Hydrophobic Interface between Two Regulators of K^+ Conductance Domains Critical for Calcium-dependent Activation of Large Conductance Ca^{2+}-activated K^+ Channels^{[5]}

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It has been suggested that the large conductance Ca^{2+}-activated K^+ channel contains one or more domains known as regulators of K^+ conductance (RCK) in its cytosolic C terminus. Here, we show that the second RCK domain (RCK2) is functionally important and that it forms a heterodimer with RCK1 via a hydrophobic interface. Mutant channels lacking RCK2 are non-functional despite their tetramerization and surface expression. The hydrophobic residues that are expected to form an interface between RCK1 and RCK2, based on the crystal structure of the bacterial MthK channel, are well conserved, and the interactions of these residues were confirmed by mutant cycle analysis. The hydrophobic interaction appears to be critical for the Ca^{2+}-dependent gating of the large conductance Ca^{2+}-activated K^+ channel.

Large conductance calcium-activated potassium (BK_{Ca})^2 channels play a key role in modulating a number of important physiological processes, such as neuronal excitability, frequency tuning of hair cells, smooth muscle contraction, and immunity (1–9). BK_{Ca} channels are activated by membrane depolarization and an increase in intracellular calcium (10–13). Thus, BK_{Ca} channels are considered to be molecular integrators of biochemical and electrical signals. Membrane depolarization and calcium binding activate BK_{Ca} channels independently via separate regions of the \(\alpha\) subunit of the channel (Slo). The transmembrane segments of the Slo channel, S1–S6, are structurally similar to those of voltage-gated potassium channels, and as in these channels, charged residues in the Slo S1–S4 segments are thought to be involved in the voltage-dependent gating of the channel (14–20). It is generally accepted that the bulky cytoplasmic C terminus of Slo is responsible for the calcium-dependent activation of the channel (21–23).

The cytoplasmic C terminus of Slo has been proposed to contain more than two Ca^{2+}-sensing sites, a high affinity site called the Ca^{2+} bowl, a low affinity site, and additional high affinity sites within a structural module known as the regulator of K^+ conductance (RCK) domain (22, 24–29). The Ca^{2+} bowl is composed of a series of Asp residues and binds Ca^{2+} with micromolar affinity. Mutations here have been shown to cause positive shifts in the conductance-voltage (G-V) relationship at constant [Ca^{2+}], which are similar to those observed with the wild-type channel when [Ca^{2+}] is lowered (22, 23, 26, 30, 31). The RCK domain is found primarily in prokaryotic ligand-gated K^+ channels and in some bacterial K^+ uptake and efflux systems, in which it is also called the \(K^+\) transport nucleotide-binding (KTN) domain (25, 32–34). The structure of the tetrameric MthK channel shows that an octameric complex could be formed by intermolecular interactions on fixed and flexible interfaces between a tetramer of dimeric RCK domains. This complex, called the gating ring, has been proposed to expand upon Ca^{2+} binding and to create a strain at the ends of the S6 helices for the opening of the channel (24, 35). In the gating ring of the MthK channel, four RCK domains are provided by the four channel subunits. The other four intracellular domains are produced as a result of translation start at downstream initiation sites.

Based on the octameric nature of the gating ring, two RCK domains have been proposed to lie in tandem within the long cytoplasmic region of the mammalian BK_{Ca} channel (24, 37, 38), and an RCK-like domain located at the proximal C terminus of the Slo protein has been characterized in detail using mutational analysis. This domain (RCK1) contains divalent cation binding sites, including the low-affinity site for Ca^{2+} (27–29). Recently, a gain-of-function mutation causing an epileptic seizure has also been localized to this region (39).

In this study, we have located the second RCK domain in the rat Slo channel using amino acid sequence alignment and investigated its functional importance in channel gating. This domain (RCK2) contains several conserved amino acids and exhibits a strong similarity in secondary structure to the crys-
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A

B
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plasmid DNA for transient transfection, the plasmid DNA was prepared using a commercial kit (Qiagen).

