperscript{2+} sensitivity solely by altering the conformation or movements of the voltage sensor. Here, to clarify this issue, we have used high-resolution Ca\textsuperscript{2+} dose-response curves to determine directly whether or not \(\beta_1\) alters the BK\(_{ca}\) channel’s affinity for Ca\textsuperscript{2+} at either of its two types of high-affinity Ca\textsuperscript{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 \(\alpha\) subunit (mslo-mbr5) (Butler et al., 1993), the mouse \(\beta_1\) 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 Lipo- vectamine 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 \(\mu\)g DNA) Lipofectamine 2000 (Invitrogen). The transfection solution was allowed to equilibrate in solutions containing between 20 \(\mu\)M and 2.5 mM free Ca\textsuperscript{2+}.

The molar ratio of \(\beta_1\) to \(\alpha\)-expressing plasmids transfected was 1:1 \(\beta_1\) 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 \(\alpha\) 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 [Ca\textsuperscript{2+}]. This is likely due to more efficient transcription and or translation of \(\beta_1\) relative to the much larger \(\alpha\) 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 \(\Omega\) 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 \(>100\) \(\mu\)M Ca\textsuperscript{2+}, 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, 22–24°C.

**Solutions**

The pipette solution for macroscopic current recordings contained the following (in mM): 118 KMeSO\(_4\), 20 N-methylglucamine-MeSO\(_4\), 2 KCl, 2 MgCl\(_2\), and 2 HEPES, pH 7.20. The pipette solution for current recordings at 0 mV contained the following (in mM): 3 KMeSO\(_4\), 135 N-methylglucamine-MeSO\(_4\), 2 KCl, 2 MgCl\(_2\), and 2 HEPES, pH 7.20. 10 \(\mu\)M GdCl\(_3\) 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 KMeSO\(_4\), 20 N-methylglucamine-MeSO\(_4\), 2 KCl, and 2 HEPES, pH 7.20. 1 mM EGTA (Fluka) was used as the Ca\textsuperscript{2+} buffer for solutions containing 3–500 nM free [Ca\textsuperscript{2+}], 1 mM HEDTA (Sigma-Aldrich) was used as the Ca\textsuperscript{2+} buffer for solutions containing 0.8–20 \(\mu\)M free [Ca\textsuperscript{2+}], and no Ca\textsuperscript{2+} chelator was used as the Ca\textsuperscript{2+} buffer in solutions containing between 20 \(\mu\)M and 2.5 mM free Ca\textsuperscript{2+}.

**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 \(\mu\)s after repolarizations to \(-80\) mV following voltage steps to the test voltage. Each G-V relation was fitted with a Boltzmann function,

\[
G = G_{\text{max}} / \left[ 1 + e^{\frac{V_{1/2} - V}{k}} \right],
\]

and normalized to the maximum of the fit.

**Single-channel Analysis**

Under conditions where the open probability (\(P_{\text{o}}\)) is small (<10\(^{-5}\)), single-channel openings were observed in patches containing hundreds of channels and \(I_0\) was measured from steadystate recordings 30 s in duration. \(P_{\text{open}}\) was determined from all-points histograms by measuring the fraction of time spent (\(P_{\text{o}}\)) at each open level \(k\) using a half-amplitude criteria and summing their contributions \(P_{\text{open}} = \sum kP_{k}\), where \(N\) is the number of channels in the patch.
**Popen versus Ca\(^{2+}\) Curves**

The effect of Ca\(^{2+}\) on Popen was determined from the ratio of NPopen at a given [Ca\(^{2+}\)] to NPopen at 0.88 or 5.4 μM 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 [Ca\(^{2+}\)] 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 [Ca\(^{2+}\)] was normalized by the NPopen value measured at 0.88 μM [Ca\(^{2+}\)]. 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 μM [Ca\(^{2+}\)], and these curves were then averaged. The curve normalized to 0.88 μM was then adjusted so as to have the same value at 0.88 μM [Ca\(^{2+}\)] as the curve normalized to 5.4 μM [Ca\(^{2+}\)]. To yield a single curve at the [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 Ca\(^{2+}\) dose–response curve with a value of 1 at 5.4 μM [Ca\(^{2+}\)].

