

Understanding the conformational motions of RCK gating rings

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Regulator of conduction of K⁺ (RCK) domains are ubiquitous regulators of channel and transporter activity in prokaryotes and eukaryotes. In humans, RCK domains form an integral component of large-conductance calcium-activated K channels (BK channels), key modulators of nerve, muscle, and endocrine cell function. In this review, we explore how the study of RCK domains in bacterial and human channels has contributed to our understanding of the structural basis of channel function. This knowledge will be critical in identifying mechanisms that underlie BK channelopathies that lead to epilepsy and other diseases, as well as regions of the channel that might be successfully targeted to treat such diseases.

Introduction

Cellular potassium homeostasis is governed by a combination of K⁺ transport proteins and ion channels, whose activity is linked to cellular metabolism and signal transduction (Hille, 2001; Kuo et al., 2005). In prokaryotes, constitutive K⁺ uptake is controlled in part by the protein complexes Trk or Ktr, which consist of “dual-pore” transmembrane and separate cytosolic regulatory domains (Albright et al., 2006, 2007; Cao et al., 2011, 2013; Vieira-Pires et al., 2013; Levin and Zhou, 2014). In contrast, K⁺ efflux is governed in part by so-called Kef proteins, which typically consist of channel-like transmembrane pores that are tethered to cytosolic regulatory domains (Jiang et al., 2002a; Roosild et al., 2004, 2009; Kuo et al., 2005; Parfenova et al., 2007; Kong et al., 2012). In each case, the cytosolic regulatory domain that controls transporter activity is a highly conserved modular domain known as the regulator of conduction of K⁺ (RCK) domain. RCK domains have also been identified in eukaryotic K⁺ channels and show a high degree of sequence conservation across phyla and kingdoms (Jiang et al., 2001, 2002a; Yuan et al., 2010, 2011; Leonetti et al., 2012; Hite et al., 2015).

In addition to Kef-like prokaryotic channels that include MthK and GsuK, RCK domains are found in eukaryotic channels of the *Slo* gene family, which includes the BK channel (Slo1, KCa1.1, and KCNMA1), Na⁺-activated K⁺ channels (Slo2.1-2.2, KCa4.1-4.2, and KCNT1-2), and a H⁺-inhibited K⁺ channel found in mammalian sperm cells (Slo3, KCa5.1, and KCNMC1; Albright et al., 2006; Ye et al., 2006; Wu et al., 2010; Yuan et al., 2011; Kong et al., 2012; Leonetti et al., 2012; Smith et al., 2012; Cao et al., 2013; Hite et al., 2015).

The molecular architecture common to prokaryotic and eukaryotic RCK-containing K⁺ channels is illustrated in Fig. 1. A Kef-like channel subunit consists of a transmembrane pore module that is tethered to an RCK domain; a second RCK domain is docked onto the tethered RCK domain to form an RCK dimer (Fig. 1 A). Four of these subunit assemblies constitute a channel, with the K⁺ permeation pathway formed at the confluence of the pore modules and with a modulatory unit, comprised of the RCK domains, assembled at the cytosolic side of the pore. Thus, this modulatory unit consists of a ring of eight RCK domains (shown in a bird's-eye view in Fig. 1 B). Because of its role in gating of channel and transporter activity, this unit has been called the gating ring (Ye et al., 2006; Wu et al., 2010; Yuan et al., 2011; Smith et al., 2012). Eukaryotic (*Slo*) channels exhibit a similar overall architecture, except for the addition of transmembrane voltage-sensing domains on each subunit and two RCK domains tethered to each subunit in tandem (Fig. 1 C). Each of these features (voltage-sensing domains and tandem RCK domains) has been observed in some prokaryotic channels as well (Wu et al., 2010; Yuan et al., 2010, 2011; Kong et al., 2012; Leonetti et al., 2012). Importantly, however, these tandem-linked RCK domains form gating rings similar to those seen in Kef-like channels (Fig. 1 D).

Each single RCK domain consists of a well-conserved N-terminal “Rossmann-fold” subdomain linked to a less well-conserved C-terminal subdomain via a helix-turn-helix segment (Fig. 2 A; Jiang et al., 2001, 2002a; Dong et al., 2005). In turn, RCK domains are paired to form a dimeric unit that is assembled in an “arm-in-arm” architecture (Fig. 2 B). In this architecture, the

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Abbreviations used: FRET, fluorescence resonance energy transfer; RCK, regulator of conduction of K⁺.