Expression of rslo Channels in Xenopus Oocytes—To measure the gating currents, wild-type and mutant rslo channels were expressed in Xenopus oocytes. The complementary DNA of rslo was subcloned into a modified pGEM expression vector for high level expression in Xenopus oocytes. Complementary RNAs were synthesized in vitro from the NotI-linearized plasmid using T7 polymerase (Ambion). ~150–200 ng of RNA were injected into oocytes, and the injected oocytes were incubated in ND96 solution at 18°C for 4–8 days.

Electrophysiological Recordings and Data Analysis—The macroscopic ionic currents carried by wild-type and mutant rslo channels were recorded in excised membrane patches of CHO-K1 cells with an inside-out configuration using an Axopatch 200B amplifier (Axon Instruments). All patch recordings were performed at room temperature at 24–48 h after transient transfection. Pipettes were prepared from thin-walled borosilicate glass (World Precision Instruments) and fire-polished to a resistance of 3–5 MΩ. Channel currents were activated by voltage pulses delivered from a holding potential of ~100 mV to test potentials ranging from ~150 mV to 200 mV in 10-mV increments. Signals were filtered at 1–2 kHz using a four-pole low pass Bessel filter digitized at a rate of 10 kHz using a Digidata 1200B digitizer (Axon Instruments) and stored in a personal computer. Commercial software packages, such as Clampex 8.1 (Axon Instruments) and Origin 6.1 (OriginLab), were used for the acquisition and analysis of macroscopic data.

For gating current measurements, patch pipettes made of borosilicate glass (VWR) were coated with sticky wax (Sticky Wax) and fire-polished to a resistance of 0.5–1 MΩ. Voltage commands were filtered at 7.5 kHz. Data were acquired with an Axopatch 200B amplifier at 100 kHz and filtered at 10 kHz. A Macintosh-based computer system equipped with an ITC-16 hardware interface (Instrutech) and Pulse acquisition software (HEKA Electronik) was used. Data analysis was performed using Clampfit 10.6 (Axon Instruments) and commercial software packages. Second RCK Domain of BK Channel

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FIGURE 3. Mutation of conserved hydrophobic residues in RCK2. Shown are the G-V relations for the wild type (WT) (n = 7) and I891A (n = 7) (A), L892A (n = 7) (B), L895A (n = 6) (C), and I891A/L895A (n = 8) (D), respectively. In each graph, the G-V curves for the wild type are shown with open symbols, and those of the mutant channels with solid symbols. The calcium concentrations are denoted by ◦ (0.5 μM), ▼ (5 μM), and ▲ (300 μM). The curves were fitted with the Boltzmann function. E-H, the half-activation voltage at each calcium concentration for the wild type and the indicated mutants. Each data point represents the mean ± S.E. Values that differed from the wild type by the paired Student’s t test at p < 0.05 (*) or p < 0.01 level (**) are indicated. In H, the sums of the V_{1/2} values for each single mutant channel are shown by half-filled symbols, ◦.

Solutions for macroscopic current recording were prepared according to Lim and Park (41). Pipette solutions contained 10 mM HEPES, 2 mM EGTA, 116 mM KOH, and 4 mM KCl. The intracellular solution for perfusing to the internal faces of excised patches was identical to the pipette solution except for the appropriate amounts of total Ca^{2+}/CaCl_2. To provide the precise free [Ca^{2+}]_i, the internal solution contained (in mM) 141 N-methyl-D-glucamine, 135 HMeSO_3, 6 HCl, 20 HEPES, 40 μM (+)-18-crown-6-tetracarboxylic acid (18c6TCA), and 5 EGTA, pH 7.2 (adjusted with HMeSO_3 or triethanolamine-OH). The internal solution contained (in mM) 141 N-methyl-D-glucamine, 135 HMeSO_3, 6 HCl, 20 HEPES, 40 μM (+)-18-crown-6-tetracarboxylic acid (18c6TCA), and 5 EGTA, pH 7.2 (adjusted with NMDG and HMeSO_3).

