

Gating and Inward Rectifying Properties of the MthK K⁺ Channel with and without the Gating Ring

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In MthK, a Ca²⁺-gated K⁺ channel from *Methanobacterium thermoautotrophicum*, eight cytoplasmic RCK domains form an octameric gating ring that controls the intracellular gate of the ion conduction pore. The binding of Ca²⁺ ions to the RCK domains alters the conformation of the gating ring, thereby opening the gate. In the present study, we examined the Ca²⁺- and pH-regulated gating and the rectifying conduction properties of MthK at the single-channel level. The open probability (P_o) of MthK exhibits a sigmoidal relationship with intracellular [Ca²⁺], and a Hill coefficient >1 is required to describe the dependence of P_o on [Ca²⁺], suggesting cooperative Ca²⁺ activation of the channel. Additionally, intracellular Ca²⁺ also blocks the MthK pore in a voltage-dependent manner, rendering an apparently inwardly rectifying I-V relation. Intracellular pH has a dual effect on MthK gating. Below pH 7.5, the channel becomes insensitive to Ca²⁺. This occurs because the gating ring is structurally unstable at this pH and tends to disassemble (Ye, S., Y. Li, L. Chen, and Y. Jiang. 2006. *Cell*. 126:1161–1173). In contrast, above pH 7.5, a further increase in pH shifts the P_o-[Ca²⁺] relation towards a lower Ca²⁺ concentration, augments P_o at saturating [Ca²⁺], and activates the channel even in the absence of Ca²⁺. Channel activity is marked by bursts of rapid openings and closings separated by relatively longer interburst closings. The duration of interburst closing and the burst length are highly Ca²⁺ and pH dependent, whereas the kinetics of intraburst events is Ca²⁺ and pH independent. The rapid intraburst openings and closings are also observed with the isolated MthK pore lacking the attached intracellular gating ring. The fast kinetic events, independent of both Ca²⁺ and pH, therefore appear to be determined by processes occurring within the ion conduction pore, whereas the slow events reflect the gating process controlled by Ca²⁺ and pH through the gating ring.

INTRODUCTION

Potassium channels control the flow of K⁺ across the cell membrane and are ubiquitously expressed in nearly all organisms ranging from simple bacteria to humans (Hille, 2001). Ligand-gated K⁺ channels open in response to the binding of ligand molecules such as neurotransmitters, secondary messengers, or signaling proteins. Various kinds of ligands require a specific channel architecture to elicit the gating response (Ashcroft and Ashcroft, 1990; Wickman and Clapham, 1995; Finn et al., 1996; Xia et al., 1998; Magleby, 2003). The majority of prokaryotic ligand-gated K⁺ channels contain a conserved C-terminal ligand-binding domain named RCK for its role in regulating the conductance of K⁺ (Jiang et al., 2001; Kuo et al., 2005). RCK domains are α-β proteins that associate as homodimers, forming a bilobed structure similar to periplasmic binding proteins (Jiang et al., 2001; Roosild et al., 2002; Dong et al., 2005). The two lobes are joined by a flexible hinge, which creates a deep cleft where ligands bind. Despite sequence and structural similarities between different RCK domains, their ligands are diverse. Some RCK domains contain a conserved sequence motif for nucleotide binding (Schlosser et al., 1993; Bellamacina, 1996),

whereas others do not. RCK domains are also widely distributed in bacterial K⁺ uptake and efflux machinery (Bakker et al., 1987; Munro et al., 1991; Schlosser et al., 1993; Nakamura et al., 1998). Sequence alignment and mutagenesis studies have shown that RCK domains also exist at the intracellular C-terminal side of the eukaryotic high-conductance Ca²⁺-gated K⁺ channels (BK or maxiK) (Jiang et al., 2001; Pico, 2003).

MthK is an RCK-regulated Ca²⁺-gated K⁺ channel from *Methanobacterium thermoautotrophicum*. Like most K⁺ channels, MthK functions as a tetramer. Each subunit contains two membrane-spanning segments that encircle the ion conduction pore, along with an RCK domain at the C terminus. The X-ray structure of the opened MthK channel in the ligand-bound state revealed that a functional MthK channel requires an additional four RCK domains to form an octameric gating ring on the intracellular side of the pore (Jiang et al., 2002a,b). These additional RCK domains are translated from the *MthK* gene using an internal translational start site (Met107) and are coassembled from the cytosol. Each RCK domain interacts with its two neighboring

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Abbreviations used in this paper: CTX, charybdotoxin; DM, *n*-decyl-β-D-maltoside; NMG, *N*-methyl-D-glucamine.

subunits through two distinct interfaces. One interface (flexible interface) is extensive and holds two RCK domains into a tightly associated dimer. Despite the extensive protein–protein contacts at this interface, the two RCK domains can move relative to each other (Dong et al., 2005; Ye et al., 2006). The second interface, initially defined as the fixed interface, was later redefined as an assembly interface for its role in assembling four RCK dimers into an octameric ring (Ye et al., 2006). Eight Ca^{2+} ions are bound in the MthK structure, one per RCK domain, and Ca^{2+} binding was proposed to cause conformational changes in the gating ring leading to pore opening.

