Mg\textsuperscript{2+} Enhances Voltage Sensor/Gate Coupling in BK Channels

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BK (Slo1) potassium channels are activated by millimolar intracellular Mg\textsuperscript{2+} as well as micromolar Ca\textsuperscript{2+} and membrane depolarization. Mg\textsuperscript{2+} and Ca\textsuperscript{2+} act in an approximately additive manner at different binding sites to shift the conductance–voltage (G\textsubscript{V}) relation, suggesting that these ligands might work through functionally similar but independent mechanisms. However, we find that the mechanism of Mg\textsuperscript{2+} action is highly dependent on voltage sensor activation and therefore differs fundamentally from that of Ca\textsuperscript{2+}. Evidence that Ca\textsuperscript{2+} acts independently of voltage sensor activation includes an ability to increase open probability (P\textsubscript{O}) at extreme negative voltages where voltage sensors are in the resting state; 2 \mu M Ca\textsuperscript{2+} increases P\textsubscript{O} more than 15-fold at −120 mV. However 10 mM Mg\textsuperscript{2+}, which has an effect on the G\textsubscript{V} relation similar to 2 \mu M Ca\textsuperscript{2+}, has no detectable effect on P\textsubscript{O} when voltage sensors are in the resting state. Gating currents are only slightly altered by Mg\textsuperscript{2+} when channels are closed, indicating that Mg\textsuperscript{2+} does not act merely to promote voltage sensor activation. Indeed, channel opening is facilitated in a voltage-independent manner by Mg\textsuperscript{2+} in a mutant (R210C) whose voltage sensors are constitutively activated. Thus, 10 mM Mg\textsuperscript{2+} increases P\textsubscript{O} only when voltage sensors are activated, effectively strengthening the allosteric coupling of voltage sensor activation to channel opening. Increasing Mg\textsuperscript{2+} from 10 to 100 mM, to occupy very low affinity binding sites, has additional effects on gating that more closely resemble those of Ca\textsuperscript{2+}. The effects of Mg\textsuperscript{2+} on steady-state activation and I\textsubscript{K} kinetics are discussed in terms of an allosteric gating scheme and the state-dependent interactions between Mg\textsuperscript{2+} and voltage sensor that may underlie this mechanism.

INTRODUCTION

The activity of BK-type Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels is important for the function of nerve, muscle, and secretory cells (Vergara et al., 1998). BK channels respond to two primary signals, membrane voltage and intracellular Ca\textsuperscript{2+}, but are also sensitive to a variety of regulatory ligands, including intracellular Mg\textsuperscript{2+} (Shi and Cui, 2001; Zhang et al., 2001), heme (Tang et al., 2003), and protons (Avdonin et al., 2003), that can influence channel gating under physiological and/or pathophysiological conditions. Therefore, to understand how BK channel activity is regulated it is important to understand how these signaling and regulatory factors affect channel gating and interact with each other. Previous studies suggest that millimolar [Mg\textsuperscript{2+}], and micromolar [Ca\textsuperscript{2+}], activate BK channels through similar functional mechanisms (Shi and Cui, 2001; Zhang et al., 2001). Here we show that the action of Mg\textsuperscript{2+} differs from that of Ca\textsuperscript{2+} in its dependence on voltage sensor activation. The results highlight that multiple pathways of communication exist between the transmembrane and cytosolic domains of BK channels.

That Ca\textsuperscript{2+} and voltage act independently to regulate opening is evident from BK channel function and consistent with channel structure. BK channels can be fully activated by membrane depolarization in the absence of Ca\textsuperscript{2+} (Cui et al., 1997), and Ca\textsuperscript{2+} facilitates opening in a nearly voltage-independent manner, indicating that Ca\textsuperscript{2+} and voltage sensor activation have energetically additive effects on opening (Cui and Aldrich, 2000; Horrigan and Aldrich, 2002). The transmembrane domain of BK channels, like voltage-gated K\textsuperscript{+} (K\textsubscript{v}) channels, includes an S5–S6 pore domain and a charged S1–S4 voltage sensor (Ma et al., 2006). Coupling between the voltage sensor and pore can occur presumably as in K\textsubscript{v} channels, via interactions within the transmembrane domain (Fig. 1 A) (Lu et al., 2002; Long et al., 2005b). In addition, BK channels contain a large C-terminal cytosolic domain that interacts with intracellular ligands and is directly attached to the S6 activation gate. The cytosolic domain of each \alpha-subunit contains two putative RCK (regulator of K\textsuperscript{+} conductance) homology domains (RCK1, RCK2), like those in the prokaryotic channel MthK (Jiang et al., 2001). In MthK, RCK domains from different subunits assemble into a gating ring structure that expands upon Ca\textsuperscript{2+} binding to pull open the gate (Fig. 1 B) (Jiang et al., 2002; Ye et al., 2006), suggesting that Ca\textsuperscript{2+} opens BK channels by increasing tension on the RCK1–S6 linker (Niu et al., 2004). Thus models of BK channel structure support that voltage and Ca\textsuperscript{2+} sensors may act independently on the gate (Fig. 1 C) to exert additive effects on steady-state activation.

Given the example of MthK, one might suppose that any ligand binding to the cytosolic domain of the BK

Abbreviations used in this paper: K\textsubscript{v}, voltage-gated K\textsuperscript{+}; RCK, regulator of K\textsuperscript{+} conductance.
channel must act, like Ca$^{2+}$, to open the gate by pulling on the RCK1–S6 linker. However this is not necessarily the case. Voltage sensors in BK channels, which are absent from MthK, are potentially available to interact with the cytosolic domain, providing an alternative pathway of communication between ligand binding sites and the transmembrane domain. The cytosolic domain of MthK lies close to the transmembrane domain, presents a relatively flat surface toward the membrane, and with a diameter of >70 Å extends laterally far beyond the pore (Fig. 1 B). All of these features, which are presumably necessary to exert lateral force on the gate via short peptide linkers, would also tend to bring the periphery of the gating ring close to the voltage sensor domain in BK channels (Fig. 1 D). Several observations suggest that interactions between cytosolic and voltage sensor domains occur. Ca$^{2+}$ has effects on gating current, consistent with a weak interaction between Ca$^{2+}$ sensors and voltage sensors (Horrigan and Aldrich, 2002).

Intracellular heme has a pronounced effect on voltage gating, weakening the allosteric coupling between voltage sensor and gate (Horrigan et al., 2005). Likewise, chemical modification of a native cysteine residue (C430) in the cytosolic domain alters voltage sensor/gate coupling (Zhang and Horrigan, 2005).

Mg$^{2+}$ has effects on BK channel gating that resemble those of Ca$^{2+}$: increasing P$_o$, slowing I$_k$ deactivation, and shifting the G$_C$V relation to more negative voltages with little change in shape (Shi and Cui, 2001; Zhang et al., 2001). Effects of Mg$^{2+}$ and Ca$^{2+}$ on the half-activation voltage (V$_{1/2}$) are approximately additive and involve different binding sites (Shi et al., 2002; Xia et al., 2002). Consequently, Mg$^{2+}$ has been proposed to act independently of Ca$^{2+}$ but through a similar functional mechanism. Effects of Mg$^{2+}$ on the G$_{V}$ relationship can be reproduced by gating schemes that assume Mg$^{2+}$ acts, like Ca$^{2+}$, to enhance channel opening independent of voltage sensor activation (Shi and Cui, 2001; Zhang et al., 2001; Hu et al., 2006). However, several lines of evidence suggest that Mg$^{2+}$ and Ca$^{2+}$ do not act through identical functional mechanisms. First, high concentrations of Ca$^{2+}$ (>100 μM), which are known to occupy Mg$^{2+}$ binding sites, have effects that differ qualitatively from those of low [Ca$^{2+}$]. While 0–100 μM Ca$^{2+}$ produces a nearly voltage-independent increase in log(P$_o$), 100–1,000 μM Ca$^{2+}$ increases log(P$_o$) in a voltage-dependent manner (Horrigan and Aldrich, 2002). In addition, mutations in the voltage sensor, including R213Q in S4, disrupt Mg$^{2+}$ sensitivity but not Ca$^{2+}$ sensitivity, suggesting that the voltage sensor plays a critical role in Mg$^{2+}$-dependent activation (Hu et al., 2003). Indeed, recent evidence indicates that Mg$^{2+}$ bound to the cytosolic domain interacts electrostatically with R213 (Yang et al., 2007).

This study examines the functional interaction between Mg$^{2+}$- and voltage-dependent activation of mSlo1 BK channels. Effects of 0–100 mM Mg$^{2+}$ on the steady-state and kinetic properties of ionic and gating currents were examined over a wide voltage range in WT channels and channels where either the voltage sensor or a putative Mg$^{2+}$-binding site in the RCK1 domain were mutated. The results show that Mg$^{2+}$ at the RCK1 site acts primarily to strengthen the allosteric coupling of voltage sensor activation to channel opening. Only at high concentrations (>10 mM), involving an additional very low affinity binding site, does Mg$^{2+}$ have direct effects on opening, like Ca$^{2+}$. Our results together with the proposed electrostatic nature of Mg$^{2+}$/voltage sensor interaction suggest that Mg$^{2+}$ and the voltage sensor must interact in multiple states to account for the effects of Mg$^{2+}$ on voltage sensor/gate coupling.

**MATERIALS AND METHODS**

**Electrophysiology and Data Analysis**

Currents were recorded using the patch-clamp technique in the inside-out configuration (Hamill et al., 1981) at 20 ± 1°C. For Ca$^{2+}$ experiments, the external solution contained (in mM) 104 KMeSO$_4$, 6 KCl, 2 MgCl$_2$, 20 HEPES, and the internal solution contained 110 KMeSO$_4$, 6 HCl, 20 HEPES, and 2 EGTA (0 Ca$^{2+}$) or 5 HEDTA (Ca$^{2+}$ solution). Ca$^{2+}$ was added as CaCl$_2$ and [Cl$^{-}$], was adjusted to 10 mM with HCl. Free [Ca$^{2+}$] for the 2 μM Ca$^{2+}$ solution was measured (1.8 μM) with a Ca$^{2+}$ electrode (Orion Research Inc.). For Mg$^{2+}$ experiments, the external solution contained (in mM) 225 KOH, 196 HCl, 2 MgCl$_2$, 10 HEPES, and internal solutions contained 225 KOH, 5 EGTA, 10 HEPES, plus MgCl$_2$ and HCl to obtain the desired [Mg$^{2+}$] with [Cl$^{-}$], = 200 mM. 100 mM Mg$^{2+}$ solution contained a total of 100 mM Mg$^{2+}$ for an estimated free [Mg$^{2+}$] of 96.1 mM. Total Mg$^{2+}$ in other solutions was adjusted to obtain the indicated free [Mg$^{2+}$] (2, 5, 10, 21 mM) as calculated by MaxChelator (http://www.stanford.edu/~cppaton/maxchelator.html) (Patton et al., 2004). Since free [Mg$^{2+}$] was not measured, the purity of MgCl$_2$ and EGTA stocks were assayed by pH-metric titration (Tsien and Pozzan, 1989) against standardized solutions of EDTA and CaCl$_2$, respectively. For gating currents (I$_g$) the external solution contained 130 tetraethyl ammonium (TEA)-OH, 100 NMDG, 196 HCl, 2 MgCl$_2$, 10 HEPES, and internal solution contained 225 NMDG-MES, 10 HEPES, 5 EGTA, plus MgCl$_2$ and HCl as above. The pH of all solutions was adjusted to 7.2 with MeSO$_4$, Internal solutions contained 40 μM (+)-18-crown-6-tetracarboxylic acid (18C6GTA) to chelate contaminant Ba$^{2+}$ (Diaz et al., 1996; Neyton, 1996).