Homology Modeling of the Dimeric Structure of RCK Domains—Homology modeling of the RCK domains in rSlo was performed using the program MODELLER8 version 1 (44) with the high resolution atomic coordinates of the MthK RCK domain in the Ca^{2+}-bound form (Protein Data Bank (PDB) code 2AEF) (35) as the template. The position-specific iterated (PSI)-BLAST alignment shown in Fig. 1B was manually modified to make the loop positions realistic. For each rSlo RCK domain, 10 three-dimensional models of each rSlo RCK domain were generated initially, and the best model with the lowest MODELLER discrete optimized protein energy restraint energy was chosen. The resulting model was further refined by energy minimization to remove clashes between atoms. The energy computations were performed with the GROMOS96 implementation of the Swiss-PdbViewer (45). The figures were prepared using PyMOL (46).

Confocal Microscopy—The wild-type (rSlo::EGFP) and RCK2 deletion mutant constructs (rSloΔRCK2::EGFP) (50–100 ng) were injected into Xenopus oocytes. The oocytes were incubated at 18 °C for 3–5 days in ND96 solution. Each oocyte was observed under a confocal microscope (Leica Microsystems), and the images were analyzed using Leica software.

Western Blotting Analysis—Cells that had been transiently transfected with the channel gene were lysed in 20 mM HEPES (pH 7.5), 120 mM NaCl, 5.0 mM EDTA, 1.0% Triton X-100, 0.5 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Roche Applied Science). Cell lysates were spun at 15,000 x g for 15 min to pellet the insoluble material. After the addition of 5× loading buffer (250 mM Tris-HCl, pH 6.8, 500 mM dithiothreitol, 10% SDS, 0.5% bromphenol blue, 50% glycerol), the mixture was incubated at 37 °C for 15 min, and ~30 μg of the protein sample was separated using SDS-PAGE (47) and transferred to a polyvinylidene difluoride membrane. Blots were blocked with 5% nonfat dry milk in 1× TBS-T containing the primary antibody (anti-hSlo monoclonal antibody, 1:250) at room temperature for 1 h with agitation, washed three times with 1× TBS-T, and incubated in 10 ml of 1× TBS-T containing the primary antibody (anti-hSlo monoclonal antibody, 1:250) at room temperature for 2 h. After washing three times with 1× TBS-T, the membranes were incubated in 10 ml of 1× TBS-T containing the secondary antibody (goat horseradish peroxidase-conjugated anti-mouse IgG, 1:3000) for 1 h. The membranes were washed as above and immersed in chemiluminescence reagent (Pierce) for 1 min. The blots were placed on plastic wrap sheets and exposed to x-ray film (Kodak) for 5 min.

RESULTS

Localization of a Second RCK Domain in the BKα Subunit—To define the location of a second RCK domain in the α subunit of the large conductance Ca^{2+}-activated K^{+} channel (Slo), we searched for RCK homologues using PSI-BLAST (48). Initially, we used the amino acid sequences of RCK1 (25) from rSlo (Gen-
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The amino acid sequences of the RCK domains of MthK, the E. coli 6TM K⁺ channel, and various orthologues of Slo are aligned in Fig. 1B, with the secondary structures of MthK indicated above the sequences (gray arrows and boxes) (49) and those predicted for RCK2 shown below the sequences (blue arrows and boxes). The RCK domain of MthK forms a typical Rossmann-folded N-lobe, with two α helices (αA and αB) on one side of six-stranded parallel β sheets (∼αB–βF and three α helices (αC, αD, and αE) on the other side. It also contains a C-lobe composed of four β strands and three α helices (αF–αI) (24). The two lobes are connected by the turn between helices αF and αG. Whereas the similarities in both amino acid sequence and the secondary structure pattern are retained through the C-lobe in RCK1, RCK2 lacks a C-lobe, and its N-lobe is directly followed by the Ca²⁺ bowl. RCK1 and RCK2 contain several conserved residues that are functionally important in almost all RCK domains. Among these are the hydrophobic residues in αD that form the dimeric interface in MthK (Fig. 1B, green) and a pair of charged residues involved in salt bridging (Fig. 1B, blue and red).

