Molecular Architecture and Divalent Cation Activation of TvoK, a Prokaryotic Potassium Channel*

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RCK (regulator of conductance of potassium) domains form a family of ligand-binding domains found in many prokaryotic K+ channels and transport proteins. Although many RCK domains contain an apparent nucleotide binding motif, some are known instead to bind Ca2+, which can then facilitate channel opening. Here we report on the molecular architecture and ligand activation properties of an RCK-containing potassium channel cloned from the prokaryote Thermoplasma volcanium. This channel, TvoK, is of an apparent molecular mass and subunit composition that is consistent with the heter-octameric configuration hypothesized for the related MthK (Methanobacterium thermoautotrophicum potassium) channel, in which four channel-tethered RCK domains coassemble with four soluble (untethered) RCK domains. The expression of soluble TvoK RCK subunits arises from an unconventional UUG start codon within the TvoK gene; silent mutagenesis of this alternative start codon abolishes expression of the soluble form of the TvoK RCK domain. Using single channel recording of purified, reconstituted TvoK, we found that the channel is activated by Ca2+ as well as Mg2+, Mn2+, and Ni2+. This non-selective divalent activation is in contrast with the activation properties of MthK, which is selectively activated by Ca2+. Transplantation of the TvoK RCK domain into MthK generates a channel that can be activated by Mg2+, illustrating that the Mg2+ binding site is likely contained within the RCK domain. We present a working hypothesis for TvoK gating in which the binding of either Ca2+ or Mg2+ can contribute ~5 kcal/mol toward stabilization of the open conformation of the channel.

Potassium channels are central to a wide range of biological processes, including electrical signaling, electrolyte homeostasis, and cell volume regulation (1). The activation states of several types of potassium channels and their relatives can be modulated by cytoplasmic ligands, such as G-proteins, calmodulin, or cyclic nucleotides; some of these ligands bind to modular domains that show remarkable sequence conservation across phyla. One such ligand-binding domain is the RCK2 (regulator of conductance of potassium) or KTN (potassium transporter nucleotide-binding) domain, which is found in many prokaryotic K+ channels and transporters, as well as the eukaryotic BK channel (2–5). Many RCK domains contain a consensus nucleotide binding motif, such as those from the KtrA and TrkA transporter-associated proteins (6–8). Another class of RCK domain, however, contains no apparent nucleotide binding motif but instead contains a Ca2+ binding site. So far, the only two channels that have been demonstrated to contain a Ca2+-binding RCK domain are the BK channel and the prokaryotic channel MthK (Methanobacterium thermoautotrophicum potassium channel) (2, 3, 9–11).

X-ray and biochemical studies have suggested that the cytoplasmic ligand binding region of MthK is formed by an octameric ring of RCK domains, with four “tethered” RCK domains that are directly connected to the transmembrane pore and four “soluble” RCK domains (without transmembrane segments) that are docked onto the tethered RCKs (2, 12). An alternating arrangement of tethered and soluble RCK domains gives rise to an octameric ring assembly, called the “gating ring” because of its role in Ca2+-dependent gating of the MthK pore (2, 12). The soluble MthK RCK domain arises from an alternative AUG start codon within the MthK gene, just upstream from the RCK domain coding sequence. Mutation of this second start codon eliminates heterologous overexpression of the MthK RCK domain in E. coli while permitting overexpression of full-length subunits with tethered RCK domains (2). However, many RCK-containing K+ channels do not contain an AUG codon upstream from the RCK domain and eliminating the internal start codon does not necessarily abolish channel function in vivo (13). Thus, it has not been entirely clear whether the octameric RCK domain architecture observed with MthK is generally applicable or whether alternative mechanisms exist to generate this architecture.