Data were acquired with an Axopatch 200B amplifier (Molecular Devices Corp.) in patch mode with the Axopatch’s filter set at 100 kHz. Currents were filtered by an 8-pole Bessel filter (Frequency Device, Inc.) at 50 kHz (I$_g$) or 20 kHz (I$_k$) and sampled at

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formed by appending the Kv1.2 voltage sensors to MthK shows how Ca$^{2+}$ and voltage may both interact with the gate to have independent effects on activation. The position of the voltage sensors was determined by superimposing the pore domains of Kv1.2 (residues 341–406) and MthK (residues 26–91) in PyMol (http://www.pymol.org). (D) The hypothetical BK channel structure also illustrates the possibility that the large cytosolic domain may interact directly with voltage sensors.

Patch-to-patch variation in $V_{0.5}$ of the G-V and Q-V relationships are observed for Slo1 channels (Stefani et al., 1997; Horrigan et al., 1999). To compensate for the effect of such variation on mean P$_{0}$-V and Q$_{0}$-V relations, $V_{0.5}$ was determined for each patch and individual relations were shifted along the voltage axis by $\Delta V_{0.5} = (V_{0.5}^p - V_{0.5})$ before averaging, where $V_{0.5}\geq$ is the mean for all experiments at the same $[Mg^{2+}]$. Patch to patch variation in $P_{0}$ at extreme negative voltages is also observed (Horrigan et al., 1999). Therefore, mean $log(P_{0})$-V and $\tau_V$-V relations for 0, 10, and 100 mM $Mg^{2+}$ were constructed using only experiments in which both $Mg^{2+}$ data and matching $0 M^{2+}$ controls exist. In this way, mean relations accurately reflect effects of $Mg^{2+}$ that were observed in individual experiments.

Gating capacitance ($C_g$) was measured using admittance analysis as previously described (Horrigan and Aldrich, 1999). In brief, gating currents were recorded in response to 0.5-s voltage ramps upon which a sinusoidal voltage command (781 Hz, 60 mV peak to peak) was superimposed. Admittance was calculated for each cycle of the sinewave, and capacitance was determined after correcting for phase shifts due to instrumentation.

**RESULTS**

**Mg$^{2+}$ and Ca$^{2+}$ Act through Different Mechanisms**

The effects of millimolar $[Mg^{2+}]$, and micromolar $[Ca^{2+}]$, on steady-state activation are compared in Fig. 2. In addition to increasing open probability, intracellular $Mg^{2+}$ rapidly blocks the pore in a voltage-dependent manner that effectively reduces single channel conductance at depolarized voltages (Ferguson, 1991). Both of these effects are evident by comparing macroscopic potassium currents ($I_K$) in the presence and absence of 10 mM $Mg^{2+}$ (Fig. 2 A). $I_K$ was evoked by 50-ms pulses to different voltages in inside-out patches from *Xenopus* oocytes.
expressing mSlo1 BK channels. Mg\textsuperscript{2+} decreases outward current at the most positive voltages owing to pore block, but increases tail currents at \textasciitilde 80 mV, indicating that P\textsubscript{O} is increased. Normalized G\textsubscript{K}-V relations, determined from tail currents exhibit a \textasciitilde 67.2 \pm 4.9 mV shift in half-activation voltage (V\textsubscript{0.5}) in response to 10 mM Mg\textsuperscript{2+}, with little change in shape, similar to the effect of 2 \mu M Ca\textsuperscript{2+} (Fig. 2 B).

Despite similar effects of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} on the G\textsubscript{K}-V relation, fundamental differences in the action of these ligands are evident when P\textsubscript{O} is plotted on a log-scale versus voltage (Fig. 2 C). P\textsubscript{O} was measured to values as low as \textasciitilde 10^{-7} by recording unitary current activity in macropatches containing hundreds of channels. Log(P\textsubscript{O}) is increased by 2 \mu M Ca\textsuperscript{2+} in a nearly voltage-independent manner; shifting the entire log(V)-V curve upward by 1.2 log units (a 15-fold increase in P\textsubscript{O}) until channels are maximally activated at positive voltages. P\textsubscript{O} is increased 15-fold even at extreme negative voltages (\textasciitilde 100 mV) where voltage sensors are not activated and log(P\textsubscript{O}) is weakly voltage dependent (Fig. 2 C, dotted lines) (Horrigan and Aldrich, 2002). By contrast, 10 mM Mg\textsuperscript{2+} has no effect on steady-state activation at extreme negative voltages and increases log(P\textsubscript{O}) in a voltage-dependent manner from \textasciitilde 80 to +40 mV such that the effects of Mg\textsuperscript{2+} and Ca\textsuperscript{2+} are similar only at more positive voltages.

Why Is the Effect of Mg\textsuperscript{2+} Voltage Dependent?

Two different mechanisms could potentially account for the voltage dependence of Mg\textsuperscript{2+} action; the effect of Mg\textsuperscript{2+} could depend on the activation state of the voltage sensor, or Mg\textsuperscript{2+} binding could be intrinsically voltage dependent. Voltage-dependent binding must be considered because if Mg\textsuperscript{2+} interacts with the voltage sensor it could also lie within the membrane electric field; and a model assuming that Mg\textsuperscript{2+} traverses a fraction of the electric field (\gamma \approx 0.2) to reach its binding site can reproduce the effect of 10 mM Mg\textsuperscript{2+} (Fig. 2, B and C, dotted curves, see legend for details). However, the effects of Mg\textsuperscript{2+} on two voltage sensor mutants (R207Q, R167E)
in Fig. 2 D indicate that voltage sensor activation is critical to the effect of Mg$^{2+}$ and probably sufficient to account for the voltage dependence of Mg$^{2+}$ action.

R207Q in S4 and R167E in S2 shift voltage sensor activation to more negative and positive voltages, respectively (Ma et al., 2006), such that log($P_O$)-V relations for these mutants are shifted along the voltage axis relative to the WT in 0 Mg$^{2+}$ (Fig. 2 D, filled symbols). R207Q also shifts the Q-V relation by more than −250 mV, based on gating current measurements, such that even at −200 mV a significant fraction (∼0.2) of voltage sensors are activated (Ma et al., 2006). Both R207Q and R167E are readily activated by 10 mM Mg$^{2+}$ at depolarized voltages (Fig. 2 D), suggesting these mutations do not disrupt Mg$^{2+}$ binding nor interactions of Mg$^{2+}$ with the voltage sensor (Hu et al., 2003; Yang et al., 2007). However the voltage dependence of Mg$^{2+}$-dependent activation for the mutants is greatly altered relative to the WT, suggesting that Mg$^{2+}$ action depends on the activation state of the voltage sensor. In the case of R167E, Mg$^{2+}$ has no effect on $P_O$ at voltages up to +60 mV where the WT is strongly activated by Mg$^{2+}$. Conversely, R207Q exhibits a robust 30-fold increase in $P_O$ at negative voltages (−120 to −200 mV) where the WT appears insensitive to Mg$^{2+}$. The response of R207Q indicates that Mg$^{2+}$ is capable of occupying its binding site even at extreme negative voltages. Thus, it is unlikely that Mg$^{2+}$ binding is intrinsically voltage dependent; and the inability of the WT to be activated at negative voltages likely reflects that Mg$^{2+}$ is incapable of increasing $P_O$ when voltage sensors are in the resting state. This hypothesis is supported by comparison of the WT and R167E data that show that channels are Mg$^{2+}$ insensitive when $P_O$ is weakly voltage dependent.

**Understanding the Action of Mg$^{2+}$ in Terms of Allosteric Mechanisms**

To determine how Mg$^{2+}$ and voltage sensors interact mechanistically we interpreted our results in terms of a well-established allosteric gating scheme that accounts for the effects voltage on steady-state activation of BK channels (Fig. 2 E, Scheme 1) (Horrigan and Aldrich, 1999; Horrigan et al., 1999). Scheme 1 asserts that the opening of the gate can be described as a concerted, weakly voltage-dependent transition between an open and closed conformation (O–C) with zero-voltage equilibrium constant $L_0$ and partial charge $z_L$. In addition, voltage sensors in each of four independent and identical subunits can undergo a transition between a resting and activated conformation (R–A) characterized by equilibrium constant $J_0$ and partial charge $z_J$. The coupling between voltage sensor and gate is described by an allosteric factor D, such that the C-O equilibrium constant increases D-fold for each voltage sensor activated and the voltage sensor equilibrium increases D-fold when the channel opens.

Based on Scheme 1 there are four distinct mechanisms by which a ligand might alter $P_O$. Ligand binding could (a) influence the intrinsic stability of the gate ($L_0$), (b) perturb the voltage sensor equilibrium ($J_0$), (c) alter voltage sensor/gate coupling (D-factor), or (d) alter the gating charge associated with voltage sensor activation ($z_L$) or channel opening ($z_J$). The action of Ca$^{2+}$ is mainly accounted for by an effect on the gate ($L_0$) with a minor impact on voltage sensor activation ($J_0$). This mechanism can be represented by an allosteric model (Fig. 2 F, Scheme 2) that successfully reproduces the effects of Ca$^{2+}$ (Fig. 2, B and C, solid lines) in terms of a ligand-binding equilibrium (X-X·M$^{2+}$) and allosteric factors C and E that describe the coupling of Ca$^{2+}$ sensors to the gate and to voltage sensors, respectively (Horrigan and Aldrich, 2002). Direct coupling between Ca$^{2+}$ sensors and gate is evident from the ability of Ca$^{2+}$ to increase $P_O$ whether or not voltage sensors are activated. Thus the insensitivity of $P_O$ to 10 mM Mg$^{2+}$ when voltage sensors are in the resting state rules out a similar interaction between Mg$^{2+}$ sensors and gate and indicates that $L_0$ is unchanged, but leaves open the possibility that Mg$^{2+}$ affects voltage sensor activation or coupling (Fig. 2 G, Scheme 3) or charge.

**Mg$^{2+}$ Has Little Effect on Voltage Sensor Activation when Channels Are Closed**

To characterize effects of Mg$^{2+}$ on the voltage sensor we recorded gating current from WT and mutant channels (Fig. 3). The results indicate that Mg$^{2+}$ has little effect on the voltage sensor equilibrium ($J_0$) or gating charge ($z_J$).