In some cases, Popen rather than (NPopen/NPopen\(_{\text{min}}\)) 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\(_{\text{min}}\)) 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 Ca\(^{2+}\) to add to internal solutions to yield the desired free Ca\(^{2+}\) concentrations was calculated using the program MaxChelator, which was downloaded from http://www.stanford.edu/~cpattom/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 β1**

The BK\(_{\text{Ca}}\) channel is both Ca\(^{2+}\) and voltage sensitive, and the effects of these stimuli are often displayed as a series of G-V relations determined over a series of Ca\(^{2+}\) concentrations. Such a series, determined from current families recorded from BK\(_{\text{Ca}}\) 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\(_{\text{Ca}}\) α subunit (mSlo1). Increasing intracellular Ca\(^{2+}\) shifts the channels’ G-V curve leftward, an effect that is generally known to be due to three types of Ca\(^{2+}\) 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 Ca\(^{2+}\) sensing (Shi et al., 2002; Xia et al., 2002). So here only the effects of Ca\(^{2+}\) binding at the channel’s high-affinity Ca\(^{2+}\) binding sites are evident. We refer to the mSlo1 channel carrying this mutation as ΔE. Increasing Ca\(^{2+}\) from 3 nM to 2.5 mM shifts the ΔE G-V relation ~200 mV leftward.

When the mouse BK\(_{\text{Ca}}\) β1 subunit is expressed with the α subunit (see currents in Fig. 1 B), the Ca\(^{2+}\)-induced leftward shifting evident in Fig. 1 C becomes more pronounced (Fig. 1 D), and thus it may be said that β1 increases the Ca\(^{2+}\) sensitivity of the BK\(_{\text{Ca}}\) channel in that it increases its G-V shift in response to a given change in [Ca\(^{2+}\)] (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 Ca\(^{2+}\) (Fig. 1 F). These data reveal that Ca\(^{2+}\) binding to the BK\(_{\text{Ca}}\) channel’s low-affinity Ca\(^{2+}\) binding sites (eliminated by the E399N mutation) is not required for β1’s effects on Ca\(^{2+}\) 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 β1**

In a previous study we found that much of β1’s effects on Ca\(^{2+}\) 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 Ca\(^{2+}\) binding (Bao and Cox, 2005). Here, to test this hypothesis we sought to estimate the channel’s Ca\(^{2+}\) dissociation constants at each high-affinity Ca\(^{2+}\) binding site in the presence and absence of β1. To do this we used high-resolution Ca\(^{2+}\) 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 [Ca\(^{2+}\)]. Fig. 2 (A and B) shows unitary currents recorded from membrane patches expressing either ΔE or ΔE + β1. 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 [Ca\(^{2+}\)], such that activity was observed as the infrequent and brief openings of single channels. Increasing intracellular Ca\(^{2+}\) then caused an increase in Popen. From data like these we derived the ΔE and ΔE + β1 channels’ Popen versus [Ca\(^{2+}\)] 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 Ca\(^{2+}\) 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 [Ca\(^{2+}\)] (determined in separate single-channel experiments; see Materials and methods). This yielded curves like those in Fig. 2 D.
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 voltage sensors (not rigorously true [Sweet and Cox, 2008], but see below), then at constant voltage the channel’s $P_{\text{open}}$ as a function of voltage can be written as

$$P_{\text{open}} = \frac{M(Ca|K_{C1}K_{C2})}{(1 + [Ca]/K_{C1}K_{C2})^2 + M(1 + [Ca]/K_{C1}K_{C2})^2},$$

where $K_{C1}$ and $K_{C2}$ represent the dissociation constants of binding sites 1 and 2 in the closed conformation, $K_{O1}$ 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 BKCa channel, $M$ is voltage dependent and incorporates all effects of voltage on opening.

Fig. 2 C shows a comparison of the $P_{\text{open}}/P_{\text{open}}\max$ relations at 0 mV of the $\Delta E$ channel in the presence (filled circles) and absence (open circles) of $\beta 1$. The magnitude of the change in $P_{\text{open}}$ 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 $\Delta E$ 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 $\Delta E$ + $\beta 1$ coexpression indicates that $\beta 1$ alters Ca$^{2+}$ binding.