The presence of an apparent RCK domain within the BK channel C terminus has prompted the use of MthK as a limited structural template for BK channels (Fig. 1) (2, 3, 9–11). This in turn has provided insight toward potential ligand activation mechanisms for BK channels. However, the correspondence between the Ca2+ binding site of MthK and those of BK channels is not clear. For example, BK channels contain multiple Ca2+ binding sites that operate in low (millimolar) and high (micromolar) affinity ranges (14–16). MthK displays millimo-

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2 The abbreviations used are: RCK, regulator of conductance of potassium; MthK, M. thermoautotrophicum potassium channel; TvoK, T. volcanium potassium channel.
lar affinity activation by Ca\(^{2+}\) at room temperature (2, 3, 9–11). However, the low affinity site of the BK channel also imparts activation by Mg\(^{2+}\), whereas MthK has been shown to be insensitive to Mg\(^{2+}\) (10, 12, 16, 17). It is possible that the Ca\(^{2+}\) binding site of MthK does not correspond to the BK channel site. Alternatively, it is possible that these Ca\(^{2+}\) binding sites are homologous structures, although structural elements that impart Mg\(^{2+}\) sensitivity are absent from the MthK site.

During a survey of prokaryotic RCK-containing proteins, we identified and cloned a putative potassium channel from Thermoplasma volcanium. This putative channel, TvoK, lacks an AUG start codon in the region preceding the RCK domain, so we wondered whether this protein might form a channel with a non-octameric architecture or whether the TvoK gene might generate a soluble RCK domain without the AUG start codon. In addition, the RCK domain of TvoK contained structural features that were in common with BK channels but absent from MthK (e.g. residues between αA and ββ and between βD and αD; Fig. 1). It would ultimately be of interest to resolve whether these structural elements contribute to ligand binding or ligand selectivity in the RCK domain.

In this report we describe the molecular architecture and function of TvoK. Although the gene does not contain a second AUG start codon preceding its RCK domain, it does contain an unconventional UUG start codon that results in efficient translation of a soluble TvoK RCK domain. The soluble RCK domain copurifies with full-length subunits, consistent with a hetero-octameric channel assembly. Using single channel recording in planar lipid bilayers, we found that reconstituted TvoK channels are activated not only by Ca\(^{2+}\) but also by Mg\(^{2+}\), Mn\(^{2+}\), or Ni\(^{2+}\), in contrast to MthK, which is activated only by Ca\(^{2+}\). In chimeric channels containing the MthK pore domain, activation by Mg\(^{2+}\) can be imparted by the TvoK RCK domain, suggesting that the RCK domain contains the Mg\(^{2+}\) activation site and that the RCK domain is modular in the sense that ligand binding to this domain can yield the opening of an otherwise different K\(^{+}\) channel pore. Finally, kinetic modeling suggests that activation of TvoK by Ca\(^{2+}\) or Mg\(^{2+}\) is energetically roughly equivalent and either Ca\(^{2+}\) or Mg\(^{2+}\) binding contributes ~5 kcal/mol toward stabilization of the open state.

**EXPERIMENTAL PROCEDURES**

**Channel Constructs**—The TvoK gene was PCR-cloned from T. volcanium genomic DNA (ATCC) using primers that added an SphI restriction site to the 5′-end of the gene, the cDNA code for a C-terminal threonyl cleavage site, and a BamHI site to the 3′-end. The PCR product was gel-purified and ligated into the pCR-Blunt vector. After removal of an internal SphI site in the coding region by silent mutagenesis, the TvoK gene was subcloned into a modified pQE-82L vector (Qiagen) (11) between the SphI and BamHI sites to generate a threonyl-cleavable hexahistidine-tagged fusion construct.

The TvoK wild-type protein could not practically be overexpressed in *E. coli* at levels sufficient for our biochemical or functional studies. We fortuitously generated a well expressing construct by deleting a region of seven residues near the outer mouth of the putative pore region (residues Leu-39 to Lys-45) after discovering that the analogous stretch of residues is apparently absent from MthK. Several other deletions in the region bounded by residues 39–45 were tested, but none could be consistently overexpressed as well as TvoKΔ-(39–45). This construct was used in all of our subsequent biochemical and functional studies of the full-length channel.