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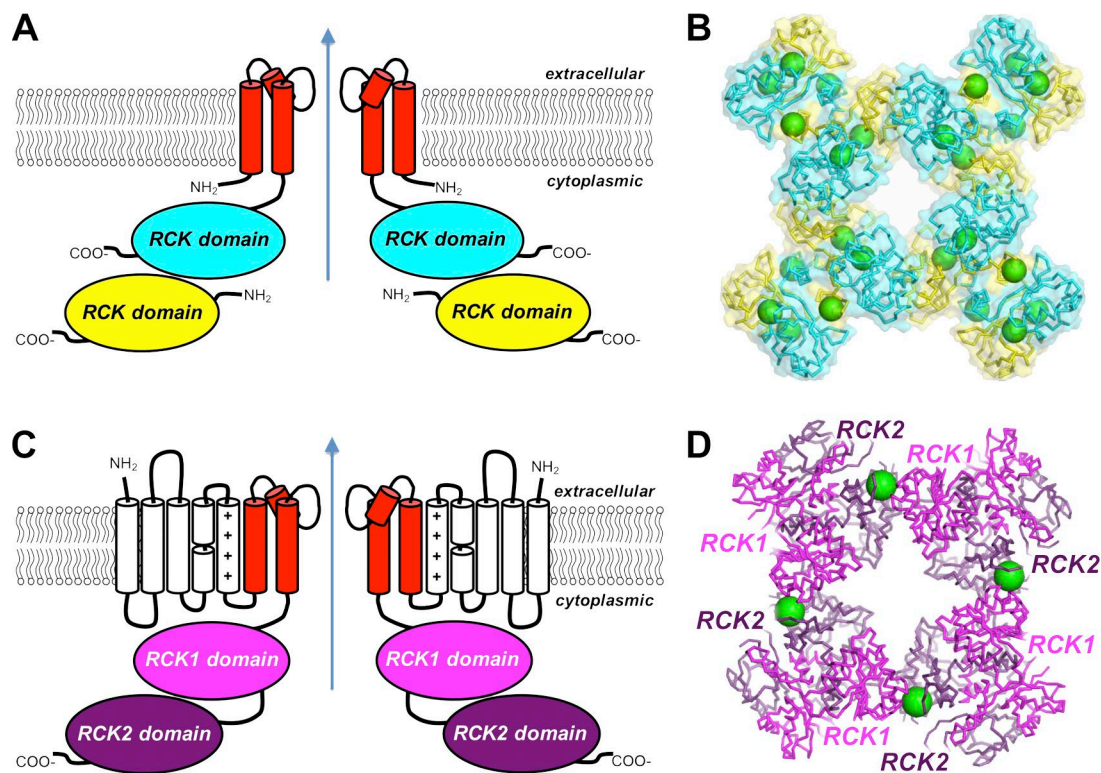


Figure 1. Molecular architecture of RCK domain-containing channels. (A) Schematic diagram of Kef-like channel subunits. Two of the four subunits are shown side by side to illustrate the K^+ permeation pathway (arrow). The pore module (red) is tethered to an RCK domain (cyan). A second, identical RCK domain (yellow) associates tightly with the tethered domain. These paired RCK domains from the four subunits form a gating ring, as shown in B. (B) Crystal structure of the gating ring from the MthK channel (PDB accession no. 3RBZ), shown as a bird's-eye view from above the membrane, with the transmembrane pore removed for clarity. Positions of Ca^{2+} ions are represented by green spheres. (C) Schematic diagram of a BK channel subunit. Each subunit contains a transmembrane voltage-sensing domain (S0–S4 helices, white), pore module (red), and two tandem RCK domains that make up the Ca^{2+} sensor (RCK1, magenta; RCK2, dark purple), with an architecture analogous to the one shown in A. (D) Crystal structure of the BK gating ring (PDB accession no. 3U6N) shown as a bird's-eye view (as in B). Positions of Ca^{2+} ions in the Ca^{2+} bowl site are represented by green spheres.

“turn” in each of the intertwined helix-turn-helix segments forms an “elbow,” although this has been observed to be a static elbow and not a flexing one among RCK domains observed in different conformations (Ye et al., 2006; Yuan et al., 2011; Smith et al., 2012, 2013). This arm-in-arm architecture is conserved among eukaryotic tandem RCK domains (Fig. 2 C).

RCK domains are known to bind a diverse range of biological ligands, including nicotinamide adenine dinucleotide, ATP, and metal cations such as Na^+ or Ca^{2+} , and to modulate transporter or channel activity (Schlösser et al., 1993; Jiang et al., 2002a; Kröning et al., 2007; Yuan et al., 2010; Kong et al., 2012; Levin and Zhou, 2014). The efforts of several laboratories have contributed to an increasingly detailed understanding of the conformational changes that lead to ligand-dependent activation of RCK domains and their energetic contribution to K^+ flux.

In this review, we focus on two exemplary ion channels, the prokaryotic MthK and eukaryotic BK channel, as excellent reviews of what RCK-regulated transporters

are available (Levin and Zhou, 2014). We first describe the conformational repertoire of RCK gating rings in bacterial MthK channels. They have constituted a key model to understand the structural basis of BK channel gating by Ca^{2+} and other cations, which is presented in the second part of the review. Additionally, we discuss the relevance of this knowledge in the context of BK channels as potential therapeutic targets.

Insights from a channel found in sludge

Initial insights toward the physical structure of the RCK gating apparatus came from the crystal structure of a prokaryotic K^+ channel, MthK, 15 years ago. The MthK channel was cloned from *Methanobacterium thermoautotrophicum*, an anaerobe that thrives in raw sewage at temperatures ranging from 40 to 70°C (Smith et al., 1997). Despite its humble origins, the MthK channel bears sequence similarity to the eukaryotic BK channel (Jiang et al., 2002a). Owing to this similarity, crystal structures from the MthK channel initially served as important guides for understanding the relation between