To analyze this effect more rigorously, $P_{\text{open}}$ versus [Ca$^{2+}$] relations were determined for the $\Delta E$ (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 $P_{\text{open}}$ as a function of voltage can be written as

$$P_{\text{open}} = \frac{M(1 + [Ca]/K_{O1})(1 + [Ca]/K_{O2})}{(1 + [Ca]/K_{C1}K_{C2})^2 + M(1 + [Ca]/K_{O1})(1 + [Ca]/K_{O2})^2},$$

where $K_{C1}$ and $K_{C2}$ represent the dissociation constants of binding sites 1 and 2 in the closed conformation, $K_{O1}$ 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 BKCa channel, $M$ is voltage dependent and incorporates all effects of voltage on opening.
In the absence of Ca\(^{2+}\), Eq. 1 reduces to:

\[
P_{\text{open}} = \frac{M}{1 + M},
\]

which can be rearranged to

\[
M = \frac{P_{\text{open}}}{1 - P_{\text{open}}}.  \tag{3}
\]

Further, at voltages where \(P_{\text{open}}\) 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

\[
M \approx P_{\text{open}}.  \tag{4}
\]

Thus, in the absence of Ca\(^{2+}\), \(M\) can be determined directly from \(P_{\text{open}}\).

Conversely, at saturating Ca\(^{2+}\), Eq. 1 reduces to

\[
P_{\text{open}} = \frac{1}{1 + \frac{1}{M} \left( \frac{1}{C_1} + \frac{1}{C_2} \right)^{\Delta E} \left( \frac{1}{C_1} + \frac{1}{C_2} \right)^{\Delta E + \beta_1}}, \tag{5}
\]

where

\[
C_1 = \frac{K_{C_1}}{K_{O_1}}, \tag{6}
\]

and

\[
C_2 = \frac{K_{C_2}}{K_{O_2}}. \tag{7}
\]

and, therefore, the top of the \(P_{\text{open}}\) versus [Ca\(^{2+}\)] curve is determined by \(M\) and the ratios (\(C_1\) and \(C_2\)) of the open- and closed-state Ca\(^{2+}\) dissociation constants at the two types of sites.

The \(\Delta E\) channel’s \(P_{\text{open}}\) versus [Ca\(^{2+}\)] curve at 0 mV (Fig. 2 D, open circles) was fitted with Eq. 1 (solid line). The fit yielded the following values:

- **Site 1:** \(K_{C_1} = 4.3 \pm 21 \mu M, K_{O_1} = 1.1 \pm 19 \mu M, C = 3.9\)
- **Site 2:** \(K_{C_2} = 7.9 \pm 20 \mu M, K_{O_2} = 1.3 \pm 24 \mu M, C = 6.1\)

\[
M = 4.7 \times 10^{-5} \pm 10^{-5}
\]

\(\Delta E\) increases the Ca\(^{2+}\) dependence of \(P_{\text{open}}\) at a constant voltage. (A and B) Outward K\(^+\) currents recorded at 0 mV and filtered at 2 kHz from macropatches containing \(\Delta E\) channels (A) or \(\Delta E + \beta_1\) channels (B) exposed to the indicated [Ca\(^{2+}\)]. These currents demonstrate that \(P_{\text{open}}\) increases in a Ca\(^{2+}\)-dependent manner when voltage is constant. A comparison of the dose–response relations for the effect of Ca\(^{2+}\) on \(P_{\text{open}}\) at 0 mV in the absence or presence of \(\beta_1\) is shown. Each point represents the average of between 7 and 30 patches at each [Ca\(^{2+}\)] tested. Error bars represent SEM. The \(P_{\text{open}}\) curves are fitted with Eq. 1 yielding values of: \(\Delta E, K_{C_1} = 4.3 \mu M, K_{O_1} = 1.1 \mu M, K_{C_2} = 7.9 \mu M, K_{O_2} = 1.3 \mu M, M = 4.7 \times 10^{-5}; \Delta E + \beta_1, K_{C_1} = 2.2 \mu M, K_{O_1} = 0.5 \mu M, K_{C_2} = 32 \mu M, K_{O_2} = 0.6 \mu M, M = 2.0 \times 10^{-8}\). Additionally, \(\Delta E\) 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 \(M = 4.2 \times 10^{-6}\).
β1 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 △E△R△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 Ca²⁺ sensing. Importantly, △E△R△B + β1 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 β1 contain Ca²⁺ binding sites of its own that are coupled to channel opening.

