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Divalent Cation Sensitivity of BK Channel Activation Supports the Existence of Three Distinct Binding Sites

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ABSTRACT Mutational analyses have suggested that BK channels are regulated by three distinct divalent cation-dependent regulatory mechanisms arising from the cytosolic COOH terminus of the pore-forming α subunit. Two mechanisms account for physiological regulation of BK channels by μM Ca2+. The third may mediate physiological regulation by mM Mg2+. Mutation of five aspartate residues (5D5N) within the so-called Ca2+ bowl removes a portion of a higher affinity Ca2+ dependence, while mutation of D362A/D367A in the first RCK domain also removes some higher affinity Ca2+ dependence. Together, 5D5N and D362A/D367A remove all effects of Ca2+ up through 1 mM while E399A removes a portion of low affinity regulation by Ca2+/Mg2+. If each proposed regulatory effect involves a distinct divalent cation binding site, the divalent cation selectivity of the actual site that defines each mechanism might differ. By examination of the ability of various divalent cations to activate currents in constructs with mutationally altered regulatory mechanisms, here we show that each putative regulatory mechanism exhibits a unique sensitivity to divalent cations. Regulation mediated by the Ca2+ bowl can be activated by Ca2+ and Sr2+, while regulation defined by D362/D367 can be activated by Ca2+, Sr2+, and Cd2+. Mn2+, Co2+, and Ni2+ produce little observable effect through the high affinity regulatory mechanisms, while all six divalent cations enhance activation through the low affinity mechanism defined by residue E399. Furthermore, each type of mutation affects kinetic properties of BK channels in distinct ways. The Ca2+ bowl mainly accelerates activation of BK channels at low [Ca2+], while the D362/D367-related high affinity site influences both activation and deactivation over the range of 10–300 μM Ca2+. The major kinetic effect of the E399-related low affinity mechanism is to slow deactivation at mM Mg2+ or Ca2+. The results support the view that three distinct divalent-cation binding sites mediate regulation of BK channels.

KEY WORDS: BK channels • Ca2+ regulatory sites • divalent cations • Slo • activation

INTRODUCTION

The sites and mechanisms by which Ca2+ binding results in regulation of Ca2+-activated and voltage-dependent BK type K+ channels (Barrett et al., 1982; Moczydlowski and Latorre, 1983; Horrigan and Aldrich, 2002; Lingle, 2002; Magleby, 2003) remain incompletely understood. Available evidence suggests that the Ca2+-regulatory machinery is contained in the extensive cytosolic COOH terminus of each BK channel α subunit (Schreiber and Salkoff, 1997; Schreiber et al., 1999; Bian et al., 2001; Bao et al., 2002; Shi et al., 2002; Xia et al., 2002, 2004), but that physiological regulation by Ca2+ is defined by multiple determinants in the COOH terminus. Specifically, mutational analyses indicate that at least three distinct Ca2+ regulatory mechanisms in each α subunit may influence activation of BK channels, two of μM affinity mediating physiological regulation by Ca2+ (Schreiber and Salkoff, 1997; Bao et al., 2002; Xia et al., 2002) and one of lower affinity perhaps providing the basis for channel regulation by cytosolic Mg2+ (Shi et al., 2002; Xia et al., 2002). However, except perhaps for the so-called Ca2+ bowl residues (Schreiber and Salkoff, 1997; Bian et al., 2001; Bao et al., 2004), the extent to which these mutationally defined loci correspond to specific divalent cation binding sites remains unclear. In addition, the extent to which the three distinct regulatory effects are coupled or independent remains unknown.

Explicit structural information about the BK channel COOH terminus remains unavailable. However, the recognition that domains within the BK channel COOH terminus exhibit homology to ligand-binding RCK (regulatory of conductance for K+) domains of bacterial K+ channels (Jiang et al., 2001, 2002) provides a substantive guide to evaluating the BK channel cytosolic structure. Specifically, the Ca2+-regulated MthK bacterial K+ channel exhibits an octameric arrangement of RCK domains, with each RCK domain proposed to contain a Ca2+-binding site (Jiang et al., 2002). Similarly, the COOH terminus of each BK channel α subunit has also been proposed to contain a pair of RCK domains (Jiang et al., 2002; Roosild et al., 2004). Whereas the homology of the first RCK domain in the BK channel α
subunit to bacterial RCK domains is fairly well defined, the second BK RCK domain is less clear (Roosild et al., 2004). Yet, the model provided by the MthK channel raises the possibility that two ligand binding sites per BK α subunit may be required to produce the conformational changes required for channel opening.

The mutational analysis, in fact, can be viewed as supporting the idea that there are separate Ca\(^{2+}\) regulatory elements at least loosely associated with each of the RCK-containing motifs of each α subunit. One of these elements is the Ca\(^{2+}\) bowl, an aspartate-rich region near the COOH terminus (Schreiber and Salkoff, 1997). The fact that Ca\(^{2+}\) bowl mutations remove only a portion of Slo1 Ca\(^{2+}\) dependence suggested that there must be additional Ca\(^{2+}\) regulatory sites. Subsequently, two separate loci in the first RCK domain were shown to disrupt a second component of Slo1 regulation by micromolar Ca\(^{2+}\) (Bao et al., 2002; Xia et al., 2002). Residues D362 and D367 were identified by a search for negatively charged residues in the first RCK domain that were shared among all Slo1 homologues, but not found in the Ca\(^{2+}\)-independent, pH-regulated Slo3 channel (Xia et al., 2002). The D362A/D367A mutation, in conjunction with mutation of the Ca\(^{2+}\) bowl, results in complete abolition of Slo1 regulation up to 1 mM Ca\(^{2+}\). Another mutation, M513L, behaved in a fashion similar to the D362A/D367A mutation (Bao et al., 2002), both mutations removing a similar component of regulation by Ca\(^{2+}\). Thus, these results suggest that distinct Ca\(^{2+}\) binding sites, associated with two distinct modules in the COOH terminus of the BK channel α subunit, may account for regulation of Slo1 current by micromolar Ca\(^{2+}\). Finally, a third set of residues, also in the first RCK domain, participates in regulation by mM Ca\(^{2+}\) or Mg\(^{2+}\) (Shi and Cui, 2001; Xia et al., 2002). While Ca\(^{2+}\) overlay assays have supported the idea that the Ca\(^{2+}\) bowl may bind divalent cations directly (Bian et al., 2001; Braun and Sy, 2001; Bao et al., 2004), at present there is no information about Ca\(^{2+}\) binding to the first RCK domain. Thus, in the absence of such information, it remains possible that the mutations in the first RCK domain do not represent a distinct binding site, but simply disruption of a component of Ca\(^{2+}\) regulation initiated by a single Ca\(^{2+}\) binding site elsewhere on the protein.

