Metal-driven Operation of the Human Large-conductance Voltage- and Ca\(^{2+}\)-dependent Potassium Channel (BK) Gating Ring Apparatus\(^*\)\(^{[S]}\)*

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Large-conductance voltage- and Ca\(^{2+}\)-dependent K\(^{+}\) (BK, also known as MaxiK) channels are homo-tetrameric proteins with a broad expression pattern that potently regulate cellular excitability and Ca\(^{2+}\) homeostasis. Their activation results from the complex synergy between the transmembrane voltage sensors and a large (\(>300\) kDa) C-terminal, cytoplasmic complex (the “gating ring”), which confers sensitivity to intracellular Ca\(^{2+}\) and other ligands. However, the molecular and biophysical operation of the gating ring remains unclear. We have used spectroscopic and particle-scale optical approaches to probe the metal-sensing properties of the human BK gating ring under physiologically relevant conditions. This functional molecular sensor undergoes Ca\(^{2+}\)- and Mg\(^{2+}\)-dependent conformational changes at physiologically relevant concentrations, detected by time-resolved and steady-state fluorescence spectroscopy. The lack of detectable Ba\(^{2+}\)-evoked structural changes defined the metal selectivity of the gating ring. Neutralization of a high-affinity Ca\(^{2+}\)-binding site (the “calcium bowl”) reduced the Ca\(^{2+}\) and abolished the Mg\(^{2+}\) dependence of structural rearrangements. In congruence with electrophysiological investigations, these findings provide biochemical evidence that the gating ring possesses an additional high-affinity Ca\(^{2+}\)-binding site and that Mg\(^{2+}\) can bind to the calcium bowl with less affinity than Ca\(^{2+}\). Dynamic light scattering analysis revealed a reversible Ca\(^{2+}\)-dependent decrease of the hydrodynamic radius of the gating ring, consistent with a more compact overall shape. These structural changes, resolved under physiologically relevant conditions, likely represent the molecular transitions that initiate the ligand-induced activation of the human BK channel.

The large-conductance voltage- and Ca\(^{2+}\) -activated K\(^{+}\) channel (BK, MaxiK, Slo1) is an important regulator of cellular function including cellular excitability, neurotransmitter release, vascular tone, and hair cell tuning (1–8). Its K\(^{+}\) conductance of \(~250\) picosiemens is an order of magnitude larger than that observed in typical voltage-gated K\(^{+}\) selective channels (9), making the BK channel a powerful regulator of the cell membrane potential. A functional BK channel is composed of four identical \(\alpha\) subunits, each consisting of a short extracellular N-terminal tail, seven transmembrane segments (S0–S6) (10, 11), and a large intracellular C-terminal domain. The four \(\alpha\) subunits likely assemble around a central symmetry axis, forming the K\(^{+}\)-selective pore, surrounded by peripheral transmembrane voltage-sensing domains (12, 13) that undergo conformational rearrangements upon membrane depolarization (14–16) to facilitate pore opening (17–20). Another pathway to BK channel activation is mediated by intracellular Ca\(^{2+}\). In the CNS, BK channels localize within Ca\(^{2+}\) nanodomains generated at the immediate proximity of voltage-gated Ca\(^{2+}\) channels as an efficient strategy to regulate BK channel activity (reviewed by Ref. 21). Intracellular Ca\(^{2+}\) sensitivity is conferred by a large “gating ring” tetrameric superstructure, which is composed of two tandem C-terminal regulators of K\(^{+}\) conductance (RCK1 and RCK2) domains from each of the four channel \(\alpha\) subunits (22–24). The gating ring apparatus regulates BK channel activation by micromolar Ca\(^{2+}\) and millimolar Mg\(^{2+}\) (4, 7, 8, 25) as it is also expected to undergo ligand-driven conformational rearrangements that in turn favor channel opening (26–28). A region containing five consecutive aspartates (Asp\(^{894}\)–Asp\(^{898}\)) termed the “calcium bowl” (29) constitutes a high-affinity Ca\(^{2+}\)-binding site (24, 30–33) located within the RCK2 domain (23, 24, 33). Consistent with evidence that the neutralization of the calcium bowl region reduces, but does not abolish, Ca\(^{2+}\) sensitivity, a second high-affinity Ca\(^{2+}\)-sensing region is thought to exist within the RCK1 domain, where residues aspartate 367, methionine 513, arginine 514, and glutamate 535 have a critical role in the Ca\(^{2+}\) sensitivity of the channel (34–38). The human BK channel mutation D434G (in RCK1), thought to pathologically enhance the Ca\(^{2+}\)-sensing

