The MlotiK1 channel transports ions along the canonical conduction pore

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Received 28 August 2007; accepted 10 September 2007

Available online 19 September 2007

Edited by Julian Schroeder

Abstract  Although the cyclic nucleotide-modulated potassium channel from Mesorhizobium loti, MlotiK1, is easily studied using a ⁸⁶Rb⁺ flux assay, its comparatively low activity raises serious concerns about the integrity of the purified protein. We investigated the pathway of uptake using a multi-pronged approach. First, we probed the conduction pathway using quaternary ammonium compounds known to block conduction in eukaryotic K⁺ channels. Second, we examined the effect of chemical modification of putative pore-lining residues. Our results are consistent with ions traversing MlotiK1 along a conduction pathway like that of the eukaryotic channels, but at a much slower rate.

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Keywords: Cysteine modification; Quaternary ammonium; Polyamine; Potassium channel

1. Introduction

Ion channel proteins modulated directly by intracellular cyclic nucleotides (CN channels) play pivotal roles in many eukaryotic cell types [1]. Genes encoding CN channels are also found in several bacterial species, but the native function(s) of prokaryotic CN channels remains unknown [2,3]. One of these proteins, the MlotiK1 channel from Mesorhizobium loti, is experimentally amenable to milligram-scale expression and purification [4]. The soluble cyclic nucleotide binding domains (CNB domains) from MlotiK1 have been crystallized in both liganded and unbound conformations [5,6], and the combination of these two structures provides an unique framework for understanding the molecular basis of modulation by cyclic nucleotide.

Prokaryotic channel function is typically studied with electrophysiological recordings or through radioactive flux assays [7–9]. MlotiK1 activity has proven stubbornly resistant to the former approach, but has yielded to the latter [4,5]. MlotiK1 mediates ⁸⁶Rb⁺ uptake into proteoliposomes, and it displays the nucleotide-dependent activity expected of a CN channel [4,6,7,10,9]. However, the kinetics of uptake are at least 60-fold slower than those of other prokaryotic K⁺ channels [8]. The slow kinetics, coupled with an inability to see activity of the purified MlotiK1 channel in planar lipid bilayers, raises a concern about the physical nature of ⁸⁶Rb⁺ translocation in the uptake assay: perhaps uptake is not through the normal conduction pathway, but rather reflects ⁸⁶Rb⁺ “sneaking” through the membrane in a MlotiK1-dependent fashion, possibly along the bilayer–protein interface.

These experiments use compounds known from functional and structural studies to act along the central symmetry axis that forms the conserved conduction pathway of potassium channels to reexamine the functional pathway of ion translocation mediated by MlotiK1.

2. Materials and methods

2.1. Materials and reagents

Decylmaltoside was obtained from Anatrace (Maumee, OH, USA), E. coli polar lipids from Avanti Polar Lipids (Alabaster, AB, USA), PA and QA compounds from Fluka (Ronkonkoma, NY, USA), ⁸⁶Rb⁺ from GE Healthcare (Piscataway, NJ, USA), cAMP, βME, DTT, and imidazole from Sigma Chemical Co. (St. Louis, MO, USA), and MTSEA from Toronto Research Chemicals (North York, ON, Canada).

2.2. Clones and mutagenesis

The WT and cysteine-free MlotiK1 genes with C-terminal hexahistidine tags in the pASK90 expression plasmid were described previously [5,6]. Cysteine mutations were generated using QuickChange mutagenesis (Stratagene, La Jolla, CA) and confirmed by DNA sequencing.

2.3. Expression and purification

Expression and purification were adjusted from [5,6] as follows: membrane protein was solubilized with 25 mM DM and bound to Ni²⁺-matrix in the presence of 25 mM imidazole (pH 7.8). Wash and elution buffers contained 5 mM DM, 25 mM imidazole (pH 7.8) or 5 mM DM, 500 mM imidazole (pH 7.0), respectively. 5 mM βME was used as the reducing agent throughout, except for studies of the A211C single cysteine mutant, where it was omitted from the final size exclusion chromatography run and all subsequent steps.