We hypothesize that if each regulatory mechanism involves a distinct divalent cation binding site, each mechanism might exhibit differences in selectivity among different divalent cations. In fact, it has been previously observed that the functional disruption of the calcium bowl does not disrupt BK channel regulation produced by Cd\(^{2+}\), leading to the suggestion that the BK channel may contain additional Ca\(^{2+}\) regulatory sites (Schreiber and Salkoff, 1997). A number of other divalent cations, such as Mn\(^{2+}\), Ni\(^{2+}\), and Co\(^{2+}\), are also known to regulate BK activation (Oberhauser et al., 1988), but how their effects relate to known regulatory mechanisms is unknown. We have therefore evaluated the divalent cation sensitivity of each of the three mutationally defined processes contributing to regulation of BK channels. Furthermore, to assess whether the regulatory sites act independently or in concert to regulate BK gating, we also examined the kinetic consequences of mutation of each regulatory site.

Our results show that each mutationally defined regulatory mechanism exhibits a sensitivity to a set of divalent cations distinct from each of the other two regulatory mechanisms. This argues strongly that each regulatory mechanism involves a specific and unique divalent cation binding site located on each α subunit, although it does not establish that the mutated residues contribute to cation coordination. Furthermore, each regulatory site exerts distinct effects on channel kinetic behavior. The results support the idea that there are three distinct divalent cation binding sites that contribute in independent ways to regulation of BK channel activation.

**MATERIALS AND METHODS**

**Generation and Expression of mSlo1 Mutants**

The wild-type construct for these studies was mouse Slo1 (mSlo1) (Butler et al., 1993), generously provided by L. Salkoff (Washington University, St. Louis, MO). Numbering used here for mSlo1 residues begins with the second potential initiation site. Construction of mutations was as previously described (Xia et al., 2002). Disruption of Ca\(^{2+}\) bowl function was accomplished by neutralization of five consecutive aspartic acid residues in that region (5D5N; mSlo1 residues 897 through 901). Mutations in the first RCK domain disrupted another high affinity Ca\(^{2+}\) regulatory effect (D362A/D367A), while a mutation in a second location in the first RCK domain (E399A) disrupted low affinity regulation by Mg\(^{2+}\) and Ca\(^{2+}\). We have also confirmed that mutation of E374A, another residue implicated in low affinity Mg\(^{2+}\) regulation (Shi and Cui, 2002), produces effects on regulation by Mg\(^{2+}\) identical to those of E399A (not depicted). Simultaneous mutation of both E374A and E399A produced no additional effect beyond that occurring with each mutation of each residue alone.

Methods of expression in Xenopus oocytes were as previously described (Zeng et al., 2001, 2003). SP6 RNA polymerase was used to synthesize cRNA for oocyte injection after DNA was linearized with M1uI. 10–50 nl of cRNA (10–20 ng/μl) was injected into stage IV Xenopus oocytes harvested 1 d before. Oocytes were used 2–7 d after injection of cRNA.

**Electrophysiological Recordings**

Channel currents were recorded from inside-out patches (Hamill et al., 1981) using Clampex (Axon Instruments), as performed routinely in our laboratory (Lingle et al., 2001; Zeng et al., 2003). During electrophysiological recordings, oocytes were maintained in ND96 bath solution (96 mM NaCl, 2.0 mM KCl, 1.8 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), 5.0 mM HEPES, pH 7.5). The pipette extracellular solution was 140 mM potassium methanesulfonate, 20 mM KOH, 10 mM HEPES, 2 mM MgCl\(_2\), 50 mM Mg\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), and Ni\(^{2+}\) solutions bathing the cytoplasmic face of the patch membrane.
contained 155 mM KF and 10 mM HEPES. Such solutions were typically prepared by adding aliquots of metal-chloride solutions to the KF solution. As noted previously (Oberhauser et al., 1988), because of the lack of solubility of CaF₂, F⁻ effectively removes free Ca²⁺ from such solutions such that the effective free Ca²⁺ concentrations can be considered <20 nM. In contrast, Cd²⁺, Mn²⁺, Co²⁺, and Ni²⁺ remain soluble. Since Sr²⁺ is insoluble in the presence of F⁻, Sr²⁺ solutions were made with 140 mM potassium methanesulfonate, 20 mM KOH, 10 mM HEPES, and buffered with 10 mM EGTA. Appropriate additions of Sr²⁺ were determined with EQCALWIN ( Biosoft). Assuming a contaminant Ca²⁺ obtained from Fisher Scientific. KF, MnCl₂, and NiCl₂ were obtained from Sigma-Aldrich. All other salts and chemicals were obtained from Sigma-Aldrich.

KOH solutions for preparation of physiological solutions was buffered with 10 mM EGTA. In all cases, error bars represent averages from a set of patches at specific divalent cation concentrations. For each patch, G–V curves were fit with the following:

\[ G(V) = G_{\text{max}} / (1 + \exp{(V-V_h)/k}) \]  

(1)

to provide estimates of \( V_h \), the voltage of half activation, and \( k \), the slope factor describing the voltage dependence of the closed-open equilibrium. For comparison of the magnitude of \( V_h \) shifts, \( \Delta V_h \) values were obtained by subtracting the \( V_h \) in the absence of specific divalent ions. Activation and deactivation time constants were obtained by fitting the original current traces by exponential functions. In all cases, error bars show SEM.

Experiments were done at room temperature (21–24°C). 1 N KOH solutions for preparation of physiological solutions was buffered with 10 mM EGTA. Appropriate additions of Sr²⁺ were determined with EQCALWIN ( Biosoft). Assuming a contaminant Ca²⁺ obtained from Fisher Scientific. KF, MnCl₂, and NiCl₂ were obtained from Alfa-Aesar. All other salts and chemicals were obtained from Sigma-Aldrich.

R E S U L T S

The results in this paper address two topics. First, the relative divalent cation selectivity of each of the mutationally defined divalent cation regulatory mechanisms is addressed. Second, the impact of each of the mutationally defined regulatory mechanisms on activation and deactivation time course is examined.

