

## Subunit-Specific Effect of the Voltage Sensor Domain on $\text{Ca}^{2+}$ Sensitivity of BK Channels

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**ABSTRACT** Large conductance  $\text{Ca}^{2+}$ - and voltage-activated  $\text{K}^+$  (BK) channels, composed of pore-forming  $\alpha$ -subunits and auxiliary  $\beta$ -subunits, play important roles in diverse physiological processes. The differences in BK channel phenotypes are primarily due to the tissue-specific expression of  $\beta$ -subunits ( $\beta 1$ – $\beta 4$ ) that modulate channel function differently. Yet, the molecular basis of the subunit-specific regulation is not clear. In our study, we demonstrate that perturbation of the voltage sensor in BK channels by mutations selectively disrupts the ability of the  $\beta 1$ -subunit—but not that of the  $\beta 2$ -subunit—to enhance apparent  $\text{Ca}^{2+}$  sensitivity. These mutations change the number of equivalent gating charges, the voltage dependence of voltage sensor movements, the open-close equilibrium of the channel, and the allosteric coupling between voltage sensor movements and channel opening to various degrees, indicating that they alter the conformation and movements of the voltage sensor and the activation gate. Similarly, the ability of the  $\beta 1$ -subunit to enhance apparent  $\text{Ca}^{2+}$  sensitivity is diminished to various degrees, correlating quantitatively with the shift of voltage dependence of voltage sensor movements. In contrast, none of these mutations significantly reduces the ability of the  $\beta 2$ -subunit to enhance  $\text{Ca}^{2+}$  sensitivity. These results suggest that the  $\beta 1$ -subunit enhances  $\text{Ca}^{2+}$  sensitivity by altering the conformation and movements of the voltage sensor, whereas the similar function of the  $\beta 2$ -subunit is governed by a distinct mechanism.

### INTRODUCTION

Large conductance  $\text{Ca}^{2+}$ - and voltage-activated  $\text{K}^+$  (BK) channels are composed of  $\alpha$ -subunits (1,2) encoded by a single *Slo1* gene and four types of auxiliary  $\beta$ -subunits ( $\beta 1$ – $\beta 4$ ) (3–8). They are activated by membrane depolarization and elevated intracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ). Due to a large single channel conductance, their opening effectively hyperpolarizes the membrane resulting in the closure of voltage-dependent  $\text{Ca}^{2+}$  channels and, hence, a decrease in  $[\text{Ca}^{2+}]_i$ . Thus, BK channels provide a negative feedback mechanism to control membrane potential and cytosolic  $[\text{Ca}^{2+}]_i$ . Based on this mechanism, BK channels modulate various physiological processes including action potential repolarization, neurotransmitter release, smooth muscle contraction, and hearing (9–12). BK currents show various phenotypes in different tissue types, which are primarily due to the tissue-specific expression of  $\beta$ -subunits ( $\beta 1$ – $\beta 4$ ) that modulate BK channel function. For example, the  $\beta 1$ -subunit, expressed primarily in smooth muscles, increases  $\text{Ca}^{2+}$  sensitivity of the channel by shifting voltage-dependent activation to a more negative voltage range in  $[\text{Ca}^{2+}]_i$  (3,13,14). Thus, the  $\beta 1$ -subunit is essential in regulating tone in the vasculature (15), trachea (16), urinary bladder (17), and uterus (18). In contrast, the  $\beta 2$ -subunit, expressed in the  $\beta$ -cells of the pancreas, ovaries, brain, and adrenal chromaffin cells, also

increases the  $\text{Ca}^{2+}$ -dependent conductance-voltage ( $G$ - $V$ ) shift and confers inactivation on the BK channel (5–7,19).

BK channel  $\beta$ -subunits share similar topology that includes two transmembrane segments (TM1 and TM2), a relatively large extracellular loop, and short cytosolic N- and C-termini (3) (Fig. 1). Nevertheless, the coexpression of different  $\beta$ -subunits with *Slo1* produces various phenotypes, suggesting that different molecular mechanisms are employed by these  $\beta$ -subunits to modify channel function. Most interestingly, the  $\beta 1$ - and  $\beta 2$ -subunits share 43% identical, or 66% conservative, residues (7), and both enhance  $\text{Ca}^{2+}$  sensitivity of channel activation by increasing a  $\text{Ca}^{2+}$ -dependent shift in the  $G$ - $V$  relationship. A previous study on the biophysical properties of the channel, however, suggested that these two  $\beta$ -subunits modulate channel activation by different mechanisms (20). By studying the effects of these  $\beta$ -subunits on ionic and gating currents of mSlo1 and fitting the results to the allosteric conceptual framework provided by Horrigan, Cui, and Aldrich (denoted here as the HCA model (21)), it has been suggested that the  $\beta 1$ -subunit may affect the apparent  $\text{Ca}^{2+}$  sensitivity by altering voltage-dependent activation (20,22–24). In contrast, the  $\beta 2$ -subunit may affect the apparent  $\text{Ca}^{2+}$  sensitivity by altering both voltage-dependent activation and  $\text{Ca}^{2+}$  binding (20). However, a recent study by Savalli et al. (25) suggests that the  $\beta 2$ -subunit may also affect BK channel activation by primarily altering voltage sensor movements. This study and others (23,26–29) have provided a solid characterization of the functional effects of  $\beta$ -subunits and important insights on the mechanisms of their action. Nevertheless, the molecular identities of residues/domains in  $\alpha$ -subunits that are directly involved in the regulation of  $\beta$ -subunits are still not known

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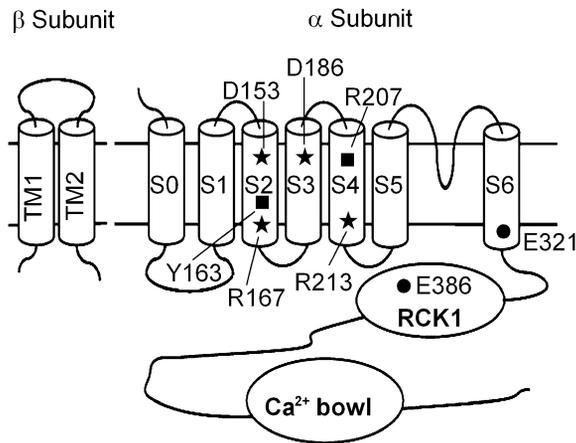


FIGURE 1 Cartoons of mSlo1  $\alpha$ - and  $\beta$ -subunits. All residues mutated in this study are shown. D153, R167, D186, and R213 (stars) are voltage-sensing residues. Y163 and R207 (squares) are nonvoltage-sensing residues in S2 and S4, respectively. E321 and E386 (circles) shift the  $G$ - $V$  relationship to negative voltages without altering the  $\text{Ca}^{2+}$  sensitivity or the  $\beta$ 1-induced enhancement of  $\text{Ca}^{2+}$  sensitivity.

fully. This lack of knowledge limits our ability to interpret results from biophysical studies and undermines our understanding of the physiological role of BK channels in various tissue types. The identifications of the molecular components that are specifically important for the function of different  $\beta$ -subunits will help remove these limitations and provide molecular targets to alter physiological and pathophysiological functions of BK channels in specific tissues.

