Mechanism of Increased BK Channel Activation from a Channel Mutation that Causes Epilepsy

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Concerted depolarization and Ca2+ rise during neuronal action potentials activate large-conductance Ca2+- and voltage-dependent K+ (BK) channels, whose robust K+ currents increase the rate of action potential repolarization. Gain-of-function BK channels in mouse knockout of the inhibitory β4 subunit and in a human mutation (D369G) have been linked to epilepsy. Here, we investigate mechanisms underlying the gain-of-function effects of the equivalent mouse mutation (αD369G), its modulation by the β4 subunit, and potential consequences of the mutation on BK currents during action potentials. Kinetic analysis in the context of the Horrigan-Aldrich allosteric gating model revealed that changes in intrinsic and Ca2+-dependent gating largely account for the gain-of-function effects. D369G causes a greater than twofold increase in the closed-to-open equilibrium constant (6.6e-7→1.65e-5) and an approximate twofold decrease in Ca2+-dissociation constants (closed channel: 11.3→5.2 μM; open channel: 0.92→0.54 μM). The β4 subunit inhibits mutant channels through a slowing of activation kinetics. In physiological recording solutions, we established the Ca2+ dependence of current recruitment during action potential–shaped stimuli. D369G and β4 have opposing effects on BK current recruitment, where D369G reduces and β4 increases K1/2 (K1/2 μM: αWT 13.7, αD369G 6.3, αWT/β4 24.8, and αD369G/β4 15.0). Collectively, our results suggest that the D369G enhancement of intrinsic gating and Ca2+ binding underlies greater contributions of BK current in the sharpening of action potentials for both α and α/β4 channels.

INTRODUCTION

Large-conductance Ca2+- and voltage-activated K+ (BK) channels open in response to additive effects of Ca2+ and voltage to contribute to action potential repolarization in neurons. It is generally assumed that outward K+ currents through BK channels repolarize the cell and reduce excitability (Faber and Sah, 2003). However, in some neurons, the sharpening of action potentials due to increased BK channel activation has been found to facilitate high frequency firing (Brenner et al., 2005; Gu et al., 2007).

The observation that increased BK channel activation increases excitability in some neurons may explain the otherwise paradoxical finding that a human BK potassium channel gain-of-function mutation (D434G) is associated with epilepsy (Du et al., 2005). The D434G mutation resides in the RCK1 domain, a putative Ca2+-binding domain within the pore-forming α subunit (Jiang et al., 2001, Bao et al., 2002, Zeng et al., 2005). In heterologous expression systems, the D434G mutation speeds channel activation, increases steady-state open probabilities, and results in Ca2+-dependent G-V shifts consistent with increased Ca2+ sensitivity (Du et al., 2005; Diez-Sampedro et al., 2006).

In the context of the Horrigan-Aldrich (HA) model (Horrigan and Aldrich, 2002), BK channel gating is determined by three equilibria: a central “closed-to-open” step (also called intrinsic gating [L]), voltage sensor activation (J), and Ca2+ binding (K). These are coupled through allosteric interactions between them (C, D, E, respectively; see Table I). Changes in closed-to-open equilibrium can alter the apparent Ca2+ sensitivity of G-V relations (Wang and Brenner, 2006). In addition, increased “Ca2+ sensitivity” could arise through either changes in Ca2+ affinity (i.e., binding) or allosteric coupling between Ca2+ binding and gating.

In addition, it was observed that the inhibitory effect of β4 is lost in the D434G mutant channels (Diez-Sampedro et al., 2006). This is quite surprising because β subunit interaction domains have not previously been mapped to this region (RCK1 domain) of the channel (Wallner et al., 1996; Qian et al., 2002; Morrow et al., 2006). Further, this implies that the D434G epilepsy phenotype may partly result from a loss of modulation by β4.

Here, we seek to gain a better understanding of the effects of D434G mutation by using the equivalent mutation in the mouse BK channel (D369G). Guided by the HA allosteric gating model for BK channels (Horrigan and Aldrich, 2002), we used recording conditions to identify specific gating parameters altered by the mutation. We further reexamined effects of the mutation on
where $V$ is the test potential of $Q_{C}$ and $Q_{O}$, respectively

$\frac{D}{V_{h}} = \exp \left(\frac{z_{J} V}{kT}\right)$

$P_{k}$ of occupying $NP_{O}$

$G / G_{max} = 1 / \left(1 + e^{V_{m}/V_{t}}\right)$

$V_{1/2}$ is the membrane potential at half-maximal conductance, $Q$ is the effective gating charge, and $F$, $R$, and $T$ are constants.