**Cd²⁺ Activates BK Channels by Acting on the D362/D367 Site but not the Ca²⁺ Bowl**

BK currents exhibit a characteristic shift in activation with elevations in cytosolic [Ca²⁺], (Cox et al., 1997). Typically, the voltage of half activation (\( V_h \)) obtained from conductance–voltage (G–V) curves shifts to more negative potentials as Ca²⁺ is elevated (Xia et al., 2002). Here, we compare the \( V_h \) shifts caused by specific divalent cations for wild-type mSlo1 BK channels and mSlo1 mutants with selective disruption of each of three putative regulatory mechanisms. The ability of mutation of a specific regulatory mechanism to alter the \( V_h \) shift produced by a given divalent cation can be taken as evidence for interaction between this cation and the binding site that mediates the regulatory effect. It does not prove that the mutated residues participate in binding site.

It has been previously reported that 60 μM Cd²⁺ activates BK currents, producing a negative \( V_h \) shift (Schreiber and Falkoff, 1997). This effect was not altered by mutation of residues in the Ca²⁺ bowl, leading to the proposal that there was a second divalent cation regulatory site (Schreiber and Falkoff, 1997). We therefore examined whether Cd²⁺ selectively activates BK channels through either of the other putative divalent cation regulatory sites.

Representative currents recorded from wild-type mSlo1, D362A/D367A, and 5D5N over a range of [Cd²⁺] up to 300 μM are shown in Fig. 1 (A–C). At [Cd²⁺] >300 μM, both steady-state current and tail current amplitudes are largely blocked and the recovery is very slow. The blocking effect of Cd²⁺ on 5D5N seems weaker than that on mSlo1, although Cd²⁺ produces similar \( V_h \) shifts for both.

For mSlo1 and 5D5N, as [Cd²⁺] is increased over the range of 0 through 300 μM, current activation is shifted to more negative activation potentials. In contrast, this is not the case for D362A/D367A. For each construct, G–V curves at different [Cd²⁺] were generated from tail currents following standard procedures (Zeng et al., 2001). Families of G–V curves represent averages from a set of patches at specific divalent cation concentrations. For each patch, G–V curves were fit with the following:

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Divalent Cation Sensitivity of BK Channel Activation

On the other hand, the ability of \([\text{Cd}^{2+}]\) to cause current activation is well preserved in 5D5N (Fig. 1, F and H), consistent with previous observations (Schreiber and Salkoff, 1997). Up through 100 \(\mu\text{M}\), \([\text{Cd}^{2+}]\) shows little effect on the voltage dependence of channel gating. However, a more shallow G–V curve is observed at 300 \(\mu\text{M}\) \([\text{Cd}^{2+}]\) for both mSlo1 (Fig. 1 D) and D362A/D367A (Fig. 1 E), probably arising in part from the voltage dependence of the mechanism of \([\text{Cd}^{2+}]\) block.

We next examined whether the E399 low affinity site might contribute to the additional \(V_h\) shift observed with 300 \(\mu\text{M}\) \([\text{Cd}^{2+}]\) in D362A/D367A (Fig. 1 G). For the E399A channel, \([\text{Cd}^{2+}]\) up through 30 \(\mu\text{M}\) had effects similar to its effects on wild-type mSlo1. However, \(V_h\) shifts at 100 and 300 \(\mu\text{M}\) \([\text{Cd}^{2+}]\) are largely reduced in the E399A channel compared with results obtained from mSlo1 (Fig. 1 I). Furthermore, for the double mutant, D362A/D367A+E399A, the ability of \([\text{Cd}^{2+}]\) to shift current activation is essentially abolished over the entire test concentration range (Fig. 1 I). The apparently additive effects of D362A/D367A and E399A suggest that these two sites act independently in regards to the ability of \([\text{Cd}^{2+}]\) to regulate BK channels.

In conclusion, it is the regulatory mechanism defined by the D362/D367 site, and not that defined by the \([\text{Ca}^{2+}]\) bowl, which mediates activation of BK channels by lower concentrations of \([\text{Cd}^{2+}]\). At higher concentrations, \([\text{Cd}^{2+}]\) also can enhance channel activation through the low affinity site defined by E399.

Mn\(^{2+}\) Enhances Activation of BK Channels through the E399 Low Affinity Site

Mn\(^{2+}\) has been shown to activate muscle membrane BK channels expressed in bilayers (Oberhauser et al., 1988). We therefore tested whether the ability of Mn\(^{2+}\) to modulate BK channels might be selectively associated with any of the three putative regulatory sites. Fig. 2 (A–C) shows currents activated in the presence of 0 through 5 \(\text{mM Mn}^{2+}\) for mSlo1, E399A, and the triple mutation (5D5N+D362A/D367A+E399A). For mSlo1, Mn\(^{2+}\) causes a leftward shift in the G–V curves with no...
change in voltage dependence (Fig. 2 D). The negative $V_h$ shift produced by 5 mM Mn$^{2+}$ relative to 0 divalent is $>$100 mV (Fig. 2 G), while 100 μM Mn$^{2+}$ produces an $\sim$50-mV leftward shift. Mutation of the low affinity site alone is sufficient to abolish most of the effect of Mn$^{2+}$ (Fig. 2, E and G). Moreover, additional mutation of the two high affinity sites together with E399A does not produce any additional effect other than that caused by E399A alone (Fig. 2, F and H). In fact, Mn$^{2+}$ loses its ability to activate current only for those constructs in which the E399A mutation is present (Fig. 2 H), while Mn$^{2+}$ produces similar current activation for all constructs containing an intact E399 site (Fig. 2 I). These results show clearly that Mn$^{2+}$ modulates BK channels predominantly through the low affinity site. Furthermore, although we were only able to examine Mn$^{2+}$ concentrations up through 5 mM, the E399A mutation appears to completely remove the approximately $-$90-mV shift produced by 5 mM Mn$^{2+}$, whereas E399A removes about half of the approximately $-$60-mV shift caused by 10 mM Mg$^{2+}$ (Xia et al., 2002).

The cumulative data in Fig. 2 I suggest that mutation of either 5D5N or D362A/D367A alone may produce a small reduction in the ability of Mn$^{2+}$ to shift activation at 100 μM Mn$^{2+}$, raising the possibility of a small effect involving the higher affinity Ca$^{2+}$ regulatory mechanisms. However, these small effects of 5D5N and D362A/D367A do not appear to be additive, and estimates of $V_h$ for constructs with very positive $V_h$ are subject to uncertainties (Fig. 2 I). Furthermore, simultaneous mutation of all three sites (the triple mutation: 5D5N/D362A/D367A/E399A) shows a behavior similar to E399A alone (Fig. 2 H). Although we cannot completely exclude the possibility that Mn$^{2+}$ may produce a small activation effect through one or both of the higher affinity sites, it is clear that in comparison to Ca$^{2+}$ or Cd$^{2+}$, the ability of Mn$^{2+}$ to shift activation via these sites is either absent or markedly reduced.