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4 The abbreviations used are: BK, large-conductance voltage- and Ca\(^{2+}\)-activated K\(^{+}\) (Slo1) channels; CTD, C-terminal domain; DLS, dynamic light scattering; RCK, regulators of K\(^{+}\) conductance; \(\lambda_{\text{exc}}\), excitation wavelength; \(\lambda_{\text{em}}\), emission wavelength.
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mechanism of the channel (39), has been linked to generalized epilepsy and paroxysmal dyskinesia (40). Low-affinity Mg supplementary sensing depends on coordinating residues within RCK1 (glutamines 374/399) (34, 41) and the transmembrane voltage sensor domain (aspartate 99, asparagine 172) (4, 7, 42, 43). The gating ring is thought to be the site of action of different cytosolic interacting partners (2, 44) as well as small signaling molecules such as reactive oxygen species, CO, hemin, and HNO (4, 5, 45, 46).

BK channels have been visualized at 17–20 Å resolution (22) and, more recently, atomic structures (3 Å resolution) of the human BK gating ring have been reported (23, 24). Thus, although the BK gating ring has a well-defined structural identity, the functional properties linked to its ligand-sensing operation have not yet been investigated. We have probed the structural and functional properties of the recombinant human BK channel C terminus under physiologically relevant conditions, providing evidence that RCK1-RCK2 complexes assemble into a tetrameric gating ring structure that undergoes metal-dependent conformational rearrangements. The resolved molecular events are likely associated with the ligand-induced signal propagation that activates the channel pore. The gating ring superstructure appears to possess the structural and functional properties of a chemo-mechanical coupler able to transduce the free energy of ligand binding into mechanical work.

EXPERIMENTAL PROCEDURES

Expression and Purification—Expression and mutagenesis corresponding to the wild-type and calcium channel-neutralized (D894A,D895A,D896A,D897A,D898A) human BK C-terminal domain (322ILE-ALK1005) were performed as described previously (33, 47). Briefly, protein fractions were solubilized in 20 mM Tris-HCl and 8 M urea (pH 8.0). The supernatant obtained after centrifugation was applied to a nickel-nitrilotriacetic acid affinity column, and the protein fractions were eluted with (in mM) 250 imidazole and dialyzed against (in mM) 25 MOPS, 2 EGTA, 120 KCl, pH 7.2. Free ion concentrations were estimated using WEBMAXC or measured as described previously (47).

Size-exclusion Chromatography—Size-exclusion chromatography experiments were performed as described previously (47). Purified BK gating ring in (in mm) 50 Tris-HCl, 2 β-mercaptoethanol, and 2 EGTA (pH 8.4) or (in mm) 20 MOPS (pH 7.2) was loaded onto a Superdex 200 10/300 column equilibrated with the same buffer with a flow rate of 0.5 ml/min. Calibration was performed using protein standards (Sigma).

Time-resolved Trp Fluorescence Spectroscopy—The time-resolved fluorescence lifetime of intrinsic Trp was measured with a Fluorolog-3 spectrofluorometer (Horiba Jobin Yvon) using time-correlated single photon counting (λex = 296 nm, λem = 340 nm) in (in mm) 25 MOPS, 120 KCl, 2 EGTA, pH 7.2. Free ion concentrations were estimated using WEBMAXC or measured as described previously (47).

The instrument response function was acquired using a non-fluorescent, light scattering sample (Ludox, colloidal silica). The instrument response function is a function of the detector characteristics and timing electronics and demonstrates the speed of the instrument and its detection properties. It is the shortest time profile that can be recorded by the instrument (49).

The fluorescence intensity decay data were fit to a sum of three exponential functions, using the DAS6 v6.4 software (Horiba Jobin Yvon)

\[
l(t) = \sum_{j=1}^{n} \alpha_j \exp(-t/\tau_j) \quad (\text{Eq. 1})
\]

where \(l\) is the fluorescence intensity and \(\alpha\) and \(\tau\) are the normalized pre-exponential factor and decay time constant, respectively. The average fluorescence lifetimes (\(\tau_{avg}\)) for three-exponential iterative fittings are calculated from the decay times and pre-exponential factors using the equation

\[
\tau_{avg} = \sum_{i=1}^{n} f_i \tau_i \quad (\text{Eq. 2})
\]

where \(f_i\) is the fractional contribution of each decay time to the steady-state intensity, which is given by
The goodness of the fit was determined from its $\chi^2$ value and the variance of the weighted residual distribution ($n = 3$, unless otherwise noted).