Voltage sensor activation in BK channels is much faster than channel opening (Stefani et al., 1997; Horrigan and Aldrich, 1999). Therefore ON gating current ($I_{gON}$) reflects primarily voltage sensor activation when channels are closed. Fig. 3 A shows the effects of Mg$^{2+}$ on $I_{gON}$ evoked by brief 0.5-ms depolarizations to +200 mV. 10 mM Mg$^{2+}$ causes an ∼20% increase in peak $I_{gON}$ with little change in kinetics (Fig. 3 A, top). Application of 100 mM Mg$^{2+}$, to activate very low affinity binding sites (Hu et al., 2006), produces a greater increase in peak current (Fig. 3 A, bottom). Mean charge–voltage relations for closed channels ($Q_c$–V), determined from $I_{gON}$ (see Fig. 3 legend) are shifted to more negative voltages by −16.7 ± 2.3 mV (r = 5) or −29.6 ± 2.6 mV (n = 3) in 10 or 100 mM Mg$^{2+}$, respectively (Fig. 3 B). This shift in half-activation voltage ($V_{hC}$) occurs without appreciable change in the shape of the $Q_c$–V relation, suggesting that Mg$^{2+}$ does not alter gating charge. Similarly, effects of 10 mM Mg$^{2+}$ on the time constant of $I_{gON}$ ($\tau_{gFast}$) for a single experiment (Fig. 3 C) reflect a small −21 mV shift in the $\tau_{gFast}$–V relation to more negative voltages.

To better quantify small changes in $V_{hC}$ ($AV_{hC}$) by Mg$^{2+}$ we examined gating capacitance ($C_g$) (Fig. 3, D–F). $C_g$ is the voltage-dependent component of membrane capacitance and reflects the derivative of gating.
charge with respect to voltage. The Cg-V relation was determined from admittance analysis by measuring Ig evoked by a sinusoidal voltage command superimposed on a 500-ms voltage ramp from −200 to +200 mV (see Materials and methods) (Horrigan and Aldrich, 1999, 2002). At voltages where steady-state Po is low (<0.1), the Cg-V relation approximates the derivative of Qc-V (Horrigan and Aldrich, 1999). Thus ∆Vhc can be determined directly from shifts in the foot of Cg-V relation (Fig. 3 D). Because Cg-V is determined rapidly and with high voltage resolution, small Vhc shifts are detectable, the effects of applying and washing out Mg2+ can be assessed rapidly, and errors in ∆Vhc due to slow changes in Vhc from channel oxidation or electrode potential drift are minimized. Cg-V analysis for the WT (Fig. 3 D) yielded ∆Vhc values (10 Mg: 17.2 ± 1.5 mV [n = 6], 100 Mg: 36.1 ± 1.6 mV [n = 6]) similar to those determined from Qc-V relations.

The effect of Mg2+ on Vhc is comparable to that of micromolar Ca2+ (Horrigan and Aldrich, 2002), consistent with a weak interaction between Mg2+ binding and voltage sensor activation in closed channels that increases the voltage sensor equilibrium constant J0 (e.g., E-factor in Schemes 2 and 3, Fig. 2, F and G). However, millimolar Mg2+ may also shift voltage-dependent parameters by nonspecific mechanisms such as screening of membrane surface charge (Hu et al., 2006). Therefore, to assess the impact of the RCK1 Mg2+ site on J0, we compared effects of Mg2+ on the WT channel to those of a mutant (E374A/E399N) whose RCK1 site is disrupted (Shi et al., 2002; Hu et al., 2006). This mutation of putative Mg2+-coordinating residues, which reduces the GK-V decay of IgON with an exponential function with time constant τgFast and integrating the area under the rising phase and fit (Horrigan and Aldrich, 1999). (C) τgFast values (10 Mg: 17.2 + 1.5 mV [n = 6], 100 Mg: 36.1 ± 1.6 mV [n = 6]) similar to those determined from Qc-V relations.

Figure 3. Effects of Mg2+ on gating current were measured in the absence of permeant ions and presence of extracellular tetraethyl ammonium (TEA) to block the pore. (A) Ig evoked by 0.5-ms pulses to +200 mV in the presence (dotted traces) or absence (solid traces) of Mg2+, from two different patches testing the effects 10 or 100 mM Mg2+. (B) Mean Qc-V relations in 0 (●), 10 (△), and 100 (◆) mM Mg2+ are fit by Boltzmann functions with identical equivalent charge (zJ = 0.58e) (Horrigan and Aldrich, 2002) and were normalized by the fit amplitude (QcMax). Qc represents the charge distribution for closed channels, determined by fitting the initial 50–100-μs decay of IgON with an exponential function with time constant τgFast and integrating the area under the rising phase and fit (Horrigan and Aldrich, 1999). (C) τgFast values for the patch in A are shifted by −21 mV in 10 mM Mg2+. Relations are fit by functions of the form τgFast = [α(V) + β(V)]−1 where α and β are forward and backward rate constants for voltage sensor activation (0 Mg: α(0) = 58600 s−1, β(0) = 1930 s−1; 10 Mg: α(0) = 45400 s−1, β(0) = 2350 s−1) (zJ = 0.58e). Shifts along the voltage axis indicate the change in Vhc (∆Vhc). Arrows indicate the upper voltage limit of the fit that was determined over a range where most channels are closed (Po < 0.1). (D) ∆Vhc = [Vhc(Mg2+) − Vhc(0 Mg2+)] for WT (●, n = 6) and E374A/E399N (◆, n = 4) determined from Cg-V relations.
Figure 4. Mg$^{2+}$ facilitates opening in a voltage-independent manner when voltage sensors are constitutively activated. (A) Mean log($P_O$)-V relations for WT (●), R207Q (▲), and R210C (▼) in 0 Mg$^{2+}$. Arrows indicate the voltage range where voltage sensors of WT, R207Q, and R210C are fully activated (see text). Dotted curve represents a Boltzmann fit to R210C with partial charge $z = 0.46e$ that describes the weak voltage dependence of channel opening in both mutants when voltage sensors are fully activated (Ma et al., 2006). (B) Mean log($P_O$)-V relations for R207Q show that 10 mM Mg$^{2+}$ (△) increases $P_O$ even when voltage sensors are fully activated. Dotted Boltzmann fits from $-20$ to $+240$ mV have identical charge ($0.46e$) but $V_{o.5}$ is shifted by $-91.6$ mV in Mg$^{2+}$, consistent with a 8.2-fold increase in the C-O equilibrium constant. (C) Mean log($P_O$)-V relations for R210C show that 10 mM Mg$^{2+}$ (▼) increases the C-O equilibrium constant in a voltage-independent manner when voltage sensors are constitutively activated. Boltzmann fits have identical charge ($0.46e$) but $V_{o.5}$ is shifted by $-122$ mV in Mg$^{2+}$, consistent with a 16.6-fold increase in the C-O equilibrium constant and a twofold increase in the allosteric coupling factor $D$.

The $-17.2$ mV $\Delta V_{hC}$ by 10 mM Mg$^{2+}$ (Fig. 3 F) can account for a similar $G_{C-V}$ shift but is much too small to explain the observed $-67$-mV shift (Fig. 2 B). By contrast, an increase in the coupling factor $D$ can shift $V_{o.5}$ without affecting $V_{hC}$. In addition, enhanced coupling should facilitate channel opening when voltage sensors are fully activated and facilitate voltage sensor activation when channels are open. These predictions are confirmed below by results in Figs. 4 and 5.

Mg$^{2+}$ Facilitates Opening When Voltage Sensors Are Constitutively Activated

Voltage sensor/gate coupling defines the extent to which $P_O$ is increased by voltage sensor activation. According to Scheme 1, the C-O equilibrium constant increases from $L_0$ when voltage sensors are in the resting state to $L_0 D^4$ when all four voltage sensors are activated. If 10 mM Mg$^{2+}$ enhances coupling ($D$) without affecting $L_0$, then the C-O equilibrium constant when voltage sensors are fully activated should increase by a factor of $\delta D^4$ where $\delta D = D(Mg^{2+})/D(control)$. This predicted facilitation of opening cannot be tested for the WT channel because in 0 Mg$^{2+}$ $P_O$ is already nearly saturated ($\sim 1$) at potentials $>250$ mV where voltage sensors are fully activated (compare $G_{C-V}$ in Fig. 2 B and $Q_{C-V}$ in Fig. 3 B). However, facilitated opening by Mg$^{2+}$ can be observed in mutants with enhanced voltage sensor activation (R207Q, R210C; Fig. 4).

Fig. 4 A compares the log($P_O$)-V relations for WT, R207Q, and R210C channels in 0 Mg$^{2+}$. R207Q shifts $Q_{C-V}$, as noted above, such that this relation is saturated and voltage sensors are fully activated at $V \geq -20$ mV (Ma et al., 2006) (Fig. 4 A, labeled arrow). Over this voltage range, $P_O$ is weakly voltage dependent and well described by a Boltzmann function (dotted curve) as expected for channels with fully activated voltage sensors undergoing a two-state C-O transition with equilibrium constant $L_0 D^4$ (Horrigan and Aldrich, 2002). Importantly, the weak voltage dependence of $L_0$ reduces $P_O$ to nonsaturating levels at potentials where the mutant voltage sensors are still fully activated (e.g., $P_O = 0.04$ at $-20$ mV). The $P_{C-V}$ relation for R210C is indistinguishable from that of R207Q at $V \geq -20$ but extends the weakly voltage-dependent phase of $P_O$ to less than $-20$ mV, suggesting that R210C voltage sensors are constitutively activated (Ma et al., 2006). The difference between WT and R207Q in Fig. 4 A is consistent with a 100-fold change in the voltage sensor equilibrium constant $J_0$ (Horrigan and Aldrich, 1999; Ma et al., 2006) and illustrates qualitatively the effect expected if Mg$^{2+}$ acts merely to promote voltage sensor activation. That is, an increase in $J_0$ can shift the steepest part of the log($P_O$)-V relation to more negative voltages but cannot increase $P_O$ above the limiting relation defined by $L_0 D^4$ (Fig. 4 A, dotted curve). By contrast, 10 mM Mg$^{2+}$ markedly increases $P_O$ for R207Q (Fig. 4 B) and R210C channels (Fig. 4 C) when voltage sensors are fully activated, indicating that $L_0 D^4$ is increased. This increase presumably reflects enhanced voltage sensor/gate coupling (increased $D$) because $L_0$, although it cannot be measured directly for R207Q or R210C channels, is insensitive to 10 mM Mg$^{2+}$ in WT and R167E (Fig. 2 D).
Enhances Allosteric Coupling in BK Channels

Analysis of the mutant data indicate that 10 mM Mg$^{2+}$ produces an approximate twofold increase in the coupling factor D. Fig. 4 C shows that R210C channels are activated by 10 mM Mg$^{2+}$ in a voltage-independent manner, consistent with the assumption that voltage sensors are constitutively activated. Mean log($P_o$)-V relations in the presence and absence of Mg$^{2+}$ were fit by Boltzmann functions with identical charge (Fig. 4 C, dotted curves), as expected if Mg$^{2+}$ increases the C-O equilibrium constant by a voltage-independent factor ($\delta D^4$) and Mg$^{2+}$ binding is not voltage dependent. A similar response is observed for R207Q at V $\geq$ -20 mV (Fig. 4 B, dotted curves). The fits to R207Q and R210C yield $\delta D^4$ values of 8.2 and 16.6, respectively, implying a 1.7–2.0-fold increase in D. A more direct estimate of $\delta D^4$ is obtained from the Mg$^{2+}$-dependent increase in $P_o$ for R210C at extreme negative voltage. Over a range of voltages ($-200$ to $-140$ mV) where $P_o$ is small ($<0.1$) and therefore approximately the C-O equilibrium constant, Mg$^{2+}$ increases $P_o$ by an average of $21.6 \pm 6.0$-fold ($\delta D^4$), indicating a $2.16 \pm 0.13$-fold increase in D, consistent with the Boltzmann fits.