In our study, we investigate the role of the voltage sensor of Slo1 in the regulation of  $\beta$ -subunits. Like all voltage-dependent  $\text{K}^+$  channels, BK channels are composed of four Slo1 proteins (30) (Fig. 1). Transmembrane segments S5–S6 form the pore domain, with part of S6 forming the activation gate, whereas S1–S4 form the voltage-sensing domain (VSD) (31–34). We hypothesize that if the  $\beta$ 1- or the  $\beta$ 2-subunit modulates BK channel activation by altering voltage sensor movements, a change in voltage sensor movements per se by mutations should affect the function of the  $\beta$ -subunits. A previous study by Ma et al. (34) demonstrated that mutations of charged residues in the VSD of Slo1 alter voltage sensor movements. In addition, the ionic currents of these mutations in various voltages and  $[\text{Ca}^{2+}]_i$  have been recorded, and the experimental data have been fitted to the HCA allosteric model (21,35). It was found that these mutations change various aspects of voltage-dependent gating of BK channels, including the number of equivalent gating charges ( $z_j$  and  $z_T$ ), the voltage dependence of voltage sensor movements ( $V_{\text{HC}}$ ), the open-close equilibrium of the channel ( $L_0$ ), and the allosteric coupling between voltage sensor movements and channel opening to various degrees ( $D$ ) (34) (see Table 1 for definition of the parameters). We studied some of these mutations (Fig. 1) to examine if they also affect the ability of the  $\beta$ -subunits to enhance  $\text{Ca}^{2+}$  sensitivity. We chose these mutations because they affect all parameters of

TABLE 1 Abbreviation and gating parameters (35)

$C, O$	Closed and open conformation of the channel.
$R, A$	Resting and activated conformation of voltage sensor.
$L$	$C$ - $O$ equilibrium constant (unliganded channel, resting voltage sensors).
$L_0$	The zero voltage value of $L$ ( $L = L_0 \exp(z_L V/kT)$ ).
$z_L$	The partial charge of $L$ .
$J$	$R$ - $A$ equilibrium constant (closed, unliganded channel).
$z_j$	The partial charge of $J$ .
$z_T$	Total gating charge ( $z_T = z_L + 4z_j$ ).
$Q_C, Q_O$	Steady-state gating charge distribution for closed or open channels.
$V_{\text{HC}}, V_{\text{HO}}$	Half-activating voltage of $Q_C$ and $Q_O$ , respectively.
$D$	Allosteric factor describing interaction between channel opening and voltage sensor activation ( $D = \exp[-z_j(V_{\text{HO}} - V_{\text{HC}})/kT]$ ).

voltage-dependent gating (Table 1) to various degrees, so that the entire spectrum of perturbations of conformation and movements of the voltage sensor and the activation gate by these mutations were covered. We found that these mutations specifically reduced the ability of the  $\beta$ 1-subunit to shift the voltage dependence of activation in  $[\text{Ca}^{2+}]_i$  but not that of the  $\beta$ 2-subunit. These results indicate that the  $\beta$ 1- and  $\beta$ 2-subunits affect distinct molecular processes during  $\text{Ca}^{2+}$ - and voltage-dependent activation, resulting in specific alterations of channel function.

## EXPERIMENTAL PROCEDURES

### Mutagenesis and channel expression

All  $\alpha$ -subunit constructs were made from the mbr5 clone of mSlo1 (36). Human  $\beta$ 1 and  $\beta$ 2 (KCNMB1 and KCNMB2; GenBank/EMBL/DBJ accession nos. U25138 and AF209747) cDNAs were subcloned into pcDNA3.1(+). The  $\beta$ 2 with N-terminus deleted ( $\beta$ 2ND)-subunit was created by removing amino acids from position 2 through 20. All mutations of mSlo1 and  $\beta$ 2ND constructs were made using polymerase chain reaction with Pfu polymerase (Stratagene, La Jolla, CA). The polymerase chain reaction-amplified regions of all constructs were verified by sequencing. mRNA was transcribed in vitro with T3 polymerase for all mSlo1 constructs and with T7 polymerase for  $\beta$ 1- and  $\beta$ 2ND-subunits (Ambion, Austin, TX). A total of 0.05–20 ng of mSlo1 mRNA or a mixture of 5–15 ng of mSlo1 and 25–50 ng of  $\beta$ -subunit mRNAs was injected into each *Xenopus laevis* oocyte 2–6 days before recording.

### Electrophysiology

Macroscopic currents were recorded from inside-out patches formed with borosilicate pipettes of 1 ~ 2 M $\Omega$  resistance. Data were acquired using a patch-clamp amplifier (Axopatch 200-B; Axon Instruments, Union City, CA) and data acquisition software (Pulse; Heka Elektronik, Lambrecht/Pfalz, Germany). Records were digitized at 20- $\mu$ s intervals and low-pass filtered at 10 kHz with Axopatch's internal filter. The pipette solution contained the following (in mM): 140 potassium methanesulfonic acid, 20 HEPES, 2 KCl, and 2 MgCl<sub>2</sub> (pH 7.20). The basal internal solution contained the following (in mM): 140 potassium methanesulfonic acid, 20 HEPES, 2 KCl, and 1 HEDTA (pH 7.20). Increasing EGTA concentration of the basal internal solution to 5 mM gave a  $[\text{Ca}^{2+}]_i$  of ~0.5 nM. For other  $[\text{Ca}^{2+}]_i$ , CaCl<sub>2</sub> was added to internal solutions to give the appropriate free  $[\text{Ca}^{2+}]_i$ . Actual free  $[\text{Ca}^{2+}]_i$  was measured using a  $\text{Ca}^{2+}$ -sensitive electrode (Orion Research, Cambridge, MA). A total of 50  $\mu$ M 18-crown-6-tetracarboxylic acid (Sigma-

Aldrich, St. Louis, MO) was added to internal solutions to prevent  $Ba^{2+}$  block. Experiments were conducted at room temperature ( $22^{\circ}C \sim 24^{\circ}C$ ).

Gating currents were recorded with inside-out patches (37). The pipette solution contained (in mM): 127 tetraethylammonium (TEA), 125 HMeSO<sub>3</sub>, 2 HCl, 2 MgCl<sub>2</sub>, and 20 HEPES (pH 7.2). The internal solution contains (in mM): 141 *N*-ethyl-D-glucamine, 135 HMeSO<sub>3</sub>, 6 HCl, 20 HEPES, and 5 EGTA (pH 7.2); MgCl<sub>2</sub> was added to the internal solution to reach 10 mM [Mg<sup>2+</sup>]<sub>i</sub>. Voltage commands were filtered at 20 kHz with an eight-pole Bessel filter (Frequency Devices, Ottawa, IL) to prevent the saturation of fast capacitive transients (37). Data were sampled at 100 kHz with an 18-bit analog/digital converter (ITC-18; Instrutech, Port Washington, NY) and filtered at 10 kHz with an internal filter (Axopatch; Axon Instruments). Capacitive transients and leak currents were subtracted using a P/5 protocol with a holding potential of  $-120$  mV.

## Data analysis and statistics

Relative conductance was determined by measuring tail current amplitudes at negative voltages as indicated for the wild-type (WT) and mutant mSlo1 channels. The gating charge movements were determined by integrating the area under the rising phase and single exponential fits to the decaying phase of  $I_{gON}$  at various voltages. The  $G$ - $V$  relationship or the charge-voltage ( $Q$ - $V$ ) relations of the WT and mutant channels were fitted with the Boltzmann equation as follows:

$$\frac{G}{G_{Max}} = \frac{1}{1 + e^{-ze(V-V_{1/2})/kT}} \quad (1)$$

or

$$\frac{Q}{Q_{Max}} = \frac{1}{1 + e^{-ze(V-V_{HC})/kT}}, \quad (2)$$

where  $z$  is the number of equivalent charges,  $V_{1/2}$  is the voltage for channel in half activation,  $e$  is the elementary charge,  $k$  is the Boltzmann constant, and  $T$  is the absolute temperature. In Eq. 2,  $z_1$  is the gating charge associated with voltage sensor movement,  $V_{HC}$  is the voltage for half of the gating charge movements at the closed conformation of the channel, and the other parameters have the same meaning as in Eq. 1. Curve fittings were done with Igor Pro software (WaveMetrics, Lake Oswego, OR) using the Levenberg-Marquardt algorithm to perform nonlinear least squares fits. The means of the data were obtained by averaging from 4 to 30 patches and error bars represent mean  $\pm$  SE. Statistics were performed using SigmaStat 3.5 software (Systat Software, San Jose, CA); one-way analysis of variance with an all pairwise multiple comparison procedure (Tukey test) was performed. A  $p$  value  $< 0.05$  was considered significant.