Functional Importance of RCK2 in Channel Gating—To examine the functional importance of the putative RCK2, this domain was deleted (rSloΔRCK2), and the activity of the mutant channel was compared with that of the wild type. Whereas the wild-type channels were robustly activated at various intracellular calcium concentrations and depolarizing membrane potentials (Fig. 2A), the mutant channels failed to generate any ionic current, even under extreme conditions (Fig. 2B). We also failed to reconstitute the functional channel by co-expressing rSloΔRCK2 and RCK2 (Fig. 2C). However, the co-expression of the Slo “Core,” containing the entire transmembrane domains and RCK1, and the Slo “Tail,” harboring RCK2, resulted in functional channels (Fig. 2D), as has been reported in previous studies (21, 23, 50).

To examine whether the nonfunctionality of rSloΔRCK2 was because of the failure of tetramer formation and/or the lack of surface expression, we performed nonreducing gel electrophoresis and immunoblot analysis (41). CHO-K1 lysates containing the exogenously expressed rSlo:EGFP or rSloΔRCK2:EGFP proteins were pretreated with nonreducing Laemmli buffer. An immunoblot with anti-hSlo antibody showed that the oligomers up to the size of the tetramer, as well as the monomer, were detected in both the wild-type and the mutant channel (supplemental Fig. 1A).

We then examined the surface expression of the RCK2 deletion mutant using confocal microscopy. EGFP signals were detected at the surface membrane in both the wild type and the deletion mutant. Soluble EGFP signals were widely distributed throughout the entire cytoplasm (supplemental Fig. 1, B–D). These results indicate that the RCK2 deletion mutant can
D927K were indistinguishable from that of the wild type (supplemental Fig. 2, A–C). At various calcium concentrations, the \( V_{1/2} \) values of the wild-type and each mutant channel were not significantly different (supplemental Fig. 2D).

The amino acid residues corresponding to the fixed interface in MthK are well conserved in both RCK1 and RCK2 (Fig. 1B, green). To examine the functional importance of these hydrophobic residues (Ile-891, Leu-892, and Leu-895) on RCK2, we compared the \( \text{Ca}^{2+} \) sensitivities of the wild-type channel and mutant channels in which each residue was individually replaced with alanine. Single alanine substitutions at either Ile-891 or Leu-895 shifted the \( G-V \) relationship in the positive direction (Fig. 3, A and C) and increased \( V_{1/2} \) values by \( \sim 30 \text{ mV} \) (Fig. 3, E and G). The mutation of Leu-892 caused an opposite effect, a negative shift in the \( G-V \) relationship by 25–40 mV (Fig. 3, B and F). When Ile-891 and Leu-895 were simultaneously mutated, however, the \( G-V \) curves were shifted to the right by as much as 100 mV (Fig. 3, D and H), which is more than the sum of the shifts of each of the individual mutations (Fig. 3H, half-filled diamonds). Thus, Ile-891 and Leu-895 appear to function synergistically in calcium-dependent gating.

To determine whether the rightward \( G-V \) shifts in the I891A/L895A mutant were caused by an alteration in voltage-dependent gating, we measured the gating current (Fig. 4). To our surprise, the \( Q-V \) relationships of the wild-type and mutant channels were superimposable, and their Boltzmann fit parameters were virtually identical. We were unable to record any channel current from the triple mutant (I891A/L892A/L895A), even under extreme conditions. Thus, it can be concluded that the mutations in the putative hydrophobic interface of RCK2 alter the \( \text{Ca}^{2+} \) -dependent gating of the Slo channel without affecting its voltage-activated gating.