For action potential–shaped stimuli, we used the physiological pipette solution described above. The action potential stimulus waveforms were ramp voltage steps designed to simulate action potentials from wild-type dentate gyrus (DG) granule neurons during 105-pA current injections (Brenner et al., 2005). Prepulse was $-80$ mV for 100 msec, followed by a 0.1-msec step to a threshold voltage of $-30$ mV. Action potential rise is from $-30$ to $+57$ mV over 0.5 msec. Repolarization is in three phases: from $+57$ to $-28$ mV over 2 msec, $-28$ to $-38$ mV for 0.56 msec, and $-38$ to $-42$ mV in 1.2 msec. To subtract capacitance and leak currents, a $P / T$ leak subtraction protocol was used from a holding potential of $-120$ mV. The action potential currents were normalized to maximal current in the same patch measured from the tail current (0 mV) after a maximal activating 40-msec square wave stimulus in 1 mM of internal calcium concentration.

**Single-Channel Analysis**

Single-channel opening events were obtained from patches containing one to hundreds of channels. Recordings were of 20 s to hundreds of seconds long. Analysis was performed using TAC and TACFIT programs (Bruxton Corporation). $NP_{O}$ was determined using either all-point amplitude histogram or by event detection using a $50\%$ amplitude criteria. The probability ($P_{O}$) of opening each open level ($k$) gave rise to $NP_{O}$. $NP_{O} = \sum_{k=1}^{K} P_{O}$ was then determined by normalizing $NP_{O}$ values by channel number ($N$).

$N$ was obtained from the instantaneous tail current amplitude during maximal opening at saturation ($Ca^{2+}$) divided by the single-channel current for each channel at the tail voltage.

**RESULTS**

D369G Increases BK Channel Opening

A mouse BK channel mutation (D369G) equivalent to $\alpha_{D434G}$ in humans (Fig. 1B) was generated and transiently
expressed in HEK 293 cells. Ionic currents were recorded using excised inside-out patches to allow control of [Ca^{2+}] at the cytoplasmic side of the membrane. Fig. 1A shows representative BK currents in response to test voltage steps in 2.1 μM of internal Ca^{2+}. Tail current amplitudes (G at −80 mV) were normalized to maximum tail current (G_{max} at −80 mV) to generate average G-V (G/G_{max} - V) relationships. Relative to α_{WT}, G-V relationships are consistently shifted to negative potentials for α_{D369G} (Fig. 1C). Plots of V_{1/2} (voltage at half-maximal G/G_{max}) versus Ca^{2+} demonstrate more dramatic shifts at intermediate Ca^{2+} (between 0.073 and 2.1 μM) and smaller shifts at low (nominal) and high Ca^{2+} (41 μM; Fig. 1D; values are listed in Table II). The D369G mutation, however, does not significantly alter the slope or the equivalent gating charge (Q) of the G-V relationship (Fig. 1E and Table II).

Effects of D369G on V_{1/2} and Q are qualitatively similar to previous descriptions of the equivalent human D434G mutation (Du et al., 2005; Diez-Sampedro et al., 2006), confirming that D369G is a gain-of-function mutation. We directly compared effects of the mutation on human (hslo) and mouse (mslo) channels at 41 and 0 μM Ca^{2+}. Although effects were qualitatively similar, D434G produced larger G-V shifts on hslo than D369G did on mslo. At 41 μM Ca^{2+}, \Delta V_{1/2} for hslo and mslo are \sim −35 and −20 mV, respectively (Fig. 1F). At 0 Ca^{2+}, \Delta V_{1/2} for hslo and mslo are \sim −36 and −16 mV, respectively (Fig. 1G). Whereas the size of G-V shift at 41 μM Ca^{2+} is comparable to previous results on the hslo mutant channel (Du et al., 2005; Diez-Sampedro et al., 2006), negative shift at 0 Ca^{2+} was not observed previously (Diez-Sampedro et al., 2006).

Increased Intrinsic Gating and Ca^{2+}-binding Affinities Underlie Increased Channel Opening of the D369G Mutation

To better understand how the D369G mutation alters BK channel gating, we used recording conditions that isolate the effects on intrinsic gating from Ca^{2+} and voltage-dependent gating. Fig. 2A illustrates how intrinsic gating (closed-to-open transition in the absence of Ca^{2+} binding and voltage sensor activation) can be examined. At 0 Ca^{2+}, we observe gating of primarily