Different Mg$^{2+}$ Sites Have Distinct Effects on Gating

To further characterize the action of Mg$^{2+}$ at both RCK1 and very low affinity binding sites we examined the effects on $P_o$ of different [Mg$^{2+}$], up to 100 mM, in WT and mutant channels (Fig. 5). The RCK1 site with an
estimated $K_{u}$ of 2.2–5.5 mM should be nearly saturated at 10 mM Mg$^{2+}$, whereas a putative very low affinity site ($K_{p}$: 40–136 mM) is not significantly affected until concentrations $>$10 mM (Hu et al., 2006). The effects of high and low concentrations of Mg$^{2+}$ appear similar in that $G_{o}$-$V$ relations are progressively shifted to more negative voltages with little change in shape (Fig. 5 A) (Shi and Cui, 2001; Zhang et al., 2001; Hu et al., 2006). Increasing Mg$^{2+}$ from 10 to 100 mM shifted the G-V by an additional $-34$ mV (Fig. 5 A). However, log($P_{o}$)-$V$ relations reveal that 100 mM Mg$^{2+}$ also increases $P_{o}$ significantly at extreme negative voltages (Fig. 5 B). Thus it appears that occupancy of very low affinity Mg$^{2+}$ sites, unlike the RCK1 site, influence the gate ($L_{0}$) to increase $P_{o}$ when voltage sensors are not activated.

The log($P_{o}$)-$V$ relation in 100 mM Mg$^{2+}$ appears to achieve a limiting slope between $-160$ and $-140$ mV, where $P_{o}$ is increased 2.9-fold relative to the control (Fig. 5 B, dotted lines), implying a 2.9-fold increase in $L_{0}$. However measurements below $-140$ mV were difficult to obtain. Therefore, to confirm that 100 mM Mg$^{2+}$ increases $L_{0}$ we also compared log($P_{o}$)-$V$ relations in 0, 10, and 100 mM Mg$^{2+}$ for two mutant channels (Fig. 5, C and D).

Mutation of the RCK1 site (E374A/E399N) largely eliminates the response to 10 mM Mg$^{2+}$ but leaves the $P_{o}$ increase by 100 mM Mg$^{2+}$ intact (Fig. 5 C). The mutation reduces the $G_{o}$-$V$ shift ($\Delta V_{G}$) by 10 and 100 Mg$^{2+}$ to $-14.0 \pm 2.0$ mV and $-34.6 \pm 1.8$ mV, respectively, similar to previous reports (Shi and Cui, 2001; Hu et al., 2006). The reduced shift allows log($P_{o}$)-$V$ to achieve a limiting slope at more positive voltages than the WT (Fig. 5 C, dotted lines), indicating a 2.9-fold increase in $L_{0}$ with 100 mM Mg$^{2+}$, like the WT.

The response of E374A/E399N is important not only in verifying that $L_{0}$ is increased by 100 mM Mg$^{2+}$ but also in ruling out that Mg$^{2+}$ occupancy of either the RCK1 site or Ca$^{2+}$-binding sites contribute to $L_{0}$. The insensitivity of $L_{0}$ to 10 mM Mg$^{2+}$ in the WT could conceivably reflect an ability of Mg$^{2+}$ at the RCK1 site to decrease $L_{0}$ that is masked by the action of Mg$^{2+}$ at other sites. However, this possibility is unlikely since $L_{0}$ is also insensitive to 10 mM Mg$^{2+}$ in E374A/E399N. In addition this result indicates that high affinity Ca$^{2+}$-binding sites, which are known to bind Mg$^{2+}$ at millimolar concentrations, are not responsible for the $L_{0}$ increase in 100 mM Mg$^{2+}$ because such sites should already be nearly saturated in 10 mM Mg$^{2+}$ (Shi and Cui, 2001; Zhang et al., 2001; Hu et al., 2006). Thus the E374A/E399N results confirm that occupancy of a very low affinity Mg$^{2+}$ site is required to increase $L_{0}$.

The R167E mutant was used to better quantify the effects of Mg$^{2+}$ on $L_{0}$ (Fig. 5, D and E). R167E is activated strongly by 100 mM Mg$^{2+}$ but achieves a limiting slope at voltages $\sim$100 mV more positive than the WT (Fig. 5 D, dotted lines). Thus $L_{0}$ can be determined at voltages that are sufficiently negative to achieve a limiting slope but not too extreme to prevent steady-state recording. The effects on $L_{0}$ of 10 and 100 mM Mg$^{2+}$ were determined from the change in NPV at $-80$ mV in patches where Mg$^{2+}$ was repeatedly applied and washed out. The results confirm that 10 mM Mg$^{2+}$ has little or no effect on $L_{0}$ ($1.06 \pm 0.05$-fold change, $n = 10$), whereas 100 mM Mg$^{2+}$ increased $L_{0}$ by a factor of 2.24 $\pm 0.23$ ($n = 5$) (Fig. 5 D, inset).

**Mg$^{2+}$ Enhances Voltage Sensor Activation when Channels Are Open**

The voltage dependence of $P_{o}$ in the WT channel provides further evidence that Mg$^{2+}$ enhances voltage sensor/gate coupling. According to Scheme 1, the equilibrium constant for voltage sensor activation when channels are open ($J_{o}D$) is directly proportional to D. Therefore the charge–voltage relation for open channels ($Q_{o}$-$V$), should be shifted to more negative voltages by Mg$^{2+}$ if coupling is enhanced. Although BK channel voltage sensors can move when channels are open, determination of $Q_{o}$ from gating currents is difficult because channels close rapidly (Horrigan and Aldrich, 1999). However, $Q_{o}$ can be assessed in a model-independent fashion from the logarithmic slope of the $P_{o}$-$V$ relation (Horrigan and Aldrich, 2002). The mean activation charge displacement ($q_{a} = kTd(ln[P_{o}]) / dV$) exhibits a bell-shaped dependence on voltage that is plotted in Fig. 5 E for 0, 10, and 100 mM Mg$^{2+}$. $q_{a}$ is expected to approximate $Q_{o}$ at voltages where both $P_{o}$ and $Q_{c}$ are small (Horrigan and Aldrich, 1999). However, $Q_{o}$ was repeatedly applied and washed out. $Q_{a}$ depends on both $D$ and $J_{o}$, whereas $Q_{c}$ depends only on $J_{o}$. Therefore the quantity $\Delta \Delta V_{CO} = [\Delta V_{hc} - \Delta V_{ho}]$, plotted in Fig. 5 F for WT and E374A/E399N channels, should depend only on $D$ (i.e., $\delta D = \exp(z_{j} \Delta \Delta V_{CO} / kT)$, based on Scheme 1). Fig. 5 F indicates $\Delta \Delta V_{CO} = 43 \pm 3$ mV in 10 mM Mg$^{2+}$, corresponding to a 2.7-fold increase in D. 100 mM Mg$^{2+}$ produces a larger $\Delta \Delta V_{CO} (56 \pm 4$ mV), corresponding to a 3.6-fold increase in D, that probably represents the effect of saturating the RCK1 site. $\Delta \Delta V_{CO}$ for the E374A/E399N mutant is not significantly different from 0 (P < 0.05, t test) in 10 or 100 Mg$^{2+}$ (Fig. 5 F), implying that all
changes in voltage sensor/gate coupling can be attributed to the RCK1 site.

**Modeling the Effects of Mg$^{2+}$ on Steady-State Activation**  
The above results suggest that 10 mM Mg$^{2+}$ acts primarily at the RCK1 binding site to enhance voltage sensor/gate coupling (D) with a minor effect on the voltage equilibrium ($J_0$), and that 100 mM Mg$^{2+}$ causes additional increases in D, $J_0$, and $L_0$ that reflect action at both RCK1 and very low affinity binding sites. To confirm that these parameter changes are sufficient to describe the effects of Mg$^{2+}$ on steady-state activation and to better quantify the changes in D we fit simultaneously the log($P_0$)-V, $q_0$-V, and $P_0$-V relations for 0, 10, and 100 mM Mg$^{2+}$ using Scheme 1, as defined by Eq. 1:

$$P_o = \frac{L(1+JD)^J}{L(1+JD)^J + (1+J)^J},$$

where $L = L_0 e^{(\frac{z_1 V}{kT})}$; $J = J_0 e^{(\frac{z_1 V}{kT})} = e^{(\frac{z_1 [V_{Hc}]}{kT})}$

The 0 Mg$^{2+}$ data were fit by setting charge parameters to values determined previously ($z_1 = 0.58 e$, $z_L = 0.3 e$) (Horrigan and Aldrich, 2002) and allowing all other parameters ($J_0$, $L_0$, D) to vary, yielding results (Table I, WT 0 Mg$^{2+}$) similar to previous reports (Horrigan and Aldrich, 2002; Ma et al., 2006). To fit the Mg$^{2+}$ data, the change in $J_0$ relative to 0 Mg$^{2+}$ was set by gating current measurements ($\Delta V_{Hc}$, Fig. 3 F), while $L_0$ and D were allowed to vary. Excellent fits to the log($P_0$)-V and $q_0$-V relations were obtained in this way (Fig. 5, B and E, dotted curves), yielding values of D (Table I, WT parameters). A similar procedure was used to fit the E374A/E399N data (Fig. 5 C, dotted curves), yielding values of D (Table I, E374A/E399N parameters) that vary by <$15\%$ relative to the control, which is within the resolution of our measurement (Ma et al., 2006). Thus, shifts in $V_{Hc}$ determined from gating currents together with an increase in $L_0$ in 100 Mg$^{2+}$ appear sufficient to account for the changes in log($P_0$)-V when the RCK1 site is disrupted.