Next, we examined the functional importance of three hydrophobic residues in RCK1, Ile-507, Met-508, and Ile-511. Each residue was mutated to alanine, and its functional activity was compared with the wild type. The \( G-V \) relation of I507A was positively shifted by \( \sim 20–60 \text{ mV} \) compared with the wild type (Fig. 5A). The difference in the \( V_{1/2} \) between the wild type and I507A decreased as the intracellular calcium concentration
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Heterodimeric Interaction of RCK1 and RCK2 via the Hydrophobic Interface—Because the individual residues on the putative fixed interfaces of both RCK1 and RCK2 were so sensitive to mutation, we questioned whether we could determine the interaction across the dimeric surface. We generated combinations of double mutations with three hydrophobic residues on each domain. Of the nine double mutants, all but M508A/I891A and M508A/L892A showed macroscopic currents large enough for accurate and reproducible recordings. The G-V curves of the functional mutants are compared with that of the wild type in Fig. 6. Whereas the G-V curves of most of the double mutant channels were positively shifted, those of I507A/L892A and M508A/L895A were negatively shifted by ~30–60 and ~5–10 mV, respectively (Fig. 6, A–G). The $V_{1/2}$ values of each mutant channel at various calcium concentrations are compared in Fig. 6, $H$ and $I$.

To examine the specific interactions between pairs of amino acid residues, we applied a systematic analysis of a “thermodynamic mutant cycle” (51, 52). This analysis quantifies the influence of one mutation on the effect of the second mutation as a pairwise coupling energy between the two mutated residues. A representative mutant cycle is shown in Fig. 7A. To calculate the coupling energy, we used $V_{1/2}$ and $z$ values from G-V curves of each single and double mutant at 5 μM calcium concentration. The coupling energy values between pairs are shown in Fig. 7B. Several pairs of amino acid residues showed significant energetic couplings. Three pairs, Ile-507:Leu-892, Ile-511:Leu-892, and Ile-511:Leu-895, had coupling energy values of 3.18 kT, 1.96 kT, and 1.98 kT, respectively. Three other pairs, Ile507:Leu891, Met508:Leu895, and Ile511:Leu891 also showed weak but appreciable couplings with coupling energy values of 1.29, 1.26, and 1.03 kT, respectively, because it is generally accepted that a coupling energy $>1.5$ kT represents a strong pairwise interaction between two amino acid residues (51). The coupling energy between Ile-507 and Leu-895 was small with only 0.36 kT. In Fig. 7C, the relative strength between the hydrophobic residues are depicted with the thickness of arrows.

Fig. 7D shows the heterodimeric structural model of the two RCK domains of Slo. We constructed homology models of both the RCK1 and RCK2 domains using the known structure of the MthK RCK domain as a template (35). The configuration of the dimeric structure was based on the fixed interface of the octameric gating ring of MthK. To show the structure in detail, the hydrophobic interface between the two RCK domains is enlarged, and each interfacial residue is shown as a ball-and-stick image.

Increased (Fig. 5D). The G-V curves of M508A were negatively shifted, similar to the shift observed in L892A (Fig. 5B). The I511A mutation caused a slight positive change in the G-V curves (Fig. 5C). The $V_{1/2}$ values of the three mutants and the wild type are compared in Fig. 5D.