**Protein Purification**—TvoKΔ-(39–45) was overexpressed overnight in *E. coli* (XL1-Blue; Stratagene) upon induction with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside at 20 °C. Cells were harvested, resuspended, and lysed by sonication in 50 mM Tris, 100 mM KCl, 10% v/v glycerol, pH 8.5, supplemented with Complete EDTA-free protease inhibitor tablets (1 tablet/50 ml lysate; Roche Applied Science) and 0.1 mM phenylmethylsulfonyl fluoride, at 4 °C. Membranes were solubilized by incubation at 4 °C for 2 h in 50 mM decyl maltoside (Anatrace). Insoluble material was pelleted by centrifugation (16,000 rpm, 45 min), and the supernatant was collected. The pH of the supernatant was adjusted to 8.5, and the supernatant was loaded onto a CoCl\(_2\)-activated nitrilotriacetic acid-HiTrap column (GE Healthcare). The column was washed with 10 volumes of buffer A containing 20 mM Tris, 100 mM KCl, 5 mM decyl maltoside, pH 8.5 followed by 10 volumes of buffer A containing 20 mM imidazole. The recombinant protein was eluted with buffer A containing 400 mM imidazole. The hexahistidine tag was removed by thrombin digestion for 2 h at room temperature. The digest was concentrated to ~10 mg/ml using an Amicon-Ultra centrifugal concentrator (molecular weight cutoff 50,000), and the oligomeric channel assembly was further isolated in buffer A by size exclusion chromatography using a Superdex-200 10/300 column (GE Healthcare). Fractions that were eluted from the Superdex-200 column between ~9.5 and 11 ml were pooled and concentrated to 10 mg/ml using an Amicon concentrator (Millipore). Expression and purification of MthK wild type or MthK-TvoK chimeric channel were performed as described previously for MthK channels (11).

**Electrophysiology**—The purified TvoKΔ-(39–45) assembly was reconstituted into liposomes composed of *E. coli* lipids (Avanti Polar Lipids) as described previously (18). The concentration of protein per lipid in the case of TvoKΔ-(39–45) and MthK-TvoK chimeric channel was 50 μg/ml and for MthK wild type 5 to 10 μg/ml. Single channel recordings were obtained using planar lipid bilayers of POPE:POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine:1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)]) (3:1) lipids (Avanti Polar Lipids) in a horizontal bilayer apparatus. Solutions in both the cis and trans chambers contained 200 mM KCl, 10 mM HEPES, pH 7.2, with the addition of either CaCl\(_2\), MgCl\(_2\), MnCl\(_2\), or NiCl\(_2\) at concentrations ranging from 0.1 to 25 mM. We estimated the concentration of contaminating Ca\(^{2+}\) in our solutions to be ~10 μM, as measured with Ca\(^{2+}\)-sensitive electrode (Orion Research). Because of the relatively high concentrations of divalent cations used, no divalent cation buffers were included in the recording solutions. All experiments were performed at room temperature (22–24 °C).

Orientation of channels in the bilayer membrane was identified using the intrinsic inward rectification property of the channel, which is similar to MthK (2). TvoKΔ-(39–45) dis-
played a form of voltage-dependent inactivation or blockade, such that observing channel activity required intermittent hyperpolarizing steps. We have not yet characterized this phenomenon in detail, except to note that it was observed at different ligand concentrations and in the presence of each of the different divalent cations used in these studies. A similar phenomenon has been observed in recordings of MthK in spheroplast membranes (19).

Electrophysiological data were analyzed using Clampfit 9.2 (Molecular Devices) and are expressed as mean ± S.E. from three to seven different experiments for each data point. Channel activity was quantified as $N/P_{o}$, and this value was in all cases divided by the number of active channels in the bilayer ($N$) to estimate the mean open probability ($P_{o}$). At low divalent ion concentrations that produced low levels of channel activation ($P_{o}/0.25$), $N$ was estimated at the end of the experiment by maximally activating the channels using 10 mM Ca$^{2+}$ added to the apparent cytoplasmic side.