Figure 1. D369G shifts mslo steady-state G-V relation to hyperpolarizing membrane potentials. (A) A family of currents from wild-type (top) or D369G mutant (bottom) BK channels composed of only the pore forming α subunits. Recorded in 2.1 μM Ca^{2+}, currents were evoked in response to 200-ms depolarizations at the indicated membrane potentials. (B) Alignment of amino acid sequence flanking the lysine (D) to glycine (G) epilepsy mutation. (C) Mean G-V relations at different Ca^{2+} for α_{WT} and α_{D369G}. Each point represents mean data from 5 to 26 experiments. Solid curves represent fits to the Boltzmann function. (D) Mean V_{1/2} and (E) mean effective gating charge (Q) values plotted as a function of Ca^{2+}. (F) D434G shifts G-V to more negative membrane potentials at 41 μM Ca^{2+} compared to D369G (hslo_α_{WT}: n = 9; hslo_α_{D434G}: n = 10; mslo_α_{WT}: n = 19; mslo_α_{D369G}: n = 18). (G) D434G shifts G-V to more negative membrane potentials at nominal Ca^{2+} compared to D369G (hslo_α_{WT}: n = 5; hslo_α_{D434G}: n = 5; mslo_α_{WT}: n = 12; mslo_α_{D369G}: n = 14). Symbols represent mean G/G_{max} data, curves represent fits to the Boltzmann function, and error bars represent SEM.
unliganded channels (i.e., with 0 Ca\(^{2+}\) bound). In the context of the HA model, these reside in 1 of 10 states: closed or open, with 0–4 voltage sensors activated (Fig. 2 A, Sub-Scheme a) (Horrigan and Aldrich, 2002). LogP\(_O\) at the limiting slope is only weakly voltage dependent, reflecting the weak voltage dependence of closed-to-open transition. Fitting the limiting slope phase of logP-V relations (Fig. 2 A, right panel) by

\[ \text{LogP}_O = \log(L_0 \exp\left(z_L V/kT\right)) \]  

The values shown are Boltzmann-fit parameters. They indicate mean ± SEM.

**Table II**

| Ca\(^{2+}\) (µM) | \( V_{1/2} \) (mV) | Q (e\(_0\)) | n | \( V_{1/2} \) (mV) | Q (e\(_0\)) | n | \( V_{1/2} \) (mV) | Q (e\(_0\)) | n |
|---|---|---|---|---|---|---|---|---|---|
| 0.006 | 177.6 ± 4.0 | 1.30 ± 0.06 | 12 | 161.5 ± 2.5 | 1.25 ± 0.05 | 14 | 246.1 ± 3.8 | 0.81 ± 0.23 | 4 |
| 0.073 | 162.5 ± 5.2 | 1.53 ± 0.12 | 13 | 126.2 ± 3.0 | 1.63 ± 0.11 | 9 | 206.4 ± 9.7 | 1.16 ± 0.16 | 6 |
| 0.363 | 130.2 ± 3.8 | 1.63 ± 0.11 | 15 | 85.6 ± 4.3 | 1.77 ± 0.14 | 16 | 155.8 ± 6.2 | 1.33 ± 0.10 | 19 |
| 2.1 | 57.3 ± 3.1 | 1.93 ± 0.08 | 26 | 16.2 ± 3.1 | 2.96 ± 0.09 | 22 | 74.5 ± 5.6 | 1.54 ± 0.06 | 19 |
| 41 | −15.7 ± 2.5 | 1.85 ± 0.09 | 19 | −34.3 ± 3.6 | 1.63 ± 0.08 | 18 | −36.9 ± 2.8 | 1.88 ± 0.04 | 38 |

Figure 2. D369G decreases the energetic barrier for channel to open. (A; left) According to the dual-allosteric mechanism (Horrigan et al., 1999; Horrigan and Aldrich, 2002), BK channel transitions between closed (C) and open (O) conformation is allosterically regulated by the state of four independent and identical voltage sensors. Sub-Scheme a represents BK channel’s gating scheme at 0 Ca\(^{2+}\). The channel resides in either the open or closed conformation, with 0–4 voltage sensors not activated), channels reside primarily in either C\(_O\) or O\(_0\) (Fig. 2 A, Sub-Scheme b) (Horrigan and Aldrich, 2002). LogP\(_O\) at the limiting slope is only weakly voltage dependent, reflecting the weak voltage dependence of closed-to-open transition. Fitting the limiting slope phase of logP-V relations (Fig. 2 A, right panel) by

\[ \text{LogP}_O = \log(L_0 \exp\left(z_L V/kT\right)) \]  