Figure 7. Mutant cycle analysis of amino acid residues on the hydrophobic interface and structural model of heterodimeric RCK domains of the Slo channel. A, representative mutant cycle showing the interaction between Ile-507 and Leu-892. The $z$ and $V_{1/2}$ values of the wild type (WT) and each mutant are from the Boltzmann-fitted G-V curves at 5 μM calcium. The measured $2zV_{1/2}$ values for each channel are shown in italics. The coupling energy ($\Delta G$) was calculated using the equation $\Delta G = \Delta G_1 - \Delta G_2 = \Delta G_3 - \Delta G_4$. A strong energetic coupling was observed between Ile-507 and Leu-892. B, the coupling energy values of possible pairs on the hydrophobic interface between RCK1 and RCK2 were calculated in a mutant cycle analysis at a calcium concentration of 5 μM and 23 °C. The pairs Met-508/Ile-891 and Met-508/Leu-892 were not determined due to the low expression of the double mutant channels M508A/I891A and M508A/L892A. C, schematic diagram showing the relative strength of the couplings. The thickness of arrows indicates the relative value of the coupling energy between the indicated residues. D, proposed dimeric structure of Slo channel RCK1 (yellow) and RCK2 (green) based on the dimeric structure from the octameric gating ring of MthK. To show the structure in detail, the hydrophobic interface between the two RCK domains is enlarged, and each interfacial residue is shown as a ball-and-stick image.
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DISCUSSION

The atomic structure of the MthK channel has been determined and a similar structural arrangement has been proposed for the mammalian BK\textsubscript{ca} channels (24, 25). Thus, the gating ring composed of eight RCK domains is a valuable structural model for Ca\textsuperscript{2+}-dependent activation of this class of K\textsuperscript{+} channels. A region homologous to bacterial RCK domains was initially identified in the cytosolic C terminus of the human BK\textsubscript{ca} channel \(\alpha\) subunit, which lies proximal to the transmembrane domains (25). Since its localization, this first RCK domain (RCK1) has been under close experimental scrutiny. It has been suggested that divalent cation binding sites are located in this RCK domain of the BK\textsubscript{ca} channel and that the energy due to calcium binding in these sites is transferred to the S6 gates by spring-like linkers (22, 23, 26–30, 53). Moreover, it has been reported that the N terminus of the RCK1 domain is important for transferring the conformational change from calcium binding to channel opening (54).

However, the tetrameric nature of the BK\textsubscript{ca} channel suggested the presence of a second RCK domain in the cytosolic domain of the channel for assembly of an octameric gating ring (24, 37, 38). In this study, we have placed the second RCK domain (RCK2) ~100 residues downstream of RCK1 and demonstrated the functional importance of this region. In addition, we have shown that the heterodimeric interaction between RCK1 and RCK2, via conserved hydrophobic residues, is critical for Ca\textsuperscript{2+}-dependent activation of the channel.

RCK2 exhibits a strong homology to other RCK domains in its N-terminal region, not only in its amino acid sequence but also in its predicted secondary structure (Fig. 1B). RCK2 also contains several key residues that confer structural integrity to RCK domains. However, there is a marked difference that distinguishes RCK2 from other RCK domains. RCK2 lacks a C-lobe, and its N-lobe, composed of a Rossmann fold, is directly linked to the highly negative Ca\textsuperscript{2+} binding site, the Ca\textsuperscript{2+} bowl. We can envision that the binding of Ca\textsuperscript{2+} to this high affinity site may evoke a conformational change of the gating ring, composed of a RCK1/2 heteroctamer, and shift the gating equilibrium to an open conformation. This mechanism is distinct from that of MthK, because the gating ring of the MthK channel is composed of eight identical RCK domains, and the conformational change is initiated by the direct binding of Ca\textsuperscript{2+} to a low affinity binding site within the RCK domains. It should be noted that the residues known to be involved in the binding of Ca\textsuperscript{2+} or other divalent cations in RCK1 (Fig. 1B, residues in yellow) are not conserved in RCK2.

