Ion sensing in the RCK1 domain of BK channels

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BK-type K+ channels are activated by voltage and intracellular Ca2+, which is important in modulating muscle contraction, neural transmission, and circadian pacemaker output. Previous studies suggest that the cytosolic domain of BK channels contains two different Ca2+ binding sites, but the molecular composition of one of the sites is not completely known. Here we report, by systematic mutagenesis studies, the identification of E535 as part of this Ca2+ binding site. This site is specific for binding to Ca2+ but not Cd2+. Experimental results and molecular modeling based on the X-ray crystallographic structures of the BK channel cytosolic domain suggest that the binding of Ca2+ by the side chains of E535 and the previously identified D367 changes the conformation around the binding site and turns the side chain of M513 into a hydrophobic core, providing a basis to understand how Ca2+ binding at this site opens the activation gate of the channel that is remotely located in the membrane.

Ca2+-activated | allosteric gating | Ca2+ binding site | Cd2+ | Slo1

Large conductance Ca2+-activated K+ (BK) channels open in response to membrane depolarization and the elevation of intracellular Ca2+ concentration ([Ca2+]i). In neurons and muscle cells, membrane depolarization activates voltage-dependent Ca2+ channels, resulting in Ca2+ entry into the cell and subsequent activation of BK channels. The K+ efflux through BK channels repolarizes the membrane, which shuts Ca2+ channels, thereby providing a negative feedback mechanism to modulate membrane excitability and [Ca2+]i. Because of this function, BK channels are important modulators of muscle contraction (1), neuronal spike frequency adaptation (2), neurotransmitter release (3), and circadian pacemaker output (4). BK channels are formed by four Slo1 subunits, which is made of two regulating domains for K+ conductance—voltage sensing domain (VSD), and a cytosolic domain (CTD) (7, 8), which is made of two regulating domains for K+ conductance (RCK1 and RCK2) (9, 10). Intracellular Ca2+ binds to the CTD to activate the channel by enhancing the open probability of the activation gate located in the membrane-spanning PGD.

Previous studies have identified two putative Ca2+ binding sites in the CTD of BK channels, one is the Ca2+ bowl located in the RCK2 domain (10–13) and the other is located in the RCK1 domain including the residue D367 (14). The existence of two distinctively different high-affinity Ca2+ binding sites that are responsible for Ca2+-dependent activation of BK channels has been demonstrated in various experimental studies (15). These studies demonstrated that Ca2+ binding to the two sites activates channel independently with only a small cooperativity (14, 16, 17), and the two sites show differences in various properties including affinities for Ca2+ (14, 17), voltage dependence (17), and the molecular mechanisms of coupling to the activation gate (18). The distinction between the properties of the two putative Ca2+ binding sites may lead to different physiological roles of these sites. For instance, a mutation in Slo1 that is associated with epilepsy and dyskinesia in human (19) specifically enhances the coupling of the RCK1 site to the activation gate to increase Ca2+ sensitivity of channel activation (18).

Although previous studies showed that the Ca2+ binding site in RCK1 is important for physiological functions, its molecular identity is less certain than that of the Ca2+ bowl. In the Ca2+ bowl, previous mutagenesis studies (13) and a recently published X-ray crystallographic structure of the Ca2+-bound Slo1 CTD demonstrate that a Ca2+ ion is coordinated by the side chains of D989 and D900 and the main chain carbonyls from Q892 and D895 (10). On the other hand, besides D367, no other residues have been identified to be part of the putative RCK1 Ca2+ binding site. Surprisingly, the same structure of CTD in high Ca2+ concentration did not identify any second Ca2+ binding site although the residue D367 is shown (10). To gain a better understanding of how the RCK1 Ca2+ binding site contributes to physiological functions and to solve the discrepancy between the structural data and the results from functional studies, further studies of this binding site are needed.