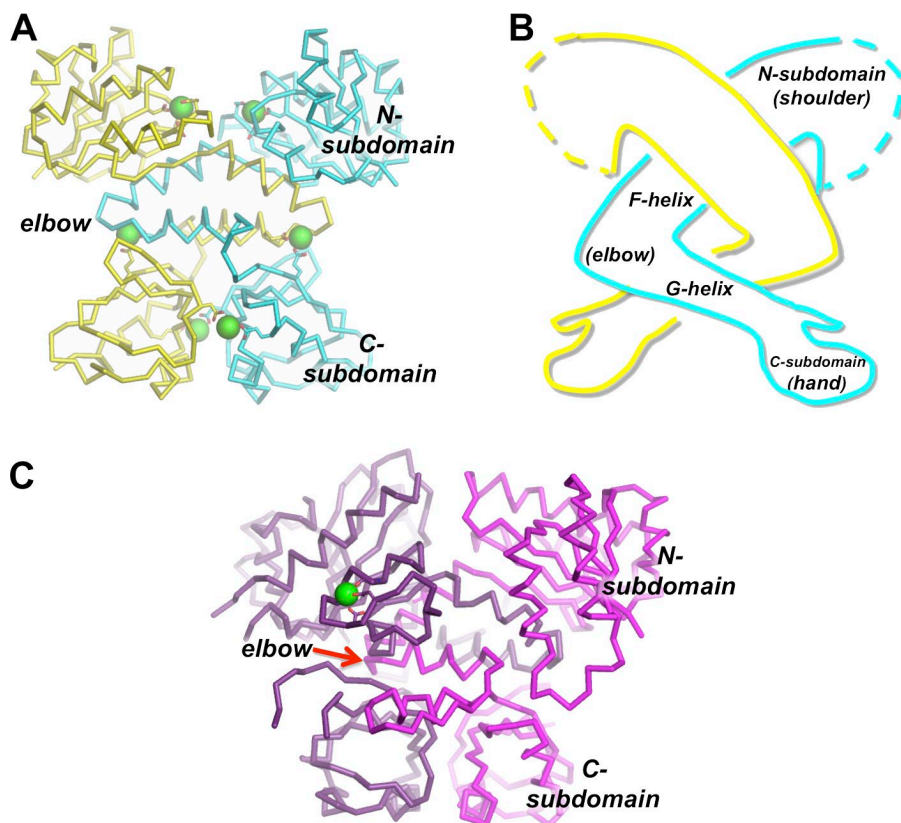


Figure 2. Structures of prokaryotic and eukaryotic RCK domains. (A) Crystal structure of an RCK domain homodimer from the MthK channel (PDB accession no. 4L73). Positions of Ca^{2+} ions are represented by enlarged green spheres. (B) Cartoon illustrating the arm-in-arm architecture of the RCK dimer with subdomain anatomy as indicated. (C) Crystal structure of the tandem RCK pseudodimer from the BK channel (PDB accession no. 3U6N). The position of the Ca^{2+} ion in the Ca^{2+} bowl site is represented by a green sphere.

RCK domain activation and gating of BK channels and continue to provide new insight toward mechanisms of channel activation (Jiang et al., 2002a,b; Bao et al., 2004; Yang et al., 2007, 2008; Hou et al., 2008; Pau et al., 2011; Smith et al., 2012, 2013; Liu et al., 2013).

Each of the MthK channel's four primary subunits contains pore-lining helices that are tethered to a pair of RCK domains at the cytoplasmic side of the channel, as illustrated in Fig. 1 A (Jiang et al., 2002a). Using single-channel recordings, several laboratories contributed to development of an allosteric gating scheme that accounts for the major features of both Ca^{2+} -dependent activation of the channel and inhibition of the channel by protonation (Zadek and Nimigean, 2006; Pau et al., 2010). Through this work and complementary structural studies by x-ray crystallography, it was deduced that the RCK domains bind at least eight Ca^{2+} ions to stabilize the open/conducting state of the channel's transmembrane pore, whereas binding of at least eight protons contributes to inhibition of Ca^{2+} -dependent gating, consistent with the idea that each of the eight RCK domains in a gating ring contributes one Ca^{2+} site and one H^+ site that control gating (Dong et al., 2005; Ye et al., 2006; Pau et al., 2010; Smith et al., 2012, 2013).

Whereas initial structural studies identified a single Ca^{2+} -binding site within the N-terminal subdomain, determined by acidic side chains of aspartate and glutamate residues (D184, E210, and E212), two experimental observations suggested that this initial view was too sim-

ple. First, MthK channels consistently display a very steep relation between open probability (P_o) and $[\text{Ca}^{2+}]$, with Hill coefficients of nine or greater (Zadek and Nimigean, 2006; Pau et al., 2010); this relation is much steeper than one predicted from a model with only eight Ca^{2+} -binding sites per channel. Second, mutation of the key Ca^{2+} -binding residues D184 and E210 reduced, but did not eliminate, Ca^{2+} -dependent activation of MthK, suggesting that there must be additional Ca^{2+} -binding sites (Pau et al., 2011).

It was later discovered that each MthK RCK domain contributed two additional Ca^{2+} -binding sites, for a total of three sites per RCK domain, each of which contributes energetically to activation of the channel (Pau et al., 2011; Smith et al., 2012, 2013). Each binding site was identified from a combination of electrophysiological and crystallographic data such that Ca^{2+} -dependent activation of the channel can be eliminated only with combined mutations at all three binding sites.