The structure of the MthK gating ring in its ligand-free closed state was determined recently and revealed a ring with a narrower diameter than that with Ca^{2+} bound (Ye et al., 2006). The expansion of the gating ring from closed to open states upon calcium binding could exert a mechanical stress on the pore-lining inner helices. The force applied would, in turn, open the intracellular gate of the pore by bending the membrane-spanning inner helices at a conserved glycine residue (gating hinge) as observed in the open MthK structure (Jiang et al., 2002a,b). A return to the ligand-free closed conformation would diminish this stress.

Crystal structures only provide snapshots of the channel in different states. A more complete understanding of the actual dynamics of channel opening and closing requires a real-time analysis of channel activity. Here we analyze two basic functional properties of MthK, ion conductance and gating, using single channel recordings in planar lipid bilayers. Our data indicate that MthK can be activated by both Ca^{2+} and pH, and the channel is inwardly rectified due to a voltage-dependent divalent cation blockage of the pore from the intracellular side. Our kinetic analyses reveal two gating processes: a slower gating process characterized by bursts of openings separated by relatively long interburst closings, and a fast gating process characterized by rapid intraburst openings and closings. Increasing $[\text{Ca}^{2+}]$ or pH primarily affects the slow gating process by increasing the duration of channel burst states and shortening the interburst closed period. These kinetic studies along with the structural information about MthK allow us to propose a minimal model to account for its gating process.

MATERIALS AND METHODS

Protein Expression and Purification

The gene coding for MthK was cloned into the pQE70 protein expression vector between Sph I and Bgl II restriction endonuclease sites with a thrombin cleavage site right before the C-terminal hexahistidine tag. The channel was expressed in *Escherichia coli* XL1-Blue or SG13009 cell cultures by induction with 0.4 mM isopropyl- β -D-thiogalactopyranoside at $A_{600} \sim 0.8$. Cells were harvested and lysed in 50 mM Tris-HCl, pH 8.0, 100 mM KCl,

containing leupeptin, pepstatin, aprotinin, and PMSF (Sigma-Aldrich) to inhibit proteases. Expressed channel was extracted directly from the cell lysate for 3 h at room temperature using 50 mM *n*-decyl- β -D-maltoside (DM from Anatrace). The extracted channel was purified on a Talon Co^{2+} affinity column (CLONTECH Laboratories, Inc.) and eluted with 5 mM DM, 20 mM Tris-HCl, pH 8.0, 100 mM KCl, and 300 mM imidazole. Protein eluted from the Co^{2+} column was incubated for 3 h at room temperature in the presence of 1.0 U thrombin (Roche) per 2.0 mg protein to remove the hexahistidine tag. The channel was then further purified on a superdex 200 (10/30) gel filtration column (GE Healthcare) in a buffer of 5 mM DM, 20 mM Tris-HCl, pH 8.0, and 100 mM KCl.

The membrane-spanning portion of the MthK channel that forms the ion conduction pore was obtained by limited trypsin digestion of the MthK channel. Trypsin was added to the purified MthK channel at a ratio of 1:50 (wt:wt). The digestion was allowed to proceed for 5 h at room temperature before the addition of trypsin inhibitor (from turkey egg white; Sigma-Aldrich) to inactivate the protease. The pore was then purified on a superdex 200 (10/30) gel filtration column (Pharmacia) in a buffer of 5 mM DM, 20 mM Tris-HCl, pH 8.0, and 100 mM KCl. The size of the pore containing residues from His11 to Arg101 was determined by MALDI time-of-flight mass spectrometry.

Protein Reconstitution and Functional Analysis

All lipids used in reconstitution were obtained from Avanti Polar Lipids. The MthK channel or its pore, purified in DM detergent, was reconstituted into lipid vesicles composed of 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE, 7.5 mg/ml) and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG, 2.5 mg/ml) using the same method as previously described (Heginbotham et al., 1999) except the following modifications. DM detergent was used to solubilize the lipid and dialysis (against a buffer of 10 mM HEPES, pH 7.2, 450 mM KCl, and 4 mM *N*-methyl-D-glucamine) was used to slowly remove the detergent from the detergent/lipid/protein mixture. To control the amount of channels in the synthetic lipid bilayers, various protein-to-lipid ratios (0.05–2 μg protein/mg lipid) were used in the reconstitution. Single channel data was recorded in a vertical lipid bilayer setup in which a planar lipid bilayer of POPE (15 mg/ml) and POPG (5 mg/ml) in decane was painted over a hole ($\sim 200 \mu\text{m}$) in a polystyrene partition separating the internal and external solutions. To induce fusion of channel-containing vesicles, the solution on the side to which vesicles were added (external side) contained 150 mM KCl, 10 mM HEPES, pH 7.2, while the opposite side (internal side) contained 15 mM KCl, 10 mM HEPES, pH 7.2. After the appearance of channels in the membrane, as monitored under voltage pulses, KCl concentration in the internal solution was raised to 150 mM. All recordings were performed with 150 mM KCl on both sides of the membrane. Channel orientation was determined from their inward rectification property. The pH of the solution on the intracellular side of the channel was adjusted by adding KOH or HCl from 1 M stock and was monitored by a pH meter. Unless otherwise specified, most recordings were performed with an intracellular pH of 7.5. Extracellular pH has little effect on channel activity, and therefore was maintained at pH 7.2 in all experiments. Ca^{2+} was added only to the intracellular side of the channel. In the study of pH activation (Fig. 4, C and D), 5 mM EGTA was added to the intracellular solution to eliminate any trace amounts of free Ca^{2+} coming from impurities in the chemicals used for solution preparation. The addition of EGTA decreased the single channel activity compared to recordings performed without EGTA.