**Steady-state Fluorescence Spectroscopy**—Steady-state Trp fluorescence emission spectra of the gating ring in (in mM) 25 MOPS, 120 KCl, 2 EGTA, pH 7.2, were acquired in the 310–400 nm range, $\lambda_{ex} = 295$ nm (5 nm excitation/emission slit width). Normalized fluorescence data were fit with a Hill function in the form

$$F = (F_{\text{max}} - F_{\text{min}})/(1 + ([M^2+] / K_1)^n) + F_{\text{min}}$$

where $F$ is the fluorescence intensity at 340 or 350 nm for WT and calcium bowl mutant gating ring, respectively; $[M^2+]$ is the concentration of $Ca^{2+}$ or $Mg^{2+}$; $K_1$ is the apparent dissociation constant; and $n$ is the Hill coefficient.

**Dynamic Light Scattering (DLS)**—DLS measurements were recorded using a NICOMP 380 submicron particle sizing system. The hydrodynamic properties of gating ring particles were determined by measuring the translational diffusion coefficient $D_T$, which is related to the frictional coefficient $f$ by the Einstein-Sutherland equation

$$D_T = K_B T / f (cm^2/s)$$

where $K_B$ is the Boltzmann constant and $T$ is the temperature in K. The frictional coefficient of a spherical particle, $f_{\text{sph}}$, is a function of the fluid viscosity, $\eta$, and the radius of the particle, $r_{\text{sph}}$. It is defined by the Stokes law.

$$f_{\text{sph}} = 6 \pi \eta r_{\text{sph}}$$

Mean particle size distribution was analyzed in volume-weighted Nicomp-fit mode. The theoretical hydrodynamic radius ($R_{\text{theo}}^H$) was calculated from the formula

$$R_{\text{theo}}^H = [(3M(V_s + h))/4\pi N_A]^{1/3}$$

where $M$ is the molecular mass of the gating ring (312,000), $V_s$ is the particle specific volume (0.73 cm$^3$ g$^{-1}$), $h$ is the hydration (0.35 g of H$_2$O (g-protein)$^{-1}$), and $N_A$ is Avogadro’s constant (50).

The Perrin, or shape factor, is the ratio of the measured frictional coefficient $f$ to the frictional coefficient $f_{\text{theo}}$ of a hypothetical sphere, where

$$f_i = \alpha_i\tau_i / \sum \alpha_i \tau_i \quad \text{(Eq. 3)}$$

The polydispersity ($P_d$) was evaluated according to the following equation

$$P_d = \sigma / D_H \cdot 100\%$$

where $\sigma$ is the standard deviation of the distribution and $D_H$ is the mean hydrodynamic diameter from DLS measurements.

**RESULTS**

**Structural Organization of the BK Gating Ring in Solution**—The intracellular C-terminal domain (CTD, 684 amino acids) of the BK channel, which accounts for $>60\%$ of the whole channel and includes the two ligand-sensing modules RCK1 and RCK2 (supplemental Fig. S1), was expressed and purified. The CTD electrophoretic mobility in SDS-PAGE corresponds to an estimated $M_r = 72,000$ (Fig. 1B), close to the theoretical molecular weight of the purified protein (78,000). The identity of the purified BK CTD was confirmed by mass spectrometry, which identified 67% of the primary sequence (supplemental Fig. S1). The secondary and quaternary structures of the protein were investigated under physiologically relevant ionic conditions similar to the intracellular environment to ascertain its proper folding and native assembly, information necessary for the validity of the results of subsequent functional assays.