During the last 2 y, we have searched residues other than D367 that may be part of the putative Ca2+ binding site in RCK1 by systematic mutagenesis. These experiments show that the mutations of E535 in the RCK1 domain produce nearly identical functional consequences on the Ca2+-dependent activation as the mutations of D367. Therefore, both E535 and D367 may be part of the Ca2+ binding site in RCK1 (10). We found that mutations of M513, some of which have been shown to reduce Ca2+ sensitivity (20), result in a different pattern of functional consequences than those of E535 and D367, which suggests that M513 may not be part of the Ca2+ binding site. We have also investigated Cd2+-dependent activation of BK channels and found that Ca2+ and Cd2+ interact with different sets of residues to activate BK channels, but some of the residues important for Ca2+-dependent activation may overlap with part of the putative Cd2+ binding site in RCK1. These results identify a cluster of residues that are important for BK channel function and further differentiate their roles in controlling channel gating. The Ca2+ binding site formed by D367 and E535 is consistent with the recently solved structures of BK channel CTD (9, 10) and provides a basis for understanding how the Ca2+ binding site couples to the activation gate during Ca2+-dependent activation.

Results and Discussion

Ca2+ ions bound to proteins are usually coordinated by oxygen-containing side chains, main chain carboxyl groups, and water molecules (21). To search for an oxygen-containing residue that may be part of the Ca2+ binding site in RCK1, we mutated most Asp, Glu, Asn, Gln, Ser, Thr, and Tyr residues in RCK1 individually (Fig. 1A), which are possibly located close to D367 in structural models of RCK1 based on the structure of the RCK domain of MthK (22). We examined the change in Ca2+ sensitivity of the channel due to these mutations, and Fig. 1B shows...
The E535A mutant channel retains part of Ca\(^{2+}\) sensitivity; the G-V relation of the channels shifts to negative voltages in response to an increase of [Ca\(^{2+}\)], from 0 to 100 \(\mu\)M (Fig. 1 C and D). An additional mutation 5D5N, which substitutes the five consecutive Asp residues in the Ca\(^{2+}\) bowl with Asn, nearly eliminates the remaining Ca\(^{2+}\) sensitivity of E535A (Fig. 1D), indicating that E535A reduces Ca\(^{2+}\) sensitivity specifically derived from the Ca\(^{2+}\) binding site in RCK1. Consistent with this result, a double mutation D367A/E535A reduces Ca\(^{2+}\) sensitivity similarly as either D367A or E535A (Fig. 1D), indicating that E535A, similar to D367A, destroys Ca\(^{2+}\) sensitivity derived from the RCK1 site.

Previous studies measured Ca\(^{2+}\)-dependent activation derived from either the RCK1 site or the Ca\(^{2+}\) bowl by mutating the other site and found that the two sites show different affinities for Ca\(^{2+}\) (14, 17). To further examine whether E535A destroys Ca\(^{2+}\) sensitivity derived from the RCK1 site, we measured G-V relations of the mutant channel at various [Ca\(^{2+}\)], from 0 to 100 \(\mu\)M (Fig. 2A). Comparing the pattern of G-V shifts at various [Ca\(^{2+}\)] among mutants D367A, E535A, and 5D5N, it is apparent that the E535A channels behave more similarly as D367A than 5D5N, with a larger reduction of Ca\(^{2+}\) sensitivity and less even distribution of G-V relations along the voltage axis (Fig. 2A and B). Fitting the G-V relations of each mutant channel with a voltage-dependent Monod–Wyman–Changeux (MWC) model (27), we obtained apparent affinity of Ca\(^{2+}\) binding sites (Fig. 2C). These results indicate that 5D5N destroys the Ca\(^{2+}\) bowl that has a higher affinity for Ca\(^{2+}\), leaving the RCK1 site intact with a lower affinity for Ca\(^{2+}\). However, both E535A and D367A destroy Ca\(^{2+}\) sensitivity derived from the RCK1 site that has a lower Ca\(^{2+}\) affinity.