## RESULTS

### Mutations of voltage-sensing residues disrupt the $\beta 1$ -induced enhancement of $Ca^{2+}$ sensitivity

Among all charged residues in the VSD of Slo1 channels, neutralization of D153, R167, D186, or R213 reduces the number of equivalent gating charge. Thus, these residues are proposed as voltage-sensing residues (34). The mutations of these residues also alter other parameters ( $V_{HC}$ ,  $L_0$ , and  $D$ ) for voltage-dependent gating, indicating that these mutations profoundly affect the conformation and movements of the voltage sensor and the activation gate. Hence, we first studied the effects of these mutations on the function of the  $\beta 1$ - and  $\beta 2$ -subunits.

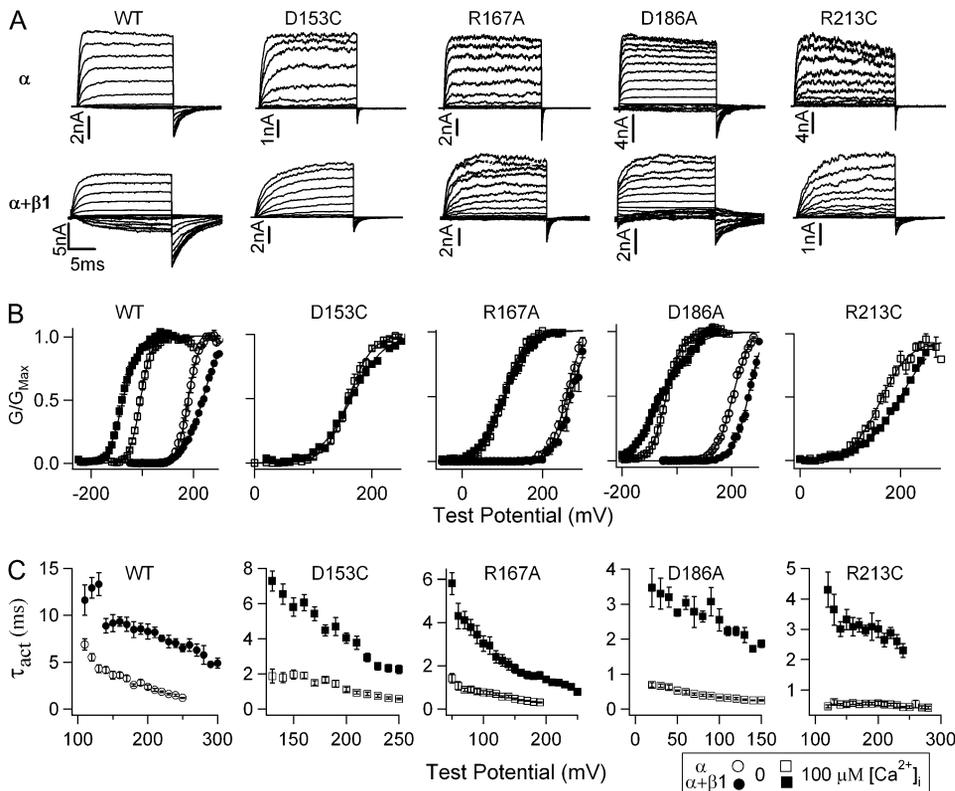
The coexpression of the  $\beta 1$ -subunit induces three major effects on the ionic currents of WT BK channels. First, the  $\beta 1$ -subunit slows the activation and deactivation kinetics in 0

(20) and saturating  $100 \mu M [Ca^{2+}]_i$  (Fig. 2, A and C). Second, the  $\beta 1$ -subunit reduces the steepness of the steady-state  $G$ - $V$  relationship (Fig. 2 B). The equivalent charge ( $z$ ) that is proportional to the steepness of the  $G$ - $V$  relationship decreases from  $1.28 \pm 0.02$  to  $0.72 \pm 0.01$  in 0  $[Ca^{2+}]_i$  and from  $1.32 \pm 0.05$  to  $0.88 \pm 0.03$  in  $100 \mu M [Ca^{2+}]_i$ . Third, the  $\beta 1$ -subunit increases the shift of  $G$ - $V$  relationships to more negative voltages (a leftward shift) in response to increasing  $[Ca^{2+}]_i$ ; the change in  $G$ - $V$  relationship is  $-83.6 \pm 5.7$  mV more with than without the coexpression of the  $\beta 1$ -subunit in  $100 \mu M [Ca^{2+}]_i$ .

Individual mutation of the four voltage-sensing residues (D153C, R167A, D186A, and R213C) reduces the  $z_T$  by 25% to 55% and shifts the  $V_{HC}$  in mSlo1 channels (34). Consequently, the voltage dependence of channel opening, measured by the position and the steepness of the  $G$ - $V$  relationship, is changed (Fig. 2 B). Among the four mutations, D153C and R213C shift the  $G$ - $V$  relationship to more positive voltages (a rightward shift) to such an extent, their macroscopic currents can only be measured in  $[Ca^{2+}]_i > 10 \mu M$  to obtain reliable  $G$ - $V$  relations (34). Therefore, we first examined if the mutations affect  $\beta 1$  function at the saturating  $[Ca^{2+}]_i$  of  $100 \mu M$ .

For the four mutant channels, the  $G$ - $V$  relationship with the  $\beta 1$ -subunit in  $100 \mu M [Ca^{2+}]_i$  is either at a similar or a more positive voltage range than without the  $\beta 1$ -subunit (Fig. 2 B, *open* and *solid squares*). However, the  $\beta 1$ -subunit still slows the rate of activation and deactivation of the mutant channels (Fig. 2, A and C), indicating that these mutations do not abolish the association with the  $\beta 1$ -subunit. In addition, the  $\beta 1$ -subunit reduces the slope of the  $G$ - $V$  relationship of D153C ( $z$ -values change from  $1.31 \pm 0.06$  to  $0.94 \pm 0.03$ ) and D186A ( $z$ -values change from  $1.05 \pm 0.06$  to  $0.52 \pm 0.07$ ) channels in  $100 \mu M [Ca^{2+}]_i$  in a manner similar to the WT channel ( $z$ -values change from  $1.32 \pm 0.05$  to  $0.88 \pm 0.03$ ) (Fig. 2 B). For mutations R167A and R213C, the  $\beta 1$ -subunit causes a relatively small reduction in the slope of the  $G$ - $V$  relationship in  $100 \mu M [Ca^{2+}]_i$  ( $z$ -values change from  $1.06 \pm 0.06$  to  $0.85 \pm 0.03$  and  $0.90 \pm 0.05$  to  $0.72 \pm 0.02$ , respectively). The  $G$ - $V$  relationships of R167A +  $\beta 1$ , D186A +  $\beta 1$  in 0  $[Ca^{2+}]_i$ , and R213C +  $\beta 1$  in  $100 \mu M [Ca^{2+}]_i$  does not reach saturation at 300 mV, and so the actual  $z$ -values would be even smaller than those calculated from these  $G$ - $V$  relations. Therefore, the mutations of the voltage-sensing residues specifically disrupt the  $\beta 1$ -induced shift of the  $G$ - $V$  relationship in high  $[Ca^{2+}]_i$  without a clear effect on the ability of the  $\beta 1$ -subunit to change activation kinetics or the steepness of the  $G$ - $V$  relationship.