**The E399 Low Affinity Site Is Partially Responsible for the Ability of Ni$^{2+}$ and Co$^{2+}$ To Activate BK Channels**

We next tested the selectivity of the three sites for Ni$^{2+}$. In contrast to its effects on activation by Mn$^{2+}$, the E399A mutation reduces, but does not fully abolish, the negative $V_h$ shifts caused by Ni$^{2+}$ on mSlo1, which is
about −160 mV with 50 mM Ni\(^{2+}\) (Fig. 3 A). However, simultaneous mutation of the two high affinity sites has no effect on the ability of Ni\(^{2+}\) to produce \(V_h\) shifts (Fig. 3 B). The important role of E399 in the effects of Ni\(^{2+}\) is also supported by the fact that all constructs containing E399A exhibit Ni\(^{2+}\)-induced shifts in \(V_h\) similar to those seen in E399A alone (Fig. 3 C). Furthermore, all constructs containing wild-type E399 exhibit Ni\(^{2+}\)-induced \(V_h\) shifts similar to that of wild-type mSlo1 (Fig. 3 D).

The experimental results from Co\(^{2+}\) are similar to those with Ni\(^{2+}\) (Fig. 3, E–H). In brief, 20 mM Co\(^{2+}\) produces an ∼150-mV negative \(V_h\) shift of mSlo1 BK channels (Fig. 3 E). All constructs containing E399A show a \(V_h\)=[Co\(^{2+}\)] relationship similar to that of the E399A mutant, in which the ability of Co\(^{2+}\) to produce a \(V_h\) shift is partially removed (Fig. 3, E and G). Single or double mutation of high affinity sites does not change the \(V_h\) shifts caused by Co\(^{2+}\) on BK channels (Fig. 3, F and H).

The results from Ni\(^{2+}\) and Co\(^{2+}\) suggest that, among the previously defined three divalent cation regulatory mechanisms, only the E399 low affinity site contributes appreciably to the activation effect conferred by Ni\(^{2+}\) and Co\(^{2+}\). However, it should be noted that for both mM Ni\(^{2+}\) and Co\(^{2+}\) some ability to shift \(V_h\) persists even after mutation of E399, similar to the effects of M\(^{2+}\) but distinct from that of Mn\(^{2+}\).

**Activation of BK Channels by Sr\(^{2+}\) Involves All Three Divalent Cation Regulatory Sites**

Representative current traces from wild-type mSlo1, D362A/D367A+E399A, D362A/D367A, and E399A over a range of [Sr\(^{2+}\)] are shown in Fig. 4 (A–D). For [Sr\(^{2+}\)] >300 µM, a strong voltage-dependent block was observed that influenced current estimates for both steady state and tail currents for all tested constructs. At very positive potentials, Sr\(^{2+}\) produces a slow, time-dependent blockade that can result in complete block of steady-state current. Unblock following repolarization is sufficiently slow that only partial recovery from block is observed during tail currents. Intriguingly, at higher [Sr\(^{2+}\)] and strong depolarizations, the tail current G–V curves show an unusual N shape, which cannot be simply described by a combination of activation and blocking processes (Fig. 4, E and F). Previous work has demonstrated that Sr\(^{2+}\) reduces BK current both by slow and fast blocking mechanisms probably involving two different positions of occupancy within the pore (Sugihara, 1998). How these two different facets of Sr\(^{2+}\) block may relate to the apparent N shape seen here is not clear. However, the N shape phenomenon is suggestive of a presumed punchthrough mechanism (Nimigean and Miller, 2002), in which depolarization might drive the blocking ion through the ion channel. An alternative explanation has also been suggested in recent work pointing out that an apparent punchthrough phenomenon may arise from interactions between blocker and permeant ions with the permeation pathway (Heginbotham and Kuthlay, 2004).

Because of the N shape of the G–V curves with Sr\(^{2+}\), we were unable to define curves reflecting solely the ability of Sr\(^{2+}\) to activate BK channels. Manipulations of stimulation protocols such as prolongation of the time between sweeps, varying the activation step duration, or changing the tail current potential had minimal effect on the essential properties of the tail current G–V curves. As a consequence, here we simply show the rela-
tive tail current G–V shifts among various Sr\textsuperscript{2+} concentrations. G–V curves were normalized to the conductance at 10 \mu M Sr\textsuperscript{2+} for each patch. Relative shifts were measured at the nominal half activation position. We must emphasize that, because of the blocking effects and the possibility that channel opening has not reached saturation at 10 \mu M Sr\textsuperscript{2+}, the real maximum conductance activated by 300 \mu M Ca\textsuperscript{2+} is much larger than the peak conductance shown in these figures. Despite the uncertainties involved in determining the true \( V_h \) for activation in Sr\textsuperscript{2+}, the relative ability of Sr\textsuperscript{2+} to shift gating in different constructs seems readily apparent.

For wild-type mSlo1, there are significant shifts in the G–V curves between [Sr\textsuperscript{2+}] of 0 to 10 \mu M (blue bars), 10 to 300 \mu M (red bars), and 300 \mu M to 20 mM (green bars) (Fig. 4 E). For D362A/D367A+E399A, in which only the calcium bowl site is intact, the relative shift between 0 to 10 \mu M Sr\textsuperscript{2+} is similar to the shift in G–V seen in mSlo1, but little additional shift is observed between 10 \mu M to 20 mM Sr\textsuperscript{2+} (Fig. 4 F). This result indicates that the calcium bowl is mainly responsible for the effect of Sr\textsuperscript{2+}.

When only the D362/D367 residues are mutated, the relative G–V shift between 10 and 300 \mu M Sr\textsuperscript{2+} is substantially reduced compared with mSlo1 (Fig. 4 G). In contrast, with the E399A mutation alone, only the relative shift between 300 \mu M and 20 mM Sr\textsuperscript{2+} is significantly decreased, with little change in the shifts that occur over the range of 0 to 300 \mu M Sr\textsuperscript{2+} (Fig. 4 H). These results suggest that the D362/D367 site accounts for Sr\textsuperscript{2+} effects from 10 to 300 \mu M, and E399 participates in a low affinity effect of Sr\textsuperscript{2+}.

Because the calcium bowl accounts for Sr\textsuperscript{2+} effects at 10 \mu M Ca\textsuperscript{2+} and below, constructs in which the calcium
bowl is mutated require higher Sr\textsuperscript{2+} before activation is observed. As a consequence, for constructs containing the 5D5N mutations, the severe blocking effects of Sr\textsuperscript{2+} at the voltages required for activation precluded our ability to study such constructs. Despite this limitation, the available results all support the conclusion that, similar to Ca\textsuperscript{2+}, Sr\textsuperscript{2+} activates BK channels through all three divalent cation regulatory mechanisms.