RESULTS

To find potentially novel putative K$^{+}$ channels containing RCK-like cytoplasmic domains, we performed a BLASTp search of archaeal genes using the putative mouse BK channel RCK domain (mslo1, residues His-344 through Cys-619) as the template sequence (2, 20, 21). Our search revealed, among others, a significant result that contained a K$^{+}$ channel selectivity filter sequence (TVGYG) and at least two putative transmembrane (TM) segments. This was a 348-amino acid putative K$^{+}$ transport protein from $T.\ volcanium$ (GenBank™ accession number NP111507), which we called TvoK. Surprisingly, MthK was not returned as a significant result under our search parameters. The search statistics revealed remarkable similarity (40%) and identity (20%) between mslo1 and TvoK within the search region, illustrated in Fig. 1. Comparison of the protein sequences of the full-length TvoK subunit and MthK are consistent with an orthologous relation between these two proteins (48% similarity, 25% identity).

Ligand Activation of TvoK

![Partial sequence alignment of MthK, TvoK, and the mouse BK channel (mslo1).](image_url) The alignment begins at the K$^{+}$ channel signature sequence (Filter). Secondary structure assignment is based on the MthK crystal structure; bars and arrows indicate $\alpha$-helices and $\beta$-strands, respectively. Gray/black shading indicates semi-conserved/conserved residues. Red indicates Glu-374 and Glu-399 in mouse BK, which are essential for BK's Mg$^{2+}$ sensing; blue indicates three Ca$^{2+}$-coordinating residues in MthK (Asp-184, Glu-210, Glu-212).
expression. Fig. 2A illustrates that the TvoKΔ-(39–45) expression products (visualized via anti-penta-His antibody) migrate on SDS-PAGE primarily in two bands, with apparent molecular masses of 38 and 26 kDa. These are consistent with the predicted masses of the full-length construct and the ~250-residue C-terminal fragment of this construct.

The TvoKΔ-(39–45) expression products were purified by metal affinity chromatography followed by gel filtration chromatography. The decyl maltoside-solubilized protein elutes with a peak at ~10.5 ml on a Superdex-200 10/300 column, consistent with a ~250 kDa assembly, estimated by comparison to elution of protein standards using this column (Fig. 2B).

The apparent 250-kDa assembly is consistent with a hetero-octamer comprised of four full-length subunits and four RCK domains, as was hypothesized for MthK (2). Consistent with this hypothetical architecture, the purified TvoK assembly partially dissociates in SDS-containing sample buffer and migrates with apparent molecular masses of ~200, 38, and 26 kDa (Fig. 2B, right). Each of these bands was identified by in-gel tryptic digest followed by mass spectrometry of the tryptic fragments, and the mass fingerprints are consistent with TvoKΔ-(39–45) (identified in the ~200- and 38-kDa bands) and the TvoK RCK domain (identified in the 26-kDa band).

Mechanism Underlying Generation of the Soluble TvoK RCK Domain—Interestingly, the TvoK gene contains no secondary AUG start codon in its TM2-RCK linker region, as is the case with MthK; thus, we were puzzled by the appearance of the soluble TvoK RCK domain. Although GUG also occasionally serves as a start codon in prokaryotes (in 14% of E. coli genes) (22), the TvoK gene contains no GUG in this region either.

Because of the importance of the soluble RCK domain in determining the molecular architecture of these channels, we explored two possibilities for the generation of this protein. One possibility is the existence of a protease-vulnerable cleavage site at the amino end of the RCK domain. We reasoned that any (or several) of the basic residues in this region could be vulnerable to cleavage by endogenous E. coli proteases.

To narrow down the array of possible cleavage sites, we first estimated the molecular mass of the purified RCK domain by electrospray ionization-mass spectrometry. The estimated mass was 26,230 Da, consistent with the mass of the TvoK RCK domain starting from Leu-115 and ending at the thrombin cleavage site (with the putative last ten residues being EEAIKGLVPR). This result suggested a possible cleavage site at the carboxyl side of Lys-114, possibly due to activity of the Lys-C protease, for example.