For the WT channels, the enhanced leftward  $G$ - $V$  shift induced by the  $\beta 1$ -subunit becomes less pronounced in reduced  $[Ca^{2+}]_i$  (Figs. 2 B and 3). In 0  $[Ca^{2+}]_i$ , the  $\beta 1$ -subunit even induces a rightward  $G$ - $V$  shift. This  $\beta 1$ -induced, greater leftward shift of the  $G$ - $V$  relationship in response to increasing  $Ca^{2+}$  concentrations has been referred to as an enhanced  $Ca^{2+}$  sensitivity of channel activation (20,22,23,



**FIGURE 2** Mutations of the voltage-sensing residues in BK channels abolish  $\beta$ 1-induced  $G-V$  shifts in 100  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$ . (A) Macroscopic current traces in 100  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$  for WT and mutant mSlo1 (upper panels) and WT and mutant mSlo1 +  $\beta$ 1 channels (lower panels). Test pulses were with 20-mV increments in the same voltage range as in panel B for corresponding constructs. Repolarization potentials for WT, WT +  $\beta$ 1, D153C, D153C +  $\beta$ 1, R167A, R167A +  $\beta$ 1, D186A, D186A +  $\beta$ 1, R213C, and R213C +  $\beta$ 1 channels were  $-100$ ,  $-160$ ,  $-80$ ,  $-80$ ,  $-100$ ,  $-80$ ,  $-100$ ,  $-100$ ,  $-80$ , and  $-80$  mV, respectively. (B) Mean  $G-V$  relationships of WT and mutant mSlo1 channels expressed with and without  $\beta$ 1-subunit in 0 and 100  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$ . The  $G-V$  relationships are fit with the Boltzmann relation (solid line, see Experimental Procedures). (C) The  $\beta$ 1-subunit prolongs the activation time course of WT and mutant mSlo1 channels. Activation time constants ( $\tau_{\text{act}}$ ) were obtained by fitting current traces at various voltages with a single exponential function.

27,38,39). Fig. 3 A shows the  $V_{1/2}-[\text{Ca}^{2+}]_i$  relationship for R167A and WT mSlo1 channels in six different  $[\text{Ca}^{2+}]_i$ . Although the  $\beta$ 1-induced leftward  $G-V$  shift for WT channels increases with increasing  $[\text{Ca}^{2+}]_i$ , mutation R167A completely abolishes the  $\beta$ 1-induced  $G-V$  shift in all  $[\text{Ca}^{2+}]_i$ . In contrast, the  $\beta$ 1-induced  $G-V$  shifts in varying  $[\text{Ca}^{2+}]_i$  for D186A mutant channels are not that simple (Fig. 3 B). If we simply compare  $V_{1/2}$ -values at each individual  $[\text{Ca}^{2+}]_i$  for D186A, the  $\beta$ 1-induced leftward shift of the  $G-V$  relationship seems to be abolished by this mutation, because the  $\beta$ 1-subunit causes either a large rightward shift or no significant shift in any specific  $[\text{Ca}^{2+}]_i$ . However, if we compare the  $V_{1/2}$  difference between 0 and 100  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$  ( $\Delta V_{1/2-\text{Ca}}$ ), i.e., the total shift of the  $G-V$  relationship caused by  $\text{Ca}^{2+}$  binding, it is  $-71.6 \pm 3.1$  mV greater with the  $\beta$ 1-subunit than without it. As a comparison, the  $\beta$ 1-subunit enhances  $\Delta V_{1/2-\text{Ca}}$  by  $-128.0 \pm 4.7$  mV for the WT channels. Therefore, the mutation D186A does not completely abolish the  $\beta$ 1-induced enhancement of  $\text{Ca}^{2+}$  sensitivity but reduces it by 45%.

Because mutations D153C and R213C shift the  $G-V$  relationship to extremely positive voltages, it is difficult to measure the effects of the  $\beta$ 1-subunit in  $[\text{Ca}^{2+}]_i$  lower than 100  $\mu\text{M}$ . Therefore, we do not know whether the lack of the  $G-V$  shift in 100  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$  is because the  $\beta$ 1-subunit loses its ability to increase  $\text{Ca}^{2+}$  sensitivity of the mutant channels or is simply because the  $\beta$ 1-subunit shifts the  $G-V$  relationship of the mutant channels to more positive voltages in all  $[\text{Ca}^{2+}]_i$ . Thus, comparing  $V_{1/2}$ -values in one specific  $[\text{Ca}^{2+}]_i$

might not be sufficient to represent the  $\beta$ 1-induced changes of the apparent  $\text{Ca}^{2+}$  sensitivity. To bring back the  $G-V$  relationship of these two mutant channels to less positive voltages, we made double mutations D153C:E386A and R213C:E321A. E321 is located at the COOH-terminus of S6 and E386 is located at the cytoplasmic domain (Fig. 1). Compared with WT channels, the single-site mutation E321A or E386A shifts the  $G-V$  relationship to more negative voltages by  $\sim -85$  or  $-65$  mV, respectively, at all  $[\text{Ca}^{2+}]_i$ -values between 0 and 100  $\mu\text{M}$  (Fig. 3, C and D). Most importantly, they have no significant effect on the  $\beta$ 1-induced enhancement of  $\text{Ca}^{2+}$  sensitivity (Fig. 3, C and D;  $p > 0.05$ ). Fig. 3, C and D, also show the  $V_{1/2}-[\text{Ca}^{2+}]_i$  relationship for D153C:E386A and R213C:E321A mutant channels. The  $\beta$ 1-subunit fails to alter the  $G-V$  shift of D153C:E386A double mutant at any  $[\text{Ca}^{2+}]_i$  between 2 to 100  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$ , consistent with the results of the single-site mutant D153C in 100  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$  (Figs. 2 B and 3 C). In the case of R213C:E321A, there is a parallel rightward shift of the  $G-V$  relationship with the  $\beta$ 1-subunit at all  $[\text{Ca}^{2+}]_i$ , which is also consistent with the result of the single-site mutant R213C in 100  $\mu\text{M}$   $[\text{Ca}^{2+}]_i$  (Figs. 2 B and 3 D). Similar to D153C and R213C currents, the activation and deactivation kinetics of the currents of double mutants slowed in the presence of the  $\beta$ 1-subunit (data not shown), indicating that the  $\beta$ 1-subunit still associated with the double mutant channels. Therefore, D153C and R213C mutations abolish the  $\beta$ 1-subunit's effect of increasing  $\text{Ca}^{2+}$  sensitivity.