Further analysis in which the MthK RCK domain was crystallized with one, two, or all three of the Ca^{2+} -binding sites occupied yielded further insight toward the contributions of individual sites to stabilization of domain conformations, as well as allosteric coupling between the sites (Smith et al., 2012, 2013). This work demonstrated that conformational changes at both N-terminal and C-terminal subdomains contribute to RCK domain activation and subsequent channel opening and that these different Ca^{2+} -bind-

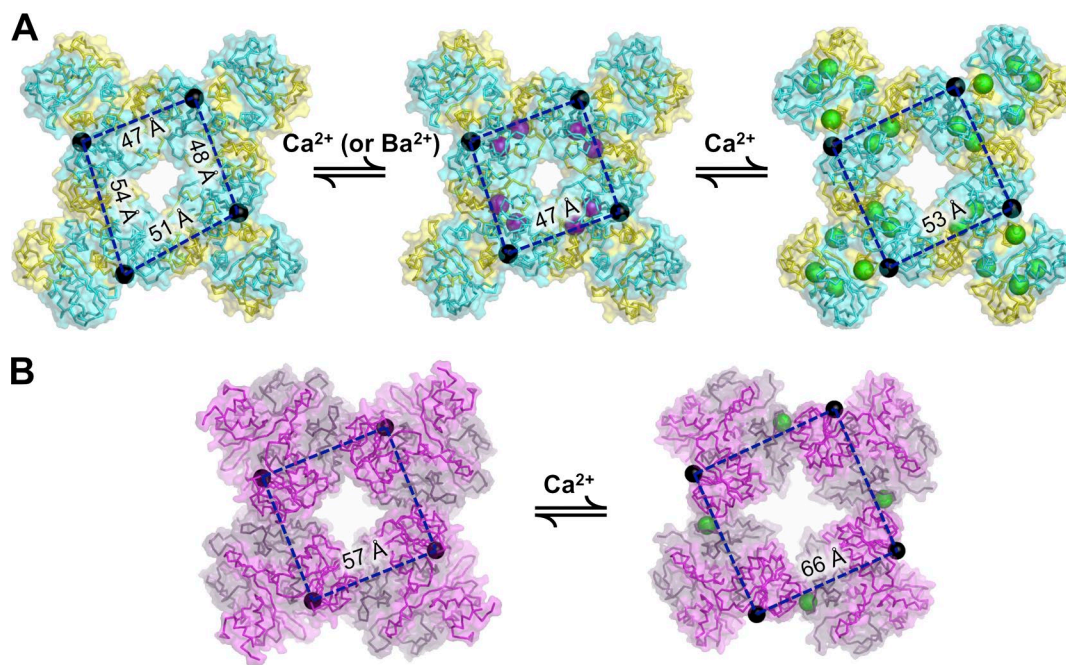


Figure 3. **Ca²⁺-dependent conformations of MthK and BK gating rings.** (A) Conformations of the MthK gating ring in (left to right) unliganded, singly liganded, and fully liganded states. N termini, which are tethered to pore-lining helices of the channel, are represented by black spheres; purple spheres represent Ba²⁺ ions in the singly liganded ring, and green spheres represent Ca²⁺ ions. RCK dimers comprising the unliganded gating ring can assume two different conformations, thus breaking fourfold symmetry. The singly liganded (Ba²⁺ bound) state stabilizes a single, symmetric conformation that facilitates channel opening, and opening is further facilitated by additional liganding. In the fully liganded state, distances between all N termini are uniformly greater than in the unliganded or singly liganded states, consistent with movements that could open the transmembrane pore. (B) Conformations of the BK gating ring in (left to right) the unliganded and Ca²⁺-bound states, in which Ca²⁺ (green spheres) is bound at the Ca²⁺ bowl. As with the MthK gating ring, binding of Ca²⁺ increases distances between the RCK domain N termini, which in turn underlies channel opening.

ing sites can interact energetically to affect channel gating. Interestingly, in the absence of ligand, the MthK gating ring can contain RCK dimers in multiple conformations, yielding gating rings that do not exhibit fourfold symmetry (Fig. 3 A; Ye et al., 2006). Binding of the divalent cations Ca²⁺ or Ba²⁺ within the N-terminal subdomain appears to affect interactions between neighboring RCK dimers in a gating ring to stabilize a fourfold symmetric conformation and facilitate channel opening (Smith et al., 2012). Binding of Ca²⁺ ions at additional sites in the C-terminal subdomain result in formation of intersubunit Ca²⁺ bridges between RCK domains within each dimer, resulting in a conformational change and further facilitation of channel opening (Dong et al., 2005; Ye et al., 2006; Pau et al., 2011; Smith et al., 2012, 2013).

Together, these structural analyses are consistent with the idea that ligand binding can stabilize activated conformations of the gating ring that in turn can stabilize the open pore, whereas in the absence of ligand, these activated conformations are much less stable. Similar interactions among multiple ligands may be at play in other RCK domain-containing K⁺ channels, and it will be important to further understand their mechanisms

in both functional and structural terms (Qian et al., 2006; Sweet and Cox, 2008; Kong et al., 2012).