β1 Alters the Affinity of the Ca²⁺ Bowl Site

We then used the mutant (E399N)(D367A), which we refer to as △E△R, to examine β1’s effect on Ca²⁺ sensing at the Ca²⁺ bowl. Fig. 4 displays BKCa currents from individual patches expressing △E△R in the absence (A) or presence (B) of β1. Each patch was held at 0 mV and exposed to various [Ca²⁺]. For the △E△R channels, application of Ca²⁺ caused an increase in *Popen*, but the increase was not as great (∼10²-fold) as it was with the △E channel (∼10⁵-fold), likely because the ΔR mutation eliminates half of the channels high-affinity Ca²⁺ binding sites. But more importantly, expression of β1 increased the Ca²⁺ dependence of *Popen* for the △E△R channels. Fig. 4 C shows a comparison of the *Popen*/*Popen*ₜₐₓ ratios for △E△R channel in the presence (filled circles) and absence (open circles) of β1 at 0 mV. Remarkably, for the △E△R channel, the change in *Popen* produced by saturating Ca²⁺ is ∼50 times larger in the presence of β1 than it is in its absence. This indicates that β1 increases C at the Ca²⁺ bowl site and therefore that it affects Ca²⁺ binding at this site.

To determine the extent to which β1 affects Ca²⁺ binding, in Fig. 4 D, the *Popen* versus [Ca²⁺] relations for the △E△R channels (open circles) and △E△R + β1 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 Ca²⁺ bowl site in the presence and absence of β1 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 Ca²⁺ binding site.

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 [Ca²⁺] curve we determined for the △E channel in the presence of β1. β1 reduces M by 235-fold, which moves the 0 [Ca²⁺] data point well down the ordinate. This indicates that some of β1’s effects are on channel properties that are unrelated to Ca²⁺ 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: $K_{C1} = 2.2 \pm 0.3 \mu M$, $K_{O1} = 0.50 \pm 2.1 \mu M$, $C = 4.4$

Site 2: $K_{C2} = 32 \pm 0.0 \mu M$, $K_{O2} = 0.56 \pm 1.9 \mu M$, $C = 57.1$

$M = 2.0 \times 10^{-2} \pm 2.1 \times 10^{-2}$

The fit is better determined than the fit to the △E (α only) curve, and it suggests that in addition to decreasing M ∼200-fold, β1 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 Ca²⁺ binding on channel opening.
Next, we fitted the $\Delta \Delta R$ 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:

$$K_c = 5.9 \pm 1.1 \, \mu M, \quad K_o = 0.7 \pm 0.13 \, \mu M, \quad C = 8.4 \quad M = 2.2 \times 10^{-6} \pm 8.8 \times 10^{-4}$$

Note that the fit is well constrained and suggests that $\beta 1$ reduces the affinity of the Ca$^{2+}$ bowl site for Ca$^{2+}$ 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 $K_o$ however, is large (2.8-fold), and it is predominately this change that increases $C$ for this site and accounts for the expanded $P_{open}$ range spanned by the $\Delta \Delta R$ channel’s Ca$^{2+}$ dose–response relation in the presence of $\beta 1$.

Thus, $\beta 1$ decreases the 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
RCK1 sites also had a dramatic effect on β1’s influence on the Ca\(^{2+}\)-independent properties of channel gating. The β1-induced change in \(M\), which was 235-fold for the \(\Delta E\) channel (Fig. 2 D), is absent in \(\Delta E\Delta R\) channel. Indeed, with the D367A mutation present, it appears \(\beta1\) no longer affects \(M\). The bottoms of the two curves in Fig. 2 D are at the same place on the log \((P_{\text{open}})\) axis. This result argues that functional RCK1 sites are required for \(\beta1\)’s effect on the intrinsic equilibrium constant between open and closed, usually referred to as \(L\) (for an analysis of mouse \(\beta1\)’s effects on \(L\) see Wang and Brenner 2006).