Fig. 2 also shows that E535A and D367A affect BK channel gating with different details. First, in 0 [Ca\(^{2+}\)], the G-V relation of D367A is shifted to less-positive voltage ranges compared with that of the WT mSlo1 or E535A (Fig. 2A and B). Second, in the intermediate [Ca\(^{2+}\)], the G-V relations of D367A show a steeper slope, which makes these G-V curves appear more shifted away from that in 0 [Ca\(^{2+}\)], as compared with those of E535A...

the results of the mutations in the C-terminal half of RCK1. The results of the mutations in the N-terminal AC region have been shown (18). In response to increases in [Ca\(^{2+}\)], the conductance-voltage (G-V) relation of BK channels shifts to more negative voltage ranges (23) (Fig. 1C). Because the effect of voltage on Ca\(^{2+}\)-dependent activation is weak (17, 24, 25), this property has been used as an effective measure of Ca\(^{2+}\) sensitivity of BK channels in most studies of BK channel function (26). Similarly, here we define Ca\(^{2+}\) sensitivity as the G-V shift in response to the [Ca\(^{2+}\)] change from 0 to the saturating 100 \(\mu\)M, \(\Delta V_{1/2} = V_{1/2} - V_{1/2,0}\) at 0 [Ca\(^{2+}\)] = \(V_{1/2,0}\) at 100 \(\mu\)M [Ca\(^{2+}\)] (Fig. 1B), where \(V_{1/2}\) is the voltage where G-V is half maximum. Mutations alter Ca\(^{2+}\) sensitivity and the change in Ca\(^{2+}\) sensitivity, \(\Delta V_{1/2} = \Delta V_{1/2,\text{mut}} - \Delta V_{1/2,\text{wt}}\) for all of the Ala scan mutations described above is shown (Fig. 1B). Similar to reported (14), D367A reduces more than half of the total Ca\(^{2+}\) sensitivity, with \(\Delta V_{1/2} = -112.8 \pm 2.3\) mV (Fig. 1B). Of all other mutations, E535A reduces Ca\(^{2+}\) sensitivity similarly as D367A (Fig. 1B) (18), suggesting that E535 may play an equivalent role as D367 in Ca\(^{2+}\) binding.
All these differences between the two mutant channels suggest that, in addition to affecting Ca\(^{2+}\)-dependent activation, D367A also alters voltage-dependent activation of BK channels. This result is consistent with the location of D367 that is close to the VSD during BK channel activation (28, 29), and such interactions may result in a weak voltage dependence of Ca\(^{2+}\) sensitivity derived from the RCK1 site (17). Thus, it is possible that the mutation D367A also alters function of the VSD, resulting in changes of voltage-dependent activation.

Because in a systematic Ala scan of oxygen-containing residues only the mutations of D367 and E535 nearly eliminate Ca\(^{2+}\) sensitivity in addition to the Ca\(^{2+}\) bowl mutation 5D5N (Fig. 1), D367 and E535 are likely to be part of the Ca\(^{2+}\) binding site in RCK1 of BK channels where the side chain of the acidic residues may coordinate Ca\(^{2+}\). To further examine whether these residues have the properties of a Ca\(^{2+}\) coordinator, we measured Ca\(^{2+}\) sensitivity of the mutations of D367 to various amino acids and found that D367E retains a Ca\(^{2+}\) sensitivity that is significantly greater than that retained by D367A (Fig. 3A; see Fig. S1A for current traces). This result is consistent with the idea that the oxygen atoms from the side-chain carboxylate groups of D367 or E537 can coordinate Ca\(^{2+}\), but because of the difference in the size of side chains, D367E may alter the structure of the binding site and, thus, impair Ca\(^{2+}\) sensitivity. Likewise, E535D also retains more Ca\(^{2+}\) sensitivity than E535A (Fig. 3B and Fig. S1B), indicating that E535 has similar properties of a Ca\(^{2+}\) coordinator.