One final aspect of the effects of Sr\textsuperscript{2+} requires mention. In both construct D362A/D367A and also D362A/D367A+E399A, there appears to be a more robust block of tail currents than in either wild-type Slo1 or in E399A, when compared at a voltage (e.g., +100 to +150 mV) where 10 μM Sr\textsuperscript{2+} appears to produce near maximal activation. This difference occurs despite the fact that both the onset of block by Sr\textsuperscript{2+} at these voltages and recovery from block at more negative potentials is largely similar for all constructs. This suggests that the voltage- and time-dependent block by Sr\textsuperscript{2+}, probably involving pore blockade, does not differ among the constructs. Yet there appears to be an additional reduction in relative tail current amplitude at higher Sr\textsuperscript{2+} that may not be explained solely by such a block. At present, we do not have an explanation for the additional blockade produced in constructs containing the D362A/D367A mutations.

In sum, each of the three mutationally defined regulatory mechanisms exhibits a unique profile of sensitivity to divalent cations. The E399A mutation defines a process that is acted upon by all divalent cations we have examined. The D362A/D367A mutations participate in a mechanism that can be regulated by Ca\textsuperscript{2+}, Gd\textsuperscript{2+}, and Sr\textsuperscript{2+}, while activation mediated by the Ca\textsuperscript{2+} bowl only occurs with Ca\textsuperscript{2+} and Sr\textsuperscript{2+}.

The Three Regulatory Sites Affect Channel Gating Kinetics in Distinct Ways

Despite the number of distinct kinetic states that are hypothesized to contribute to BK channel gating (Rothberg and Magleby, 1999, 2000; Zhang et al., 2001; Horrigan and Aldrich, 2002), the kinetic relaxations associated with BK current activation and deactivation are remarkably simple, being reasonably well characterized by a single exponential time course (Horrigan and Aldrich, 1999, 2000; Zhang et al., 2001). The existence of a single primary kinetic relaxation in the gating behavior of BK channels has been generally explained by the idea that the rate-limiting relaxation in the gating process is a central closed–open equilibrium (C-O) (Horrigan and Aldrich, 2002).

Given that three distinct divalent cation-dependent mechanisms appear to regulate BK gating, here we evaluate whether any of these mechanisms are associated with distinct effects on channel gating kinetics. Because Ca\textsuperscript{2+} interacts with both high affinity sites and Mg\textsuperscript{2+} only acts at the low affinity site (Shi et al., 2001; Zhang et al., 2001; Shi et al., 2002; Xia et al., 2002), we have compared gating behavior at 0, 1, 10, and 300 μM Ca\textsuperscript{2+} to examine the kinetic roles of the calcium bowl and D362/D367 residues, while 20 mM Mg\textsuperscript{2+} was applied to evaluate the kinetic effects of the E399 residue.

Fig. 5 (A and B) shows example traces used for measurement of activation and deactivation time course from wild-type mSlo1 at different [Ca\textsuperscript{2+}]. Voltage commands ranged from −180 to +190 mV for activation and from +180 to −180 mV for deactivation. For mSlo1, deactivation slows with increases in [Ca\textsuperscript{2+}], while activation is accelerated as [Ca\textsuperscript{2+}] is elevated over the range of 1 to 300 μM (Fig. 5 C). At positive activation potentials, there is also an unusual slowing of activation at 1 μM Ca\textsuperscript{2+} relative to 0 Ca\textsuperscript{2+} (Fig. 5 C), which has been noted in previous work (Zhang et al., 2001).

For channels in which the calcium bowl is mutated (5D5N), the increase in activation rate between 0 and 10 μM Ca\textsuperscript{2+} is largely reduced (compare sample traces on the right of Fig. 5, C and D), while the deactivation process is less strongly influenced by Ca\textsuperscript{2+} bowl mutation at Ca\textsuperscript{2+} up to 300 μM (Fig. 5, compare C with D). In contrast, for channels with simultaneous mutation of D362A/D367A+E399A, such that only the calcium bowl site is functional, the increase in activation rate between 0 and 10 μM Ca\textsuperscript{2+} is comparable with that in wild-type mSlo1 BK channels (Fig. 5, compare C with E). Yet, in this construct, the increase in activation rate between 10 and 300 μM Ca\textsuperscript{2+} and the slowing of deactivation is almost abolished (Fig. 5 E). These results convincingly demonstrate that the main kinetic contribution of the calcium bowl is to increase the activation rate for <10 μM Ca\textsuperscript{2+}, while the Ca\textsuperscript{2+} bowl plays little role in effects of Ca\textsuperscript{2+} on deactivation.

In contrast, for channels in which the D362/D367 site alone is mutated, increases in [Ca\textsuperscript{2+}] from 0 to 300 μM produce only a small slowing of deactivation in marked contrast to wild-type Slo1 (compare Fig. 6 A with Fig. 5 C). Similarly, the increase in activation rate seen in wild-type Slo1 between 10 and 300 μM Ca\textsuperscript{2+} is also largely reduced by the D362A/D367A mutation (Fig. 6 A). On the other hand, for channels in which only the D362D367 site is functional (e.g., 5D5N+E399A; Fig. 6 B), the extent of slowing of deactivation produced by Ca\textsuperscript{2+} up through 300 μM and the acceleration of activation between 10 and 300 μM Ca\textsuperscript{2+} are comparable with those in mSlo1 (compare Fig. 5 C with Fig. 6 B). Consistent with these observations, 300 μM Ca\textsuperscript{2+} confers little kinetic effect on channels in
which both high affinity sites are mutated simultaneously (5D5N/D362AD367A; Fig. 6 C). Taken together, these results indicate that the D362/D367 site is primarily responsible not only for the slowing of deactivation caused by Ca\(^{2+}\)/H11001 up through 300 \(\mu\)M, but also for the acceleration of activation from 10 to 300 \(\mu\)M Ca\(^{2+}\).

Thus, the Ca\(^{2+}\)/H11001 bowl site and the D362/D367A site each influence distinct kinetic aspects of BK channel function. It should also be noted that the slight slowing of activation that is observed in wild-type Slo1 by increases in Ca\(^{2+}\)/H11001 from 0 to 1 \(\mu\)M appears to be absent in constructs containing the D362A/D367A mutations.