To test for the presence of one or more protease-vulnerable cleavage sites near this residue, we generated site-specific mutants of Lys → Asn or Arg → Asn at positions Arg-111, Lys-114, and Lys-117, overexpressed the mutants in E. coli, and probed the lysate for hexahistidine-tagged proteins by Western blot. If any of these residues were important for recognition or cleavage by a Lys or Arg protease, then mutation to Asn should eliminate or reduce cleavage and thus result in a reduction in the 26-kDa band. We observed that neither the K114N nor the K117N mutations reduce the relative intensity of the 26-kDa band compared with wild type (not shown). However, R111N did reduce the relative intensity of the 26-kDa band (Fig. 2). If this were due to a reduction of proteolytic cleavage at this site, however, then one would have expected the mass of the wild-type RCK fragment to be significantly larger than the estimated 26,230 Da.

We therefore considered an alternative possibility, that the TvoK gene contains a second start codon that is neither AUG nor GUG, but UUG, which is rarely used as a start codon in many prokaryotes (23, 24). Such a UUG codon (normally translated as Leu) does exist at the 3′-side of the codon for Lys-114, consistent with the presumed N terminus of the soluble RCK domain. This codon is preceded by an optimal Shine-Dalgarno ribosome binding sequence (AGGAGG) that overlaps with the codons for Arg-111 and Arg-112; thus, the reduction of the 26-kDa band observed with the R111N mutation is consistent with a start codon for Arg-112. The putative start codon is followed by an optimal Shine-Dalgarno ribosome binding sequence (AGGAGG) and the mass fingerprints are consistent with TvoKΔ-(39–45) (identified in the ~200- and 38-kDa bands) and the TvoK RCK domain (identified in the 26-kDa band).
FIGURE 3. *TvoK*-Δ(39–45) can be activated by Ca$^{2+}$ or Mg$^{2+}$. A and B, representative current traces of single *TvoK*-Δ(39–45) channels, recorded at −200 mV in 200 mM KCl, 10 mM HEPES, pH 7.2, containing Ca$^{2+}$ or Mg$^{2+}$, as indicated. *P*$_{o}$ increased with increasing [Ca$^{2+}$] or [Mg$^{2+}$]; single channel current amplitude decreased, consistent with fast block. C, single channel current-voltage curves for *TvoK*-Δ(39–45) (black circles) and MthK (black triangles) in symmetrical bath solutions with 200 mM KCl, 10 mM HEPES, 5 mM Ca$^{2+}$, pH 7.2. D, *TvoK*-Δ(39–45) *P*$_{o}$ as a function of [Ca$^{2+}$] (black circles) or [Mg$^{2+}$] (white squares). *P*$_{o}$ was determined from three to five different bilayer recordings at each condition, plotted as mean ± S.E. Experimental data were fitted with Hill equations, yielding an EC$_{50}$ of 2.9 mM and Hill coefficient of 1.7 for Ca$^{2+}$ and EC$_{50}$ of 2.0 mM and Hill coefficient of 2.2 for Mg$^{2+}$.

To determine the activation mechanism of the putative *TvoK* channel, purified *TvoK*-Δ(39–45) was reconstituted in liposomes, which in turn were incorporated into planar lipid bilayers to obtain single channel recordings. We observed little consistent channel activity in solutions containing only 200 mM KCl and 10 mM HEPES, pH 7.2, at both sides of the membrane. However, we observed that channel activity consistently increased when CaCl$_2$ was included in the recording solutions (Fig. 3A). Using recordings obtained under bi-ionic conditions (150 mM NaCl and 50 mM KCl cis/200 mM KCl trans, with 5 mM CaCl$_2$ on both sides), we found that *TvoK*-Δ(39–45) is selective for K$^+$ over Na$^+$ by a ratio of at least 100:1, as determined by the shift of the reversal potential to *E*$_R$ (not shown). In symmetrical 200 mM KCl, 5 mM CaCl$_2$, and 10 mM HEPES, pH 7.2, *TvoK* displays inward rectification similar to that observed with the closely related MthK, with a chord conductance of 160 ± 9.0 pS at −200 mV (Fig. 3C).