The values shown are Boltzmann-fit parameters. They indicate mean ± SEM.
provides an estimate of intrinsic gating ($L_0$) and the weak voltage dependence due to the closed-to-open transition ($Z_L$) (Horrigan and Aldrich, 2002). Fig. 2B shows representative currents recorded at 0 Ca$^{2+}$ for estimation of these parameters. Although these membrane patches contain tens to hundreds of channels, only single-channel openings were observed under these conditions. There is a striking drop in the voltage dependence of $P_o$ below $-20$ mV, which likely reflects relaxation of voltage sensors (and thus "intrinsic" gating). This is reflected in the average logP$_o$-V relationships that show a similar limiting slope voltage dependence ($Z_L$, dashed line) in both $\alpha_{WT}$ and $\alpha_{D369G}$ channels (Fig. 2C). However, $\alpha_{D369G}$ has an increased logP$_o$ relative to $\alpha_{WT}$ in this portion of the curve, indicating an increased intrinsic gating (Fig. 2C). Fitting of the logP$_o$-V relationship at the limiting slope (using Eq. 1) estimates a greater than twofold increase in intrinsic gating equilibrium constant for $\alpha_{D369G}$ over $\alpha_{WT}$ ($2.49 \pm 0.08 \times 10^{-6}$ vs. $1.12 \pm 0.02 \times 10^{-6}$, respectively; see Table III). This could contribute to the leftward G-V shift for $\alpha_{D369G}$ seen in nominal Ca$^{2+}$ (Fig. 1C). In addition, the observation that logP$_o$ deflects from the limiting slope at similar voltages ($\pm 20$ mV; Fig. 1C) for $\alpha_{WT}$ and $\alpha_{D369G}$ suggests that open-channel voltage sensors are not altered by the D369G mutation (Wang and Brenner, 2006).

To understand how the mutation might affect Ca$^{2+}$ sensitivity, we recorded current at very negative voltages, but over a range of Ca$^{2+}$ (Fig. 3, A and B). At very negative

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**TABLE III**

| Parameter | $\alpha_{WT}$ | $\alpha_{D369G}$ |
|-----------|--------------|------------------|
| $L_0$     | $1.12 \pm 0.02 \times 10^{-6}$ | $2.49 \pm 0.08 \times 10^{-6}$ |
| $K_C$ (µM) | $8.7 \pm 0.8$ | $3.8 \pm 1.0$ |
| $K_O$ (µM) | $1.0 \pm 0.2$ | $0.5 \pm 0.1$ |
| $C$       | $8.7 \pm 0.8$ | $7.5 \pm 0.8$ |
voltages where voltage sensors are not activated, the channel resides in 1 of 10 states: closed or open, with 0–4 subunits bound to Ca$^{2+}$ (Fig. 3 A, Sub-Scheme c). The limiting slope log$P_O$-V was fitted using

$$\log P_O = \log(L_0' \exp(Z_i V / kT))$$

(2)

to obtain an estimate of channel open probability at a corresponding Ca$^{2+}$ in the absence of voltage sensor activation (Horrigan and Aldrich, 2002). The limiting slope $P_O$'s at 0 mV ($L_0'$) were plotted as a function of Ca$^{2+}$ concentration and fitted using

$$\log L_0' = \log[I_0'\left(\frac{K_C}{[Ca^{2+}]^4} + 1\right)]$$

(3)

to estimate open- and closed-channel Ca$^{2+}$-binding affinity of $K_O$ and $K_C$, respectively, and coupling between Ca$^{2+}$ binding and gating ($C = K_C / K_O$) (Horrigan and Aldrich, 2002). For the $\alpha_{D369G}$ channels, the log($L_0'$) versus [Ca$^{2+}$] curve is shifted to lower Ca$^{2+}$ concentrations compared with $\alpha_{WT}$ channels, with a small decrease in the slope (Fig. 3 C). Fitting of the data using Eq. 3 indicates that the D369G mutation does not alter the coupling between Ca$^{2+}$ binding and gating ($C_{D369G} = 7.5 \pm 0.8$; $C_{WT} = 8.7 \pm 0.8$), but increases apparent Ca$^{2+}$-binding affinities of both the closed ($K_C_{D369G} = 3.8 \pm 1.0 \mu M$; $K_C_{WT} = 8.7 \pm 0.8 \mu M$) and open channels ($K_O_{D369G} = 0.5 \pm 0.1 \mu M$; $K_O_{WT} = 1.0 \pm 0.2 \mu M$; 2.3- and twofold, respectively; see Table III). In summary, the major effects of the D369G mutation are increases in apparent Ca$^{2+}$-binding affinities and intrinsic gating, both of which contribute to higher $P_O$.

To determine whether changes in intrinsic gating ($L_0$) and Ca$^{2+}$ affinities ($K_C$ and $K_O$) are sufficient to account for differences between $\alpha_{WT}$ to $\alpha_{D369G}$, we simultaneously fitted $P_O$ and log($P_O$)-V data (Fig. 4) using the allosteric gating model. The voltage-dependent parameters were fixed at $\alpha_{WT}$ values to determine if changes in other parameters are sufficient to fit the data. The resulting parameters are very similar to those obtained from the above measurements (Table IV) and fit the data reasonably well (Fig. 4, A and B). There is a predicted 2.5-fold increase in $L_0$ and a 2.2- and 1.7-fold increase in closed- (1/$K_C$) and open-channel (1/$K_O$) Ca$^{2+}$-binding affinity, respectively. These results indicate that changes in these parameters can largely account for the increased channel openings observed in the $\alpha_{D369G}$ mutant channels.