A previous study showed that the mutation M513I in the RCK1 domain reduces Ca\(^{2+}\) sensitivity (20) (Fig. 3C and Fig. S1C). The double mutation M513I/D367A does not cause any reduction of Ca\(^{2+}\) sensitivity in addition to D367A, but M513I/5D5N reduces Ca\(^{2+}\) sensitivity more than 5D5N alone (Fig. 1D), indicating that M535I affects the Ca\(^{2+}\) sensitivity derived from the RCK1 site. Likewise, M513I/E535A does not cause any reduction of Ca\(^{2+}\) sensitivity in addition to E535A (Fig. 1D), further supporting that E535 is part of the Ca\(^{2+}\) binding site in RCK1. Ca\(^{2+}\) prefers to bind to hard oxygen-containing ligands; the soft sulfur in the side chain of Met residues usually is not found to coordinate Ca\(^{2+}\) in other Ca\(^{2+}\) binding proteins (21, 30). If M513 coordinates Ca\(^{2+}\) in BK channels, a change of M513 to an oxygen-containing residue is expected to retain or even enhance Ca\(^{2+}\) sensitivity. We mutated M513 to Ile, Cys, Asp, and Glu residues and found that M513D reduces Ca\(^{2+}\) sensitivity more than any other mutations, whereas M513C reduces Ca\(^{2+}\) sensitivity the least (Fig. 3C). These results are in contrast to the profile of mutational results on D367 or E535, indicating that M513 does not have the properties of a Ca\(^{2+}\) coordinator. Therefore, the mutations of M513 may reduce Ca\(^{2+}\) sensitivity of BK channel by altering the structure of the Ca\(^{2+}\) binding site in RCK1 (see below).

Besides Ca\(^{2+}\), other divalent cations, including Cd\(^{2+}\), also activate BK channels (31). Previous studies suggested that Cd\(^{2+}\) might interact with the Ca\(^{2+}\) binding site in RCK1 but not the Ca\(^{2+}\) bowl because the double mutation D362A/D367A nearly abolished Cd\(^{2+}\) sensitivity (32) (Fig. 4; see Fig. S2 for current traces), whereas mutations of the Ca\(^{2+}\) bowl had no effect on Cd\(^{2+}\) sensitivity (11, 32). To examine whether the same residues are important for both Ca\(^{2+}\)- and Cd\(^{2+}\)-dependent activation, we examined the effect of mutations D362A, D367A, M513I, and E535A on Cd\(^{2+}\)-dependent activation. The results show that the effect of these mutations on Cd\(^{2+}\) sensitivity is not correlated with the effect on Ca\(^{2+}\)-dependent activation (Fig. 4A and B). First, none of the individual mutations abolishes Cd\(^{2+}\) sensitivity, although D367A and E535A nearly abolish the Ca\(^{2+}\) sensitivity sensitivity.

![Fig. 3](https://www.pnas.org/cgi/doi/10.1073/pnas.1010124107 Zhang et al.)

**Fig. 3.** Properties of E535 and D367 but not M513 are consistent with calcium binding coordinators. Left are G-V relations of D367E (A), E535D (B), and M513D (C) in [Ca\(^{2+}\)] from nominal 0 (<0.5 nm) (●), 1 μM (▲), 2 μM (▲), 5 μM (▲), 10 μM (▲), 30 μM (▲), and 100 μM (▲). Fitted with the Boltzmann relation (filled lines), and Right are Ca\(^{2+}\) sensitivity of D367 (A), E535 (B), and M513 (C) mutated to various amino acids. ΔV\(_{1/2}\) is defined in the text. *Ca\(^{2+}\) sensitivity of the mutated channel is significantly larger than that of the mutation to Ala (P < 0.005).

![Fig. 4](https://www.pnas.org/cgi/doi/10.1073/pnas.1010124107 Zhang et al.)