We quantified the relation between *P*$_{o}$ and [Ca$^{2+}$] and found that Ca$^{2+}$ activates the channel over the mM range (Fig. 3, A and D). Fitting these data with a Hill equation yielded an EC$_{50}$ of 2.9 mM and a Hill coefficient of 1.7, consistent with the binding of at least two Ca$^{2+}$ ions/channel to promote opening. If *TvoK* assumes the same molecular architecture as MthK with eight RCK domains containing one Ca$^{2+}$ binding site each, then the Hill coefficient of 1.7 would be consistent with a relatively weak positive cooperativity among subunits to stabilize the open state.

Although both MthK and the mammalian BK channel display low affinity activation by Ca$^{2+}$, Ca$^{2+}$ activation in MthK appears to be relatively selective, i.e. Mg$^{2+}$ does not activate MthK, and biochemical assays suggest that Mg$^{2+}$ does not stabilize the MthK RCK gating ring (10, 12). To determine whether ligand activation of *TvoK* shows the same selectivity as MthK and thus (possibly) a single binding site of roughly the same properties, we tested the activation properties of the channel in the presence of other divalent cations. We found that *TvoK* is activated by Mg$^{2+}$ in the millimolar range (Fig. 3, B and D). We then quantified the relation between *P*$_{o}$ and [Mg$^{2+}$] and found that Mg$^{2+}$, similar to Ca$^{2+}$, activates the channel with an EC$_{50}$ of 2.0 mM and a Hill coefficient of 2.2.

*TvoK*-Δ(39–45) Can Be Activated by Mn$^{2+}$ and Ni$^{2+}$.—Investigations of BK channel modulation have revealed that a
number of divalent cations, like Mn$^{2+}$, Ni$^{2+}$, Co$^{2+}$, or Sr$^{2+}$, activate the channel through a low affinity site defined in part by Glu-374 and Glu-399, located in the RCK domain of the channel (Fig. 1) (16, 25). To determine whether a similar non-selective divalent cation activation site may exist in the TvoK RCK domain, we obtained recordings from TvoK channels in the presence of either 10 mM Mn$^{2+}$ or 10 mM Ni$^{2+}$.

Fig. 4 shows that TvoK can be activated to near maximal levels by either 10 mM Mn$^{2+}$ or Ni$^{2+}$. Thus Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, and Ni$^{2+}$ may bind to either an identical or overlapping activation site on the TvoK RCK domain; alternatively, there may be multiple distinct sites that coordinate subsets of these ions.

The TvoK RCK Domain Can Impart Mg$^{2+}$-dependent Activation to an MthK Pore—It was observed in previous studies that MthK is activated by Ca$^{2+}$, but not by Mg$^{2+}$ (10). To determine whether the Mg$^{2+}$ activates TvoK by binding to a site on its RCK domain, we exploited the Mg$^{2+}$ insensitivity of MthK and generated a chimeric channel containing the MthK transmembrane domains with the TvoK RCK domain. Fig. 5, top panel, illustrates that adding Mg$^{2+}$ at the cytoplasmic side of the TvoK channel results in a rapid increase in $P_o$, concomitant with a decrease in single channel conductance that is consistent with fast divalent blockade (9, 26). In experiments like those shown in Fig. 5, 10 mM Mg$^{2+}$ at the cytoplasmic side of the channel was sufficient to produce near maximal activation ($P_o = 0.80 \pm 0.03$). In contrast, addition of Mg$^{2+}$ had no effect on MthK $P_o$ but did decrease the single channel conductance.

Adding Mg$^{2+}$ at the cytoplasmic side of the MthK-TvoK chimera results in a rapid increase in $P_o$ consistent with a Mg$^{2+}$ activation site on the TvoK RCK domain (Fig. 5, bottom panel). However, with the addition of 10 mM Mg$^{2+}$ the $P_o$ of the chimera did not reach the same level as that observed with TvoK.

This could be because of differences between MthK and TvoK in the mechanical work required to open the different pores, resulting in less efficient coupling between ligand binding and channel opening in the chimeric channel. Similar results were obtained using Ca$^{2+}$ to activate the chimeric channel instead of Mg$^{2+}$ (not shown).