2.4. Functional assays

Flux assays were performed as described [6] on MlotiK1 reconstituted into E. coli polar lipid at 5 μg protein per mg lipid. Time course data are presented as means ± S.D., n = 3, from a single day, with similar data obtained on three separate days. All other data are shown as means ± S.E., performed in triplicate 2–9 times. Where not visible, error bars are smaller than symbols. Different preparations displayed variability in the “leakiness” of control vesicles (in the absence of protein) and the total fraction of vesicles that contain MlotiK1. By focusing on either protein-dependent or cAMP-dependent activity, our experiments avoided complications due to this variability.

Channel blockers were added to extraliposomal solutions from 100× stocks dissolved in sorbitol buffer immediately prior to addition of ⁸⁶Rb⁺. Uptake was monitored after 1.5 h.
For MTSEA experiments, vesicles containing A211C were reacted with 1 mM MTSEA for 30 min prior to exchanging extraliposomal KCl with sorbitol.

3. Results

3.1. Permeation through MlotiK1

MlotiK1 activity is studied using a radioactive flux assay similar to that previously used with other purified and reconstituted channels including KcsA and CIC-0 [4,5,10–12]. In brief, an uneven distribution of K+ across the liposomal membrane generates a diffusion potential that drives 86Rb+ uptake (Fig. 1A). As shown in Fig. 1B, MlotiK1 displays basal activity in the absence of exogenous ligand that is stimulated roughly 2-fold by the addition of a saturating concentration of cAMP. In the experiments that follow, we consider the different components of activity in detail. We use the term “basal activity” to refer to that induced by MlotiK1 protein reconstituted in the absence of cAMP, and define it experimentally as the difference in uptake between control liposomes and vesicles containing MlotiK1 (Fig. 1B). In contrast, “cAMP-dependent uptake” is used to describe the uptake induced by addition of exogenous cAMP to the uptake assay, and is practically defined as the increase in uptake observed with the addition of maximally-stimulating concentrations of cAMP (Fig. 1B).

Even in the presence of saturating cAMP uptake does not saturate over the 3 h time period, raising the question of whether transport occurs along a properly folded and functional pore.

3.2. Inhibition by pore-blocking compounds

K+ channels are commonly inhibited by two different classes of compounds, QAs and PAs. Both act by physically occluding the conduction pore, and are thus well-suited for examining permeation through MlotiK1. The functional effects of the compounds on uptake are easily examined by adding them to the outside of vesicles immediately prior to the addition of 86Rb+.

We first examined the effect of symmetric QAs on both basal and cAMP-stimulated activity. As shown in Fig. 2A and B, the potency of the QA compounds increased with their physical size; in both cases, TBA was the most potent, followed by TPrA, and TEA had no effect over the concentration range tested. Although cAMP-dependent activity was completely inhibited by high concentrations of TBA (Fig. 2B), roughly 30% of the basal activity remained in the presence of saturating TBA (Fig. 2A).

The PAs putrescine, cadaverine and spermidine (Fig. 3A) were tested at a single concentration (100 μM) on total uptake observed in the presence of cAMP. All three compounds dramatically reduced flux by comparison with that observed in control vesicles (Fig. 3B).
3.3. Covalent modification of A211C

The pores of even distantly related channels contain substantial homology at the level of primary sequence (Fig. 4A). We used this homology, together with the KcsA and MthK crystal structures ([13,14] thought to represent closed and open channel conformations, respectively) as a basis for predicting the disposition of specific MlotiK1 residues (Fig. 4A). Specifically, A211 should lie within the cytoplasmic vestibule of MlotiK1 (Fig. 4B). Working with a cysteine-less MlotiK1 construct, we generated the A211C mutation to test whether chemical modifications of this side chain would affect channel function.

Methanethiosulfonate (MTS) reagents have proven utility in mapping the surface of the conduction pathway in many ion channels. MTSEA is membrane-permeant, and therefore particularly suitable for use on MlotiK1 protein reconstituted into vesicles, where the absolute orientation of channel remains unknown. Incubation of 1 mM MTSEA with vesicles containing the A211C mutant protein in the presence of cAMP reduced uptake to that of control vesicles containing no protein (Fig. 4C). Although unreacted MTS reagent is not removed from the interior of the vesicle sample in these experiments, two control experiments demonstrate that MTSEA acts through a covalent modification of the cysteine side chain. First, activity of MTSEA-treated A211C protein was recovered by a 30 min incubation with the membrane-permeant reducing agent DTT (Fig. 4C), and second, the effect of MTSEA requires the cysteine sidechain, as MTSEA has no effect on uptake by the cysteine-free MlotiK1 channel (Fig. 4D).