TABLE IV

| Gating Parameters Based on $P_O$ and log$P_O$ |
|-----------------------------------------------|
| $\alpha_{WT}$ | $\alpha_{D369G}$ |
| $L_0$ | 6.6e$^{-7} \pm 3e^{-8}$ | 1.65e$^{-7} \pm 3e^{-8}$ |
| $K_C$ (µM) | 11.3 ± 0.2 | 5.2 ± 0.1 |
| $K_O$ (µM) | 0.92 ± 0.02 | 0.54 ± 0.01 |
| $z_L$ (e$^{-}0$) | 4.9 ± 0.2 | 7.6 ± 0.2 |
| $z_J$ (e$^{-}0$) | 0.25 | 0.58 |
| $V_{hC}$ (mV) | 181.7 | |
| $V_{hO}$ (mV) | 14.8 | |

$\beta$4 Modulation of Steady-state and Kinetic Properties of D369G Channels

A previous study indicated that the BK channel’s accessory $\beta$4 subunit has little effect on the $V_{1/2}$ of human D434G channels (Diez-Sampedro et al., 2006). This raised the possibility that D369G perturbs co-assembly or functional interactions with the $\beta$4 subunit. In addition, it suggests that the D369G mutation may cause more severe defects in $\beta$4-expressing neurons, where $\beta$4 otherwise inhibits channel opening. We tested these ideas by coexpressing $\alpha_{WT}$ or $\alpha_{D369G}$ with or without $\beta$4 (Fig. 5 A).
We observed that coexpression of the β4 subunit resulted in shifted G-V relations in D369G mutant channels (Fig. 5 B and Table II), indicating that β4 does indeed modulate steady-state gating of the mutant. Similar to wild-type channels (Fig. 5 D, top), β4 causes a negative G-V shift at high Ca^{2+} (41 μM Ca^{2+}) and positive G-V shifts at lower Ca^{2+} concentrations (<2.1 μM Ca^{2+}; Fig. 5 C, top) (Wang et al., 2006). Also, β4 reduces the equivalent gating charge (slope of G-V relation) of D369G channels (Fig. 5 C, bottom), similar to wild-type channels (Fig. 5 D, bottom) (Wang et al., 2006).

The effects of β4 on the gating kinetics of α_{D369G} channels are also similar to its effects on α_{WT}. At 2.1 μM, step depolarization from −80 to +80 mV activates α_{D369G}/β4 channels visibly slower compared with α_{D369G} channels (Fig. 6 A, left). Similarly, α_{D369G}/β4 channels deactivate more slowly compared with α_{D369G} channels (Fig. 6 A, right). At 41 and 2.1 μM Ca^{2+}, we observed qualitatively similar effects of β4 on α_{WT} and α_{D369G} channel–gating kinetics (Fig. 6, B–E); β4 coexpression results in slowed activation kinetics, and this effect is greater at lower Ca^{2+} compared with higher Ca^{2+} (Fig. 6, F and G). In summary, these results indicate that effects of β4 on steady-state opening of the epilepsy gain-of-function mutation are larger for mslo compared with hslo. However, activation kinetics of mslo and hslo mutant channels are dramatically slowed by β4, suggesting that the β4 subunit does interact with human α_{D434G} channels as suggested by the dramatic slowing of activation and deactivation kinetics (Fig. 6, F and G). In summary, these results indicate that effects of β4 on steady-state opening of the epilepsy gain-of-function mutation are larger for mslo compared with hslo. However, activation kinetics of mslo and hslo mutant channels are dramatically slowed by β4, suggesting...
that recruitment of both channels during action potentials would be reduced by β4.

The D369G Mutation Alters the Ca²⁺ Dependence of BK Currents during Action Potential-shaped Voltage Commands

Recruitment of BK channel current during an action potential depends critically on the time course of channel opening as a function of membrane voltage and Ca²⁺. To consider the relative current activation during action potentials, we used voltage commands designed to mimic those of action potentials measured in neurons (hippocampus DG granule cells, where the β4 subunit is expressed) (Brenner et al., 2005). The recordings were conducted using physiological ionic concentrations of K⁺ and Na⁺ to mimic a physiological K⁺-driving unit is expressed) (Brenner et al., 2005). The recordings show a graded increase with increasing Ca²⁺. Fig. 7 C shows averaged BK currents at the indicated Ca²⁺ where BK current sizes follow the trend: αD369G > αD369G/β4 = αWT > αWT/β4. As predicted by the gating kinetics in Fig. 6, β4 inhibition of both αWT (thin red vs. thin black) and αD369G (thick red vs. thick black) current recruitment is much greater at low than at higher Ca²⁺ concentration (Fig. 7 C). The increase of current activation by the D369G mutation in the presence (thick vs. thin red) or absence (thick vs. thin black) of β4 subunits is also greater at lower [Ca²⁺] (Fig. 7 C).