Membrane voltage was controlled and current recorded using an Axopatch 200B amplifier with a Digidata 1322A analogue-to-digital

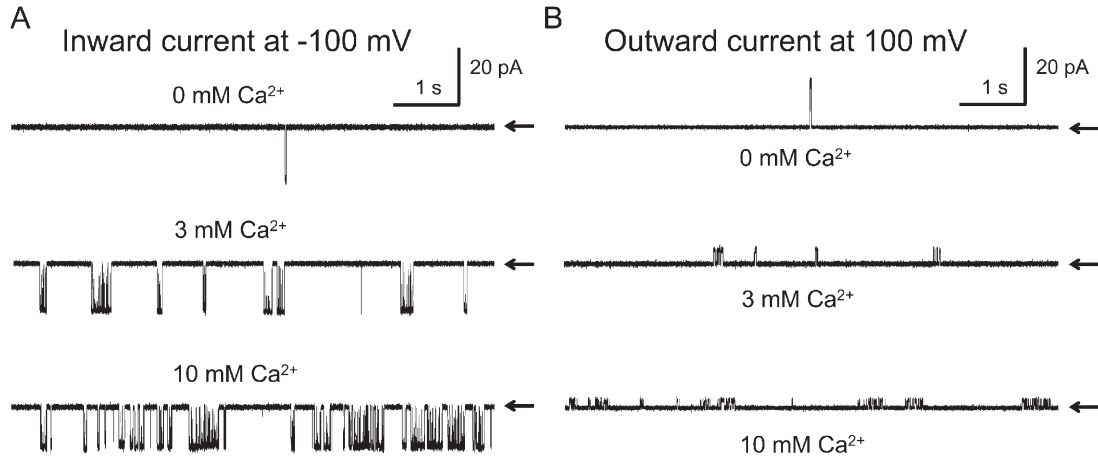


Figure 1. Single channel activity of MthK in the presence of 0, 3, and 10 mM Ca^{2+} . Currents were recorded with 150 mM symmetrical KCl at pH 7.5. Arrows to the right mark the zero current level. (A) Membrane voltage was -100 mV so that channel openings cause an inward current. (B) Outward currents recorded at 100 mV.

converter (Axon Instruments). Currents were low-pass filtered at 1–2 kHz and sampled at 10 kHz.

Data Analysis

Data analysis was performed using the QuB (Qin et al., 1996) and TAC (Bruxton) programs. All data are presented as mean \pm SEM. Inward currents were used to analyze the open probability and kinetics of the channel. Channel activity (NP_o) was measured as the mean current divided by the single channel current amplitude. The open probability (P_o) was determined by normalizing NP_o to the apparent number of channels (maximum number of superimposed events) observed in each recording. At pH ≥ 7.5 , the open probabilities of MthK as a function of $[\text{Ca}^{2+}]$ were fit with Hill equation:

$$P_o = P_{\max} / (1 + (K_{1/2} / [\text{Ca}^{2+}])^n), \quad (1)$$

where n is the Hill coefficient and $K_{1/2}$ is the Ca^{2+} concentration required for P_o to reach half maximum. In the analysis of voltage-dependent Ca^{2+} blocking in MthK, the fraction of unblocked outward current (I/I_o) at a specific voltage as a function of $[\text{Ca}^{2+}]$ was fitted with the following empirical Hill equation:

$$I/I_o = 1 / (1 + ([\text{Ca}^{2+}] / K_i)^n), \quad (2)$$

where I_o is the outward current at 0 mM $[\text{Ca}^{2+}]$, K_i is the inhibition constant which is equal to the concentration of the blocker required to achieve 50% reduction in current, and n is the Hill coefficient. The value of K_i obtained as a function of voltage was fit to the equation:

$$K_i(V) = K_i(0) \exp(-2\delta FV / RT), \quad (3)$$

where $K_i(0)$ is the inhibition constant at 0 voltage, δ is the electrical distance, and R , T , and F have their usual meanings. Only the inhibition constants obtained at voltages < 100 mV were used to fit the equation.

For analyses of single channel kinetics, burst states were defined as a sequence of openings separated by closings longer than a time chosen to equalize the number of misclassifications of the two closed states. The 50% threshold criterion was used to detect opening and closing events. To ensure a proper analysis of fast intraburst closing time with a mean duration of ~ 0.5 ms, we also performed kinetic analysis on the data that were low-pass filtered

at 10 kHz and obtained a time constant similar to that from data filtered at 1–2 kHz.