CD spectra were acquired to probe the secondary structure composition of the CTD in solution (Fig. 1C) and were subsequently analyzed using algorithms of the CDP software (48). The experimental (black) and calculated (red, CONTINLL algorithm) spectra are shown superimposed. The CD spectral analysis revealed a CTD secondary structure composition of $\sim 29\%$ $\alpha$-helix, $\sim 22\%$ $\beta$-strand, organized in 22 $\alpha$-helices and 30 $\beta$-strands, and $\sim 49\%$ turn and unordered structure (supplemental Fig. S2A). Although a direct comparison of secondary structure composition across a crystal structure study is not straightforward because of differences in the regions purified and/or resolved, reasonable correlation exists (supplemental Fig. S2).

The oligomeric state of the BK CTD was assessed by size-exclusion chromatography. A characteristic column elution and corresponding calibration curve is shown in Fig. 2, $A$ and $B$. 

![Amino acid sequence, purification, and secondary structure analysis of the recombinant BK CTD.](image)
represents the average fluorescence lifetime ($\tau_{avg}$) of the gating ring, which was 2.6 ± 0.010 ns. Increasing free Ca$^{2+}$ accelerated the decay of fluorescence intensity, as shown by the progressive reduction of $\tau_{avg}$ to a limiting value of 1.6 ± 0.0027 ns at 35 μM free Ca$^{2+}$ (Fig. 3A and supplemental Table S1), indicating a change within the immediate environment of one or more Trp residues.

We found that the Trp fluorescence lifetime of the gating ring was also sensitive to Mg$^{2+}$ (Fig. 3B) at concentrations in the millimolar range. As [Mg$^{2+}$] was incrementally raised from 0 to 12 mM, $\tau_{avg}$ progressively decreased to the same limiting value obtained for Ca$^{2+}$ (1.6 ± 0.015 ns; supplemental Tables S1 and S2). Thus, the binding of both Ca$^{2+}$ and Mg$^{2+}$ to the gating ring induced structural transitions at physiologically relevant concentrations, revealing the divergent metal cation-sensing properties of the isolated gating ring apparatus in solution.

The Structural Rearrangements of the Gating Ring Are Ion-specific—The following experiments were designed to estimate the apparent binding constants and assess the specificity of the BK gating ring for divalent cations, based on steady-state fluorescence spectroscopy of intrinsic Trp. The intensity of the fluorescence emission spectra of the gating ring ($\lambda_{ex} = 295$ nm) was progressively reduced upon free [Ca$^{2+}$] elevation in the buffer solution up to 35 μM, as shown in Fig. 4A. The [Ca$^{2+}$]-dependent quenching of the fluorescence intensity at 340 nm was well described by the linear combination of two Hill functions with apparent binding constants $K_{1/2} = 0.29 ± 0.0043 \mu M$ ($n_1 = 3.4 ± 0.16$) and $K_{2/2} = 3.5 ± 0.51 \mu M$ ($n_2 = 2.1 ± 0.35$) (Fig. 4D), suggesting at least two Ca$^{2+}$-binding sites and positive cooperativity. This apparent Ca$^{2+}$ affinity is relevant to BK channel activation (4, 54). $K_{1/2}$, which relates to Ca$^{2+}$ binding to the RCK2 domain at the calcium bowl site, whereas the higher $K_{2/2}$ value likely reflects Ca$^{2+}$ binding to the RCK1 domain, which has a lower apparent affinity in solution (33, 47), also inferred from electrophysiological investigations (34–37).

The apparent Mg$^{2+}$ affinity, $K_{1/2} = 154 ± 20.7 \mu M$ ($n = 2.02 ± 0.44$) was estimated by fitting to a Hill function the fluorescence intensity at 340 nm acquired at different [Mg$^{2+}$] (Fig. 4, B and D). Saturating [Mg$^{2+}$] produced only an overall ~15% decrease in fluorescence intensity as compared with a 30% reduction observed with saturating [Ca$^{2+}$].

Previous investigations have shown that Ba$^{2+}$ cannot activate reconstituted BK channels (55, 56); however, excluding Ba$^{2+}$-dependent BK activation is not trivial due to the blocking action of Ba$^{2+}$ at micromolar concentrations (56) on the internal side of the BK channel selectivity filter (57, 58). We probed whether Ba$^{2+}$ can bind and induce conformational changes to the isolated BK gating ring by measuring intrinsic Trp fluorescence while progressively increasing [Ba$^{2+}$]. The addition of Ba$^{2+}$ up to 13 mM did not alter the Trp fluorescence intensity of
the gating ring (Fig. 4, C and D). Nevertheless, when Ca\(^2+\) was added at the end of the Ba\(^{2+}\) experiment, the Trp fluorescence was quenched, confirming that protein activity was intact (Fig. 4C, red trace). Thus, the isolated gating ring exhibits cationic ligand selectivity, undergoing structural changes upon Ca\(^2+\) and Mg\(^{2+}\) binding with different apparent affinity, whereas not apparently responding to Ba\(^{2+}\).