The eukaryotic BK channel

Similar to the MthK channel, an intracellular gating ring formed by eight RCK domains is present in the eukaryotic BK channel (Fig. 1 D). However, whereas in MthK (and other prokaryotes), the RCK domains forming the gating ring are identical to one another, each BK channel subunit contains two nonidentical RCK domains (namely RCK1 and RCK2) that are linked in tandem (Fig. 1 C; Yuan et al., 2010). Thus, in the BK channel, four RCK1–RCK2 tandems form an intracellular gating ring that is nonetheless remarkably similar in overall structure to the gating ring of MthK (Fig. 1). In addition, BK channel gating is regulated by voltage, which is sensed by charged amino acids in transmembrane segments S2, S3, and S4 (Stefani et al., 1997; Díaz et al., 1998; Horrigan and Aldrich, 1999; Horrigan et al., 1999; Ma et al., 2006; Lee and Cui, 2010; Pantazis et al., 2010). The mechanism by which BK functionally integrates these two stimuli has been described by allosteric models (McManus and Magleby, 1991; Cox et al., 1997; Rothberg and Magleby, 1998, 1999; Horrigan and Aldrich,

2002). The common feature of these models is that the individual structural modules sensing Ca^{2+} (gating ring) and voltage (transmembrane voltage-sensing domain) undergo structural rearrangements between resting and activated conformations, leading to channel opening. Either the Ca^{2+} or voltage sensors can open the channel independently as well as synergistically as a result of allosteric interactions between them (Horrigan and Aldrich, 2002; Latorre et al., 2017). The ability of BK channels to be regulated by Ca^{2+} and voltage turns these channels into essential physiological couplers of Ca^{2+} and membrane voltage signaling, providing a negative feedback mechanism controlling Ca^{2+} influx to the cell. Consequently, BK channels are key regulators of neuronal action potential firing, neurotransmitter release, or smooth muscle contractile tone. Inherited defects in BK channel function lead to disease, including high blood pressure, seizure and epilepsy, or urinary incontinence (Latorre et al., 2017). The gating ring constitutes an essential structure involved in the coupling between Ca^{2+} ligation and channel activation.

Structures of gating rings isolated from eukaryotic BK channels were first solved in unliganded and Ca^{2+} -bound forms, corresponding to two gating ring conformations (Fig. 3 B; Wu et al., 2010; Yuan et al., 2011). Remarkably, Ca^{2+} binding to the BK channel underlies a similar “expansion” of the gating ring seen in activation of the MthK gating ring (Fig. 3), although this is achieved by ligand binding at completely different loci. Specifically, the BK channel gating ring contains a key Ca^{2+} -binding site (the “ Ca^{2+} bowl”) at the interface between adjacent RCK dimers (discussed below); thus, Ca^{2+} binding at this site has a fundamentally distinct impact on BK gating ring structure from that seen in the MthK gating ring. In addition, the BK gating ring has additional Ca^{2+} -binding sites aside from the Ca^{2+} bowl (Schreiber and Salkoff, 1997; Bao et al., 2002; Xia et al., 2002; Sweet and Cox, 2008; Zhang et al., 2010). The structural impact of these sites has been recently revealed in the published structure of the full-length BK channel from *Aplysia californica* (Hite et al., 2017; Tao et al., 2017) as described below. This review compares RCK domains in three different organisms: mouse (m), *A. californica* (a), and human (h), which often have different residue numbers for the same functional amino acids or for amino acids in the same key structural locations, so the species will be indicated for every residue mentioned.

Role of the Ca^{2+} bowl

The high calcium sensitivity of BK channels has been attributed to a binding site known as the Ca^{2+} bowl, which contains a stretch of aspartate residues within RCK2 (Wei et al., 1994; Schreiber and Salkoff, 1997; Schreiber et al., 1999). Two of these aspartates (hD895 and hD897 and mD898 and mD900; Table 1) are each

critical for both Ca^{2+} -dependent activation through this site as well as direct coordination of Ca^{2+} , whereas other aspartate residues in this segment appear to provide structural stability through interactions with surrounding side chains (Bao et al., 2004; Yuan et al., 2010). These interactions may depend on occupancy of the Ca^{2+} bowl; for example, the hD892 side chain (mD895) appears to form a hydrogen bond with the side chain of hQ907 when the bowl is unoccupied, whereas hD894 (mD897) appears to form salt bridges with hR1018 and hK1030 when Ca^{2+} is bound (Wu et al., 2010; Yuan et al., 2010). These localized Ca^{2+} -dependent changes at the Ca^{2+} bowl may contribute to Ca^{2+} -dependent structural rearrangements that underlie gating, as suggested by moderately reduced BK Ca^{2+} sensitivity in the mD895A and mD897A mutants (Bao et al., 2004). The structure of the complete Slo1 channel from *A. californica*, in which the Ca^{2+} ion is coordinated by residues aD905, aD907, aQ899, and aD902 of the RCK2 subunit, is consistent with previous work performed with channels from higher organisms (Hite et al., 2017; Tao et al., 2017).

The Ca^{2+} bowl is located very close to the intersubunit interface in the gating ring structure, in a different location than that of any of the Ca^{2+} -binding sites of MthK. In the context of the gating ring, the Ca^{2+} ion within the Ca^{2+} bowl appears to be additionally coordinated by the side chain of hN449 in the RCK1 region of the adjacent subunit (aN438; Yuan et al., 2011; Hite et al., 2017; Tao et al., 2017). Based on the observation that the overall Ca^{2+} -dependent conformational change in the gating ring involves relative movements of the subunits at the RCK1– Ca^{2+} bowl interface, the nexus formed by the hN449 side chain may be an important component of the gating machinery (Hite et al., 2017). Consistent with this idea, mutations at hN449 diminish Ca^{2+} -dependent intersubunit interactions and Ca^{2+} sensitivity of the channel (Vouga et al., 2016). It will be important to determine the energetic contributions of other components of this interface.