**DISCUSSION**

X-ray studies of the MthK channel and its isolated RCK domain have revealed the molecular details of Ca$^{2+}$ coordination and thus provide unprecedented insight toward activation mechanisms in RCK-containing K$^+$ channels (2, 12). It is important, however, to gain further insight by bridging the phylogenetic gap between MthK and its eukaryotic relative, the BK channel, as well as other members of the slo gene family. Our approach was to mine the genomic data base, using the RCK (or RCK1) domain of the mammalian BK channel as bait. In this study, we report on the architecture and activation mechanism of TvoK, an MthK orthologue that is activated by Ca$^{2+}$ and several other divalent cations, including Mg$^{2+}$, Mn$^{2+}$, and Ni$^{2+}$. Because the BK channel RCK domain contains a putative binding site that imparts activation by Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, and Ni$^{2+}$, it seems that further study of TvoK may provide insight toward mechanisms underlying ligand gating of BK channels (16, 27).
Architecture of Prokaryotic RCK-containing Channels—We were intrigued by the absence of a methionine residue in the post-TM2/pre-RCK linker region of TvoK (Fig. 1), because of the apparently critical role of an internal AUG start codon in generating soluble RCK domain for the assembly of MthK and its relatives (2, 13, 28). After we determined that TvoK contained a rare UUG start codon in this region, we conducted a BLAST search of RCK-containing K^+ channels using the TvoK sequence as bait and found several other examples of pre-RCK putative rare start codons (either UUG or the less-rare GUG). These include putative K^+ channels from Treponema denticola (NP973037), Bacillus licheniformis (YP092836), Oceanobacillus iheyensis (NP693255), Bacillus subtilis (NP391010 and CAB07933), Bacillus sp. NRRL B-14911 (ZP01173121), Deinococcus radiodurans (NP296057), and Synechocystis sp. PCC 6803 (NP440478). It therefore seems likely that in many prokaryotes the genes encoding such RCK-containing K^+ channels contain an alternative start codon, resulting in translation of both membrane-tethered and soluble RCK domains that assemble to form hetero-octameric channels.

Although UUG is relatively rarely used as a start codon in bacteria (used in ~3% of E. coli genes, compared with 14% for GUG and 83% for AUG), it is used more commonly in archaean genes and is the most common start codon found in genes from the archaea Aeropyrum pernix (42%, compared with 31% using AUG) (22, 29, 30). This may be an important consideration in analyses of genes encoding membrane proteins, such as RCK-containing channels and transporters.

We have been able to construct a functional chimeric channel using the transmembrane and cytoplasmic linker region of MthK and the RCK domain of TvoK that qualitatively preserves ligand activation properties of TvoK. This demonstrates that, at least for these similar channels, the RCK domain is modular, and it implies that a qualitatively similar ligand-dependent conformational change occurs in the two RCK domains that can be coupled to opening of the MthK pore.

**Allosteric Mechanism and the Energetic Contributions of Divalent Binding to Channel Opening**—We have observed that the TvoK RCK domain is modular, in the sense that it can be transplanted onto an MthK pore and confer upon this pore the Ca^{2+} and Mg^{2+} activation properties of TvoK. This result implies that both the Ca^{2+} and Mg^{2+} binding sites are located within the TvoK RCK domain and that ligand binding in the RCK domain is coupled to channel gating via an allosteric mechanism.

If we assume that the channel contains eight Ca^{2+} binding sites, consistent with one Ca^{2+} binding site within each RCK domain, then we can describe the TvoK P_o versus Ca^{2+} relation (corresponding to Scheme 1) using Equation 1

\[ P_o = \frac{1}{1 + (1 + K)^n} \]

where \( K = [\text{Ca}^{2+}] / K_m \) is the equilibrium constant for channel opening in the absence of ligand, and C is the allosteric coupling factor for Ca^{2+} binding and channel opening.