Effect of Calcium Bowl Neutralization on Divalent Cation Sensitivity—BK channels possess a high-affinity Ca\(^{2+}\)-binding site consisting of five consecutive aspartate residues (Asp\(^{693}\)–Asp\(^{698}\)) called the calcium bowl (29). To investigate the role of the calcium bowl in the sensitivity of the isolated gating ring for cations, we expressed and purified a gating ring carrying the calcium bowl-neutralizing mutations D894A,D895A,D896A,Asp898) called the calcium bowl (29). To investigate the role of

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FIGURE 4. The divalent cation selectivity of the purified BK gating ring. A, normalized representative steady-state emission spectra of the gating ring in solution when excited at 295 nm, acquired under increasing [Ca\(^{2+}\)]. Note the dose-dependent quenching effect. B, as in A, except for increasing [Mg\(^{2+}\)] in a nominally Ca\(^{2+}\)-free buffer. C, as in B, except for increasing [Ba\(^{2+}\)] up to 13 mM. Note that the lack of Ba\(^{2+}\) effects on the gating ring intrinsic Trp fluorescence. After adding Ba\(^{2+}\), the [Ca\(^{2+}\)] was increased to 1.2 \(\mu\)M to ascertain gating ring functionality (red trace). D, the fluorescence intensity (at 340 nm) from experiments as in A–C is plotted versus the concentration of divalent cations (●, Ca\(^{2+}\); □, Mg\(^{2+}\); ▲, Ba\(^{2+}\)). The experimental points were fit to double (Ca\(^{2+}\)) or single (Mg\(^{2+}\)) Hill functions. The apparent affinity of the gating ring for Ca\(^{2+}\) was: \(K_{1/2} = 0.29 \pm 0.0043 \\mu\text{M} (n = 3.4 \pm 0.16)\) and \(K_{2/2} = 3.5 \pm 0.51 \\mu\text{M} (n = 2.1 \pm 0.35)\), and for Mg\(^{2+}\): \(K_{1/2} = 154 \pm 20.7 \mu\text{M} (n = 2.02 \pm 0.44)\) (\(n = 3\)).
free gating ring (23) has a torus-like shape, with radius $\sim 4.5-6$ nm, height $\sim 5$ nm, and a 2-nm internal aperture (Fig. 6A). As this shape is not spherical, gating ring particles in solution are expected to exhibit a larger $R_g$ than $R_H$. Indeed, the dominant distribution (71 $\pm$ 4.3%) of purified gating ring particles in nominally Ca$^{2+}$-free solution was estimated to have a mean $R_H$ of 10 $\pm$ 0.41 nm (Fig. 6B). The particles outside the dominant distribution had $R_H > 60$ nm (most of them around 400 nm) and probably correspond to protein aggregates that were not investigated. The polydispersity of the dominant distribution (i.e. the standard deviation divided by mean hydrodynamic diameter) was 12 $\pm$ 0.53%, within the range (10–15%) of homogeneous, monodisperse solutions (59).

Notably, when [Ca$^{2+}$] was elevated to 35 $\mu$M, the gating ring $R_H$ was reduced to 7.6 $\pm$ 0.14 nm (Fig. 6B), which corresponds to a 27 $\pm$ 3.5% $R_H$ decrease (Fig. 6D). This result suggests that the Ca$^{2+}$-bound gating ring particles diffuse faster in solution; their shape has less surface-area-to-volume ratio, i.e. they are more compact. This Ca$^{2+}$-induced structural compaction is reversible; decreasing free [Ca$^{2+}$] from 35 to 0.07 $\mu$M by the addition of EGTA almost fully restored the gating ring hydrodynamic radius (9.4 $\pm$ 0.47 nm). Mg$^{2+}$ induced a much smaller $R_H$ decrease (7.0 $\pm$ 3.5%; Fig. 6, C and D).