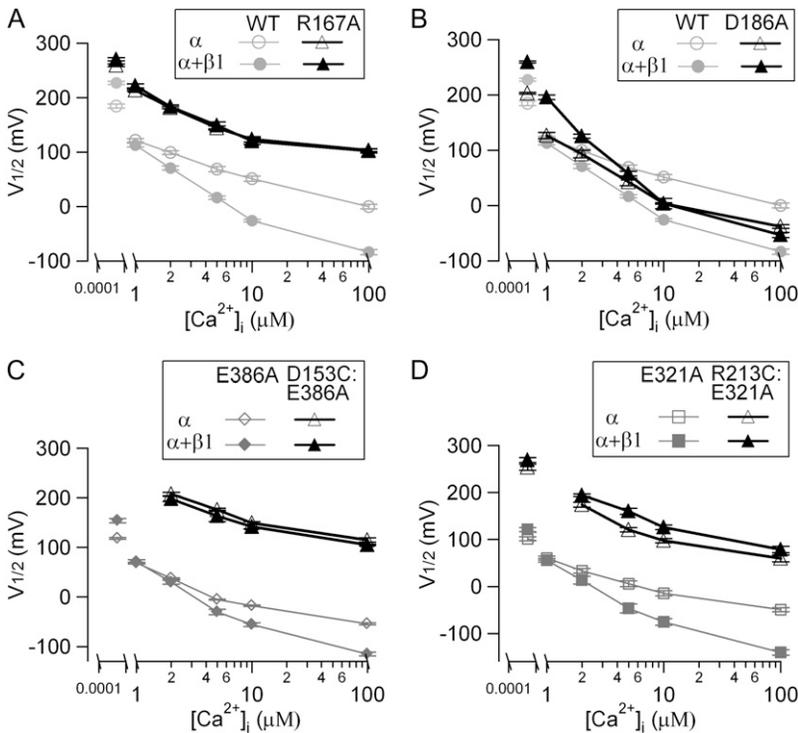


FIGURE 3 Mutations of voltage-sensing residues significantly reduced the  $\beta 1$ -induced enhancement of  $Ca^{2+}$  sensitivity. (A and B)  $V_{1/2}$ - $[Ca^{2+}]_i$  relationships of WT, R167A, and D186A mSlo1 channels with and without  $\beta 1$ -subunit. (C)  $V_{1/2}$ - $[Ca^{2+}]_i$  relationship of E386A and D153C:E386A mSlo1 channels with and without  $\beta 1$ -subunit. The  $G$ - $V$  relationship of D153C:E386A is shifted too far rightward to record any measurable currents at 0.5 nM  $[Ca^{2+}]_i$ . (D)  $V_{1/2}$ - $[Ca^{2+}]_i$  relationship of E321A and R213C:E321A mSlo1 channels with and without  $\beta 1$ -subunit.

### Mutations of voltage-sensing residues do not reduce the $\beta 2$ -induced enhancement of $Ca^{2+}$ sensitivity

The coexpression of the  $\beta 2$ -subunit with WT BK channels induces inactivation and, similar to the  $\beta 1$ -subunit, a larger leftward shift of the  $G$ - $V$  relationship in response to in-

creasing  $Ca^{2+}$  concentrations, which is referred to as the enhancement of  $Ca^{2+}$  sensitivity (20,38–40). To examine the role of voltage-sensing residues in the  $\beta 2$ -induced  $Ca^{2+}$  sensitivity enhancement, we coexpressed the N-terminus deleted  $\beta 2$ -subunit ( $\beta 2ND$ -subunit, see Experimental Procedures), which removes the inactivation peptide (4,20,26, 41), with the mutations of all voltage-sensing residues,

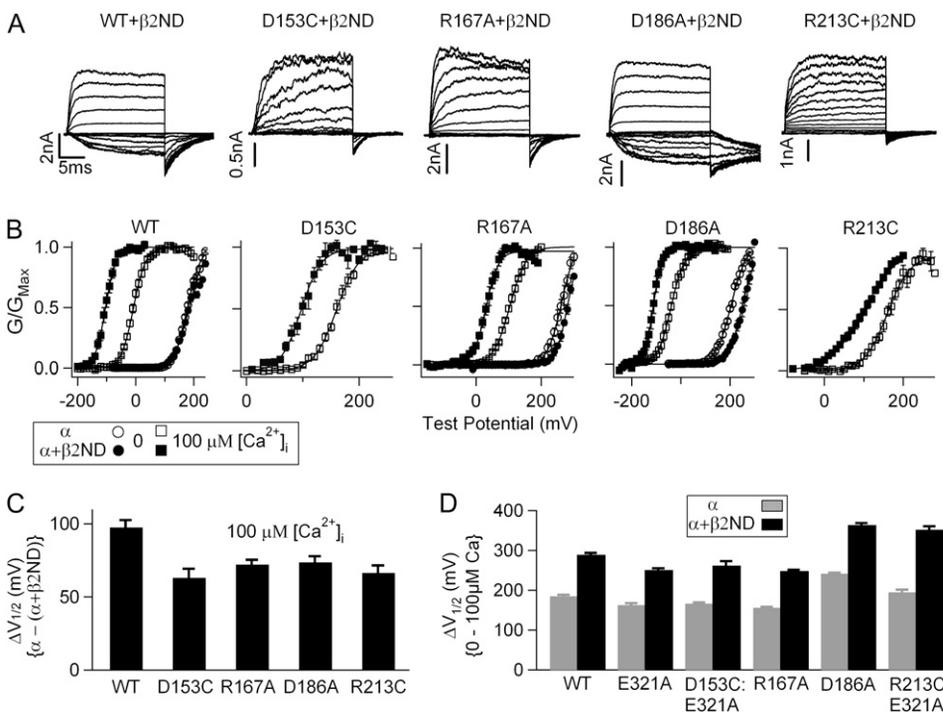


FIGURE 4 Mutations of voltage-sensing residues do not reduce the  $\beta 2$ -induced enhancement of  $Ca^{2+}$  sensitivity. (A) Macroscopic current traces in 100  $\mu M$   $[Ca^{2+}]_i$  of WT and mutant mSlo1 +  $\beta 2ND$ . Test pulses were with 20-mV increments. Repolarization potentials for WT +  $\beta 2$ , D153C +  $\beta 2$ , R167A +  $\beta 2$ , D186A +  $\beta 2$ , and R213C +  $\beta 2$  channels were -160, -100, -100, -120, and -100 mV, respectively. (B) Mean  $G$ - $V$  relationships of WT and mutant mSlo1 channels expressed with and without the  $\beta 2ND$ -subunit in 0 and 100  $\mu M$   $[Ca^{2+}]_i$ .  $G$ - $V$  relationships are fit with the Boltzmann relation (solid line). (C)  $\beta 2ND$ -induced  $V_{1/2}$  shifts of WT and mutant channels in 100  $\mu M$   $[Ca^{2+}]_i$ . (D)  $V_{1/2}$  shifts from 0 to 100  $\mu M$   $[Ca^{2+}]_i$  for WT and mutant channels with and without  $\beta 2ND$ -subunit.

D153C, R167A, D186A, and R213C, respectively. Similar to the effects of the  $\beta 1$ -subunit on the WT channels, the  $\beta 2$ ND-subunit slows down the activation and deactivation kinetics of these mutant channels (Fig. 4 A). The  $\beta 2$ ND-subunit also induced a leftward shift of the  $G$ - $V$  relationship for each of the mutant channels in  $100 \mu\text{M} [\text{Ca}^{2+}]_i$  (Fig. 4, B and C). Fig. 4 D plots  $\Delta V_{1/2-\text{Ca}}$  between 0 and  $100 \mu\text{M} [\text{Ca}^{2+}]_i$  for WT and mutant channels with and without the  $\beta 2$ ND-subunit. Because mutations D153C and R213C shift the  $G$ - $V$  relationship to extremely positive voltages, it is difficult to measure the effects of the  $\beta 2$ -subunit in 0  $[\text{Ca}^{2+}]_i$ . Therefore, we studied the D153C:E321A and R213C:E321A channels, for which we could record currents in both 0 and  $100 \mu\text{M} [\text{Ca}^{2+}]_i$  with and without the  $\beta 2$ ND-subunit (Fig. 4 D). The  $\beta 2$ ND can enhance the  $\text{Ca}^{2+}$  sensitivity for all four mutant channels, as it does for WT channels. Taken together, the disruption of the VSD movements by mutating voltage-sensing residues does not significantly influence the effect of  $\beta 2$ ND on  $\text{Ca}^{2+}$  sensitivity. The differential effects of the mutations on the  $\beta 1$ - and  $\beta 2$ -subunits suggest that these two subunits modulate  $\text{Ca}^{2+}$ -dependent activation of BK channels through different molecular mechanisms.