Mg\(^{2+}\), which enhances BK channel activation through the E399 low affinity site (Shi et al., 2002; Xia et al., 2002), only contributes to a slowing of deactivation of Slo1, with little effect on the activation kinetics of channel gating (Fig. 7 A). The slowing of deactivation is completely abolished in channels with the E399A mutation (Fig. 7 B). Therefore, the mechanism of channel regulation involving the low affinity Mg\(^{2+}\)/H11001 site involves a slowing of deactivation, with little effect on activation. This conclusion is also supported by the properties of the construct in which the E399 site is intact, but both higher affinity sites are mutated, i.e., 5D5N+D362A/D367A. In the absence of the two high affinity sites, Mg\(^{2+}\) exerts the same effects on channel gating behavior as on wild-type BK channels (Fig. 7 C). Thus, the slowing of deactivation exerted by mM Mg\(^{2+}\) or Ca\(^{2+}\) is disrupted solely by mutation of the E399 residue.

**DISCUSSION**

The main conclusions of this work are the following.

(1) The calcium bowl site is activated by Ca\(^{2+}\) and Sr\(^{2+}\),

**Figure 5.** The calcium bowl is mainly responsible for the acceleration of activation from 0 to 10 mM Ca\(^{2+}\). (A) Typical currents used for measurement of current activation time constants are shown for mSlo1 with 0, 1, 10, and 300 \(\mu\)M Ca\(^{2+}\) along with the voltage activation protocol. (B) Examples of Slo1 tail currents used for measurement of deactivation time constants are given for the indicated \([\text{Ca}^{2+}]\]. Voltage steps were from −180 to +180 mV in 20-mV increments (only every 40 mV is shown in the displayed protocol). (C) Activation (filled symbols) and deactivation (open symbols) time constants for Slo1 (\(n = 6\) ) are plotted at various \([\text{Ca}^{2+}]\) showing the slower deactivation and faster activation produced by Ca\(^{2+}\). Traces on the right (top pair of traces) show normalized current activation at +190 mV with 0 \(\mu\)M Ca\(^{2+}\) (green line is fitted single exponential, 1.00 ms) and 10 \(\mu\)M Ca\(^{2+}\) (red fitted exponential, 0.227 ms). Bottom pair of right-hand traces show current deactivation at −180 mV for 0 \(\mu\)M Ca\(^{2+}\) (green fitted line, 0.041 ms) and 300 \(\mu\)M Ca\(^{2+}\) (blue fitted line, 0.107 ms). Both activation and deactivation examples are from the same patch. For activation time courses, only every 10th digitized current value is displayed. (D) Time constants are plotted as in C, but for 5D5N (\(n = 6\)). Traces on the right are identical in format to those in C. Activation \(\tau\): 0 \(\mu\)M Ca\(^{2+}\), 0.996 ms; 10 \(\mu\)M Ca\(^{2+}\), 0.771 ms. Deactivation \(\tau\): 0 \(\mu\)M Ca\(^{2+}\), 0.041 ms; 300 \(\mu\)M Ca\(^{2+}\), 0.107 ms. (E) Time constants are plotted as in C, but for D362A/D367A+E399A (\(n = 8\)). Activation \(\tau\): 0 \(\mu\)M Ca\(^{2+}\), 1.61 ms; 10 \(\mu\)M Ca\(^{2+}\), 0.254 ms. Deactivation \(\tau\): 0 \(\mu\)M Ca\(^{2+}\), 0.074 ms; 10 \(\mu\)M Ca\(^{2+}\), 0.080 ms.
Divalent Cation Sensitivity of BK Channel Activation

**Figure 6.** The D362/D367 site slows deactivation and accelerates activation in the range of 10 to 300 μM Ca²⁺. Activation and deactivation time courses were determined as in Fig. 5 at 0, 1, 10, and 300 μM Ca²⁺. (A) Effects of Ca²⁺ on activation and deactivation time constants are plotted as a function of command voltage for D362A/D367A (n = 6). Open symbols were measured from deactivation protocols and filled symbols from activation protocols. Representative normalized current traces for activation (at +190 mV) and deactivation (at −180 mV) are shown on the right, along with lines showing single exponential fits. Activation time constants: 0 Ca²⁺ (green), 1.74 ms; 10 μM Ca²⁺ (red), 0.34 ms; 300 μM Ca²⁺ (blue), 0.27 ms. Deactivation time constants: 0 Ca²⁺ (green), 0.104 ms; 300 μM Ca²⁺ (blue), 0.129 ms. (B) Time constants for the construct with both the Ca²⁺ bowl and E399 mutated (5D5N+E399A; n = 7) are plotted with representation current traces and fitted exponentials on the right. Activation time constants: 0 Ca²⁺, 1.22 ms; 10 μM Ca²⁺, 0.74 ms; 300 μM Ca²⁺, 0.26 ms. Deactivation time constants: 0 Ca²⁺, 0.065 ms; 300 μM Ca²⁺, 0.130 ms. (C) The dependence of activation and deactivation time constants is plotted at various Ca²⁺ concentrations. Open symbols were measured from deactivation protocols and filled symbols from activation protocols. Representative normalized current traces for activation (at 180 mV) and deactivation (at −180 mV) are shown on the right, along with lines showing single exponential fits. Activation time constants: 0 Ca²⁺, 1.69 ms; 10 μM Ca²⁺, 1.67 ms; 300 μM Ca²⁺, 2.91 ms. Deactivation time constants: 0 Ca²⁺, 0.079; 300 μM Ca²⁺, 0.631 ms.

Figure 7. The E399 low affinity site slows deactivation. Activation and deactivation time courses were determined as in Fig. 5 but either in the absence or presence of 20 mM Mg²⁺. (A) Effect of 20 mM Mg²⁺ on activation time constants or deactivation time constants in wild-type Slo1 (n = 6). Open symbols, deactivation; filled symbols, activation. Traces on the right show normalized deactivation currents at −180 mV with either 0 Mg²⁺ (red fitted line, 0.061 ms) or 20 mM Mg²⁺ (blue fitted line, 0.107 ms). (B) Effect of 20 mM Mg²⁺ on activation and deactivation time constants with the E399A mutation (n = 7). Traces on the right are as in A. τₐ: 0 μM Mg²⁺, 0.055 ms; 20 mM Mg²⁺, 0.066 ms. (C) Effect of 20 mM Mg²⁺ in the construct with both higher affinity sites mutated (5D5N+D362A/D367A; n = 7). Traces on the right are as in A. τₐ: 0 μM Mg²⁺, 0.076 ms; 20 mM Mg²⁺, 0.170 ms. Circles, 0 μM Mg²⁺; diamonds, 20 mM Mg²⁺.