RESULTS

Functional Characterization of MthK in Lipid Bilayers

Purified MthK was reconstituted into lipid vesicles and its functional properties were studied in a vertical lipid bilayer membrane. Channels can be activated by adding Ca^{2+} to the intracellular side (Fig. 1). Single channel activity was marked by bursts of rapid openings and closings separated by relatively longer interburst closings. Initial recordings performed at pH 7.0 showed that the channel open probability measured from different bilayers varies significantly even at the same Ca^{2+} concentration. This variability has also been reported in a recent study on MthK by Zadek and Nimigean (2006). At this pH, the channel remains silent most of the time with occasional bursts of openings. Ca^{2+} can increase the burst events but the overall open probability of the channel remains low. Increasing intracellular pH to 7.5 gives rise to single channel activity with higher and more consistent sensitivity to $[\text{Ca}^{2+}]$. This pH-dependent Ca^{2+} activation can be attributed to the pH-dependent gating ring stability as we will discuss later.

The conductance of MthK exhibits inward rectification. In the inward direction, only one conductance state (~ 220 pS in the absence of Ca^{2+}) was observed under our experimental conditions (150 mM KCl on both sides of the membrane). At higher intracellular Ca^{2+} concentrations, there is a subtle decrease of the channel conductance, presumably due to rapid blocking by Ca^{2+} (Fig. 1 A), a phenomenon that has been observed in BK channels (Moczydlowski and Latorre, 1983; Vergara and Latorre, 1983; Cox et al., 1997). Channel conductance in the outward direction, on the other hand, has a strong

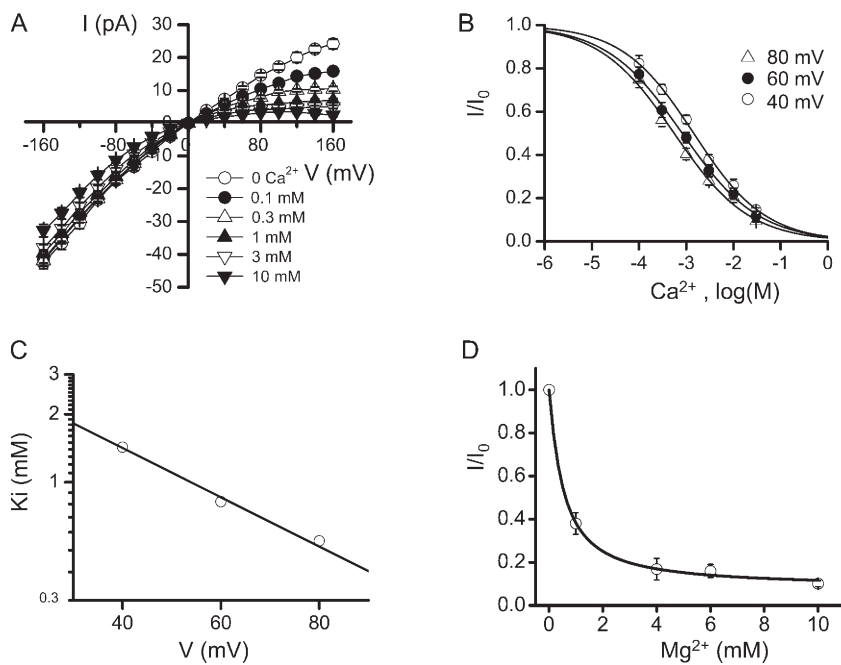


Figure 2. MthK is an inward rectifier K^+ channel. (A) I-V curve of MthK at various Ca^{2+} concentrations. Data points are mean \pm SEM from 5–7 separate bilayers. (B) $[Ca^{2+}]$ -dependent inhibition of outward currents recorded at 40, 60, and 80 mV. Curves are least square fits to Eq. 2 with Hill coefficients of 0.57 at 40 mV, 0.54 at 60 mV, and 0.55 at 80 mV. (C) Plot of inhibition constant (K_i) as a function of membrane voltage. Line is least square fit to Eq. 3. (D) $[Mg^{2+}]$ -dependent inhibition of outward currents recorded at 100 mV ($n = 5$). Curve is a least square fit to Eq. 2 with a Hill coefficient of 0.8.

$[Ca^{2+}]$ dependency. Increasing intracellular $[Ca^{2+}]$ reduces the outward conductance, the hallmark of an inward rectifier channel (Fig. 1 B).