**DISCUSSION**

Recently resolved atomic structures of the human BK channel intracellular ligand-sensing apparatus have revealed that it is a hetero-octameric assembly of RCK domains (a pair of RCK1 and RCK2 domains from each subunit) arranged to form a gating ring structure (23, 24). This work, which capitalizes on these structural data, is the first attempt to shed light on the molecular mechanisms by which the free energy from ions binding to the human BK channel gating ring structure is transduced into conformational rearrangements required to open the pore. The presumption of this study is that ligands binding to the gating ring induce detectable conformational changes. We have probed for and resolved ligand-induced conformational rearrangements under physiologically relevant conditions. The significant findings of this investigation are as follows. 1) Purified human BK C termini self-assemble under physiologically relevant conditions into tetramers, likely corresponding to gating rings. 2) Ca$^{2+}$ and Mg$^{2+}$ specifically induce conformational changes in the isolated gating ring, whereas Ba$^{2+}$ does not. 3) Neutralization of key residues within the calcium bowl significantly reduces the Ca$^{2+}$ effect on the gating ring rearrangements and eliminates Mg$^{2+}$ sensitivity. 4) Upon Ca$^{2+}$ binding, gating ring particles undergo a fully reversible reduction in their hydrodynamic radii, consistent with a compaction of their structure, whereas Mg$^{2+}$ has a modest effect on the gating ring shape. Crucially, the structural and functional features of gating ring parallel the Ca$^{2+}$–dependent activation properties of the native BK channel (4, 7, 8, 25, 27, 34, 52, 53).

The Cationic Ligand Selectivity of the Gating Ring—Ca$^{2+}$, Mg$^{2+}$, and Ba$^{2+}$ were used to probe the metal-sensing properties of the purified gating ring using its intrinsic Trp fluorescence intensity decay of a gating ring carrying the D894A,D895A,D896A,D897A,D898A neutralization within the RCK2 domain, recorded in nominal zero (blue) or 35 $\mu$M free Ca$^{2+}$. Increasing the [Ca$^{2+}$] to 35 $\mu$M Ca$^{2+}$ produced a modest change in the $\tau_{av}$ as compared with the wild-type gating ring (Fig. 3A). IRF, instrument response time. B and C are fluorescence emission spectra of the calcium bowl mutant gating ring in solution excited at 295 nm. B, the Trp fluorescence intensity decreased as the free [Ca$^{2+}$] of a solution containing the calcium bowl mutant gating ring was increased from nominal zero to 35 $\mu$M. C, unlike the wild-type gating ring, the calcium bowl mutant did not exhibit Mg$^{2+}$ sensitivity, up to 12 mM. D, the fluorescence intensity at 350 nm from experiments in panels B and C is plotted versus the concentration of divalent cations (○, Ca$^{2+}$; ■, Mg$^{2+}$). The experimental points are fit to a Hill function with $K_{1/2} = 2.0 \pm 0.19 \mu$M and $n = 2.1 \pm 0.35$.

**FIGURE 5.** Neutralization of the calcium bowl region reduces the Ca$^{2+}$ sensitivity of the gating ring and abolishes Mg$^{2+}$ sensitivity. A, the intrinsic Trp fluorescence intensity decay of a gating ring carrying the D894A,D895A,D896A,D897A,D898A neutralization within the RCK2 domain, recorded in nominal zero (blue) or 35 $\mu$M free Ca$^{2+}$. Increasing the [Ca$^{2+}$] to 35 $\mu$M Ca$^{2+}$ produced a modest change in the $\tau_{av}$ as compared with the wild-type gating ring (Fig. 3A). IRF, instrument response time. B and C are fluorescence emission spectra of the calcium bowl mutant gating ring in solution excited at 295 nm. B, the Trp fluorescence intensity decreased as the free [Ca$^{2+}$] of a solution containing the calcium bowl mutant gating ring was increased from nominal zero to 35 $\mu$M. C, unlike the wild-type gating ring, the calcium bowl mutant did not exhibit Mg$^{2+}$ sensitivity, up to 12 mM. D, the fluorescence intensity at 350 nm from experiments in panels B and C is plotted versus the concentration of divalent cations (○, Ca$^{2+}$; ■, Mg$^{2+}$). The experimental points are fit to a Hill function with $K_{1/2} = 2.0 \pm 0.19 \mu$M and $n = 2.1 \pm 0.35$. 