The D362/D367 site is activated by Ca²⁺, Sr²⁺, and Cd²⁺, but not smaller divalent cations. Disruption of this site has little effect on current deactivation, but this site plays a role in Ca²⁺-dependent increases in activation rate <10 μM Ca²⁺. (2) The D362/D367-related site is activated by Ca²⁺, Sr²⁺, and Cd²⁺, but not smaller divalent cations. Disruption of this site has little effect on current deactivation, but this site plays a role in Ca²⁺-dependent increases in activation rate <10 μM Ca²⁺.
At Least Three Divalent Cation Binding Sites Regulate BK Gating

The possible presence of two RCK domains associated with each BK α subunit (Jiang et al., 2002; Roosild et al., 2004) naturally suggests that a Ca\(^{2+}\) regulatory site on each RCK domain on a single BK channel α subunit may be required for proper function of the cytosolic structure of the BK channel. As summarized in Introduction, mutational analyses have supported this idea that two distinct Ca\(^{2+}\)-dependent regulatory mechanisms sensitive to μM Ca\(^{2+}\) may participate in BK channel regulation. For the Ca\(^{2+}\) bowl regulatory mechanism, Ca\(^{2+}\) binding studies to fusion peptides containing the Ca\(^{2+}\) bowl (Bian et al., 2001; Braun and Sy, 2001) and more detailed mutagenesis in conjunction with both physiological and binding assays (Bao et al., 2004) have bolstered the idea that the Ca\(^{2+}\) bowl does, in fact, represent a likely Ca\(^{2+}\) binding site. However, for the high affinity mechanism identified by D362A/D367A (or M513), no specific evidence supports the idea that any of these residues participate in coordination of Ca\(^{2+}\) ions. Based on mutagenesis alone, it has remained a possibility that each α subunit contains only a single Ca\(^{2+}\) binding site, but that mutations at different positions in the COOH terminus can disrupt independently acting components of the regulatory apparatus.

To provide independent evidence that each mutationally defined regulatory effect corresponds to a distinct divalent cation binding site, the present results have shown that each higher affinity Ca\(^{2+}\) regulatory site exhibits a unique profile of divalent cation sensitivity. An important point is that, for either the 5D5N mutations or D362AD367A mutations, the consequences of a given mutation are the same, irrespective of whether other sites are simultaneously mutated. This argues against the possibility that there is only a single high affinity site that exhibits altered binding/selectivity properties after mutation. Furthermore, the results suggest that the particular profile of divalent cation sensitivity observed for each putative regulatory mechanism reflects the intrinsic properties of a unique binding site and that each site acts largely independently of each other. Thus, two distinct higher affinity Ca\(^{2+}\) binding sites on each Slo1 α subunit contribute to regulation of BK channels by divalent cations. The specific residues that coordinate ion binding for each of these sites remain to be identified.

The presence of two higher affinity Ca\(^{2+}\) regulatory domains in each α subunit supports the idea that COOH terminus involves a modular structure with a Ca\(^{2+}\)-dependent regulatory site in association with each of the two RCK domains. The D362D367 residues are, in fact, positioned in the first BK RCK domain within loops homologous to those known to contain ligand coordinating residues in the RCK domains of bacterial K\(^{+}\) channels (Jiang et al., 2001). The position of the Ca\(^{2+}\) bowl with respect to the hypothesized second RCK domain is less clear, but is consistent with the idea that a second RCK-containing module in the BK channel COOH terminus also contains a Ca\(^{2+}\)-binding site that regulates channel gating. Intriguingly, the idea that the cytosolic structure of each BK channel α subunit contains two modular elements, perhaps similar to the MthK bacterial channel (Jiang et al., 2002), was supported by earlier work indicating that functionally normal channels could arise from the separate expression of cDNA encoding a peptide corresponding to the second RCK domain (and Ca\(^{2+}\) bowl) along with cDNA encoding a peptide corresponding to the first RCK domain attached to the pore module (Wei et al., 1994). The point of separation between the two components of the COOH terminus in these earlier experiments was essentially a linker between the two RCK domains.

The present results also suggest that there is a second low affinity site in addition to that defined by E374 and E399. Even at 10 mM Mg\(^{2+}\), an appreciable V\(_h\) shift (~20–30 mV) is still observed with either the E374A (Shi et al., 2002) or E399A (Xia et al., 2002) mutations. Here, following mutation of E399, similar residual shifts were observed with mM concentrations of Ni\(^{2+}\) and Co\(^{2+}\). Furthermore, with the E399A mutation, effects of Ni\(^{2+}\) and Co\(^{2+}\) were observed at concentrations even as low as 1 mM, despite the fact that neither cation has appreciable effects on the higher affinity sites, suggesting the presence of an additional low affinity site. Given that the E399A mutation completely abolishes the effects of 5 mM Mn\(^{2+}\), this putative second low affinity site may exhibit a selectivity distinct from that of E399. Thus, whatever the basis for the residual effect of mM concentrations of divalent cations following mutation of E399, there are probably specific residues on the BK α subunit that mediate this effect.

Defining the Ion Selectivity of an Allosteric Regulatory Site

A previous study (Oberhauser et al., 1988) of skeletal muscle BK channels in bilayers reported that Ca\(^{2+}\), Cd\(^{2+}\), Sr\(^{2+}\), Mn\(^{2+}\), Fe\(^{2+}\), and Co\(^{2+}\) were effective at activating channels while Mg\(^{2+}\), Ni\(^{2+}\), Ba\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Hg\(^{2+}\), and Sn\(^{2+}\) were ineffective. However, Ni\(^{2+}\) and Mg\(^{2+}\), when studied in the presence of Ca\(^{2+}\), produced an additional enhancement of activation, while Ba\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Hg\(^{2+}\), and Sn\(^{2+}\) remained ineffective. These results were interpreted in terms of two kinds of effects: first, direct channel activation at a Ca\(^{2+}\) activation site and, second, an allosteric effect mediated by some divalent cations that enhanced the effectiveness of Ca\(^{2+}\). Now, within the three binding site perspective, those cations that were observed to cause activation (Oberhauser et al., 1988) correspond either to those acting on one or both of the high affinity sites (Ca\(^{2+}\), Sr\(^{2+}\), and
Cd$^{2+}$ or to those (Mn$^{2+}$ and Co$^{2+}$) that were studied at concentrations at which effects on the low affinity site would begin to be observed. Here, because of higher concentrations and more positive activation potentials, Mg$^{2+}$ and Ni$^{2+}$ were also observed to activate channels directly through the lower affinity site.