Inward Rectification of MthK Is due to Ca^{2+} Blocking from the Intracellular Side

The inward rectification of MthK is due to voltage-dependent Ca^{2+} blocking from the intracellular side. As shown in the I-V curve of MthK at various Ca^{2+} concentrations, the blocking occurs at positive voltages and the rectification increases with increasing intracellular $[Ca^{2+}]$ (Fig. 2 A). Fig. 2 B plots the fraction of unblocked outward current (I/I_0) as a function of $[Ca^{2+}]$ at three different voltages. The least squares fits to Eq. 2 give rise to inhibition constants (K_i) of 1.43 mM at 40 mV, 0.82 mM at 60 mV, and 0.55 mM at 80 mV. Plotting the inhibition constants as a function of voltage (V) yields $K_i(0)$ of 3.96 mM and electrical distance (δ) of 0.32 from Eq. 3 (Fig. 2 C). Mg^{2+} has a similar blocking effect on MthK. A graph of the fraction of unblocked outward current at 100 mV as a function of intracellular $[Mg^{2+}]$ gives rise to a $K_i(100 \text{ mV})$ of ~ 0.45 mM (Fig. 2 D). The inward rectification of MthK is reminiscent of some eukaryotic inward rectifier K^+ (Kir) channels such as ROMK, whose rectification can be caused by voltage-dependent Mg^{2+} blocking from the intracellular side (Horie et al., 1987; Matsuda et al., 1987; Vandenberg, 1987). It is worth noting that the hydrophilic residue of the inner helix that conveys the rectification property on Kir channels (Lu and MacKinnon, 1994) is replaced by a hydrophobic residue (Phe87) in MthK. Further studies will be required to determine whether the inward rectification caused

by divalent cation blocking in MthK and Kir channels has the same molecular basis.

Voltage-dependent divalent cation (Mg^{2+}) blockage of outward K^+ currents from the intracellular side was also observed in the BK channel (Ferguson, 1991; Laver, 1992; Morales et al., 1996). A recent study on the BK channel suggested that the ring of eight negatively charged residues (two from each subunit, E321 and E324) at the intracellular entrance to the inner vestibule of BK channels facilitates Mg^{2+} blockage through a preferential electrostatic attraction of Mg^{2+} over K^+ (Zhang et al., 2006). The acidic residues also exist in MthK at equivalent positions (E92 and E96 from each subunit). Whether these acidic residues play a similar role in divalent cation blockage in MthK requires further investigation.

In our study, Ca^{2+} blocking appears to be the main cause of channel rectification. In the absence of Ca^{2+} , MthK exhibits weak rectification with an inward conductance of 220 pS and an outward conductance of 170 pS in 150 mM symmetrical KCl. This observed rectification is smaller than what was reported in a recent study on MthK by Zadek and Nimigean (2006). We believe this discrepancy is due to the presence of EDTA in their study, since we also observed decreased outward conductance when EDTA was added to the intracellular side.

Influence of the Gating Ring on Ion Conduction

Through limited trypsin digestion we were able to isolate the pore of MthK. This allowed us to analyze the conduction properties of the MthK pore without the attachment of the gating ring. The purified pore,