The Cationic Ligand Selectivity of the Gating Ring—Ca$^{2+}$, Mg$^{2+}$, and Ba$^{2+}$ were used to probe the metal-sensing properties of the purified gating ring using its intrinsic Trp fluores-
Ca²⁺ and Mg²⁺, known regulators of the BK channel, significantly affected the average lifetime of Trp fluorescence (Fig. 3, A and B and supplemental Tables S1 and S2), suggesting conformational rearrangements. These results are also supported by steady-state fluorescence measurements, suggesting at least two distinct high-affinity Ca²⁺-binding sites with $K_{\text{d}}$ 0.29 and 3.5 μM (Fig. 4, A and D), in agreement with the electrophysiologically inferred apparent Ca²⁺ affinity of whole BK channels (4, 34, 35, 37).

The gating ring exhibited significantly less affinity for Mg²⁺ ($K_{\text{d}} = 154$ μM, Fig. 4, B and D) and was apparently insensitive to Ba²⁺ (Fig. 4, C and D), defining the cationic ligand selectivity of the BK metal-sensing apparatus. The lack of detectable Ba²⁺-induced conformational changes is consistent with previous work on reconstituted BK channels that did not show evidence for Ba²⁺-dependent BK channel activation (55, 56).

However, the well-characterized block effect of Ba²⁺ at the BK channel pore (55–58) could confound attempts to characterize an alternative effect of Ba²⁺ on channel activation (56). Because we did not resolve Ba²⁺-driven conformational changes in the isolated gating ring, we speculate that if Ba²⁺ can activate BK channels, other protein domain(s), in addition to the gating ring, may be required.

**How Many High-affinity Ca²⁺-binding Sites Exist within the Gating Ring?**—A recent crystal structure of the BK gating ring in the presence of Ca²⁺ (Protein Data Bank code 3MT5) definitively demonstrated that the calcium bowl region in RCK2 is a Ca²⁺-binding site (24). This finding was in agreement with previous electrophysiological investigations (29, 34, 35) as well as biochemical investigations of the calcium bowl region (30, 32, 60) and the whole RCK2 domain (33). Although it has yet to be confirmed in atomic structures, electrophysiological investigations support the existence of a second micromolar-affinity Ca²⁺-binding site in the RCK1 domain (34–38); indeed, purified RCK1 domains in solution assemble into homo-octameric gating ring-like structures that sense micromolar Ca²⁺ in solution (47).

The Ca²⁺-induced conformational changes of the purified gating ring detected spectroscopically in this work exhibit a composite Ca²⁺ dependence with $K_{\text{d}} = 0.29$ and 3.5 μM (Fig. 4, A and D), which implies the existence of more than one type of high-affinity Ca²⁺-binding site. Furthermore, neutralization of the five consecutive aspartates that comprise the calcium bowl significantly reduced the overall extent of the detectable fluorescence changes (Fig. 5, B and D) but did not completely eliminate Ca²⁺ sensitivity; calcium bowl gating ring mutants exhibit a single transition with an apparent Ca²⁺ affinity of 2 μM (Fig. 5D).

We propose that the transition with higher affinity ($K_{\text{d}} = 0.29$ μM) is relevant to Ca²⁺ binding to the RCK2 calcium bowl site, whereas the second transition ($K_{\text{d}} = 3.5$ μM, Fig. 4D) arises from Ca²⁺ binding to the RCK1 domain, which has less apparent affinity (34, 35, 37). Supporting this view, the homo-RCK1 gating ring exhibited an apparent Ca²⁺ affinity of 1.7 μM.
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(47), similar to the lower Ca\(^{2+}\) affinity component of the wild-type gating ring (3.5 \(\mu M\)) and the residual Ca\(^{2+}\) sensitivity of the calcium bowl mutant (2 \(\mu M\)). Electrophysiological investigations have highlighted residues Asp\(^{362}\), Asp\(^{367}\), Met\(^{511}\), Arg\(^{514}\), and Glu\(^{385}\) as candidates for Ca\(^{2+}\) coordination in RCK1 (34–38). Consistent with this view, the Ca\(^{2+}\) binding properties of isolated RCK1 domains in solution suggest that an RCK1 Ca\(^{2+}\)-binding site could exist with multiple Ca\(^{2+}\)-coordinating residues (47).