Summary data across a broad range of Ca²⁺ for different channels is plotted in Fig. 7 D. D369G and the β4 subunit are expressed) (Brenner et al., 2005). Representative recordings from a single αD369G/β4 patch at various internal Ca²⁺ is shown in Fig. 7 B. BK currents were activated with a small delay after the voltage command. The normalized BK currents show a graded increase with increasing Ca²⁺. Fig. 7 C shows averaged BK currents at the indicated Ca²⁺ where BK current sizes follow the trend: αD369G > αD369G/β4 = αWT > αWT/β4. As predicted by the gating kinetics in Fig. 6, β4 inhibition of both αWT (thin red vs. thin black) and αD369G (thick red vs. thick black) current recruitment is much greater at low than at higher Ca²⁺ concentration (Fig. 7 C). The increase of current activation by the D369G mutation in the presence (thick vs. thin red) or absence (thick vs. thin black) of β4 subunits is also greater at lower [Ca²⁺] (Fig. 7 C).

Figure 6. Effects of β4 on D369G BK channel gating kinetics. (A; left) Compare activation kinetics. αD369G and αD369G/β4 currents at 2.1 μM Ca²⁺. Patches were held at −80 mV and stepped to +80 mV for 200 ms. Superimposed on the current traces are the single-exponential fits to the activation time courses (αD369G: τ = 1.1 ms; αD369G/β4: τ = 11.7 ms). (B) Comparison of αWT and αWT/β4 channel kinetics at 41 μM Ca²⁺ (αWT activation: n = 8–26; αWT deactivation: n = 12); αWT/β4 activation: n = 13–35; αWT/β4 deactivation: n = 16–21). (C) Comparison of αWT and αWT/β4 channel kinetics at 2.1 μM Ca²⁺ (αWT activation: n = 17; αWT deactivation: n = 6–30; αWT/β4 activation: n = 7–22; αWT/β4 deactivation: n = 11–12). (D) Comparison of αD369G and αD369G/β4 channel kinetics at 41 μM Ca²⁺ (αD369G activation: n = 5–18; αD369G deactivation: n = 13–14; αD369G/β4 activation: n = 5–16; αD369G/β4 deactivation: n = 15). (E) Comparison of αD369G and αD369G/β4 channel kinetics at 2.1 μM Ca²⁺ (αD369G activation: n = 5–23; αD369G deactivation: n = 13–19; αD369G/β4 activation: n = 6–34; αD369G/β4 deactivation: n = 8–20). (F) Comparison of αD364G and αD364G/β4 channel kinetics at 41 μM Ca²⁺ (αD364G activation: n = 11; αD364G deactivation: n = 7; αD364G/β4 activation: n = 11; αD364G/β4 deactivation: n = 10). (G) Comparison of αD364G and αD364G/β4 channel kinetics at 2.1 μM Ca²⁺ (αD364G activation: n = 7; αD364G deactivation: n = 5; αD364G/β4 activation: n = 9; αD364G/β4 deactivation: n = 9). Filled symbols represent measurements obtained from tail currents (deactivation time constant), and empty symbols represent measurements obtained from activation time constant.
the dose–response of α and αβ4 channels toward lower Ca2+. Indeed, there is a roughly twofold increase in apparent K1/2 for D369G channels and a twofold decrease in apparent K1/2 for β4-containing wild-type channels (Fig. 7 D). Interestingly, the effect of the D369G mutation is to shift αD369G/β4 channels to a dose–response overlapping αWT channels lacking the β4 subunit. In addition, αD369G channels have a higher maximal current integral at saturating Ca2+ (Fig. 7 D). The relative effects of the D369G mutation and β4 are quantified by comparing relative ratio of current recruitment as a function of the D369G mutation (Fig. 7 E) or as a function of the β4 subunit (Fig. 7 F). The results clearly indicate that the effect of D369G and β4 are most dramatic at low Ca2+ and are Ca2+ independent at Ca2+ > 18 μM. For example, there is an approximately four- to sixfold increase in BK current with the D369G mutation (Fig. 7 E) and an approximately three- to fourfold reduction of BK current with β4 subunit (Fig. 7 F) at 3.4 μM Ca2+. At lower Ca2+, the current recruited is too small for estimation of fold changes.