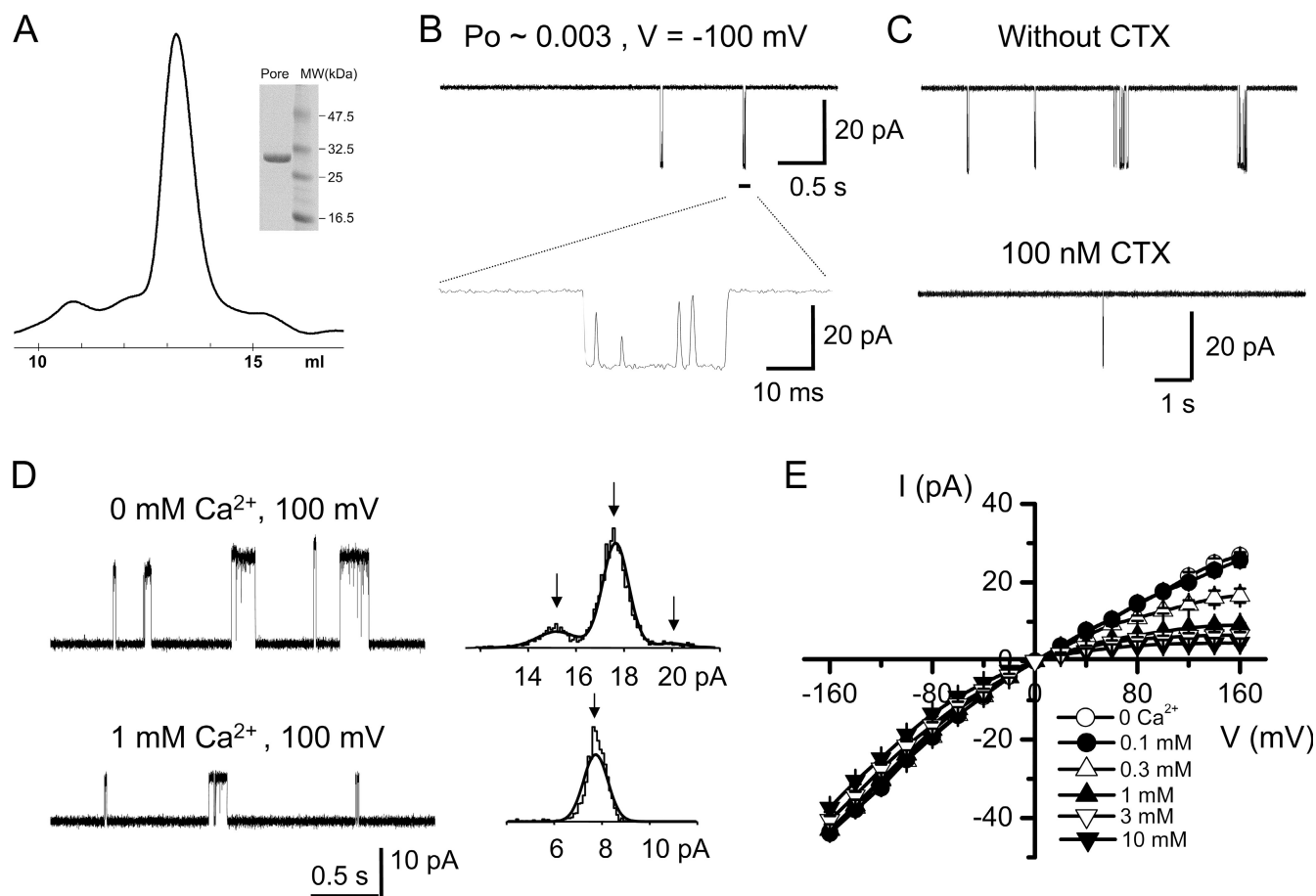


Figure 3. Conduction properties of the isolated MthK pore are like those of the wild-type channel. (A) Gel filtration (superdex 200, 10/30 column) profile and SDS-PAGE of the MthK pore purified in DM detergent. (B) A sample trace of the MthK pore recorded at -100 mV. The channel pore has low open probability ($NP_o \sim 0.003$ for this trace) and its activity exhibits the same burst behavior as the wild-type channel. The inward conductance of the pore is ~ 250 pS with 150 mM symmetrical KCl. (C) Single channel traces recorded at -100 mV in the absence and presence of 100 nM CTX at the extracellular side. Due to the extremely low open probability of the pore, high protein-to-lipid ratio was used in the reconstitution in order to have multiple channels in the bilayer. This allowed us to record a sufficient number of opening events and observe the CTX block. (D) The outward current of the MthK pore at 100 mV in the absence (upper trace) and presence (lower trace) of 1 mM Ca^{2+} . Amplitude histograms of the ionic current are shown beside each trace and the arrows indicate the peak currents. High protein-to-lipid ratio was used in the reconstitution in order to increase the frequency of opening events. (E) I-V curve of MthK pore at various intracellular $[Ca^{2+}]$. Data points are mean \pm SEM from five separate bilayers.

containing residues from His11 to Arg101 as indicated by mass spectrometry, elutes at a position equivalent to a tetramer on gel-filtration column (Fig. 3 A). Like KcsA, the MthK pore is very stable and remains a tetramer even on SDS-PAGE (Fig. 3 A).

The purified pore was reconstituted into lipid vesicles and its functional properties were studied in lipid bilayers. The activity of the pore was also characterized by short bursts of rapid openings and closings separated by long interburst closings (Fig. 3 B). The open probability of the pore was extremely low ($NP_o \sim 0.003$ for the single channel trace shown in Fig. 3 B), indicating that the channel pore is stable in its closed state. Similar to the wild-type MthK, the isolated pore can be blocked by charybdotoxin (CTX), a K^+ channel blocker, from the extracellular side (Fig. 3 C). Adding $[Ca^{2+}]$ or changing pH has no obvious effect on the single channel activity

of the pore, confirming that the intracellular gating ring is responsible for the Ca^{2+} - and pH-regulated gating of MthK.

The conductance of the pore in the inward direction was ~ 250 pS with 150 mM KCl on both sides, only slightly larger than that of the wild-type channel, indicating that the attachment of the gating ring has a small effect on channel conductance. In the absence of Ca^{2+} , the outward conductance of the pore was not homogeneous. Three conducting states were observed, with a state of 180 pS being predominant (Fig. 3 D, top trace). Adding Ca^{2+} to the intracellular side eliminated the heterogeneity of the conductance (Fig. 3 D, bottom trace) and increasing $[Ca^{2+}]$ decreased outward conductance just as in the wild-type channel (Fig. 3 E). Although we are unable to identify the cause of the heterogeneity of the outward conductance in the absence of Ca^{2+} , it is