### Differential effects of mutating nonvoltage-sensing residues on the $\beta 1$ - and $\beta 2$ -induced enhancement of $\text{Ca}^{2+}$ sensitivity

The mutations of the voltage-sensing residues affect the voltage sensor in two major aspects: they reduce the number of  $z_j$  and shift  $V_{\text{HC}}$  to more positive ranges. These similar effects suggest that the mutations of the voltage-sensing residues may cause similar perturbations of the conformation and movements of the voltage sensor, whereas mutations of nonvoltage-sensing residues that do not cause the same functional effects may perturb the conformation and movements of the voltage sensor differently. To examine how these mutations affect the function of  $\beta$ -subunits, we studied R207Q and Y163E, which do not reduce  $z_j$  but shift  $V_{\text{HC}}$  to either negative or positive voltage ranges (34).

R207 is one of three Arg residues in S4, whereas Y163 is located in S2 (Fig. 1). Similar to the mutations of the voltage-sensing residues, in 0  $[\text{Ca}^{2+}]_i$ , mutation R207Q reduced the steepness of the  $G$ - $V$  relationship of mSlo1 channels ( $z = 0.57 \pm 0.03$  vs.  $1.28 \pm 0.02$  for WT) (34,42) (Fig. 5 A). To the contrary, Y163E increased the steepness of the  $G$ - $V$  relationship ( $z = 1.68 \pm 0.10$ ) (Fig. 5 B). They also shifted

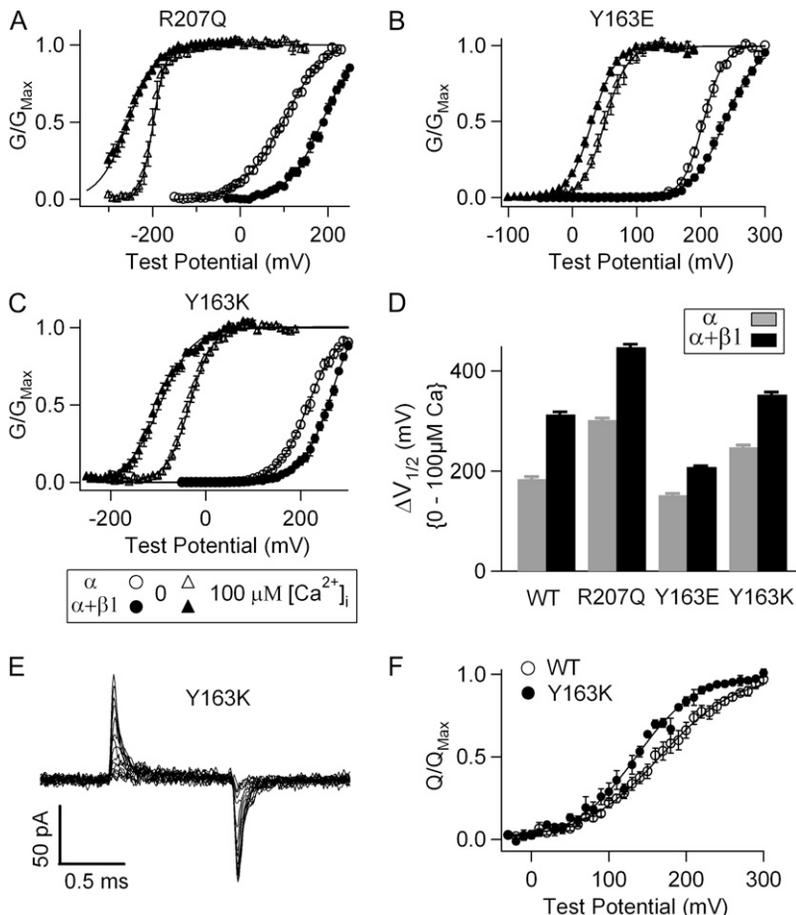


FIGURE 5 Mutations of nonvoltage-sensing residues R207 and Y163 exhibit differential effects on the  $\beta 1$ -induced enhancement of  $\text{Ca}^{2+}$  sensitivity. (A–C) Mean  $G$ - $V$  relationships of R207Q, Y163E, and Y163K channels with and without  $\beta 1$ -subunit in 0 and  $100 \mu\text{M} [\text{Ca}^{2+}]_i$ . The  $G$ - $V$  relationships are fit with the Boltzmann relation (solid lines). (D)  $V_{1/2}$  shifts from 0 to  $100 \mu\text{M} [\text{Ca}^{2+}]_i$  for WT, R207Q, Y163E, and Y163K channels with and without  $\beta 1$ -subunit. (E) Gating current traces for Y163K channels. Test pulses were from  $-20$  to  $280$  mV with  $20$ -mV increments. (F)  $Q_c$ - $V$  relations for WT and Y163K channels.  $Q_c$  was obtained by integrating the on-gating current over time. Solid lines are fits to the Boltzmann relation.

the  $G$ - $V$  relationship in opposite directions at 0  $[Ca^{2+}]_i$ . R207Q shifted  $V_{1/2}$  to hyperpolarizing potentials by  $-80.8 \pm 3.6$  mV, whereas Y163E resembled the mutations of the voltage-sensing residues by shifting  $V_{1/2}$  to depolarizing potentials by  $+23.7 \pm 3.2$  mV (Fig. 5, *A* and *B*).

The  $\beta$ 1-subunit enhances  $\Delta V_{1/2-Ca}$  between 0 and 100  $\mu M$   $[Ca^{2+}]_i$  by  $146.1 \pm 5.1$  mV for R207Q channels and by  $55.5 \pm 3.0$  mV for Y163E (Fig. 5), either an increase or a decrease from the  $\beta$ 1-induced enhancement of  $\Delta V_{1/2-Ca}$  for WT channels ( $128.0 \pm 4.7$  mV). Given these results, it seems that the  $\beta$ 1-induced enhancement of  $Ca^{2+}$  sensitivity is not correlated with only a change of the shape or position of the  $G$ - $V$  relationship or the reduction of the number of equivalent gating charges. However, all the mutations that reduce or abolish the ability of the  $\beta$ 1-subunit to enhance  $Ca^{2+}$  sensitivity also shift  $V_{HC}$  to positive voltage ranges (Figs. 3 and 5) (34).

To further test if a positive shift of  $V_{HC}$  is correlated to the reduction of  $\beta$ 1-induced enhancement of  $Ca^{2+}$  sensitivity, we studied mutation Y163K. Unlike the mutation of the same residue (Y163E), Y163K does not reduce  $\beta$ 1-induced enhancement of  $Ca^{2+}$  sensitivity significantly ( $p > 0.05$ ) (Fig. 5, *C* and *D*). The  $\beta$ 1-subunit enhances  $\Delta V_{1/2-Ca}$  between 0 and 100  $\mu M$   $[Ca^{2+}]_i$  by  $105.5 \pm 5.0$  mV, similar to that of the WT mSlo1 ( $128.0 \pm 4.7$  mV) (Fig. 5 *D*). The effect of Y163K on  $V_{HC}$  has not been reported previously. Therefore, we measured the gating currents of Y163K at various voltages (Fig. 5 *E*) and plotted the relationship between the on gating charge and voltage ( $Q_C$ - $V$ ; Fig. 5 *F*) (21). The  $Q_C$ - $V$  relation was fit with a Boltzmann equation (21) to obtain the number of equivalent gating charge ( $z_j = 0.65 \pm 0.04$ ) and  $V_{HC}$  ( $136.1 \pm 3.0$  mV). Opposite to mutation Y163E, Y163K shifted  $V_{HC}$  to a less positive voltage. Thus, for the two mutations of the same residue, a larger reduction of  $\beta$ 1-in-

duced enhancement of  $Ca^{2+}$  sensitivity is correlated with a larger positive shift of  $V_{HC}$ . This result further suggests that  $\beta$ 1 regulation is sensitive to a specific change of the conformation and movements of the voltage sensor.