In the MthK gating ring, two different interfaces were initially noted, the fixed interface composed of four \(\alpha\) helices (\(\alpha D\) and \(\alpha E\)) of two different RCK domains and the flexible interface formed by \(\alpha F\), \(\alpha G\), and the C-lobe (24). The hydrophobic residues comprising the fixed interface of MthK are well conserved in both RCK1 and RCK2 of the Slo channels (Fig. 1B, residues in green), suggesting a hydrophobic interface between RCK1 and RCK2, and the formation of heterodimer via the interface. The hydrophobic residues of both RCK1 and RCK2 were highly sensitive to mutational substitution. Whereas the individual replacement of the four flanking residues (Ile-507 and Ile-511 of RCK1; Ile-891 and Leu-895 of RCK2) with alanine shifted the 
G–V curve to a positive voltage, indicating the stabilization of a closed conformation (Fig. 5, A and C; Fig. 3, A and C), alanine substitution of the two middle residues (Met-508 of RCK1 and Leu-892 of RCK2) resulted in negative shifts (Figs. 3B and 5B). Moreover, the mutational effects of multiple residues were synergistic rather than additive. Although the triple mutant failed to evoke any measurable current (data not shown), the cooperative contributions of individual mutations strongly suggest the functional significance of the hydrophobic interactions.

In an attempt to reveal the pairwise interaction across the RCK1–RCK2 interface, we employed mutant cycle analysis on conserved hydrophobic residues (Fig. 7). First of all, the interaction between the two middle residues (Met-508 and Leu-892) appears to be critical for functional channels, because no macroscopic currents were recorded from the M508A/L892A double mutant. In addition, in several dozen trials, we only detected 1–3 functional channels for M508A/L891A. Significant energetic couplings were detected from several pairs, e.g. I507:L892, I511:L895, and I511:L892. Although it is difficult to predict the precise arrangement of the interactive surface because of the extreme sensitivity of hydrophobic interactions to side-chain geometry and local environments, we were able to apply our results to the structure of the fixed interface in MthK. The small couplings revealed for I507:L895 and I511:L891 are in good agreement with the structural model of the dimeric interface based on MthK (Fig. 7D). It should be noted that the mutational analysis utilized in this study has unavoidable shortcomings, in that such analysis represents the changes in overall equilibrium between a closed conformation and an open conformation rather than the changes in intrinsic binding energy between the two RCK domains due to the specific mutations.

Recently, new crystal structures of MthK RCK were determined in the absence and the presence of Ca\textsuperscript{2+} (35, 36). Subtle, but significant, movement of the fixed interface in the octameric gating ring was noticed during the conformational change of closed to open. Thus, the hydrophobic interface between RCK1 and RCK2 in Slo channel may not be entirely fixed, and such a relative movement may be involved in the conformational coupling of Ca\textsuperscript{2+} binding and opening of the channel conduction pore.

A second interface known as “flexible interface” may also connect RCK1 and RCK2. This interface is formed, in part, by \(\alpha G\) and \(\alpha F\) as well as by the C-terminal lobe. It has been proposed that the relative movement of this interface is the main conformational change induced by Ca\textsuperscript{2+} binding in MthK (24). Because RCK2 is truncated at the seventh \(\alpha\) helix (\(\alpha G\)) and lacks the C-terminal subdomain protruding from the gating ring, however, it is difficult to envision that such an interface is strong enough to hold the two domains together. Thus, it remains to be seen whether the flexible interfaces found in MthK also exist in the heteromeric gating ring comprised of RCK1 and RCK2. It is also important to characterize the structural and functional connections among the three distinct modules, RCK1, RCK2, and the Ca\textsuperscript{2+} bowl, in the context of Ca\textsuperscript{2+}-dependent conformational change.

In summary, we have localized the second RCK domain in the BK\textsubscript{ca} channel and provided experimental evidence sup-
porting the functional importance of RCK2 in Ca\(^{2+}\)-dependent gating of the channel. We propose that RCK2 plays a critical role in the functional integrity of the Slo channel via heteromeric interaction with RCK1 and transmits the conformational changes evoked by Ca\(^{2+}\) binding to the transmembrane gate.

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