These experiments were conducted in the presence of cAMP, but the finding that MTSEA reduces uptake to the level observed in the no-protein control indicates that it must inhibit both basal and cAMP-dependent uptake.

If A211C is indeed readily reactive, then we should be able to observe its reaction with MTSEA directly during the course of an experiment. As expected, acute treatment of vesicles with MTSEA stalls subsequent uptake (Fig. 4E), while MTSEA-inhibited uptake can be recovered by the addition of 10 mM DTT (Fig. 4F). These results indicate that the MTSEA reaction and subsequent reduction by DTT are both rapid processes compared to the slow time-course of Rb⁺ uptake.

4. Discussion

We used two parallel approaches, functional studies of channel inhibitors and site-specific chemical modification, to verify that ³⁶Rb⁺ uptake proceeds along the central conduction pathway.

QAs and PAs are distinct classes of potassium channel inhibitors. With the notable exception of TEA, these compounds inhibit K⁺ channels only from the intracellular side [1,15]. Structure–function studies in numerous K⁺ channels firmly establish that both QA and PA compounds bind within the conduction pathway and physically occlude ion translocation [16–18]. QA compounds are generally more specific for potassium channels, while PAs inhibit a variety of eukaryotic channels, including inward rectifier K⁺ channels, cyclic-nucleotide gated channels, ionotropic glutamate receptors, and Na⁺ and Ca²⁺ channels [19]. The effects of QAs on MlotiK1-mediated flux exactly mirror their actions on the related eukaryotic channels. TEA typically has modest (mM) affinity in K⁺ channels; we did not observe inhibition up to the practical limitation of this assay of 100 µM. Other QA compounds display the same order of efficacy in MlotiK1 as they do at the internal vestibule of eukaryotic K⁺ channels [20], with Kᵢ decreasing as hydrophobicity increases. Moreover, the actual values for Kᵢ observed in MlotiK1 (2 µM and 70 µM for TBA and TPRA, respectively) are even lower than those found in eukaryotic channels (with apparent dissociation constants of 32 µM and 650 µM, respectively in the delayed rectifier K⁺ channel of the squid giant axon [20]).

The effect of polyamines in MlotiK1 is particularly reminiscent of that observed in the cyclic nucleotide-gated channel CNGA1 [21]: inhibition occurs in the micromolar concentration range, and spermidine acts more strongly than putrescine. The selectivity and affinity of the QA effects are most easily explained if, like in traditional K⁺ channels, we did not observe inhibition up to the pratical limitation of this assay of 100 µM. Other QA compounds display the same order of efficacy in MlotiK1 as they do at the internal vestibule of eukaryotic K⁺ channels [20], with Kᵢ decreasing as hydrophobicity increases. Moreover, the actual values for Kᵢ observed in MlotiK1 (2 µM and 70 µM for TBA and TPRA, respectively) are even lower than those found in eukaryotic channels (with apparent dissociation constants of 32 µM and 650 µM, respectively in the delayed rectifier K⁺ channel of the squid giant axon [20]).

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Our observation of inhibition of A211C mutant protein by MTSEA provides independent evidence of functional similarity between MlotiK1 and related channels. MTS compounds have been used extensively to examine gating and conduction in numerous channels homologous to MlotiK1. In the three eukaryotic channels Shaker, CNGA1, and SpHCN, there are functional consequences of modifying the residue analogous to A211 in MlotiK1 (V478 in Shaker, G395 in CNGA1, T464 in SpHCN) [22–24].