**Fig. 4.** The effects of mutations on Cd\(^{2+}\) sensitivity are not correlated with that on Ca\(^{2+}\) sensitivity. (A) G-V relations in 0 (open circles) and 100 μM (filled circles) [Ca\(^{2+}\)] and 0 (open triangles) and 100 (filled triangles) [Cd\(^{2+}\)], fitted with the Boltzmann relation (filled lines). (B) Effects of Ca\(^{2+}\) (filled bars) and Cd\(^{2+}\) (open bars) on the WT and mutant channels. (Left) ΔV\(_{1/2}\) = V\(_{1/2}\) at 0 [Ca\(^{2+}\)] (or [Ca\(^{2+}\)]) – V\(_{1/2}\) at 100 μM [Ca\(^{2+}\)] (or [Ca\(^{2+}\)]). (Right) ΔΔV\(_{1/2}\) = ΔV\(_{1/2}\) of mutations – ΔV\(_{1/2}\) of WT.
derived from the RCK1 site (Fig. 1). Second, D362A reduces Ca$^{2+}$ sensitivity by the smallest fraction among all individual mutations but reduces Cd$^{2+}$ sensitivity by the largest fraction. On the other hand, M513I and E535A have large effects on Ca$^{2+}$ sensitivity but small effects on Cd$^{2+}$ sensitivity. Thus, only D367 is important for both Ca$^{2+}$- and Cd$^{2+}$-dependent activations, whereas other residues are important for either Ca$^{2+}$- or Cd$^{2+}$-dependent activation. Therefore, Cd$^{2+}$ does not seem to bind to the same site as Ca$^{2+}$. Additionally, because D362A and D367A both reduce approximately half of Cd$^{2+}$ sensitivity ($\Delta V_{1/2}^{\text{mut}} - \Delta V_{1/2}^{\text{wt}} = -35.5 \pm 5.1$ mV and $-32.8 \pm 8.1$ mV, respectively; Fig. 4B) and the effects of the two individual mutations add up to that of the double mutation D362A/D367A ($\Delta V_{1/2}^{\text{mut}} - \Delta V_{1/2}^{\text{wt}} = -84.6 \pm 6.6$ mV), D362 and D367 seem to affect Cd$^{2+}$ sensitivity independently and neither seem to be necessary for Cd$^{2+}$ binding.

Recently, two crystal structures of the gating ring from the human BK channel were experimentally determined, representing two different conformations of the gating ring: One is the Ca$^{2+}$-bound conformation crystallized in the presence of 50 mM [Ca$^{2+}$] ($\text{Ca}^{2+}$-bound Ca$^{2+}$-bound crystal structure; PDB ID code: 3MT5) (10), and the other is the Ca$^{2+}$-free structure (PDB ID code: 3NAF) (9) (Fig. 5A). However, interestingly, only one Ca$^{2+}$ is present at the Ca$^{2+}$-bound Ca$^{2+}$-bound crystal structure, whereas no Ca$^{2+}$ is observed at the putative D367/E535 site. Based on the Ca$^{2+}$-free structure, it was predicted that D367 and E535 could form a Ca$^{2+}$ binding site (9). However, the structure is in the Ca$^{2+}$-free conformation, and the side chains of D367 and E535 in the structure are not close enough to coordinate a Ca$^{2+}$ ion. Comparisons between the RCK1 domains of the Ca$^{2+}$-bound and Ca$^{2+}$-free structures show that, although the overall architectures of the two conformations are similar, the conformations differ significantly around the D367/E535 binding site. This flexible region includes three segments: the loop between $\alpha A$ and $\beta B$, the loop between $\alpha G$ and $\alpha I$, and the linker between $\alpha H$ and $\beta G$ (Fig. 5A).

The conformational changes suggest flexibility around the D367/E535 binding site, which binds and releases Ca$^{2+}$ during the functional cycle of the BK channel. In other words, in addition to the two conformations shown by the crystal structures, there should be a third conformation of RCK1 that allows binding of Ca$^{2+}$ at the D367/E535 site. Here, we model the conformation of RCK1 bound with Ca$^{2+}$ near D367/E535 based on the Ca$^{2+}$-free crystal structure (9), in which the side chain of the critical residue D367 points outward to the solvent and, thus, better resemble the Ca$^{2+}$-bound conformation because Ca$^{2+}$ ions stay in the solvent before binding.

Fig. 5B shows two Ca$^{2+}$ ions binding to the modeled RCK1-RCK2 structure of the BK channel. It can be seen that the Ca$^{2+}$ ion that binds at the D367/E535 site is coordinated by one main-chain carbonyl oxygen atom of R514 and four oxygen atoms from the side-chain carboxylate groups of D367 and E535, which is consistent with the previous study (14) and our experimental results (Figs. 1–3) (also see SI Results and Discussion and Fig. S3). Because only the main-chain carbonyl oxygen atom of R514 coordinates the binding of the Ca$^{2+}$ ion, it is expected that mutants of this residue would not have much effect on atomic coordination for Ca$^{2+}$ and, therefore, the Ca$^{2+}$ sensitivity of the BK channel.