Analysis of the G-V and τs-V relations (with the physiological ionic conditions used in the experiments above) indicates that activation kinetics determine the relative current recruitment during action potential stimuli. For example, at 7.3 μM Ca2+, steady-state open probability is maximal for all channels at the peak action potential voltage of +50 mV (boxed in Fig. 8 A, top). Rather, the relative activation taur at +50 mV (boxed in Fig. 8 A, bottom) correlate well with the current recruitment during the action potential stimulus (Fig. 7 F). Thus, β4-mediated slowing of activation (8.1 ± 1.2 msec) effectively precludes αWT channel opening during the relatively short action potential time window. In contrast, β4 slows αD369G to time constants (2.9 ± 0.4 msec) that allow significant BK channel activation. This is consistent across Ca2+ concentrations, which show high steady-state conductance at +50 mV for either αWT or αD369G channels, with or without β4 (Ca2+ > 0.9 μM; Fig. 8 B). Nevertheless, αD369G, which displayed the fastest gating kinetics, showed the largest action potential-evoked BK currents (Fig. 8 B). αD369G/β4 and αWT channels have intermediate activation rates and current recruitment. Finally, the slowest gating αWT/β4 has the least current recruitment.

**DISCUSSION**

In the context of the HA allosteric gating model, our analysis suggest that mslo D369G is a gain-of-function mutation affecting two aspects of gating. It favors opening independently of Ca2+ and voltage sensor activation, with an approximate twofold increase of the closed-to-open

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### Figure 7. Ca2+-dependent effects of the mutation on BK channel recruitment by spike-shaped depolarization.

- **A** Voltage command of the spikeshaped depolarization (dashed line) approximating average DG granule cell action potentials (trace). (B) Representative patch showing αD369G/β4 current evoked by spike depolarization and various intracellular Ca2+. (C) Average BK current for different channels at 41 μM Ca2+ (αWT: n = 19; αWTβ4: n = 16; αD369G: n = 22; αD369G/β4: n = 18), 7.3 μM Ca2+ (αWT: n = 10; αWTβ4: n = 11; αD369G: n = 16; αD369G/β4: n = 9), and 3.4 μM Ca2+ (αWT: n = 9; αWTβ4: n = 8; αD369G: n = 12; αD369G/β4: n = 7). Currents in B and C were normalized to maximal current size obtained from 0 mV tail current (0 mV) at saturating (1 mM) Ca2+.

- **D** Average current integral as a function of intracellular Ca2+ concentration. Error bars represent SEM. Curves represent fits to Hill equations (αWT: K1/2 = 13.7, n = 1.6; αWTβ4: K1/2 = 24.8, n = 1.9; αD369G: K1/2 = 6.3, n = 0.9; αD369G/β4: K1/2 = 15.0, n = 1.5). (E) Fold increase in current resulting from the D369G mutation measured from ratio values (from D) of αD369G/αWT (red) and αD369Gβ4/αWTβ4 (green). (F) Fold increase in current resulting from channels lacking β4 measured from ratio values (from D) of α/αWTβ4 (green) and αD369G/αD369Gβ4 (blue).
equilibrium constant ($L_0$). It increases the channel’s Ca$^{2+}$ sensitivity by an approximate twofold increase of the Ca$^{2+}$-binding equilibrium constant (K). Given that D369 is located in RCK1, a putative Ca$^{2+}$-sensing domain (Zeng et al., 2005), it was not surprising to see that the mutation affects Ca$^{2+}$ binding. The D369G mutation does not greatly alter the allosteric interaction between channel opening and Ca$^{2+}$ binding (C), nor voltage-dependent gating.

Similar gating effects may be shared by the hslo (D434G) mutation. Changes in G-V slopes were not observed in the mutation (Du et al., 2005; Diez-Sampedro et al., 2006). In addition, the mutation does not alter Mg$^{2+}$ facilitation of opening (Diez-Sampedro et al., 2006), which is highly dependent on voltage sensor activation (Yang et al., 2007; Horrigan and Ma, 2008). These observations suggest that D434G has little effects on voltage-dependent gating. Negative G-V shift by the mutation in 0 Ca$^{2+}$ was observed in our study, suggesting that D434G increases channels’ closed-to-open equilibrium constant. Finally, consistent with the possibility that D434G alters Ca$^{2+}$ binding instead of coupling, shifts of G-V by D434G are greater at intermediate Ca$^{2+}$ compared with nominal and saturating Ca$^{2+}$ (Du et al., 2005; Diez-Sampedro et al., 2006).

One difference between studies was that the 0 Ca$^{2+}$ G-V shift by hslo D434G mutation was not previously observed (Diez-Sampedro et al., 2006). A possible explanation is that the previous study may have overestimated $G/G_{max}$ (Diez-Sampedro et al., 2006). Whereas $\beta_4$ slows the activation of both hslo D434G and mslo D369G at low Ca$^{2+}$, at high Ca$^{2+}$ (41 μM) $\beta_4$ slows the activation of D434G (threefold), but not mslo D369G. As a consequence, one may expect that $\beta_4$ reduces recruitment of the D434G mutation more than that of D369G at high Ca$^{2+}$. These findings suggest that at some Ca$^{2+}$, $\beta_4$ may have differing effects on human versus mouse neurons containing the epilepsy mutation.