By fitting the single channel data with Equation 1, we can use the fitted parameters to obtain estimates of the free energy difference between open and closed states with a given number of Ca^{2+} bound to the channel, using Equation 2

\[ \Delta G_{\text{Ca}^{2+}} = -RT \ln(C) \]

\[ P_o \text{ data measured from channels over a range of Ca}^{2+} \text{ from 0.1 to 25 mM were fitted with Equation 1, which yielded parameter estimates of } K_m = 1.3 \text{ mM, } L = 0.0018, \text{ and } C = 2.6. \text{ These are consistent with net free energy changes of } +3.7 \text{ kcal/mol for transition to the open state for unliganded channels and } -0.8 \text{ kcal/mol for fully liganded channels (from Equation 2). Thus, each Ca}^{2+} \text{ binding provides a net contribution of roughly 0.57 kcal/mol toward stabilization of the open state (total of } \sim 4.6 \text{ kcal/mol for eight Ca}^{2+}. \text{ From Equation 2, these parameters suggest that the binding of at least seven out of eight Ca}^{2+} \text{ is required to render the open state energetically more favorable than the closed state.}

The apparent effectiveness of Mg^{2+} for channel activation was similar to that of Ca^{2+} and yielded similar estimates for the energetic contribution to open state stabilization. For fits with Mg^{2+}, we constrained the estimate of \( L = 0.0018 \) to agree with the estimate of \( L \) obtained from fits with Ca^{2+}, and this fit gave a \( K_m \) for Mg^{2+} of 1.3 mM and \( C = 2.9 \), consistent with a net energetic contribution of 0.63 kcal/mol/Mg^{2+} toward stabilization of the open state.

Because these parameter and energetic estimates depend on the estimate of \( L \) and we have been unable to directly measure \( P_o \) in the absence of ligand for these channels, we analyzed further the effect of \( L \) on our energetic estimates. Because 0.0018 is likely to be an overestimate of the true value of \( L \), we performed this analysis by setting \( L \) to a range of values <0.0018 and obtaining new estimates of \( C \) for each value of \( L \). For Mg^{2+}, we found that for \( L = 1.8 \times 10^{-4}, C = 3.9 \), and for \( L = 1.8 \times 10^{-5}, C = 4.9 \), consistent with contribution of 0.80 kcal/mol/Mg^{2+} and 0.94 kcal/mol/Mg^{2+} toward stabilization of the open state. Similarly, we found that for Ca^{2+}, with \( L = 1.8 \times 10^{-4}, C = 3.4 \), and for \( L = 1.8 \times 10^{-5}, C = 4.5 \), consistent with contribution of 0.72 kcal/mol/Ca^{2+} and 0.89 kcal/mol/Ca^{2+} toward stabilization of the open state.
Relation between the MthK Ca\(^{2+}\) Binding Site and the TvoK RCK Domain and Possible Mechanisms Underlying Divalent Ion Selectivity—In the MthK RCK domain, Ca\(^{2+}\) is bound to seven oxygen ligands, consisting of 1) the two oxygens of the Asp-184 side chain, 2) one oxygen from each of Glu-210 and Glu-212, and 3) three waters of hydration, one of which appears to be stabilized through hydrogen bonding with a nearby serine hydroxyl group (2, 12). Sequence alignment suggests that at least two of these side chain ligands may be present in the TvoK RCK domain, at positions Asp-192 and possibly Glu-226, which align closely with the MthK residues Asp-184 and Glu-210 (Fig. 1). The TvoK residue that aligns with Glu-212, however, is not a glutamate but an arginine (Arg-228).

Might Glu-212 be critical for the selectivity of Ca\(^{2+}\) over Mg\(^{2+}\) in MthK? At the MthK Ca\(^{2+}\) binding site, a divalent cation exchanges four hydration waters for protein oxygens (12); although this dehydration may be energetically favorable for Ca\(^{2+}\), it may not be favorable for Mg\(^{2+}\) (31). In TvoK, the absence of a glutamate at this position might allow either Ca\(^{2+}\) or Mg\(^{2+}\) to occupy the site in a more hydrated state.

Alternatively, sequence differences between TvoK and MthK could generate a difference in the spatial arrangement of side chains at the binding site and thus a difference in ligand selectivity between MthK and TvoK at this site. It is also possible that TvoK may contain a different complement of ligand-coordinating groups within this binding pocket or it may contain an additional binding pocket at a different location.

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