β1 also alters the affinities of the RCK1 sites

To examine \(\beta1\)’s effect on Ca\(^{2+}\) 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\(_{\text{Ca}}\) currents from individual patches expressing \(\Delta E\Delta B\) channels in the absence (A) or presence of \(\beta1\) (B) are displayed. Patches were held at 0 mV and exposed to various [Ca\(^{2+}\)]. For \(\Delta E\Delta B\) channels application of Ca\(^{2+}\) caused an increase in \(P_{\text{open}}\), but again the increase is not as great (\(\sim10^5\)-fold) as it is with the \(\Delta E\) channel (\(\sim10^3\)-fold), presumably because the \(\Delta E\Delta B\) channel had lost half of its high-affinity Ca\(^{2+}\) binding sites. More importantly, however, coexpression of \(\beta1\) increased the maximal effect of Ca\(^{2+}\) on \(P_{\text{open}}\). Fig. 5 C shows a comparison of the \(P_{\text{open}}/P_{\text{open, min}}\) versus [Ca\(^{2+}\)] relations for the \(\Delta E\Delta B\) channel in the presence (filled circles) and absence (open circles) of \(\beta1\). The effect of \(\beta1\) on Ca\(^{2+}\) sensing via the RCK1 sites is substantial. For the \(\Delta E\Delta B\) channel, the change in \(P_{\text{open}}\) produced by saturating Ca\(^{2+}\) is \(\sim400\) times larger in the presence of the \(\beta1\). This again requires that \(\beta1\) increase \(C\) (\(K_{C1}/K_{O1}\)) at the RCK1 site and therefore that it affects Ca\(^{2+}\) binding at this site as well.

To determine how much each dissociation constant is affected, we plotted (Fig. 5 D) the \(P_{\text{open}}\) versus [Ca\(^{2+}\)] relations for the \(\Delta E\Delta B\) (open circles) and \(\Delta E\Delta B + \beta1\) (filled circles) channels and fit these relations with Eq. 8. The affinities of the intact RCK1 sites in the presence and absence of \(\beta1\) were then determined from the fits. The fit to the \(\Delta E\Delta B\) data (solid line) yielded the following values (see also Table 1):

\[
K_C = 15.8 \pm 3.1 \ \mu M, \ K_O = 2.1 \pm 0.43 \ \mu M, \ C = 7.5
M = 1.8 \times 10^{-5} \pm 0.45 \times 10^{-5}
\]

And the fit to the \(\Delta E\Delta B + \beta1\) data (dashed curve) yielded the following values (see also Table I):

\[
K_C = 18.5 \pm 4.4 \ \mu M, \ K_O = 0.52 \pm 0.07 \ \mu M, \ C = 36
M = 1.5 \times 10^{-7} \pm 0.5 \times 10^{-7}
\]

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 Ca\(^{2+}\) bowl. Most important, however, the changes are diametric and consequently the change in \(C\) is large (7.5→36), and it is this change that drives the expansion of the channel’s Ca\(^{2+}\) dose–response curve along the ordinate in Fig. 5 C upon \(\beta1\) coexpression. Interestingly, the \(\beta1\)-induced change in \(M\) is \(\sim200\)-fold for the \(\Delta E\Delta B\) channel, similar to what is observed with the \(\Delta E\) channel. Thus, unlike functional RCK1 sites, functional Ca\(^{2+}\) bowls are not required for \(\beta1\)’s effects on the Ca\(^{2+}\)-independent gating properties of the BK\(_{\text{Ca}}\) channel.

Although \(\beta1\) has very large effects on the \(\Delta E\Delta B\) channel’s Ca\(^{2+}\) dose–response relation, there is a complicating factor that makes our conclusions about the effects of \(\beta1\) on this site less than definitive. Our analysis assumes that there are no direct interactions between Ca\(^{2+}\) binding sites and voltage sensors. We have however shown previously (Sweet and Cox, 2008) that Ca\(^{2+}\) 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 Ca\(^{2+}\) binding site. Further, we have estimated the allosteric factor by which voltage sensor activation alters the equilibrium constant for Ca\(^{2+}\) binding at the RCK1 site (\(E\)) to be \(\sim2.8\), and Horrigan and Aldrich (2002) arrived at a similar value, 2.4, based on the effect of Ca\(^{2+}\) 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 \(V_{\text{hr}}\), and \(V_{\text{hr}}zJ\), and \(zJ\) (Horrigan and Aldrich, 2002). Furthermore, if \(\beta1\) changes any of these