We also coexpressed the  $\beta$ 2ND-subunit with mutations R207Q, Y163E, and Y163K. The effects of the  $\beta$ 2ND-subunit on gating properties of the channel were not affected significantly by these mutations (Fig. 6). Importantly, the  $\beta$ 2ND-subunit did not reduce the  $\Delta V_{1/2-Ca}$  between 0 and 100  $\mu M$   $[Ca^{2+}]_i$  compared with the WT mSlo1 (Fig. 6 *D*). Thus, unlike the effects of the  $\beta$ 1-subunit, the effects of the  $\beta$ 2ND-subunit are not sensitive to alterations of the conformation and movements of the voltage sensor and the activation gate caused by the VSD mutations.

## DISCUSSION

The fine-tuning of BK channel properties by  $\beta$ -subunits greatly diversifies physiological roles of BK channels in various tissues. Each type of  $\beta$ -subunits may have been evolved to target different parts of BK channel  $\alpha$ -subunit to modulate various channel properties. Previous studies have shown that the N-terminus and the S0 transmembrane segment of Slo1 are important for the  $\beta$ 1-induced  $G$ - $V$  shift (27,28). In this study, we found that the disturbance of voltage sensor movements by mutations disrupted the  $\beta$ 1-induced enhancement of  $Ca^{2+}$  sensitivity. For all the mutations, the reduction of the  $\beta$ 1-induced enhancement of  $Ca^{2+}$  sensitivity is correlated quantitatively with the positive shift of  $V_{HC}$  (Fig. 7 *A*). These results provide direct evidence that the effect of the  $\beta$ 1-subunit on  $Ca^{2+}$  sensitivity relies on the conformation and movements of the VSD. In contrast, the  $\beta$ 2-induced enhancement of  $Ca^{2+}$  sensitivity was not re-

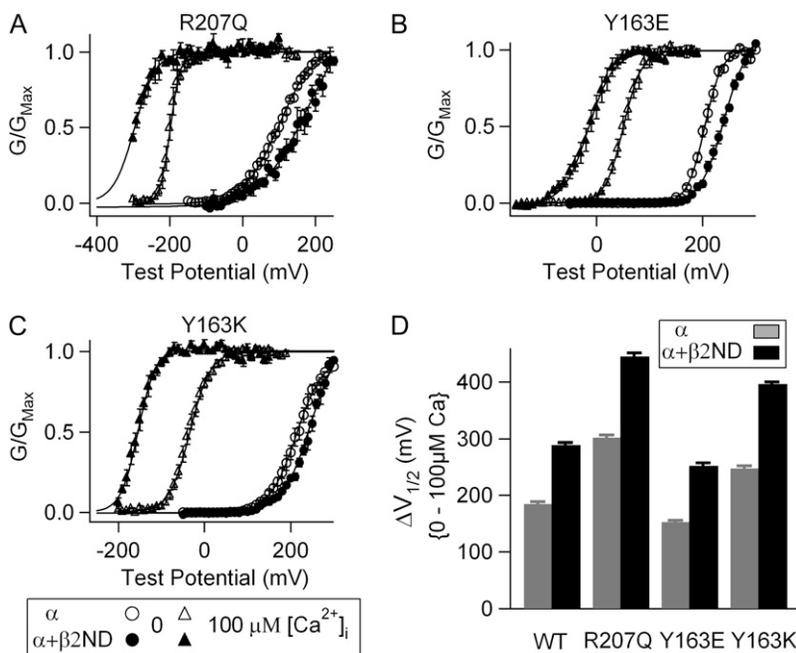


FIGURE 6 Mutations of nonvoltage-sensing residues R207 and Y163 do not reduce the  $\beta$ 2-induced enhancement of  $Ca^{2+}$  sensitivity. (A–C) Mean  $G$ - $V$  relationships of R207Q, Y163E, and Y163K channels with and without the  $\beta$ 2ND-subunit in 0 and 100  $\mu M$   $[Ca^{2+}]_i$ . The  $G$ - $V$  relationships are fit with the Boltzmann relation (solid line). (D)  $V_{1/2}$  shifts from 0 to 100  $\mu M$   $[Ca^{2+}]_i$  for WT and mutant channels with and without  $\beta$ 2ND-subunit.