Our results also support the earlier conclusion (Oberhauser et al., 1988) that selectivity for channel activation is correlated with cation size. The Ca$^{2+}$ bowl interacts with the two largest cations we have examined, Ca$^{2+}$ and Sr$^{2+}$. The D532/D537 site interacts with the three largest cations (Ca$^{2+}$, Sr$^{2+}$, and Cd$^{2+}$), while the lower affinity site interacts with all cations we have examined. On the other hand, the differential effects of Ca$^{2+}$ and Cd$^{2+}$ on the Ca$^{2+}$ bowl points out that size per se is not sufficient for defining the effects of a given cation, but that other physico-chemical properties of a given ion are important. Yet, the fundamental importance of ionic radius is clear and, in general, it is the case that the binding sites of greater specificity are those that prefer ions of larger radii. This seems somewhat surprising, given that, in a site preferring larger ions, ions of smaller radii are also likely to pass in and out of the general coordinates of that site. Two factors may contribute to the lack of effect of smaller ions. One possibility is that, similar to one model of ion permeation (Doyle et al., 1998), precise coordination distances between ion occupancy positions and oxygen donors may be critical for defining the residence time of an ion at a given site. Thus, the specific chemical nature and resulting strength of interactions between a given divalent cation and ion-coordinating residues would impact on this residence time. A second factor is that our analysis compares selectivity for activation, and not selectivity for binding. When selectivity is being compared by a measurement of a response that depends on an allosteric mechanism, the ability of a cation to cause activation depends not only on its ability to occupy the binding site, but also on its ability to bind more tightly to the open state of the channel. This latter point is, in fact, the crux of the idea of allosteric regulation. The measurements used here provide no direct information about the ability of any given ion to occupy the presumed binding sites, except as indicated indirectly by current activation. Thus, some cations might effectively occupy a binding site, but exhibit no difference in binding between closed and open conformations. Cations acting in such a fashion would be expected to exert inhibitory effects on activation by Ca$^{2+}$.

An interesting case is the apparent lack of effect of Cd$^{2+}$ on the Ca$^{2+}$ bowl site, despite their similarity in ionic radii (Table I). Does Cd$^{2+}$ act as a blocker of the Ca$^{2+}$ bowl site? Although specific experiments to test this possibility will need to be done, some aspects of the data seem consistent with this possibility. In the D532A/D537A construct containing a functional Ca$^{2+}$ bowl, Cd$^{2+}$ produces a small rightward shift in the G–V curve at 100 μM (Fig. 1 E). One interpretation of such an effect is that Cd$^{2+}$ may bind with reasonable affinity to the binding site corresponding to calcium bowl, but the binding affinity is slightly higher in the closed state than in open state. Despite their similarity in ionic radii, it has been noted that Ca$^{2+}$ and Cd$^{2+}$ differ appreciably in their coordination chemistry (Oberhauser et al., 1988), with Ca$^{2+}$ more likely to form ionic bonds with carboxyl and carbonyl groups, whereas Cd$^{2+}$ has a higher affinity for nitrogen and sulfur moieties. Perhaps the Ca$^{2+}$ bowl lacks the groups appropriate for tighter binding of Cd$^{2+}$ to the open channel conformation. Experiments that examine competition between Cd$^{2+}$ and Ca$^{2+}$ in particular constructs might illuminate this issue.
Similar considerations to those just mentioned pertain to evaluation of the selectivity of the site defined by the D362/D367 residues. The D362/D367 related site responds to Ca\(^{2+}\), Cd\(^{2+}\), and Sr\(^{2+}\), but not appreciably to four cations, Ni\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), and Mg\(^{2+}\), all with ionic radii appreciably smaller than Ca\(^{2+}\) (Table 1). Again, it seems likely that smaller cations would enter and transiently occupy the D362/D367 site, but that the closed and open channel conformations bind these ions with a similar affinity, such that binding does not favor channel opening but perhaps may inhibit activation by Ca\(^{2+}\). Previous results have, in fact, suggested that Mg\(^{2+}\) may competitively inhibit some component of activation produced by Ca\(^{2+}\) perhaps by acting at the D362/D367 site (Shi and Cui, 2001; Zhang et al., 2001).

**Multiple Binding Sites and Allosteric Models of BK Channel Activation**

BK channel activation at \(\mu\text{M} \text{Ca}^{2+}\) has been effectively described by allosteric gating models (Horrigan et al., 1999; Rothberg and Magleby, 2000; Horrigan and Aldrich, 2002; Magleby, 2003) in which a central closed–open equilibrium is independently regulated by either voltage-sensor movement or by Ca\(^{2+}\) binding. The ability of Ca\(^{2+}\) binding to favor channel activation has been proposed to involve a simple mechanical coupling mediated by a linker connecting the cytosolic structure to the pore-linking S6 helix (Niu et al., 2004). To date, the most extensive models have considered a single high affinity Ca\(^{2+}\) regulatory site per \(\alpha\) subunit (Rothberg and Magleby, 2000; Horrigan and Aldrich, 2002; Magleby, 2003), although the consequences of including a lower affinity site on each subunit have also been considered (Shi and Cui, 2001; Zhang et al., 2001; Horrigan and Aldrich, 2002). The present results require an extension of the previous model (Horrigan and Aldrich, 2002) to include two high affinity sites along with the low affinity site. Although the apparent independence of these putative binding sites suggests that the allosteric coupling between each of the binding constants should be close to 1, further work will be required to define the allosteric interaction factors between each of the components of this complex channel.

The present results also show that mutation of a given regulatory site alters channel gating kinetics in a stereotypical fashion irrespective of whether any or all of the other sites have also been mutated. This also supports the idea that each regulatory mechanism acts independently of each other to influence distinct aspects of the overall conformational equilibria between closed and open states. For example, both high affinity sites influence transitions that contribute to Ca\(^{2+}\)-dependent increases in channel activation rate, although over different concentration ranges. In contrast, the low affinity site defined by E399 has no effects on channel activation rates, but plays a prominent role in slowing the return of channels to closed states. Together, these results support the view that there is a complex set of conformational steps in the cytosolic structure of the BK channel that are regulated in a largely independent fashion by ligation of divalent cations to three distinct sites.

We thank Lynn Lavack and Yimei Yue for preparation and injection of oocytes.

This work was supported by the National Institutes of Health grant GM066215 to C. Lingle, by the Digestive Diseases Research Core Center at Washington University (DK52574), and an AHA-Heartland affiliate fellowship to X. Zeng.

Olaf S. Andersen served as editor.

**Submitted: 13 December 2004**

**Accepted: 21 January 2005**

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