Other binding sites outside the Ca^{2+} bowl

Two additional independent sites have been proposed to bind Ca^{2+} within the RCK1 domain. In the isolated gating ring structures obtained in high Ca^{2+} concentrations, no electron density attributable to Ca^{2+} was found at either of these sites, and a model was proposed to accommodate a high-affinity binding site at the RCK1 domain (Zhang et al., 2010). In the full channel *A. californica* Slo1 structure, strong density is detected consistent with a Ca^{2+} ion coordinated by side chains contributed by aD356 and aE525 and main-chain oxygens from aR503, aG523, and aE591 (Tao et al., 2017). Among these, residues aD356, aE525, and aR503 (mD367, mE535, and mR514) are highly conserved among BK channels and were previously identified from rigorous

Table 1. Residues relevant to gating ring function reported in the literature

<i>Aplysia</i> Slo1	mBK	hBK	Localization/role	References and notes
D86 ^a	D99	D99	Mg ²⁺ -binding site/stabilization of binding site	Yang et al., 2008, 2013; Tao et al., 2017
N161 ^a	N172	N172	Mg ²⁺ -binding site/Mg ²⁺ coordination	Yang et al., 2008, 2013; Tao et al., 2017
G302 ^b	A313	A313	S6/pore function; bending residue of S6 in <i>Aplysia</i> Slo1 Ca ²⁺ -bound structure	Zhou et al., 2011; Chen et al., 2014; Tao et al., 2017
P309 ^a	P320	P320	S6/pore function; bending residue of S6 in <i>Aplysia</i> Slo1 EDTA structure	Zhou et al., 2011; Tao et al., 2017
D356 ^a	D367	D367	RCK1 Ca ²⁺ -binding site/Ca ²⁺ coordinating residue and gating	Xia et al., 2002; Tao et al., 2017
E363 ^a	E374	E374	Mg ²⁺ -binding site/Mg ²⁺ coordination	Shi et al., 2002; Yang et al., 2006, 2008; Tao et al., 2017
T385 ^b	T396	E396	Mg ²⁺ -binding site/Mg ²⁺ coordination	Tao et al., 2017; note that Yang et al. (2006) identified Q397 as a residue involved in Mg ²⁺ sensitivity
E388 ^a	E399	E399	Mg ²⁺ -binding site/Mg ²⁺ coordination	Shi et al., 2002; Xia et al., 2002; Yang et al., 2006, 2008; Tao et al., 2017
N438 ^a	N449	N449	Ca ²⁺ bowl/ Ca ²⁺ coordination, gating, intersubunit cooperativity	Yuan et al., 2011; Vouga et al., 2016; Tao et al., 2017; note that mutation of the neighboring residue K448 (K448A mutants) was studied in Xia et al. (2002)
R503 ^a	R514	R514	RCK1 Ca ²⁺ -binding site/Ca ²⁺ coordination, intrasubunit cooperativity	Tao et al., 2017; note that M513 had been related to RCK1 Ca ²⁺ -binding site function (Bao et al., 2002) but also see Zhang et al. (2010)
G523 ^b	S533	S533	RCK1 Ca ²⁺ -binding site/Ca ²⁺ coordination	Tao et al., 2017; note that S533 mutation did not reduce Ca ²⁺ sensitivity in the study by Zhang et al. (2010)
E525 ^a	E535	E535	RCK1 Ca ²⁺ -binding site/Ca ²⁺ coordination	Zhang et al., 2010; Tao et al., 2017
E591 ^b	S602	S602	RCK1 Ca ²⁺ -binding site/Ca ²⁺ coordination	Tao et al., 2017; note that mutations of this residue were not included in the study by Zhang et al. (2010)
Q899 ^a	Q892	Q889	Ca ²⁺ bowl/Ca ²⁺ coordination	Bao et al., 2002, 2004; Tao et al., 2017
D902 ^a	D895	D892	Ca ²⁺ bowl/Ca ²⁺ coordination	Bao et al., 2002, 2004; Tao et al., 2017
E912 ^a	E905	E902	Ca ²⁺ bowl/intrasubunit cooperativity	Bao et al., 2002, 2004; Tao et al., 2017
Y914 ^a	Y907	Y904	Ca ²⁺ bowl/intrasubunit cooperativity	Bao et al., 2002, 2004; Tao et al., 2017
D904–D908 ^a	D897–D901	D894–D898	Ca ²⁺ bowl/Ca ²⁺ coordination	Bao et al., 2002, 2004; Xia et al., 2002; Tao et al., 2017

Equivalent amino acid numbers are shown for *A. californica* (*Aplysia* Slo1), mouse (mBK), and human BK (hBK). Table rows are color coded according to residue location: S6 (red), Mg²⁺-binding site (orange), RCK1 Ca²⁺-binding site (gray), and Ca²⁺ bowl (blue). Sequence alignment was performed using the Clustal Omega tool from EMBL-EBI and the following protein sequences: *A. californica* high conductance calcium-activated potassium channel (GenBank accession no. AAR27959.1), *Mus musculus* mbr5 mslo (GenBank accession no. AAA39746.1), and *Homo sapiens* calcium-activated potassium channel subunit α -1 isoform b (GenBank accession no. NP_002238.2).

^aConserved residue.

^bNonconserved residue.

functional studies as being important for Ca²⁺ sensing (Xia et al., 2002; Zhang et al., 2010). Additionally, the *A. californica* Slo1 structure reveals that the conserved aR503 side chain forms key interactions with highly conserved residues in the Ca²⁺ bowl site (mE905 and mY907; Table 1), providing a structural basis for cooperative interactions between the two Ca²⁺ sites, as noted below (Rothberg and Magleby, 1998, 1999; Qian et al., 2006).