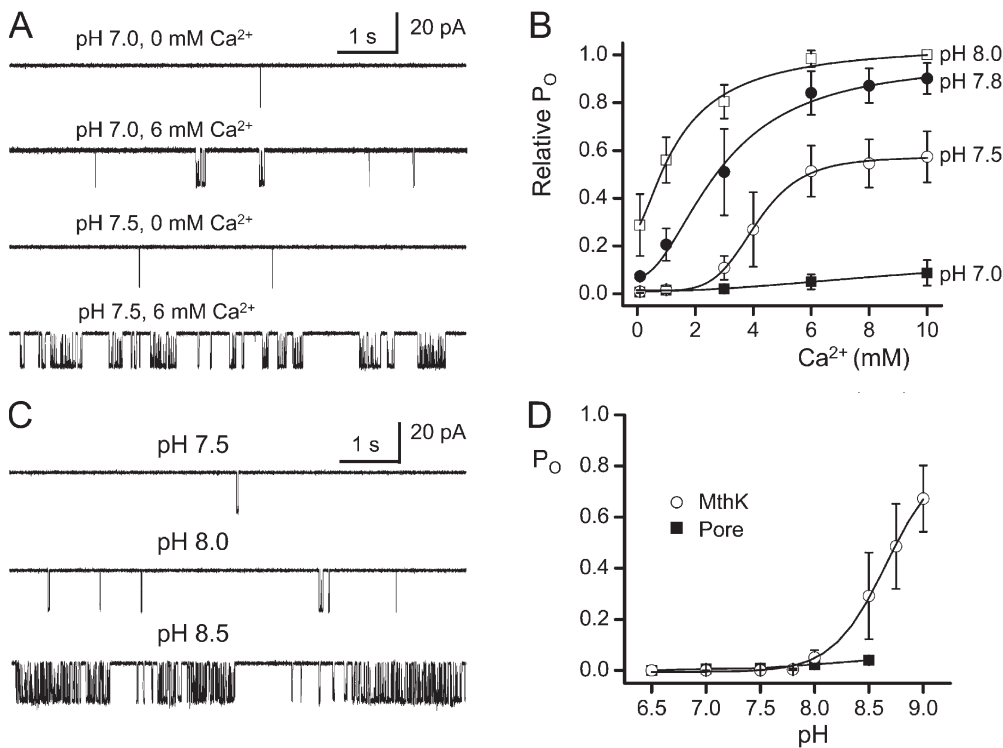


Figure 4. MthK can be activated by both Ca^{2+} and pH. (A) Single channel traces of MthK recorded at pH 7.0 (top two traces) and 7.5 (bottom two traces). (B) Plots of channel open probability as a function of $[Ca^{2+}]$ at various intracellular pHs. P_o of each measurement was normalized against the measurement of the same bilayer after adjusting the pH to 8.0 and $[Ca^{2+}]$ to 10 mM on intracellular side. Data are mean \pm SEM of four to six measurements. Fits of the data recorded at pH \geq 7.5 with the Hill equation yields Hill coefficients (n) of 4.3 at pH 7.5, 2.2 at pH 7.8, and 1.7 at pH 8.0; and $K_{1/2}$ of 4.1 mM at pH 7.5, 2.9 mM at pH 7.8, and 1.4 mM at pH 8.0. (C) Single channel activity of MthK at various intracellular pHs in the absence of Ca^{2+} . Sample traces were recorded from the same bilayer and intracellular solution contained 5 mM EGTA to eliminate trace amount of free Ca^{2+} . (D) Open probability of MthK and its pore at various intracellular pHs. Data are mean \pm SEM of four measurements.

clear that the MthK pore exhibits the same rectification as the wild-type channel. This confirms that the rectification is caused by voltage-dependent Ca^{2+} blocking of the pore from the intracellular side.

Activation of MthK is both pH and $[Ca^{2+}]$ Dependent

The presence of eight Ca^{2+} binding sites in the crystal structure of the open MthK channel (Jiang et al., 2002a) and the coordinated MthK movements of the gating ring inferred from the gating ring structures of closed and open conformations (Ye et al., 2006) suggest a cooperative activation of MthK by Ca^{2+} . However, this cooperative activation requires a stably assembled gating ring. Biochemical and structural studies have shown that the stability of the gating ring is pH dependent; the assembly of a stable octameric gating ring by eight RCK domains occurs near or above physiological pH (pH \sim 7.5), whereas lower pH destabilizes the protein-protein contacts at the assembly interface and disrupts the gating ring (Dong et al., 2005). The disruption of the gating ring will desensitize the channel to Ca^{2+} activation and result in a long period of silence even in the presence of Ca^{2+} (Ye et al., 2006). Indeed, Ca^{2+} activation of the channel exhibits the same pH dependence as the bio-

chemical stability of the gating ring; at pH 7.0, where RCK domains cannot form stable octameric gating rings, Ca^{2+} has a weak effect on channel open probability and the channel remains closed most of the time; at pH 7.5, where RCK domains are able to form the gating rings, Ca^{2+} increases the channel open probability in a concentration-dependent manner (Fig. 4 A), confirming the requirement of a preassembled gating ring for Ca^{2+} activation.