Although the fits from Scheme 1 reproduced the effects of Mg$^{2+}$ on log($P_0$) and $q_0$, they overestimated the shift in $V_{0.5}$ especially in 100 Mg$^{2+}$ (Fig. 5 A, dotted curves). $V_{0.5}$ can be fit at the expense of underestimating the shift in the log($P_0$)-V relation by reducing the Mg$^{2+}$-dependent change in D as illustrated by alternative fits in Fig. 5 (A and B, thick solid curves) where D was increased by factors of 1.97 and 2.26 in 10 and 100 mM Mg$^{2+}$, respectively (Table I, WT$B$ parameters). These parameters reasonably approximate the 10 Mg$^{2+}$- and 100 Mg$^{2+}$-V and log($P_0$)-V relations but overestimate the steepness of $P_0$-V in 100 Mg$^{2+}$ (Fig. 5 A). This discrepancy reflects a decrease in the slope of $P_0$-V as Mg$^{2+}$ is increased from 10 to 100 mM Mg$^{2+}$, a feature of the data that is not reproduced by Scheme 1.

To account explicitly for the effects of Mg$^{2+}$ binding on the parameters in Scheme 1 we modeled the effects of very low affinity and RCK1 binding sites in terms of Schemes 2 and 3, respectively (Fig. 2, F and G). These allosteric mechanisms assume that Mg$^{2+}$ can bind to any state of the channel defined by Scheme 1 and that effects of Mg$^{2+}$ on the relative stability of different states therefore reflect the relative affinity of Mg$^{2+}$ for these states. This principal is illustrated in Fig. 5 (G and H) by alternative representations of Schemes 2 and 3 that include the four states (CR, OR, CA, OA) and equilibrium constants defined by Scheme 1 for a single subunit, where boxes and associated allosteric factors indicate the states that are stabilized relative to the CR state by ligand binding. In Scheme 2, the action of Mg$^{2+}$ is defined by its dissociation constant for the CR state ($K_0$) and two allosteric factors (C and E). C defines the increase in affinity associated with channel opening, such that $K_0$ is reduced C-fold for the OR and OA states (Fig. 5 G, labeled box). Likewise, E defines the change in $K_0$ associated with voltage sensor activation (Fig. 5 G, CA and OA states). These changes in affinity can account for effects of Mg$^{2+}$ on the equilibrium for channel opening ($L_0$) and voltage sensor activation ($J_0$). However, voltage sensor/gate coupling (D) is unchanged in Scheme 2 because the effects of C and E are independent and neither alters binding in a manner that depends on the state of both the voltage sensor and gate. By contrast, in Scheme 3, D

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Parameters for Eq. 1 that were varied to fit WT and E374A/E399N data in 0–100 mM Mg$^{2+}$ (Fig. 5, A, B, C, and E). $V_{Hc}$ in 10 and 100 mM were determined as $V_{Hc}(0) + \Delta V_{Hc}(Mg^{2+})$ where $V_{Hc}(0)$ was determined from the 0 Mg$^{2+}$ fit and $\Delta V_{Hc}(Mg^{2+})$ from gating current (Fig. 3 F). $J_0$ is determined from $V_{Hc}$ ($J_0 = \exp(-V_{Hc}/kT)$). Charge parameters were fixed ($z_1 = 0.58 e$, $z_L = 0.30 e$).
is increased F-fold upon ligand binding by assuming that the affinity of the open-activated state (OA) is increased relative to all other states by an allosteric factor F. Scheme 3 also includes an independent effect of voltage sensor activation on Mg$^{2+}$ affinity (E-factor) as in Scheme 2, to account for effects on $J_0$.

The data in Fig. 5 (A and B) were fit (red curves, Table II parameters) by a two-site model that assumes the channel contains a single RCK1 (site 1, Scheme 3) and a very low affinity (site 2, Scheme 2) binding site in each of four identical subunits, yielding Eq. 2 (see below), where $L$, $J$, and $D$ are defined as in Eq. 1: $E_1$, $F_1$ and $C_2$, $E_2$ are allosteric factors for sites 1 and 2, respectively; and $K_i = [Mg^{2+}] / K_{D_i}$, $(i = 1,2)$, where $K_{D_i}$ is dissociation constant for each binding site in the CR state.

The fits of Eq. 2 to $P_O$ and log($P_O$)-$V$ relations in 0, 10, and 100 mM Mg$^{2+}$ were almost identical to those obtained with Scheme 1 (Fig. 5, A and B) and predict shifts in $V_{HC}$ for both WT and E374A/E399N (Table II) similar to those measured from gating current (Fig. 3 F). Parameters for site 1 (RCK1) were well constrained by fitting $P_O$-$V$ relations in 2, 5, 10, and 21 mM Mg$^{2+}$ (Fig. 5 A) and yielded a $K_0$ for the CR state of 5.0 mM (the $K_0$ of other states are indicated in Table II). A small $E_1$-factor (1.35) was sufficient to explain the $\Delta V_{HC}$ attributed to the RCK1 site, whereas a larger $F_1$-factor of 2.0 was required to explain the enhancement of voltage sensor/gate coupling. The site 2 (very low affinity) parameters were not as well constrained because few concentrations $> 10 \text{ mM}$ were tested and the effect of saturating this site was not determined.

Effects of Mg$^{2+}$ on $I_g$ Kinetics and the Deactivation Pathway

Although gating current is relatively insensitive to Mg$^{2+}$ when channels are closed (Fig. 3), the ability of Mg$^{2+}$ to shift the $Q_o$-$V$ relation implies that $I_g$ kinetics must be altered when channels are open. Such an effect is evident from OFF gating currents ($I_{g,OFF}$) following pulses of different duration (0.05–20 ms, +200 mV) in 0 and 10 mM Mg$^{2+}$ (Fig. 6 A). Normalized $I_{g,OFF}$ traces (Fig. 6 B) show that OFF kinetics following brief pulses ($\leq 0.1 \text{ ms}$, red traces), that are comparable to the delay in $I_K$ activation (Horrigan et al., 1999) and open few channels, are similar in 0 or 10 Mg$^{2+}$. However, as pulse duration increases and channels open, voltage sensor deactivation is slowed, reflecting that $Q_o$-$V$ is shifted to more negative voltages than $Q_o$-$V$ (Horrigan and Aldrich, 1999). The slowing of $I_{g,OFF}$ occurs in 0 Mg$^{2+}$ but is more prominent in 10 Mg$^{2+}$ (Fig. 6 B) because Mg$^{2+}$ increases the difference between $Q_O$ and $Q_C$ (Fig. 5 F) and the fraction of channels that open at +200 mV (Fig. 2 B).

To distinguish effects of Mg$^{2+}$ on voltage sensor deactivation kinetics and $P_O$, OFF kinetics were analyzed by plotting the time course of OFF charge movement ($Q_{OFF}$) on a semi-log scale (Fig. 6 C) and comparing the effect of brief (“closed”) and prolonged (“open”) depolarizations. The decay of $Q_{OFF}$ can be described by three exponential components ($\tau_{Fast}$, $\tau_{Med}$, $\tau_{Slow}$) reflecting the predicted pathways of voltage sensor deactivation (Horrigan and Aldrich, 1999). Scheme 1* (Fig. 6 D) is a kinetic scheme that depicts the 10 states and transitions defined by Scheme 1, representing the O and C conformation with zero to four activated voltage sensors. $Q_{OFF}$ following a brief depolarization is well described by a single exponential function ($\tau_{Fast}$) representing the deactivation of voltage sensors in closed channels ($Q_o$ to $Q_C$ transitions in Scheme 1*). $Q_{OFF}$ for open channels is characterized by a component representing the deactivation of voltage sensors when channels are open (Fig. 6 D, Med) and another that is rate limited by channel closing (Fig. 6 D, Slow). Therefore, $Q_{OFF}$ following prolonged depolarizations to +200 mV (0, 10 Mg [open], Fig. 6 C) are well fit by triple exponential functions where the fast component is small because most channels are open. This analysis confirms that voltage sensor deactivation in open channels ($\tau_{Med}$, Fig. 6 C, dotted red lines) is slowed (2.3-fold) by 10 mM Mg$^{2+}$. In addition $\tau_{Slow}$ (Fig. 6 C, solid red lines) is increased 1.5-fold, consistent with a slowing of channel closing by Mg$^{2+}$.

Further insight into the mechanism of Mg$^{2+}$ action is provided by plotting the amplitude of the three OFF

\[
P_o = \frac{L[(1 + K_1)(1 + K_2C_2) + JD(1 + K_1E_1F_1)(1 + K_2C_2E_2)]^4}{L[(1 + K_1)(1 + K_2C_2) + JD(1 + K_1E_1F_1)(1 + K_2C_2E_2)]^3 + [(1 + K_1)(1 + K_2) + J(1 + K_1E_1)(1 + K_2E_2)]^4},
\]