The Calcium Bowl Binds Mg\(^{2+}\) with Low Affinity—A low-affinity Mg\(^{2+}\)-binding site known to modulate channel activity (61, 62) is likely located at the interface between the RCK1 domains (Glu\(^{374}\), Glu\(^{399}\)) and the voltage sensors (Asp\(^{99}\), Asn\(^{172}\)) (4, 7, 34, 41–43), enhancing the efficiency of the voltage sensor-gate coupling (63). Because the purified gating ring does not include transmembrane domains, the effects observed in this study (Fig. 4, B and D) likely arise from Mg\(^{2+}\) binding to alternative sites. We found that calcium bowl neutralization completely eliminated the Mg\(^{2+}\) effect on the endogenous Trp fluorescence of the gating ring (Fig. 5, C and D), suggesting that the calcium bowl can bind Mg\(^{2+}\) with much less affinity than Ca\(^{2+}\). The addition of Mg\(^{2+}\) to the gating ring in the presence of 35 \(\mu M\) free Ca\(^{2+}\) induced no further spectroscopically resolvable conformational changes (supplemental Fig. S4), supporting the view that in the isolated gating ring, Mg\(^{2+}\) acts through the calcium bowl site as an inefficient Ca\(^{2+}\) mimetic, rather than an allosteric ligand. These results recapitulate previous electrophysiological experiments (4, 61, 62, 64), where it was demonstrated that Mg\(^{2+}\) competes with Ca\(^{2+}\) for the high-affinity Ca\(^{2+}\)-binding sites.

The Gating Ring Apparatus Operates as a Selective Chemomechanical Transducer of Metal Binding—In this work, we have demonstrated Ca\(^{2+}\)-dependent structural rearrangements of the whole human BK channel gating ring apparatus, resolved by the fluorescence emission of native Trp residues and, from a whole-particle perspective, by dynamic light scattering. The latter showed that the gating ring reshapes in response to [Ca\(^{2+}\)] binding, toward a “more spherical” or compact conformation characterized by a reduced surface-area-to-volume ratio. As these conformational rearrangements were induced by [Ca\(^{2+}\)] relevant to BK channel activation, we propose that they likely constitute the prime molecular events that propagate to the BK channel pore, to increase open probability. In contrast, Mg\(^{2+}\) binding produced much smaller changes in the shape of the gating ring (Fig. 6, C and D), which suggests that the Mg\(^{2+}\)-induced perturbations in the microenvironment of Trp residues (\(\approx 15\%\) maximal decrease in fluorescence intensity as compared with a 30% reduction observed with saturating [Ca\(^{2+}\)]) do not tightly correlate with the global structural rearrangements of the gating ring. Possibly, Trp residues report changes to their microenvironment, which may not reflect the extent of macroscopic structural rearrangements. The binding of Mg\(^{2+}\) to the Ca\(^{2+}\)-binding sites does not activate the channel (61, 62); therefore, the modest compaction of the gating ring caused by saturating [Mg\(^{2+}\)] is likely to be insufficient to facilitate pore opening. Finally, the lack of spectroscopic responses of the gating ring to Ba\(^{2+}\) further defined its selectivity for divalent metal cations.

In this work, we probed the divalent metal-sensing properties of the BK channel gating ring under physiological conditions using a combination of spectroscopic (time-resolved and steady-state spectroscopy) and particle-scale (DLS) optical approaches. We have demonstrated that the gating ring responds to elevations in [Ca\(^{2+}\)] and, with much less affinity, [Mg\(^{2+}\)]. Micromolar Ca\(^{2+}\) can induce robust microscopic and macroscopic conformational rearrangements compatible with a compaction of the gating ring superstructure, which likely facilitate pore opening in the whole BK channel. The micromolar sensitivity of the gating ring to Ca\(^{2+}\) persists after neutralization of the calcium bowl site, confirming the existence of more high-affinity Ca\(^{2+}\)-binding sites. In contrast, millimolar Mg\(^{2+}\) can induce changes to a smaller extent than Ca\(^{2+}\), and these changes are probably insufficient to facilitate channel activation in vivo. Finally, mirroring the selectivity of the channel to divalent metal agonists, the gating ring underwent no resolvable conformational rearrangements upon [Ba\(^{2+}\)] elevation up to millimolar levels. Thus, we have demonstrated that the BK gating ring operates as a selective chemo-mechanical coupler that transduces the free energy of ligand binding into mechanical work that ultimately activates the channel.

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