### Table I

| 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 (\(\Delta E\Delta R\)) |                         |                     |                     |                      |                     |
| \(\alpha\)         | 0                       | 2.1 ± 0.28          | 0.55 ± 0.08         | 2.8 ± 5.3            | 3.8                 |
| \(\alpha + \beta1\) | 0                       | 5.9 ± 1.1           | 0.70 ± 0.13         | 2.2 ± 8.8            | 8.4                 |
| RCK1 (\(\Delta E\Delta B\)) |                     |                     |                     |                      |                     |
| \(\alpha\)         | 0                       | 15.8 ± 3.1          | 2.10 ± 0.40         | 18 ± 4.5             | 7.5                 |
| \(\alpha + \beta1\) | 0                       | 18.5 ± 4.4          | 0.52 ± 0.07         | 0.15 ± 0.05          | 36                  |
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 $P_{\text{open}}$ at 3 nM [Ca\(^{2+}\)] is too low to measure well, making this approach impractical. However, we examined whether the effects of $\beta_1$ on the BK$_{Ca}$ channel’s voltage sensing parameters could account for its effects on the $\Delta E\Delta B$ channel’s $P_{\text{open}}$ versus [Ca\(^{2+}\)] curve as follows. We simulated $\Delta E\Delta B$ $P_{\text{open}}$ versus [Ca\(^{2+}\)] 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, $z_f = 0.58$, $z_L = 0.41$, $L_0 = 2 \times 10^{-6}$, and $E = 2.8$) and Ca\(^{2+}\) dissociation constants we determined for the RCK1 site ($K_C = 23.2$ μM and $K_O = 4.9$ μM). This led to the $P_{\text{open}}$ versus [Ca\(^{2+}\)] 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 $2 \times 10^{-6}$ to $2 \times 10^{-9}$ to account for the large decline in $M$ ($P_{\text{open}}$ at 3 nM Ca\(^{2+}\)), and we altered $V_{ho}$ to +80 mV and $V_{hc}$ to −34 mV, parameters we have determined previously for α+$\beta_1$ 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 Ca\(^{2+}\) on $P_{\text{open}}$. The change in $P_{\text{open}}$ brought about by saturating [Ca\(^{2+}\)] increased 1.55-fold. Thus, some of the changes we have observed in the $P_{\text{open}}$ versus [Ca\(^{2+}\)] curve upon $\beta_1$ expression likely arise from $\beta_1$’s effects on voltage sensing rather than Ca\(^{2+}\) binding. However, as seen in Fig. 6 A, $\beta_1$ increases the maximum effect of Ca\(^{2+}\) on $P_{\text{open}}$ 708-fold—far greater than the effect we anticipate due to the linkage between Ca\(^{2+}\) binding and voltage sensing (1.55-fold). In fact the anticipated effect is <1% of the true effect of $\beta_1$. Thus, this complication notwithstanding, it does appear that $\beta_1$ alters the true Ca\(^{2+}\) affinities of the RCK1 sites as well as the Ca\(^{2+}\) 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
allowed us to determine \( \text{Popen} \) over seven orders of magnitude. To be as model independent as possible, we made measurements at constant voltage, and where possible low \( \text{Popen} \), such that the amplitudes and shapes of the resulting \( \text{Ca}^{2+} \) dose–response curves were dependent primarily, if not exclusively, on the channel’s \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) bound, an idea that is consistent with a great many single-channel and macroscopic BK channels 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 \( \text{Ca}^{2+} \) bowl (Niu and Magleby, 2002), and given the four-fold 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 \( \text{Ca}^{2+} \) binding and voltage sensor movement.

We found that the 0 mV \( \text{Ca}^{2+} \) dose–response curve of BK 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 \( \mu \text{M} \) when the channel is closed and 2.1 \( \mu \text{M} \) when the channel is open. These values produced a \( C \) value of 7.5, which allows us to calculate that each \( \text{Ca}^{2+} \) bound to a \( \text{Ca}^{2+} \) 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 \( \mu \text{M} \), 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 \( \text{Ca}^{2+} \) on \( \text{Popen} \) brought about by \( \beta 1 \) in the \( \Delta \text{DEAB} \) channels. As discussed above, however, our estimates of these values are complicated by an interaction between \( \text{Ca}^{2+} \) binding and voltage at the RCK1 sites. But we have simulated the influence that this complication will likely have on \( \text{Ca}^{2+} \) 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 \( K_o \).