Comparisons between our modeled RCK1 conformation and the two crystal structures (9, 10) show that our RCK1 model experiences larger conformational changes around the D367/E535 binding site and better coordinates the Ca$^{2+}$ ion (Fig. 5C). In addition to the directly interacting residues D367, R514, and E535 of Ca$^{2+}$, another notable residue involved in the conformational change is M513. In the crystal structures, the side chain...
of M513 points toward the Ca$^{2+}$ binding pocket. However, in our modeled structure, the side chain of M513 adopts a completely different orientation and points toward the protein due to the conformational change induced by Ca$^{2+}$ binding in the loop where D367 locates (Fig. 5C). Further examinations on the surrounding environment of M513 in our model reveal that M513 would contribute to Ca$^{2+}$ binding in the following way: The hydrophobic side chain of M513 points toward the interface between RCK1 and RCK2, forming a hydrophobic cluster core with the residues A508, F511, Y528, and V532 from RCK1 and residues Y904 and F909 from RCK2 (Fig. 5D). This hydrophobic core would be critically important for stabilizing the interface between RCK1 and RCK2 and the subdomain from αG to βG that contains the coordinating residues R514 and E535 of Ca$^{2+}$. In other words, to allow binding of Ca$^{2+}$, it would be necessary for the side chain of M513 to face the hydrophobic core at the interface between RCK1 and RCK2 so as to maintain the local structural integrity of the Ca$^{2+}$ binding site, as shown in our modeled dimeric structure (Fig. 5D). Although both Met and Ile are hydrophobic residues, the side chain of Ile is shorter and bulkier than that of Met, which may affect the crowded hydrophobic core that contains multiple aromatic residues. On the other hand, the hydrophobic Cys residue better resembles Met despite its shorter length than Met. Therefore, the Ca$^{2+}$ binding site would be affected more by charged mutations M513D, M513E, and M513H than M513C (Fig. 3). Fig. 1B shows that Y528, one of the critical residues in the above hydrophobic core (Fig. 5D), is also important to Ca$^{2+}$ binding because Y528A significantly reduces Ca$^{2+}$ sensitivity of the BK channel. In contrast, the neighboring residue Y527, which points to the solvent and is not part of the hydrophobic core (Fig. 5D), has little effect on the Ca$^{2+}$ sensitivity when it is mutated to Ala (Fig. 1B).

Previous studies indicated that E374 and E399 in the cytosolic RCK1 form a Mg$^{2+}$ binding site with D99 and N172 in the membrane-spanning VSD in BK channels (29). The Mg$^{2+}$ ion bound to the interface between the VSD and RCK1 activates the BK channel by an electrostatic repulsion to the S4 segment (28). These results suggest that RCK1 is located close to the VSD and the two domains interact intimately, which is corroborated by an electron cryomicroscopy (cryo-EM) structure of BK channels (33). D367 is situated in a loop between the membrane-spanning VSD in BK channels (29). The Mg$^{2+}$ ion binding constants: Effects of membrane voltage.

Materials and Methods

All mutations were generated from the mbr5 splice variant of mouse Slo1 (mSlo1) (8) by using overlap-extension PCR (35) and verified by sequencing. Xenopus laevis oocytes were injected with 0.05–20 ng of cRNA/oocyte, and currents were recorded in 2–4 d.

Macroscopic currents were recorded from inside-out patches. The data acquisition and analyses, solutions for Ca$^{2+}$-dependent activation, and model fitting are the same as described (36) and can be found in SI Materials and Methods. In experiments on Ca$^{2+}$-dependent activation, the internal solution contained 150 mM KF, 20 mM Hepes, and 2 mM MgCl$_2$ with either 0 or 100 μM CdCl$_2$ as the 0 or 100 μM Cd$^{2+}$, solution. Ca$^{2+}$-sensitive currents were recorded in 2–4 d.