TABLE II

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<th>$E_i$</th>
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Parameters that were varied to fit Mg$^{2+}$ data (Fig. 5, A and B) for WT (Eq. 2) and E374A/E399N (Eq. 2 with $K_0 = 0$). Remaining parameters ($\zeta_i$, $K_i$, $L$, $J$, $D$) were identical to those for 0 Mg$^{2+}$ in Table I. These parameters predict $\Delta V_{HC}$ values of $-15.7 \text{ mV}$ (WT, 10 Mg), $-36.9 \text{ mV}$ (WT, 100 Mg), $-6.5 \text{ mV}$ (E374A/E399N, 10 Mg), $-24.4 \text{ mV}$ (E374A/E399N, 100 Mg); similar to those measured in Fig. 3 F.
Colored arrows represent different pathways of deactivation and $Q_{OFF}$ components for open channels in 0 Mg$^{2+}$ (top) or 10 mM Mg$^{2+}$ (bottom) at $-80$ mV. In 10 mM Mg$^{2+}$, channels can close from open states other than $O_0$ because Mg$^{2+}$ allows voltage sensors in open channels to remain activated at $-80$ mV ($Q_o$, Fig. 5 E). The figure illustrates one possible pathway, through the $O_2$ state. After channels close, voltage sensors can completely deactivate in 10 mM Mg$^{2+}$ ($Q_r$, Fig. 3 B). This charge movement is limited by the closing rate and therefore contributes to the Slow component of $Q_{OFF}$. (E) The amplitude of the three OFF components (Fast, Med, Slow) for the experiment in A are plotted vs. pulse duration and fit by exponential functions (0 Mg$^{2+}$, $\tau_{Fast} = 24.9$ ms; 10 Mg$^{2+}$, $\tau_{Fast} = 16.3$ ms). The "closed" traces, representing the average of 0.05 and 0.1-ms records, have an exponential time course with almost identical kinetics ($\tau_{Fast}$) in 0 Mg$^{2+}$ (15.5 $\mu$s) or 10 mM Mg$^{2+}$ (16.3 $\mu$s). The open traces, representing the average of 10, 15, and 20-ms records, were fit by triple exponential functions (0 Mg$^{2+}$: $q_{Fast} = 6.2$ fC, $\tau_{Fast} = 15.5 \mu$s, $q_{MED} = 24.9$ fC, $\tau_{MED} = 54.1 \mu$s, $q_{SLOW} = 11.2$ fC, $\tau_{SLOW} = 500 \mu$s; 10 Mg$^{2+}$: $q_{Fast} = 2.6$ fC, $\tau_{Fast} = 16.3 \mu$s, $q_{MED} = 10.6$ fC, $\tau_{MED} = 127 \mu$s, $q_{SLOW} = 39.1$ fC, $\tau_{SLOW} = 756 \mu$s). The Med and Slow components of the fits are shown as dotted and solid lines respectively. (D) Scheme 1* depicts the five closed ($C_i$) and open ($O_i$) states defined by Scheme 1, where $i$ represents the number of activated voltage sensors ($i = 0$–4). $\alpha$ and $\beta$ are forward and reverse rate constants for channel opening while $\delta$ and $\gamma$ are for voltage sensor activation when channels are closed, and $D = \sqrt{D}$. Components ($Q_{Fast}$, $Q_{MED}$, $Q_{SLOW}$) versus pulse duration (Fig. 6 E). As pulse duration increases, $Q_{Fast}$ is reduced while $Q_{MED}$ and $Q_{SLOW}$ increase, following the exponential time course of channel opening (solid curves Fig. 6 E) (Horrigan and Aldrich, 1999, 2002). These kinetics are similar in the presence and absence of Mg$^{2+}$ because 10 mM Mg$^{2+}$ has little effect on activation kinetics (Shi and Cui, 2001; Zhang et al., 2001). However the relative amplitudes of $Q_{MED}$ and $Q_{SLOW}$ are reversed by Mg$^{2+}$. The change in relative amplitude of $Q_{MED}$ and $Q_{SLOW}$ by Mg$^{2+}$ is qualitatively similar to the effect of recording $I_{OFF}$ at a more positive voltage in 0 Mg$^{2+}$ (Horrigan and Aldrich, 1999), supporting that Mg$^{2+}$ shifts $Q_o$ to more negative voltages. In the absence of Mg$^{2+}$, $Q_{MED}$ is greater than $Q_{SLOW}$ because voltage sensors can completely deactivate at $-80$ before channels close (Fig. 6 D, 0 Mg$^{2+}$, light blue arrow). That is, the $Q_o$-V relation in 0 Mg$^{2+}$ indicates that voltage sensors equilibrate to the resting state at $-80$ mV (Fig. 5 E). By contrast, in 10 Mg$^{2+}$ more than 25% of voltage sensors should remain activated at $-80$ mV when channels are open (10 Mg$^{2+}$, $Q_o$-V, Fig. 5 E). Consequently, channels can close from open states other than $O_0$ (e.g., $O_2$ in Fig. 6 D, 10 Mg$^{2+}$), and voltage sensor deactivation becomes rate limited by closing (Fig. 6 D, 10 Mg$^{2+}$ pink arrow) such that the ratio of $Q_{SLOW}$ to $Q_{MED}$ is increased.

\[ Q_{OFF}(t) = q_{MED} \tau_{MED}^{-1} e^{-t/\tau_{MED}} + q_{SLOW} \tau_{SLOW}^{-1} e^{-t/\tau_{SLOW}} \]

\[ Q_{OFF} = \frac{q_{MED}}{\tau_{MED}} e^{-t/\tau_{MED}} + \frac{q_{SLOW}}{\tau_{SLOW}} e^{-t/\tau_{SLOW}} \]
Effects of Mg$^{2+}$ on $I_K$ Kinetics

The ability to slow channel closing is a characteristic of Mg$^{2+}$ action at the RCK1 site that is evident by comparing $I_K$ tail currents at $-80$ mV in 0 and 10 mM Mg$^{2+}$ (Fig. 2 A). Time constants of $I_K$ relaxation ($\tau_K$) plotted versus voltage in Fig. 7 A for 0, 10, and 100 mM Mg$^{2+}$ indicate that 10 mM Mg$^{2+}$ slows $I_K$ deactivation approximately twofold over a wide voltage range ($-380$ to $+280$ mV) with little impact on activation kinetics at very positive voltages ($+160$ to $+280$ mV). By contrast, increasing Mg$^{2+}$ from 10 to 100 mM to occupy very low affinity sites speeds activation with no additional effect on deactivation at extreme negative voltages (Fig. 7 A) (Zhang et al., 2001; Hu et al., 2006).

That 10 and 100 mM Mg$^{2+}$ have distinct effects on $\tau_K$ is not surprising given their action at different binding sites. However, the effects of Mg$^{2+}$ on deactivation kinetics are remarkable in comparison to the effects on steady-state activation. Macroscopic $I_K$ kinetics are rate limited by the C-O transition (Horrigan and Aldrich, 1999) and at sufficiently negative voltages by the O$_6$ to C$_0$ rate constant (Fig. 6 D, top). Thus, the ability of 10 Mg$^{2+}$ to slow deactivation at extreme negative voltages is surprising given that 10 mM Mg$^{2+}$ has no detectable effect on the equilibrium constant for the C$_0$-O$_6$ transition ($I_0$). Similarly, the failure of 100 Mg$^{2+}$ to slow deactivation relative to 10 Mg$^{2+}$ is notable because 100 Mg$^{2+}$ increases $I_0$.

Although previous studies have shown that Mg$^{2+}$ slows deactivation at voltages as low as $-200$ mV, measurements at more negative voltage were required to confirm that the O$_6$ to C$_0$ transition rate is altered. Because Mg$^{2+}$ shifts the Q-V relation, channels can close by pathways involving different C-O transitions in the presence and absence of Mg$^{2+}$ (e.g., Fig. 6 D). This difference contributes to the slowing of tail currents in Mg$^{2+}$ at $-80$ mV. However at sufficiently negative voltages, $\tau_K$ should be limited only by the O$_6$ to C$_0$ transition whether or not Mg$^{2+}$ is present. Consistent with this prediction, at voltages less than $-240$ mV the difference in $\tau_K$ between 10 and 100 mM Mg$^{2+}$ is almost eliminated and $\tau_K$ achieves a limiting voltage dependence that is identical in the presence or absence of Mg$^{2+}$ (Fig. 7 A, dotted lines). Normalized $\tau_K$-V relations (Fig. 7 B) illustrate that a major effect of Mg$^{2+}$ is to shift the $\tau_K$-V relation along the voltage axis, consistent with effects on Q$_6$. However, the ability of 10 mM Mg$^{2+}$ to increase $\tau_K$ 1.53-fold at V $<-240$ mV confirms that the O$_6$ to C$_0$ rate is also reduced.
That the kinetics of the $C_0-O_0$ transition are affected by 10 mM Mg$^{2+}$ without altering the equilibrium constant suggests that both the forward and reverse rate constants for this transition must be slowed. To test this hypothesis we analyzed unitary current kinetics from a macropatch containing R167E channels at $-80$ mV where voltage sensors should be in the resting state. Although the patch contained an estimated 10,000 channels, $>99\%$ of openings were to the single channel level because $P_0$ at this voltage is low ($4 \times 10^{-7}$ in 0 Mg$^{2+}$, Fig. 5 D). Both open and closed dwell times histograms were reasonably fit by single exponential components as illustrated for 0 Mg$^{2+}$ in Fig. 7 C, consistent with a two-state $C_0-O_0$ transition. Changes in $N P_0$ (Fig. 7 D) and mean open and closed times (Fig. 7 E) are plotted as $[\text{Mg}^{2+}]$ was changed in sequence from 100 mM to 0, 10, and back to 0 Mg$^{2+}$. The results show that the change from 0 to 10 mM Mg$^{2+}$ increases both open and closed times, consistent with a similar slowing of both forward and backward rates of channel opening that leaves $N P_0$ unchanged. Open times were brief but comparable to the $\sim100$-µs time constant of macroscopic $I_n$ measured for this patch at $-80$ mV in 0 Mg$^{2+}$. The frequency of channel openings was reversibly reduced by 10 mM Mg$^{2+}$ as expected from the effect on mean closed times. In a patch with N independent channels and brief openings the mean closed time should equal $N/\text{opening rate}$, implying that the mean closed time of individual channels was $\sim3$–4 min. Increasing Mg$^{2+}$ from 0 to 100 mM only slightly increased open times but markedly decreased closed times, consistent with the ability of 100 Mg$^{2+}$ to increase $N P_0$ without changing $\tau_K$ relative to 10 mM Mg$^{2+}$ at negative voltages. These conclusions about the $C_0-O_0$ transition from single-channel analysis are consistent with effects of Mg$^{2+}$ on rate constants in Scheme 1* ($\delta_0$, $\gamma_0$) derived by fitting the macroscopic data (Fig. 7 A, solid curves, Table III parameters).

The effect of Mg$^{2+}$ on the kinetics of the $C_0-O_0$ transition is important in providing mechanistic information that is not evident from the steady-state data. In particular, the ability of 10 mM Mg$^{2+}$ to slow opening and closing rates demonstrates that Mg$^{2+}$ remains bound to the RCK1 site and can influence channel gating when voltage sensors are in the resting state even though $P_0$ is insensitive to Mg$^{2+}$ under these conditions.

### DISCUSSION

Our results show that intracellular Mg$^{2+}$ activates Slo1 BK channels through a fundamentally different mechanism from Ca$^{2+}$. While micromolar [Ca$^{2+}$], promotes channel opening independent of voltage sensor activation, millimolar [Mg$^{2+}$], acting at a putative binding site in the cytosolic RCK1 domain has effects on opening and voltage sensor activation that are highly interdependent. Mg$^{2+}$ promotes opening only when voltage sensors are activated and facilitates voltage sensor activation mainly when channels are open. Because Mg$^{2+}$ action depends on the conformation of both voltage sensor and gate, the primary effect on gating is to enhance voltage sensor/gate coupling such that open-activated states are stabilized. A two-site allosteric model that incorporates this mechanism reproduces the major effects of Mg$^{2+}$ on steady-state activation. As discussed below, these results support that multiple pathways of communication exist between the cytosolic and transmembrane domains of BK channels. Our data also constrain the possible physical mechanisms that underlie Mg$^{2+}$-dependent activation and voltage sensor/gate coupling, and suggest that the physiological impact of Mg$^{2+}$ could differ qualitatively from that of Ca$^{2+}$.

### Communication between Cytosolic and Transmembrane Domains

Ca$^{2+}$ has no effect on voltage sensor/gate coupling (Horrigan and Aldrich, 2002) and is thought to promote channel opening by pulling on the RCK1–S6 linker (Niu et al., 2004). Therefore, the ability of Mg$^{2+}$ to enhance coupling suggests that the cytosolic domain can communicate with the transmembrane domain through pathways other than the RCK1–S6 linker, presumably involving direct or indirect interactions with the voltage sensor (e.g., Fig. 1 D). A similar conclusion was drawn from the effects of intracellular heme (Horrigan et al., 2005).