Another site at the RCK1 exhibits millimolar affinity for divalent cations and is thought to underlie activation of the channel by Mg²⁺ (Oberhauser et al., 1988). This site has been proposed to be formed at the interface between the gating ring and the transmembrane domains (mD99 and mN172 from the transmembrane region and mE374 and mE399 at the RCK1 region),

constituting an interacting site between the voltage-sensing domain and the gating ring (Yang et al., 2007, 2008; Zhang et al., 2010). Consistent with the functional studies, the structure of the full-length Slo1 channel from *A. californica* shows a density peak at the interface between the gating ring and the transmembrane domain, most likely corresponding to a Mg²⁺ ion coordinated by residues aE363, aE388, aT385, and aN161 and a water molecule. Interestingly, the residue equivalent to mD99 (aD86 in *A. californica* Slo1) seems not to participate in direct coordination of the divalent cation, although it may be involved in maintaining the site stability (Tao et al., 2017). Interestingly, Mg²⁺ has been also proposed to interact with the gating ring through the Ca²⁺ bowl site (Javaherian et al., 2011; Miranda et al., 2016).

Ca²⁺-binding sites in the gating ring are not equivalent. In addition to different binding affinities for Ca²⁺, the structural differences among these sites are made evident by differential interactions with other chemically distinct divalent cations. For example, Ba²⁺ selectively interacts with the Ca²⁺ bowl (Zeng et al., 2005), whereas Cd²⁺ appears to act through the RCK1 domain-binding site (Zeng et al., 2005; but see Zhang et al., 2010). Other divalent cations of smaller ionic radius, such as Mn²⁺, Co²⁺, Mg²⁺, and Ni²⁺, bind uniquely to the low-affinity binding RCK1 site (Zhou et al., 2012). Although these cations may not have physiological relevance to BK channel function, they are useful tools to assess the properties of different cation binding sites as well as their independent role in the conformational rearrangements of the gating ring (see below).

RCK movements in functional BK channels

How is Ca²⁺ binding mechanically transduced into pore opening? Comparison of isolated gating ring x-ray structures obtained in the absence and presence of high Ca²⁺ shows little change in the layer formed by the RCK2 domains, whereas that formed by the four RCK1 domains seems to be expanded by >10 Å (Yuan et al., 2011). Large conformational changes induced by Ca²⁺ have also been measured in isolated tetrameric gating rings in solution (Javaherian et al., 2011). Because this region of the gating ring is directly linked to the channel's pore-forming helices, this expansion could represent the direct link between Ca²⁺ binding and the opening of the pore in BK channels (Savalli et al., 2006; Yusifov et al., 2008, 2010; Lee and Cui, 2010). However, the physiological relevance of these crystal structures and biochemical studies was limited by the fact that only part of such a complex channel was studied. Cryo-electron microscopy structures of the full-length Slo1 channel from *A. californica* have been recently obtained both in an EDTA-treated, ligand-free conformation and in a Ca²⁺- and Mg²⁺-bound conformation, leading the authors to propose a structure-based mechanism of channel activation involving Ca²⁺ binding-induced tilting of the RCK1 N lobe regions (also known as the AC regions, βA-αC; Hite et al., 2017). Interestingly, this mechanism entails two pathways, one through direct pulling of the S6 helices via the RCK1-S6 polypeptide linkers and the other via noncovalent protein-protein interfaces between the gating ring and the transmembrane domains as well as the S4-S5 linker. This is an interesting finding because the existence of a covalent linkage between membrane domains and the RCK domains forming the gating ring had led to the idea that variations in the tension of such linkers may be the major mechanism transducing ligand-induced rearrangements of the gating ring into pore opening. Although some experimental work supported this notion (Niu et al., 2004), the lack of mechanical linkages be-

tween the gating ring and membrane domains in some bacterial transporters (i.e., KtrAB) suggested that other interactions between the RCK domains and the gating pore may be relevant for function (Lingle, 2007).

Conformational changes between subunits at the level of the gating ring during activation of functional BK channels have been studied experimentally using patch-clamp fluorometry on membrane patches containing fluorescently labeled BK channels (Giraldez et al., 2005; Miranda et al., 2013, 2016). Large changes in fluorescence resonance energy transfer (FRET) were observed upon Ca²⁺ binding and channel activation. Simultaneous FRET and electrophysiological recordings using two different locations of the fluorescent probes showed that the linkers between RCK1 and RCK2 domains (around hH667 sites) from adjacent subunits get closer upon Ca²⁺ binding, whereas the regions close to the Ca²⁺ bowl (hN860) are relatively moved apart. This movement is dependent on Ca²⁺ binding to the specific sites, as mutation of the Ca²⁺ bowl and/or the lower affinity Ca²⁺-binding sites gradually impairs state-dependent FRET changes. Surprisingly, the FRET changes detected by Miranda et al. (2013) do not directly track with open probability. The electrophysiological data were well described by the standard allosteric model (Sweet and Cox, 2008); however, this model could not describe the electrophysiological and fluorescence data simultaneously, even after adding a large number of modifications to it (Miranda et al., 2013). With the available data, the authors concluded that, to explain simultaneously the movement of the gating ring and the channel gating, the standard model would need to be extended, implying that the conformational change in the gating ring has a more complex relation to pore opening than previously thought (Miranda et al., 2013). These observations cannot be fully correlated with the recently available full-length structures, which have been obtained only in extreme conditions of low and high Ca²⁺ and Mg²⁺ (Hite et al., 2017; Tao et al., 2017). These structures provide the framework to design new experiments toward full understanding of the dynamic structural rearrangements occurring in the gating ring of intact BK channels.