\( \text{Ca}^{2+} \) binding to the \( \text{Ca}^{2+} \) bowl is not voltage sensitive (Sweet and Cox, 2008); thus, our conclusions drawn from data for the \( \text{Ca}^{2+} \) bowl sites are more clear: We found that the \( \text{Ca}^{2+} \) bowl’s dose–response curve at 0 mV could be well fitted by supposing that each \( \text{Ca}^{2+} \) bowl independently influences opening, and that each site has a dissociation constant for \( \text{Ca}^{2+} \) of 2.1 \( \mu \text{M} \) when the channel is closed and 0.56 \( \mu \text{M} \) when the channel is open. These values produce a \( C \) of 3.75, which allows us to calculate that each \( \text{Ca}^{2+} \) bound at a \( \text{Ca}^{2+} \) 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 \( K_o \) for this site. Xia et al. (2002) estimated \( K_c = 4.5 \pm 1.7 \mu \text{M} \) and \( K_o = 2.0 \pm 0.7 \mu \text{M} \) (C = 2.25), and Bao et al. (2002) estimated \( K_c = 3.8 \pm 0.2 \mu \text{M} \) 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 \( K_o = 0.88 \pm 0.06 \mu \text{M} \) (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 \( \Delta \text{DEAR} \) channel’s \( \text{Popen} \) versus \([\text{Ca}^{2+}]\) relation in a manner that indicated that the affinity of the \( \text{Ca}^{2+} \) bowl site for \( \text{Ca}^{2+} \) is changed in the presence of \( \beta 1 \). In the presence of \( \beta 1 \), the \( \Delta \text{DEAR} \) channel’s dose–response curve at 0 mV could be well fitted by supposing that each \( \text{Ca}^{2+} \) bowl independently influences opening, and that each site has an affinity of 5.9 \( \mu \text{M} \) when the channel is closed and 0.67 \( \mu \text{M} \) 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 Ca\textsuperscript{2+} bound at a Ca\textsuperscript{2+} bowl decreases the energy difference between open and closed by 5.3 KJ/mol.

These numbers are difficult to compare with previous estimates of the affinities of the BK\textsubscript{Ca} channel in the presence of \(\beta 1\). Bao and Cox (2005) estimated the affinities of the channel in the presence of \(\beta 1\) to be \(K_0 = 3.71 \mu M\) and \(K_0 = 0.88 \mu M\) (\(C = 4.2\)) for site 1 and \(K_0 = 5.78 \mu M\) 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 (2000a) observed that the affinity of the channel for Ca\textsuperscript{2+} in the open conformation must not change upon expression of \(\beta 1\), as \(\beta 1\) does not alter the critical [Ca\textsuperscript{2+}] 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 \(\beta 1\) does not appreciably alter \(K_0\) for the higher-affinity site (Ca\textsuperscript{2+} bowl: \(K_0 = 0.56 \mu M\), \(K_0,\beta 1 = 0.67 \mu M\)).

In considering the effects of \(\beta 1\) on Ca\textsuperscript{2+} binding, it is interesting to note that the BK\textsubscript{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\textsuperscript{2+} by interacting directly with Ca\textsuperscript{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 \(\beta 1\)'s effects on Ca\textsuperscript{2+} binding, and in agreement with the previous findings of Wang and Brenner (2006), we found that mouse \(\beta 1\) reduces \(P_{open}\) at 0 mV in the absence of Ca\textsuperscript{2+}, presumably by lowering \(L\). Strikingly, our results show that a mutation at a Ca\textsuperscript{2+} binding site (D367A) not only eliminates Ca\textsuperscript{2+} binding at the RCK1 sites, but it also eliminates \(\beta 1\)'s ability to reduce \(P_{open}\) at 0 mV in the absence of Ca\textsuperscript{2+}. That is, a single Ca\textsuperscript{2+} binding site mutation affects both the mechanism by which the channel senses Ca\textsuperscript{2+} and also the mechanism by which \(\beta 1\) influences the intrinsic energetics of opening.