The data from both inhibitor and chemical modification experiments of MlotiK1 closely mirror that from homologous K⁺ channels. We do not yet understand the structural basis of basal activity observed in the absence of exogenous cAMP; it may reflect a very low open probability, an open state with a
lo conductance, or both. The finding that a substantial fraction of the basal activity and all the cAMP-dependent component are blocked by QA ions and MTS reagents is most simply explained if the principal transport pathway through MlotiK1 for basal and cAMP-mediated uptake occurs along the canonical conduction pore conserved in the related K⁺ channels. The

Fig. 4. Chemical modification of MlotiK1. (A) Sequence alignment of selectivity filter and adjacent transmembrane helix from related channels. The conserved glycine hinge is highlighted. A211 in MlotiK1 is shown with a ★. Sequences and their accession numbers are: MlotiK1 (gi: 14023393), KcsA (gi: 1089905), Drosophila Shaker, (dShaker; gi: 157064), rat Kv2.1 (rKv2.1; gi: 698120), MthK (gi: 2622632), human Slo (hSlo; gi: 4639628), bovine CNGA1 (bCNGA1; gi: 27805875), bovine CNGA2 (BCNGA2; gi: 287744), and sea urchin HCN (SpHCN; gi: 3242324). (B) The structures of KcsA (left) and MthK (right), with front and rear subunits removed for clarity. β-Carbons of residues analogous to A211 shown in CPK (KcsA, A111; MthK, L95). Structures were drawn using Molscript from PDB files 1BL8 (KcsA) and 1LNQ (MthK). (C,D) Uptake into liposomes in the presence of 0.1 mM cAMP containing A211C (C) or cysteine-less (D) MlotiK1. An aliquot of liposomes was pretreated with MTSEA prior to the uptake assay. In the case of A211C, an MTSEA-treated aliquot was further incubated with 5 mM DTT for 30 min prior to use. (E) Time course of MTSEA inhibition of uptake into vesicles containing A211C mutant protein in the presence of 0.1 mM cAMP containing A211C (C) or cysteine-less (D) MlotiK1. An aliquot of liposomes was pretreated with MTSEA prior to the uptake assay. In the case of A211C, an MTSEA-treated aliquot was further incubated with 5 mM DTT for 30 min prior to use. (E) Time course of reversing MTSEA inhibition in the presence of 0.1 mM cAMP. Vesicles containing A211C MlotiK1 were treated with MTSEA prior to initiation of uptake. At the time indicated by the arrow, DTT was added to one aliquot (■) at a final concentration of 10 mM from a 1 M stock solution, while the other untreated aliquot served as a control (▲).
question of where the small fraction of basal uptake which is resistant to QA ions originates remains open; it seems unlikely to proceed through the conduction pore, but may instead come from ion movement along the lipid–protein interface.

If MlotiK1 conducts ions along the same pathway as other K⁺ channels, it is unclear why conduction through MlotiK1 is so slow. In the experiments shown here at least part of the answer may reflect our use of ⁸⁶Rb⁺ as the tracer. Both K⁺ and Rb⁺ traverse the same permeation pathway in K⁺ channels [25], and K⁺ channels are generally equally permeant to Rb⁺ and K⁺ under biionic conditions [26,27]. Yet kinetically there are often differences between the two ions: Rb⁺ can have a 10–15-fold lower conductance than K⁺ [27]. We used ⁸⁶Rb⁺ for practical reasons; our experiments focused on the physical nature of the permeation pathway and not the kinetics of the permeation process, and ⁸⁶Rb⁺ is far more experimentally suited than ⁴₂K⁺ (with a half-life of ~19 days as compared to ~12 h for ⁴₂K⁺).

Finally, we wonder whether polyamines might modulate prokaryotic channels in vivo. The physiological function of the related inward-rectifier K⁺ (KIR) channels depends on the related inward-rectifier K⁺ (KIR) channels depends on intracellular block by the native polycationic polyamines spermine, spermidine, putrescine, and cadaverine [19]. PAs are clearly important in prokaryotic physiology, where they play roles in cell division and proliferation, and protein synthesis [28,27,26]. The compounds are found in relatively high abundance: levels of spermidine and putrescine are 20- and 8000-fold higher, respectively, in E. coli than in eukaryotic brain tissue [28,29]. Similar PA concentrations in Mesorhizobium loti would undoubtedly affect the native activity of MlotiK1.

Acknowledgements: We thank S. Altieri, M. Maduke and J. Morais-Cabral for helpful comments on an early version of the manuscript and G. Clayton for Molscript tutoring. Supported in part grants from the National Institutes of Health (GM61747 to L.H.) and the American Heart Association (0425831T to W.S.).

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