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5. Shen KZ, et al. (1994) Tetraethylammonium block of Ca$^{2+}$-activated K$^{+}$ currents were recorded in 2–4 d.


Supporting Information

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SI Results and Discussion

An inspection of the Ca\textsuperscript{2+}-bound structure of the BK channel CTD (3MTS) (1) shows that OE1 and OE2 of the E535 side chain are only 4.6 and 3.7 Å from NZ of K366, respectively (Fig. S3A), which suggests that E535 and K366 might form a salt bridge during Ca\textsuperscript{2+}-dependent activation (2). Therefore, an alternative explanation for the effects of E535 mutations could be that these mutations destroy the salt bridge, thereby destabilizing the conformation of the channel to reduce Ca\textsuperscript{2+} sensitivity. To examine this hypothesis, we made mutation K366A and found that it has little effect on Ca\textsuperscript{2+} (e.g., Fig. S3 B and C). This result indicates that either E535 may not form a salt bridge with K366, consistent with the Ca\textsuperscript{2+}-free structure (3NAF) (3) in which OE1 and OE2 of E535 are farther away from NZ of K366 (11.5 and 9.5 Å, respectively), or the salt bridge formation does not affect Ca\textsuperscript{2+}-dependent activation.

SI Materials and Methods

Mutagenesis and Expression. All mutations were made by using overlap-extension PCR (4) with Pfu polymerase (Stratagene). The PCR-amplified regions for all constructs were verified by sequencing. cRNA was transcribed in vitro by using T3 polymerase (Ambion). cRNA (0.05–20 ng) was injected into each Xenopus laevis oocyte. Oocytes were incubated in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 5 mM Hepes at pH 7.6) at 18 °C for 2–4 d before recording.

Electrophysiology. Macropscopic currents were recorded from inside-out patches formed with borosilicate pipettes of 0.9–1.5 MΩ resistance. The data were acquired by using an Axopatch 200-B patch-clamp amplifier (Axon Instruments) and Pulse acquisition software (HEKA Electronik). Recordings were digitized at 20-μs intervals and low-pass filtered at 10 kHz with the four-pole Bessel filter built in the amplifier. Capacitive transients and leak currents were subtracted by using a P75 preset. Experiments were conducted at room temperature (20–22 °C). The pipette solution contained 140 mM KMeSO\textsubscript{4}, 20 mM Hpes, 2 mM KCl, and 2 mM MgCl\textsubscript{2} at pH 7.2. The internal solution contained 140 mM KMeSO\textsubscript{4}, 20 mM Hpes, 2 mM KCl, and 1 mM Na\textsubscript{2}(2-hydroxyethyl)ethylenediamine-N,N,N-triacetic acid (HEDTA) at pH 7.2. CaCl\textsubscript{2} was added to the internal solution to give the appropriate free [Ca\textsuperscript{2+}]\textsubscript{i}, which was measured with a calcium-sensitive electrode (Orion Research). The 18-crown-6-tetracarboxylic acid (50 mM; Sigma-Aldrich) was added to internal solutions to chelate Ba\textsuperscript{2+}. For nominal 0 mM [Ca\textsuperscript{2+}]\textsubscript{i}, the same internal solution was used except that HEDTA was substituted by 5 mM EGTA and no CaCl\textsubscript{2} was added. The free [Ca\textsuperscript{2+}] in nominal 0 mM [Ca\textsuperscript{2+}] solution is 0.5 nM.

Data Analysis. The relative conductance was determined by measuring tail current amplitudes at indicated voltages. The conductance-voltage (G-V) relations were fitted with the Boltzmann equation:

\[
\frac{G}{G_{\text{max}}} = \frac{1}{1 + \exp\left(-\frac{V - V_{1/2}}{\kappa}\right)}
\]

where \(G/G_{\text{max}}\) is the ratio of conductance to maximum conductance in the same [Ca\textsuperscript{2+}]\textsubscript{i}, \(z\) is the number of equivalent charges, \(V_{1/2}\) is the voltage at which the channel is 50% activated, \(\kappa\) is the elementary charge, \(k\) is Boltzmann’s constant, and \(T\) is the absolute temperature. Curve fittings were done with Igor Pro software (WaveMetrics) by using the Levenberg–Marquardt algorithm to perform nonlinear least squares fits. Statistics were performed by using Origin 6.1 (OriginLab); independent/unpaired Student’s \(t\) test was performed with Bonferroni and Šidák corrections for multiple comparisons.