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**Table III**

<table>
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<th><a href="mM">Mg$^{2+}$</a></th>
<th>$\tau_0$</th>
<th>$\delta_0$</th>
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</table>

Rate constants for the $C_i-O_i$ transitions in Scheme 1* (Fig. 6 D) determined from fits to WT $\tau_K$V relations in 0-100 mM Mg$^{2+}$ (Fig. 7 A), using equilibrium parameters from Table I (WT, WT$\alpha$). Since voltage sensor activation equilibrates rapidly, $\tau_K$ was fit by $\tau_K = \left[ \sum_i \left( \delta_i p C_i + \gamma_i p O_i \right) \right]^{-1}$, where $i$ represents the number of activated voltage sensors, $\delta_i$ and $\gamma_i$ are forward and backward rate constants for the $C_i-O_i$ transitions and $p C_i$, $p O_i$ are conditional occupancies of the open and closed states ($p C_i = p(C_i|C)$, $p O_i = p(O_i|O)$) (Horrigan et al., 1999). Forward rates ($\delta_i$) were varied while backward rates were $\gamma_i = \delta_i/L_0\delta^i$ and partial charges associated with these rate constants were $z_0 = 0.165\epsilon$ and $z_i = -0.135\epsilon$ (Horrigan and Aldrich, 2002).
However, the action of Mg\(^{2+}\) is remarkable in its simplicity and specificity relative to heme. While heme weakens voltage sensor/gate coupling, it also reduces the efficacy of Ca\(^{2+}\) and increases P\(_O\) at extreme negative voltages (i.e., increases L\(_0\)). These diverse effects on gating, together with a slow onset of action (minutes), suggest that heme binding may perturb the normal conformation of the cytosolic domain such that tension in the RCK1–S6 linker is increased while multiple allostERIC interactions are also disrupted (Horrigan et al., 2005). In contrast, 10 mM Mg\(^{2+}\) does not alter Ca\(^{2+}\) sensitivity (Shi and Cui, 2001; Zhang et al., 2001) and acts rapidly (<1 s) at the RCK1 binding site to enhance voltage sensor/gate coupling without affecting the intrinsic stability of the gate (L\(_0\)). The effect of Mg\(^{2+}\) is complimentary to that of Ca\(^{2+}\), which increases L\(_0\) with no effect on coupling (Horrigan and Aldrich, 2002). Thus Mg\(^{2+}\) and Ca\(^{2+}\) appear to independently regulate two different pathways of communication, consistent with their additive effects on channel activation. Unlike heme, the effects of Mg\(^{2+}\) do not suggest a widespread structural perturbation, and can be understood in terms of stabilizing particular channel states.

### The Energetic Effects of Mg\(^{2+}\)

Mg\(^{2+}\) at the RCK1 site acts primarily to stabilize the open-activated (OA) state relative to the other three states (CR, CA, and OR) defined by the voltage sensor and gate conformation in each subunit (Fig. 5 H; Table II). We reached this conclusion by measuring independently each of the equilibrium constants between these states, in effect determining their relative free energies in the presence and absence of Mg\(^{2+}\), as specified by three independent parameters (L\(_0\), J\(_0\), and D). The CR-OR equilibrium constant (L\(_0\)) is not altered. The CR-CA equilibrium constant (J\(_0\)) is only increased slightly. However, the CA-OA and OR-OA equilibria are substantially altered, consistent with an approximate twofold increase in the voltage sensor/gate coupling factor D. By comparing the effects of Mg\(^{2+}\) on WT and mutant (E374A/E399N) channels, we showed that all changes in voltage sensor/gate coupling and a fraction of the changes in J\(_0\) can be attributed to the RCK1 site. Modeling the effects of Mg\(^{2+}\) in terms of a two-site allosteric scheme (Table II) suggests that saturating the RCK1 site (site 1) stabilizes the OA state relative to CR by \(\Delta G_{\text{CR}}^{OA} = kT \ln(E_{F_1}) = 0.60\text{ kcal mol}^{-1}\), while stabilizing the CA state by \(\Delta G_{\text{CR}}^{CA} = kT \ln(E_{F_1}) = 0.18\text{ kcal mol}^{-1}\). Thus the energetic effect of Mg\(^{2+}\) is weak compared with that of Ca\(^{2+}\) binding or voltage sensor activation (1.25 or 1.91 kcal mol\(^{-1}\) per subunit) (Horrigan and Aldrich, 2002).

### Parallel Pathways of Voltage Sensor/Gate Coupling

While the interactions that couple voltage sensor activation to channel opening are well defined energetically, they could reflect many different physical mechanisms. The coupling represents a differential effect of voltage sensor conformation on the free energy of closed and open states. Therefore voltage sensor and gate must interact in a state-dependent manner that depends on the conformation of both domains. Such interactions could represent a stabilizing interaction (e.g., bond formation) between voltage sensor and gate that occurs only in the OA state, or destabilizing interactions (e.g., steric or repulsive) that occur in every state but OA. Alternatively, sensor and gate could be coupled in all states by a spring-like connection whose tension varies with both voltage sensor activation and channel opening.

Any of the above or similar mechanisms, alone or in combination, could contribute to the allosteric coupling factor D. Thus the ability of Mg\(^{2+}\) to increase D does not require that interactions that normally mediate coupling in the absence of Mg\(^{2+}\) are enhanced. Indeed, two lines of evidence suggest that the effect of Mg\(^{2+}\) represents a parallel pathway of coupling, independent of that in the transmembrane domain (Fig. 8 A). First, Mg\(^{2+}\) in the RCK1 binding site appears to interact with a part of the voltage sensor that is not important for voltage sensor/gate coupling in the absence of Mg\(^{2+}\). Interactions of the S4–S5 linker near S5 with S6 are proposed to mediate coupling in Kv channels (Lu et al., 2002; Long et al., 2005b). Although mutations in the S4–S5 linker of Slo1 influence Mg\(^{2+}\) sensitivity (Hu et al., 2003), they are at sites near S4 (E219, Q222) that are unlikely to interact directly with S6. In addition, mutations of R213 in S4 virtually abolish Mg\(^{2+}\) sensitivity (Hu et al., 2003; Yang et al., 2007), but have no effect on voltage sensor/gate coupling in 0 Mg\(^{2+}\) (Ma et al., 2006). Second, interactions between the voltage sensor and Mg\(^{2+}\)-binding site should be sufficient to influence voltage sensor/gate coupling, because the binding site is in a cytosolic domain attached to S6 and therefore is connected to the gate.

### Physical Mechanism of Mg\(^{2+}\) Action

Since Mg\(^{2+}\) stabilizes primarily the OA state, thermodynamics require that Mg\(^{2+}\) affinity is greatest in the OA state, as in Scheme 3. However, the conclusion that Mg\(^{2+}\) binding is favored in a particular state does not fully describe how and when Mg\(^{2+}\) interacts with its binding site and the voltage sensor. If we suppose that the interactions of Mg\(^{2+}\) with its binding site are purely stabilizing, then interactions must exist in the OA state that are stronger or more numerous than in other conformations. Such a situation could arise if both the cytosolic and voltage sensor domains contribute to the Mg\(^{2+}\) binding site, but are favorably aligned with each other only when the voltage sensor is activated and the channel is open. However, this hypothesis appears too simple given recent evidence that a destabilizing electrostatic interaction exists between Mg\(^{2+}\) and the voltage sensor (Yang et al., 2007); and physical models that take this into account are necessarily more complex, as discussed below.
The effects on Mg\(^{2+}\) sensitivity and gating of charge-altering mutations in the voltage sensor and RCK1 domain strongly suggest that Mg\(^{2+}\) activates the channel through a long-range electrostatic interaction with R213 in S4 (Yang et al., 2007). Addition of positive charge at position 397 near the RCK1 site (Yang et al., 2006), by either mutation (Q397K, Q397R) or chemical modification, mimics the major effects of Mg\(^{2+}\) on channel gating; whereas negative charge at this position (Q397E, Q397D) inhibits activation. Conversely a positive charge at position 213 appears necessary for sensing either Mg\(^{2+}\) or charge at position 397. Finally, the effects on gating of Mg\(^{2+}\) or Q397K but not Ca\(^{2+}\) are strongly inhibited in high ionic strength solutions (1 M KCl), consistent with a long-range electrostatic interaction between Mg\(^{2+}\) and the voltage sensor. Based on these results, Yang et al. propose that Mg\(^{2+}\) bound to the RCK1 domain alters the local electric field sensed by R213 in S4 and thereby promotes voltage sensor activation. That is, electrostatic repulsion between Mg\(^{2+}\) and R213 destabilizes the resting conformation of the voltage sensor. This interaction is presumably state dependent since voltage sensor activation is enhanced by Mg\(^{2+}\) primarily when the channel is open.

The observations of Yang et al. (2007) together with our functional analysis place important constraints on the physical nature and state dependence of the interactions underlying Mg\(^{2+}\)-dependent activation. Most importantly, the ability of R213 mutants to virtually eliminate Mg\(^{2+}\) sensitivity suggests that the electrostatic interaction between R213 and Mg\(^{2+}\) accounts for the major effects of Mg\(^{2+}\) on gating, and therefore must act on balance to stabilize the OA state. This conclusion has two key implications. First, the coordination of Mg\(^{2+}\) at its binding site cannot be strongly state dependent. If it were, then the channel’s Mg\(^{2+}\) affinity should change in a state-dependent manner even in the absence of electrostatic interaction with S4; and gating should therefore remain Mg\(^{2+}\) sensitive when R213 is mutated. Although subtle effects of this sort cannot be ruled out, R213 mutations largely eliminate the G\(_{m,V}\) shift and slowing of I\(_{K}\) deactivation by Mg\(^{2+}\) (Hu et al., 2003; Yang et al., 2007). Thus it appears unlikely that conformational changes in the binding site associated with channel opening favor Mg\(^{2+}\) binding and contribute to Mg\(^{2+}\)-dependent activation. This conclusion is supported by the observation that a permanent positive charge at position 397 mimics the effects of Mg\(^{2+}\) (Yang et al., 2007). Second, if Mg\(^{2+}\)
stabilizes the OA state via a destabilizing electrostatic interaction, then the interaction must be weakest in the OA state and of similar strength in all other states (CR, CA, OR). That is, Mg$^{2+}$ must destabilize all states relative to OA.

That Mg$^{2+}$ interacts with the voltage sensor in multiple states including closed states may seem counterintuitive since Mg$^{2+}$ has little effect on voltage sensor activation when channels are closed. Indeed, interaction in a single state, open resting (OR), would be sufficient to account for the ability of Mg$^{2+}$ to promote voltage sensor activation when channels are open, by destabilizing OR relative to OA. However, this simple model cannot account for the effects of Mg$^{2+}$ on channel opening. If the OR state is destabilized without also destabilizing the CR state then channel opening should be inhibited when voltage sensors are in the resting state, inconsistent with our finding that $L_0$ is unaffected by 10 mM Mg$^{2+}$. Conversely, a lack of interaction between Mg$^{2+}$ and the activated voltage sensor would be inconsistent with the ability of Mg$^{2+}$ to facilitate opening of R210C channels when voltage sensors are constitutively activated. Thus accounting for effects of Mg$^{2+}$ on both channel opening and voltage sensor activation requires that multiple states are destabilized.