Interaction of the gating ring with the voltage sensors

Fluorescence-based studies indicate that the structural rearrangements of the gating ring show voltage dependence, but not in all regions (Miranda et al., 2013, 2015, 2016). Specifically, Ca²⁺-dependent FRET signals from the RCK1-RCK2 linkers region (hH667) have voltage dependence, whereas those of hN860 sites at the RCK2 do not. These differences in voltage dependence may reflect distinct interactions of different regions of the gating ring with the voltage-sensing domain during channel activation (Yang et al., 2007, 2008; Sweet and Cox, 2008; Savalli et al., 2012; Miranda

et al., 2015). Consistently, with this hypothesis, the structure from *A. californica* Slo1 shows a large specific protein–protein interface between the gating ring and the transmembrane region containing the voltage sensors and S4–S5 linkers connecting the sensors to the pore. Together with other structural observations, this has led Hite et al. (2017) to highlight the relevance of this interface in the channel activation mechanism by Ca^{2+} . The structures suggest that Ca^{2+} -induced rearrangements of the RCK1 N lobe directly produce a displacement of the voltage sensors away from the pore axis, which in turn induces an equivalent displacement of the S5 helices near the plasma surface, favoring pore opening (Hite et al., 2017).

Cooperativity between Ca^{2+} -binding sites

Functional studies have demonstrated the existence of cooperative interactions between the Ca^{2+} -binding sites (Rothberg and Magleby, 1999; Qian et al., 2006; Sweet and Cox, 2008; Savalli et al., 2012). The cryo–electron microscopy structure of the open *A. californica* Slo1 channel shows that the Ca^{2+} ion within the Ca^{2+} bowl is additionally coordinated by the side chain of aN438 (m449; Table 1) from the RCK1 N lobe region of the adjacent subunit. At the RCK1 site, the Ca^{2+} ion coordination is made complete by interaction with the aD356 from the RCK1 N lobe. Thus, the tilting on the RCK1 N lobe would simultaneously complete coordination of the cations at both the RCK1 site and the Ca^{2+} bowl, providing a structural basis for Ca^{2+} -binding cooperativity. Additionally, intrasubunit cooperativity may also arise from the observed interaction of the aR503 residue from the RCK1 site with amino acids aE912 and aY914 from the Ca^{2+} bowl within the same subunit (Tao et al., 2017).

By taking advantage of the specific effects of different divalent cations, fluorescence studies have recently shown that activation of single high-affinity binding sites (either the Ca^{2+} bowl or the RCK1 site) by cations other than Ca^{2+} (i.e., Mg^{2+} , Cd^{2+} , and Ba^{2+}) evoked significantly smaller conformational changes than those observed when both sites are occupied with Ca^{2+} (Miranda et al., 2016). This result indicates that both RCK domains can move independently when their specific binding sites are occupied by cations. Simultaneous occupation of both RCK1 and RCK2 sites by distinct cations is additive, emulating the effect of fully occupied Ca^{2+} -binding sites (Miranda et al., 2016). It is tempting to speculate that binding of cations different from Ca^{2+} may not attain a complete coordination in the specific sites, thus yielding different levels of cooperativity between sites or no cooperativity at all.

Other players controlling the gating ring

The tissue-specific functional diversity of BK channels arises in part from the association of the pore-forming α

subunits with any of four β and four γ regulatory subunits (Brenner et al., 2000a,b, 2005; Rothberg, 2012; Yan and Aldrich, 2012; Gonzalez-Perez et al., 2015), as well as the potential for multiple RCK domain structures and gating phenotypes through alternative splicing (Tseng-Crank et al., 1994; Glauser et al., 2011; Johnson et al., 2011; Shelley et al., 2013) or posttranslational modifications, such as palmytoylation (Shipston and Tian, 2016). Additionally, another regulatory mechanism may occur in macrocomplexes formed by BK and other ion channels, such as voltage-gated Ca^{2+} channels (Berkefeld and Fakler, 2013; Singh et al., 2016; Latorre et al., 2017). Much remains to be known about the molecular mechanisms underlying the effect of regulatory subunits on BK channel gating, some of which may include a direct interaction with the gating ring (Qian et al., 2002; Lee et al., 2010).

Conclusions and future perspectives

Our understanding of the structural basis of gating by RCK domains is rapidly evolving to the point where we can begin to tease apart the roles of chemical interactions between amino acids within the domains with great precision. We now have access to gating ring structures in the context of full-length channels, bringing the research field to a new knowledge level and setting the stage for new functional studies, having supported or confirmed much of the electrophysiological work performed over the past 30 years.

Untangling gating ring function may provide a starting point for computational drug design studies or genetic approaches to understand and treat BK channelopathies including epilepsy, ischemic heart disease, pulmonary disease, erectile dysfunction, and bladder instability. The search for drugs that activate BK channels has constituted an important goal for many years, although with only moderate clinical success. This could be attributed to many potential factors, such as low selectivity of compounds in vivo, because of the ubiquitous nature and functional diversity of BK channels (Bentzen et al., 2014). It will thus become important to study gating ring function in conjunction with regulatory subunits and other important players to better understand the impacts of these components on channel function and pharmacology.

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