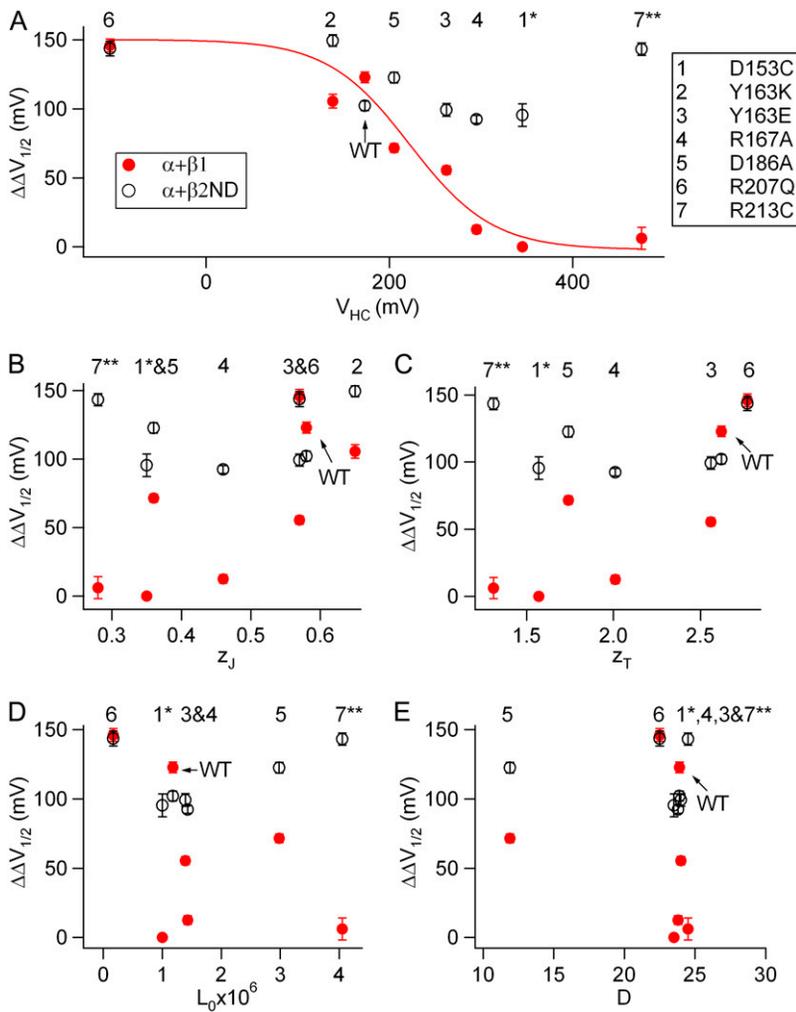


FIGURE 7 Relationship of the  $\beta$ 1- and  $\beta$ 2ND-induced enhancement of  $\text{Ca}^{2+}$  sensitivity with the parameters of HCA model for mutant mSlo1 channels (A,  $V_{\text{HC}}$ ) (B,  $z_j$ ) (C,  $z_T$ ) (D,  $L_0$ ) and (E and D). The parameters of the HCA model (Table 1) for the WT and mutant mSlo1 channels except for Y163K were adapted from Ma et al. (34). The mutations are represented by numbers (panel A) in all panels.  $\Delta\Delta V_{1/2}$  is the  $\beta$ 1- or  $\beta$ 2-induced enhancement of  $\text{Ca}^{2+}$  sensitivity from 0 to 100  $\mu\text{M}$  [ $\text{Ca}^{2+}$ ];  $\Delta\Delta V_{1/2} = \Delta V_{1/2 - \text{mSlo1} + \beta (0\text{Ca} - 100\text{Ca})} - \Delta V_{1/2 - \text{mSlo1} (0\text{Ca} - 100\text{Ca})}$ .  $\Delta\Delta V_{1/2}$  (induced by the  $\beta$ 1-subunit)- $V_{\text{HC}}$  relationship is empirically fit with the Boltzmann function (panel A, solid line). (1\*) The  $\beta$ 1-induced  $\Delta\Delta V_{1/2}$  for D153C was assumed to be 0 according to the result of D153C:E386A channels (Fig. 3 C); and the  $\beta$ 2-induced  $\Delta\Delta V_{1/2}$  was for D153C calculated from the result of D153C:E321A channels (Fig. 4 D). (7\*\*) The  $\beta$ 1- and  $\beta$ 2-induced  $\Delta\Delta V_{1/2}$ -values for R213C were calculated from the results of R213C:E321A channels (Figs. 3 D and 4 D).

duced significantly by these mutations regardless of how voltage sensor movements were altered (Fig. 7), suggesting that the voltage sensor movements are not directly linked to the function of the  $\beta$ 2-subunit. These results suggest that the  $\beta$ 2-subunit may interact with a different molecular domain of Slo1 to alter the responses of the channel to  $\text{Ca}^{2+}$  binding.

The  $\beta$ 1- and  $\beta$ 2-subunits share substantial structural similarities, with 43% identical or 66% conservative residues (7). They also share similar function by enhancing the apparent  $\text{Ca}^{2+}$  sensitivity of activation. Therefore, it is remarkable that distinct molecular mechanisms may underlie the regulation of  $\text{Ca}^{2+}$  sensitivity by these two  $\beta$ -subunits. According to the study by Orio and Latorre (20), one of the most prominent differences between the  $\beta$ 1- and  $\beta$ 2-subunit is that, whereas the effects of the  $\beta$ 1-subunit can be explained by changes in voltage sensor movements, the effects of the  $\beta$ 2-subunit can only be determined if the allosteric factor for  $\text{Ca}^{2+}$  dependence also is altered. Similar to this result, our finding shows that some mutations of voltage-sensing residues are sufficient to abolish the effect of  $\beta$ 1 in enhancing  $\text{Ca}^{2+}$  sensitivity but have a much smaller or a complete lack of impact on the  $\beta$ 2

effect. These results suggest that the  $\beta$ 1-subunit may affect the voltage sensor to alter  $\text{Ca}^{2+}$  sensitivity; whereas the  $\beta$ 2-subunit may influence  $\text{Ca}^{2+}$  dependent mechanism more directly. It should be noted that such a mechanistic distinction may explain the primary effects of the two  $\beta$ -subunits, although each  $\beta$ -subunit may use both mechanisms for their total effects. For example, it has been suggested that the  $\beta$ 1-subunit enhances  $\text{Ca}^{2+}$  sensitivity by affecting voltage sensor movements; in addition, however, it also changes  $\text{Ca}^{2+}$  binding (22,24). Likewise, it has been proposed that the  $\beta$ 2-subunit alters voltage sensor movements. In a recent voltage clamp fluorometry study (25), Savalli et al. demonstrated that the  $\beta$ 2-subunit induces a leftward shift on the voltage axis of the fluorescence versus voltage (F(V)) relations when the S3-S4 linker of the  $\alpha$ -subunit was labeled with thiol-reactive fluorescent dyes. The authors suggested that this result indicated an alteration of the voltage sensor movements due to the association of the  $\beta$ 2-subunit. Notwithstanding these complexities, our results indicate that various structural domains of both the  $\alpha$ -subunits and the  $\beta$ -subunits can interact to modulate different molecular processes during channel gating and produce similar functional changes. Our results

also provide the basis to further identify these structural domains and molecular processes that are important for the regulation of BK channels by  $\beta$ -subunits.

How does the  $\beta 1$ -subunit alter the voltage-dependent gating to enhance  $\text{Ca}^{2+}$  sensitivity (i.e., the  $\beta$ -subunit-induced larger leftward shift of  $G$ - $V$  relationship in response to increasing  $\text{Ca}^{2+}$  concentrations)? Orio and Latorre (20) found that the association of human  $\beta 1$  reduced the number of  $z_j$ , decreased the intrinsic  $L_0$ , and increased the allosteric coupling between voltage sensor movements and channel opening ( $D$ ). The reduction of  $z_j$  was proposed as the primary cause for an enhanced  $\text{Ca}^{2+}$  sensitivity. In a study of bovine  $\beta 1$ , Bao and Cox (22) found that a shift of the voltage sensor movements to more negative voltages ( $V_{\text{HC}}$ ) induced by the  $\beta 1$ -subunit was the primary reason for an enhanced  $\text{Ca}^{2+}$  sensitivity. More recently, Wang and Brenner (23) found that mouse  $\beta 1$  shifted  $V_{\text{HC}}$  to negative voltages and reduced  $L_0$ , but the enhancement of  $\text{Ca}^{2+}$  sensitivity was primarily due to the changes in  $L_0$ . These studies have made it clear that the enhancement of  $\text{Ca}^{2+}$  sensitivity by the  $\beta 1$ -subunit is accompanied by significant changes in voltage-dependent gating, suggesting that the voltage sensor is a critical structural component for  $\beta 1$  regulation. However, although the  $\beta 1$ -subunits from three different species all enhance  $\text{Ca}^{2+}$  sensitivity, this enhancement may not be the consequence of changes in any particular voltage-dependent parameters. No single gating parameter was significantly altered by all three  $\beta 1$  orthologs. These results seem to suggest that the enhancement of  $\text{Ca}^{2+}$  sensitivity and the changes in voltage dependence are all the consequences of a change in channel conformation and movements during gating, but that none of the phenomenological changes in gating parameters is the cause of others.

The results of this study demonstrate that mutating the voltage sensor can alter the enhancement of  $\text{Ca}^{2+}$  sensitivity by the  $\beta 1$ -subunit. These mutations also change voltage dependence of channel gating (34). Therefore, our results indicate that the voltage sensor is the primary target regulated by the  $\beta 1$ -subunit for all the functional consequences of  $\beta 1$  association. We compared the changes in the  $\text{Ca}^{2+}$  sensitivity caused by  $\beta 1$  to the changes in voltage dependence caused by these mutations and found that the changes in the enhancement of  $\text{Ca}^{2+}$  sensitivity is quantitatively correlated with the shift of  $V_{\text{HC}}$  but not with other parameters (Fig. 7). These comparisons indicate that only a specific perturbation of the conformation and movements of the voltage sensor would disrupt the ability of the  $\beta 1$ -subunit to enhance  $\text{Ca}^{2+}$  sensitivity. Such a specific perturbation also alters the stability of the voltage sensor at activated state. Further study is needed to identify the molecular mechanism for this specific perturbation and its correlation with  $V_{\text{HC}}$ .

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