The conformational changes that allow Mg$^{2+}$ to interact with the voltage sensor in a state-dependent manner are not known in detail. However, a speculative model consistent with our results is illustrated in Fig. 8 B. The model assumes that Mg$^{2+}$ and the voltage sensor interact electrostatically in all states but that the interaction is weakest in the OA state (as indicated by red boxes). Consequently, the OA state has the highest affinity for Mg$^{2+}$ because the voltage sensor repels Mg$^{2+}$ the least. For illustration purposes, interaction strength is assumed to vary with changes in the distance between Mg$^{2+}$ and the charged R213 sidechain, although changes in local dielectric environment could also be involved. Fig. 8 B shows how the distance between Mg$^{2+}$ and R213 could remain relatively constant in all states other than OA if the position of the Mg$^{2+}$-binding site moves with channel opening and R213 moves with voltage sensor activation.

The insensitivity of $P_o$ to Mg$^{2+}$ at extreme negative voltages is accounted for in Fig. 8 B by assuming the Mg$^{2+}$-binding site in the closed and open conformation occupies two different locations that are equidistant from R213 in the resting state. In this way the CR and OR states are equally destabilized. Interestingly, this mechanism is also consistent with our finding that Mg$^{2+}$ slows both opening and closing rates when voltage sensors are in the resting state. This kinetic effect implies that Mg$^{2+}$ destabilizes a transition state between CR and OR. In Fig. 8 B, movement of the Mg$^{2+}$ binding site between closed and open positions would allow Mg$^{2+}$ to approach R213 more closely in a transition state than in either the CR or OR states, thereby destabilizing the transition state.

Although the details of Fig. 8 B are speculative, they are consistent with known features of BK and related channels. The location and movement of the Mg$^{2+}$-binding site relative to R213 is based on the crystal structures of MthK and Kv1.2. Residues in MthK (E138, N158), corresponding to E374 and E399 in the putative Mg$^{2+}$ binding site of Slo1, are on the surface of the gating ring facing the membrane at a distance from the central axis of the channel (∼31 Å) that roughly matches the radial position of the S4 segment in Kv1.2 (Yang et al., 2007). The Mg$^{2+}$-binding site is shown to move away from the central axis upon channel opening, consistent with an expansion of the cytosolic domain as in MthK (Jiang et al., 1999; Ye et al., 2006). Voltage sensor activation is depicted as a tilt and outward translation of S4 that is qualitatively consistent with recent models of Kv channel activation (Ruta et al., 2005; Campos et al., 2007; Pathak et al., 2007). We have assumed that movement of R213 perpendicular to the membrane is small such that interaction with Mg$^{2+}$ in the CA state is not disrupted. This assumption is consistent with the fact that Slo1 is weakly voltage dependent and neutralization of R213 reduces the gating charge associated with voltage sensor activation ($z_j$) by only 0.3 $e$ (Ma et al., 2006).

One possibility that was not incorporated into Fig. 8 B is that the position of R213 in the transmembrane domain may change with channel opening. Previous results suggest that R213 moves relative to the membrane electric field during opening because mutations at this position reduce the charge associated with opening ($z_j$) (Ma et al., 2006). If the position of R213 depends on the conformation of both voltage sensor and gate then the requirement that the Mg$^{2+}$-binding site move during channel opening may be reduced. Therefore, Fig. 8 B does not represent a unique solution, but it does illustrate the constraints that must be satisfied to account for the experimental data in terms of electrostatic interactions.

Very Low Affinity Mg$^{2+}$ Binding Sites
Divalent cation binding sites with lower affinity for Mg$^{2+}$ than the RCK1 site are proposed to exist in BK channels because G$_{K-V}$ shifts produced by increasing [Mg$^{2+}$] or [Ca$^{2+}$] from 10 to 100 mM appear too large to represent surface charge screening (Zhang et al., 2001) and are nearly independent of mutations that eliminate responses to lower concentrations of Mg$^{2+}$ or Ca$^{2+}$ (Hu et al., 2006). The location, number, and physiological significance of these very low affinity sites are unknown. However, our results provide new information about their mechanism of action.

We find that 100 mM Mg$^{2+}$, unlike 10 mM Mg$^{2+}$, increases $L_0$ and causes a shift in $V_{h}$ that is only partially
eliminated by mutating the RCK1 site. These effects can be reproduced by an allosteric mechanism (Scheme 2) similar to that used to describe effects of micromolar Ca$^{2+}$. However, very low affinity sites and Ca$^{2+}$ sites differ greatly in the relative magnitude of their effects on the gate and voltage sensor represented by allosteric factors C and E, respectively. The \( G_{K-V} \) shift by Ca$^{2+}$ is dominated by a C-factor of 8, which results in a 4,000-fold increase in \( P_0 \) at \(-120 \text{ mV} \) (Horrigan and Aldrich, 2002). By contrast the very low affinity site \((C = 1.6)\) increases \( P_0 \) \((-120 \text{ mV})\) approximately threefold in 100 mM Mg$^{2+}$, sufficient to account for only one third of the G-V shift from \(10 \text{ to } 100 \text{ mM Mg}^{2+}\). The majority of the G-V shift in high [Mg$^{2+}$] reflects a change in \( V_{hC} \), which was attributed to an E-factor of 2.3.

The ability of 100 mM Mg$^{2+}$ to increase \( L_0 \) supports that the effects of high [Mg$^{2+}$] reflect mechanisms other than surface charge screening. However, the possibility remains that surface charge screening contributes to \( V_{hC} \). Hu et al. (2006) estimated, based on surface potential calculations, that such a mechanism could shift \( V_{hC} \) by \( \sim -9 \text{ mV} \) from 10 to 100 mM Mg$^{2+}$, which is roughly half of the observed \( \Delta V_{hC} \) \((-19 \text{ mV})\) for the WT over this concentration range. Therefore, although we were able to model the effects of Mg$^{2+}$ in terms of a single very low affinity site per subunit this is likely an oversimplification. The E-factor in our model could represent a combination of allosteric and surface charge effects. In addition, we observed a decrease in \( G_{K-V} \) slope from 10 to 100 Mg$^{2+}$ that was not accounted for by our model.

Relation to Previous Studies and Physiological Implications

Since Mg$^{2+}$ and Ca$^{2+}$ act through distinct mechanisms, it seems surprising that previous studies were able to model effects of Mg$^{2+}$ on the \( G_{K-V} \) relation, in the presence and absence of Ca$^{2+}$, by assuming Mg$^{2+}$ and Ca$^{2+}$ act through the same mechanism (Shi and Cui, 2001; Zhang et al., 2001; Hu et al., 2006). This comparison raises several questions addressed below. First, can our model account for previous observations that the \( G_{K-V} \) shift by Mg$^{2+}$ is similar in the presence or absence of Ca$^{2+}$? Second, were conclusions of previous studies affected by the use of a Ca$^{2+}$-like model of Mg$^{2+}$ action? Third, is the difference in mechanism of Mg$^{2+}$ and Ca$^{2+}$ action physiologically significant?

Although our experiments were performed in 0 Ca$^{2+}$, we simulated the effect of saturating Ca$^{2+}$ on our two-site model of Mg$^{2+}$-dependent activation (Eq. 2) by increasing the values of \( L_0 \) (4096-fold) and \( J_0 \) (2.4-fold) as predicted from previous work on Ca$^{2+}$-dependent activation (Horrigan and Aldrich, 2002). This simulation predicts that an increase in [Mg$^{2+}$] from 0 to 100 mM will shift \( V_{0.5} \) by \(-105 \text{ mV} \) and \(-78 \text{ mV} \) in 0 and saturating Ca$^{2+}$, respectively, similar to the experimental results measured from Zhang et al. (2001) in 0 Ca$^{2+}$ \((-104 \text{ mV} \) and \(300 \text{ mM Ca}^{2+} \) \((-72 \text{ mV})\). Thus our model appears to reproduce the combined effects of Mg$^{2+}$ and Ca$^{2+}$ on steady-state activation.

Conclusions about the Mg$^{2+}$ affinity of the RCK1 site should be relatively unaffected by the incorrect assumption that Mg$^{2+}$ acts through a Ca$^{2+}$-like mechanism. Previous studies assumed that Mg$^{2+}$ increases the C-O equilibrium constant \( L_0 \) by binding with higher affinity to the open rather than closed conformation (Shi and Cui, 2001; Zhang et al., 2001; Hu et al., 2006). Similarly, our model of the RCK1 site (Scheme 3) assumes the Mg$^{2+}$ affinity of the open-activated (OA) state is greater than any other state. That is, Mg$^{2+}$ does bind better to the open conformation than the closed, so long as the probability that an open channel is in the OA state is high. This probability is indicated by the \( Q_o-V \) relation, which is saturated at voltages where the G-V is usually measured (Fig. 5, compare A and E). Therefore, Scheme 3 affects the G-V relation similar to models that assume Mg$^{2+}$ binds equally well to all open states. Consistent with this similarity a previous study using a two-site Ca$^{2+}$-like model of Mg$^{2+}$ action estimated \( K_0 \) values for the RCK1 site of 5.46 mM (closed) and 2.25 mM (open) (Hu et al., 2006), comparable to our estimates based on Eq. 2 \( (K_0(CR) = 5 \text{ mM}, K_0(CA) = 3.7 \text{ mM}, K_0(OA) = 1.85 \text{ mM})\). The physiological impact of Mg$^{2+}$-dependent BK channel activation has yet to be defined. However, our results raise the possibility that Mg$^{2+}$ could have a differential impact on BK-dependent processes such as action potential shaping in neurons and the control of vascular smooth muscle tone. Both these processes require an increase in [Ca$^{2+}$], to open BK channels that are poorly activated by resting [Ca$^{2+}$], at physiological voltages. During an action potential, Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels together with membrane depolarization activates BK channels to regulate spike duration and the fast afterhyperpolarization (Faber and Sah, 2003). By contrast, BK channels in vascular smooth muscle can contribute to resting membrane potential and myogenic tone by activating at hyperpolarized voltages, near \(-40 \text{ mV} \), in response to local Ca$^{2+}$ release from internal stores (Ca$^{2+}$ sparks) (Brayden and Nelson, 1992; Wellman and Nelson, 2003). Since physiological concentrations of free Mg$^{2+}$ (0.4–3 mM) (Gupta et al., 1984; Romani and Scarpa, 1992) are sufficient to shift the \( G_{K-V} \) relation (Hu et al., 2006) and the effects of Mg$^{2+}$ and Ca$^{2+}$ on \( V_{0.5} \) are approximately additive, one might suppose that Mg$^{2+}$ simply increases \( P_o \) during any Ca$^{2+}$ transient; enhancing apparent Ca$^{2+}$ sensitivity. However, our results suggest this is not the case. The effect of Mg$^{2+}$, unlike Ca$^{2+}$, depends on voltage sensor activation and is greatly diminished at hyperpolarized potentials (Fig. 2 C). Therefore, Mg$^{2+}$ might be expected to have a smaller impact on vascular smooth muscle tone than on action potential shaping. Of course
determining the magnitude of such differences and the overall contribution of Mg\(^{2+}\) to BK channel physiology must await further investigation.

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