BK channels have very depolarized G-V relations in the absence of Ca$^{2+}$ and require micromolar Ca$^{2+}$ to open at physiological membrane potentials (i.e., neuronal voltages between −100 to +60 mV) (Cui et al., 1997). BK channels are often colocalized with Ca$^{2+}$ sources such as voltage-dependent Ca$^{2+}$ channels, ryanodine receptors, and NMDA receptors (Davies et al., 1996; Marrion and Tavalin, 1998; Prakriya and Lingle, 2000; Isaacson and Murphy, 2001; Parsons et al., 2002; Berkefeld et al., 2006; Berkefeld and Fakler, 2008). Using the short time window of action potential–like voltage commands reveals the relatively high Ca$^{2+}$ concentrations required to activate BK channels during neuronal action potential firing. Little BK current is observed below 2.1 and 3.4 μM Ca$^{2+}$, with $K_{1/2}$ of 13.7 and 24.8 μM, respectively, for wild-type BK/α and BK/αβ4 channels. These high μM Ca$^{2+}$ concentrations suggest that neuronal BK channels are especially suited to respond to high local Ca$^{2+}$ rises; much greater than global Ca$^{2+}$ generally attains (Fakler and Adelman, 2008).

Our Ca$^{2+}$ dose–response curves may also provide some footing for estimation of local Ca$^{2+}$ that BK channels sense in neurons. Previous studies in DG granule neurons indicate that $\beta_4$ reduces BK channel activation during the action potential, whereas knockout of $\beta_4$
allows greater BK channel contribution to action potential repolarization (Brenner et al., 2005). The Ca$_{\text{2+}}$ dose–response curves (Fig. 7 D) suggest that β4 inhibition most effectively occurs at Ca$_{\text{2+}}$ below 41 μM during action potential–type stimuli. This is somewhat lower than the estimated 40–50 μM of calcium nanodomain that BK channels experience during action potentials of DG neurons (Muller et al., 2007). The discrepancies may be due to the fact that we used constant buffered Ca$_{\text{2+}}$ to activate BK channels in our experiments, whereas neuronal BK channels experience dynamic changes of Ca$_{\text{2+}}$ in response to voltage-dependent Ca$_{\text{2+}}$ channel activation and deactivation during an action potential. Thus, in neurons, BK channels may sense somewhat larger peak calcium increases but over a more transient time course. In addition, the BK channel and cellular environment may be different between neurons and HEK cells due to differences in BK channel phosphorylation state, alternative splicing, or redox status. In other ways, however, experiments performed in HEK cells provide certain advantages. Analyzing isolated macroscopic BK currents in excised patches takes into account both changes in G–V relationship and gating kinetics during action potential–type stimuli, without the requirement for imperfect pharmacological inhibitors or mathematical modeling of action potential currents.

By slowing the BK channel’s activation rate beyond the time scale of action potentials (i.e., > 5 msec), our study shows that β4 effectively limits BK channel recruitment at low and moderate Ca$_{\text{2+}}$ (~3.4–18 μM). In DG neurons from β4 knockout mice, increased BK channel activation sharpens the repolarization phase of the action potentials. The resulting briefer action potential may reduce both activation of voltage-dependent Ca$_{\text{2+}}$ channels during the action potential (Lo et al., 2001), as well as recruitment of Ca$_{\text{2+}}$-activated SK-type channels that act to limit firing frequency; these effects likely contribute to high frequency neuronal firing and epilepsy (Brenner et al., 2005). Opposing β4 effects, D369G facilitates BK channel opening by lowering K_0/2 to 6.3 and 15.0 μM for α and α/β4 channels, respectively. The D369G enhancement of Ca$_{\text{2+}}$ affinity appears to reduce the slow-gating β4 brake on BK channel activation in a similar manner as high calcium’s effect on α$_{\text{sk}}$/β4 channels. Increased DG firing in D434G humans may contribute to epilepsy, similar to the mouse β4 knockout (Brenner et al., 2005). However, increased BK channel activity associated with increased neuronal excitability may not be limited to hippocampal DG neurons. A previous study has shown that a maladaptive gain-of-function of BK channels underlies elevated firing of neocortical pyramidal neurons in a picrotoxin-induced spontaneous seizure mouse model (Shruti et al., 2008). It will certainly be interesting to establish a causal relationship between BK channel gain-of-function and epilepsy by generating a tissue-specific knockin mouse model of the D369G mutation.

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