We also found that when we combine mutations at all three types of Ca\textsuperscript{2+} binding sites—E399N (low-affinity site), D367A (RCK1 site), and D898/D900 (Ca\textsuperscript{2+} bowl)—we see no effect of Ca\textsuperscript{2+} on channel gating in the \(\alpha\)-only channel, and we see no restoration of an effect of Ca\textsuperscript{2+} on channel gating by \(\beta 1\). This in contrast to the work of Qian and Magleby (2003), who found that coexpression of \(\beta 1\) restored some Ca\textsuperscript{2+} sensitivity to a similar triple mutant. We do not know why we did not see the restorative effect of \(\beta 1\) observed by Qian and Magleby. The most straightforward explanation is that the Ca\textsuperscript{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 mV, whereas theirs were conducted at ±50 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\textsuperscript{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\textsuperscript{2+}]. This result suggests that by altering just voltage sensor movement one can disrupt the \(\beta 1\)-induced enhancement of Ca\textsuperscript{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\textsuperscript{2+} binding at both binding sites, unless these processes are physically more intertwined than previously thought.

Perhaps rather than affecting Ca\textsuperscript{2+} binding directly, the \(\beta 1\) subunit might be affecting a linkage between Ca\textsuperscript{2+} binding and voltage sensing, which in turn affects Ca\textsuperscript{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\textsuperscript{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\textsuperscript{2+} bowl sites.

In conclusion, we have shown that the BK \(\beta 1\) subunit has effects on the affinities of the BK\textsubscript{Ca} channel's high-affinity Ca\textsuperscript{2+} binding sites. It primarily increases the affinity of the RCK1 sites when the channel is open, and it primarily decreases the affinity of the Ca\textsuperscript{2+} bowl sites when the channel is closed. Both of these modifications increase the difference in affinity between open and closed, such that Ca\textsuperscript{2+} binding at either site has a larger effect on channel opening when \(\beta 1\) is present.

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REFERENCES

Bao, L., and D.H. Cox. 2005. Gating and ionic currents reveal how the BKCa channel’s Ca2+ sensitivity is enhanced by its β1 subunit. J. Gen. Physiol. 126:393–412.

Bao, L., A.M. Rapin, E.C. Holmstrand, and D.H. Cox. 2002. Elimination of the BKCa 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 BKCa 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 the 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 β1 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. Intrinsical voltage dependence and Ca2+ regulation of mslolarge 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.

McManus, O.B., and K.L. Magleby. 1991. Accounting for the Ca2+ dependent kinetics of single large-conductance Ca2+-activated K+ channels in rat skeletal muscle. J. Physiol. 443:739–777.

McManus, O.B., L.M. Helms, L. Pallanck, B. Ganezyzki, 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 sub-units (KVCa 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. 267:633–637.

Nimigean, C.M., and K.L. Magleby. 1999a. β subunits increase the calcium sensitivity of mSlo by stabilizing bursting kinetics. Biophys. J. 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 retarding the gating in the bursting states. J. Gen. Physiol. 113:425–440.

Nimigean, C.M., and K.L. Magleby. 2000. Functional coupling of the β1 subunit to the large conductance Ca2+-activated K+ channel in the absence of Ca2+. Increased Ca2+ sensitivity from a Ca2+-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.

Orío, P., and R. Latorre. 2005. Differential effects of β1 and β2 subunits on BK channel activity. J. Gen. Physiol. 125:395–411.

Orío, P., Y. Torres, P. Rojas, I. Carvacho, M.L. García, L. Toro, M.A. Vabrele, 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 ryanoide 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. Walschutz, M. Gollasch, H. Haller, F.C. Luft, H. Ehmke, and O. Pongs. 2000. Mice with disrupted BK channel beta1 subunit gene feature abnormal Ca2+ 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 114:357).

Rothberg, B.S., and K.L. Magleby. 2000. Voltage and Ca2+ activation of single large-conductance Ca2+-activated K+ channels described by a two-tailed allosteric gating mechanism. J. Gen. Physiol. 116:75–99.

Schreiber, M., and L. Salkoff. 1997. A novel calcium-sensing domain in the BK channel. Biochem. J. 323:1355–1363.

Shen, K.Z., A. Lagrutta, N.W. Davies, N.B. Standen, J.P. Adelman, and R.A. North. 1994. Tetraethylammonium block of Slowpoke calcium-activated potassium channels expressed in Xenopus oocytes: evidence for tetramer 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 BKCa 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 beta1-subunit regulation in high-conductance voltage-activated and Ca2+-sensitive K+ channels: an additional transmembrane region at the N terminus. Proc. Natl. Acad. Sci. USA. 93:14922–14927.

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.