MWC Model. MWC model fits were performed by using the following equation:

\[
P_{\text{open}} = \frac{1}{1 + L(0) e^{-\frac{|V|}{kT}}} \left[\frac{|V|}{kT}\right]^t.
\]

where \(P_{\text{open}}\) is the channel’s open probability, \(L(0)\) is the steady-state equilibrium constant from open to close channels ([Ca\textsuperscript{2+}]\textsubscript{i}/[Ca\textsuperscript{2+}]\textsubscript{o}), in the absence of Ca\textsuperscript{2+} binding at 0 mV, \(z, e, k,\) and \(T\) are as same as in the Boltzmann equation (see above), \(K_c\) and \(K_o\) are the dissociation constants of Ca\textsuperscript{2+} in the closed and open states, respectively. MWC model code was written and executed in MATLAB V7.4 (MathWorks).

Molecular Modeling. Specific details for remodeling the three flexible segments around the D367 binding site using the LOOPY modeling program are as follows. For each of the three flexible loops (i.e., L364-Q372, S512-E520, and S533-Y537), 100 possible conformations were modeled with the LOOPY program by using the default parameters except for the force field parameters, which were set to CHARMM force fields in heavy atom representation. This process yielded a total of 1 million combinations, corresponding to 1 million possible RCK1 structures/conformations with modeled loops. Then, these modeled RCK1 structures were evaluated by our ITScore/PP scoring function to remove the high-energy structures with atomic clashes or too close atomic contacts. Because of the large number of combinatorial conformations, docking a Ca\textsuperscript{2+} ion to each of the modeled RCK1 protein structure with a regular docking program is computationally impractical. Therefore, we have written a computer program to automatically place/dock the Ca\textsuperscript{2+} ion between OD1/OD2 of D367 and OE1/OE2 of E535 for each protein conformation and to evaluate the coordination number/binding energy score between the Ca\textsuperscript{2+} and the protein. The coordination with the best protein-Ca\textsuperscript{2+} coordination/score was selected as the modeled RCK1 structure. Comparison between the modeled and crystal structures showed that two of the three flexible loops around D367 site, i.e., L364-Q372 and S512-E520, experienced significant conformational changes to coordinate the bound Ca\textsuperscript{2+} (e.g., Fig. SC).

**Fig. S1.** Macroscopic current traces from inside-out patches expressing the D367E (A), E535D (B), and M513D (C) mutant channels elicited by voltage pulses from −150 to 190 mV at 20-mV increment.

**Fig. S2.** Macroscopic current traces from inside-out patches expressing the WT, D362A, D367A, D362A/D367A, M513I, and E535A mutant channels recorded in 0 (Left) and 100 μM Cd²⁺ (Right) on the same patches.
Fig. S3.  E535 may not form a salt bridge with K366 or the salt bridge formation does not affect Ca\(^{2+}\)-dependent activation. (A) The RCK1 Ca\(^{2+}\) binding site of the 3MT5 crystal structure where the protein is shown in ribbon and two notable residues E535 and K366 are displayed in stick mode and colored by atom type. (B) Macroscopic current traces from inside-out patches expressing K366A mutant channels. Currents were elicited in [Ca\(^{2+}\)]  of nominal 0 (≈0.5 nM; Left) by voltage pulses from −30 to 250 mV and 100 μM (Right) by voltage pulses from −150 to 190 mV at 20-mV increment. The voltages before and after the pulses were −50 and −80 mV, respectively. (C) G-V curves for WT and K366A mutation channels in [Ca\(^{2+}\)] of nominal 0 (≈0.5 nM; open symbols) and 100 μM (filled symbols). The solid lines are fittings of the Boltzmann relation.