Between pH 7.5 and 8.0, the open probability (P_o) of MthK exhibits a sigmoidal relationship with $[Ca^{2+}]$, a characteristic of cooperative binding of ligand to a protein with multiple binding sites (Fig. 4 B). Fits of the data with the Hill equation yield Hill coefficients of 4.3 at pH 7.5, 2.2 at pH 7.8, and 1.7 at pH 8.0; and $K_{1/2}$ of 4.1 mM at pH 7.5, 2.9 mM at pH 7.8, and 1.4 mM at pH 8.0. Higher pH not only shifts the P_o - $[Ca^{2+}]$ relation toward lower concentrations of Ca^{2+} but also increases the channel open probability at saturating $[Ca^{2+}]$. At pH 8.0 with 10 mM Ca^{2+} , the channel exhibits an extremely high open probability ($P_o \sim 0.96$) with long bursts that last several seconds separated by very short interburst closings. The increased channel open probability at saturating $[Ca^{2+}]$ from pH 7.5 to 8.0 may simply arise from

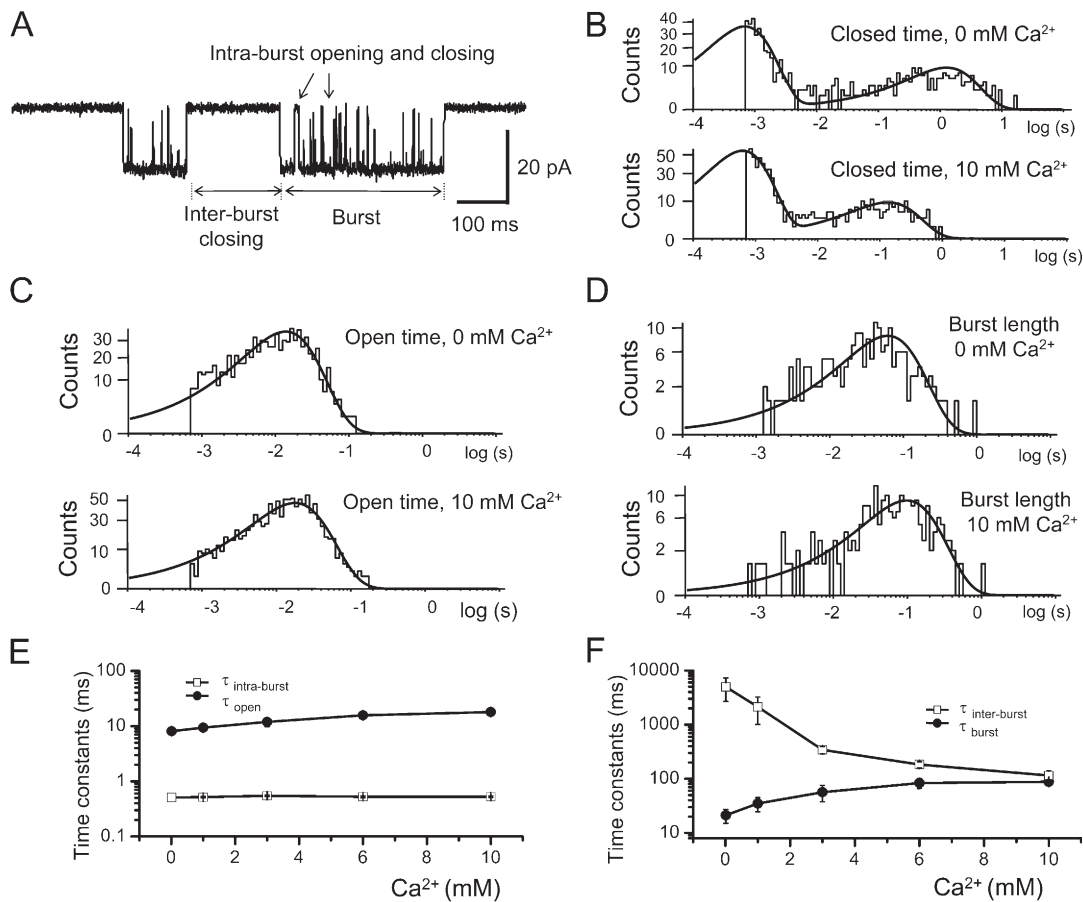


Figure 5. Kinetics analysis of Ca²⁺ activation at pH 7.5. (A) Illustration of burst, interburst closing, and intraburst opening and closing on a single channel trace. (B–D) Dwell time histogram of closed (B), open (C), and burst states (D) at 0 mM and 10 mM Ca²⁺. The number of counts (N) is scaled as a square root and the duration time is log binned (Sigworth and Sine, 1987). (E) Time constants of intra-burst opening (τ_{open}) and closing ($\tau_{\text{intra-burst}}$) at various [Ca²⁺]. (F) Time constants of burst length (τ_{burst}) and interburst closing ($\tau_{\text{interburst}}$) at various [Ca²⁺]. All data in E and F are mean \pm SEM from three separate single channel bilayers.

the stabilization effect of pH on gating ring assembly. It is likely that the gating ring at pH 7.5 is less stable than that at pH 8.0 and therefore has a higher probability to disassemble, leading to a longer closing of the channel and decreased channel open probability.

It is interesting to note that higher pH increases the Ca²⁺ sensitivity ($K_{1/2}$ decreases from 4.1 mM at pH 7.5 to 1.4 mM at pH 8.0) but decreases the cooperativity of Ca²⁺ activation as indicated by the Hill coefficients. This can be attributed to the fact that in addition to stabilizing the gating ring, pH can also activate the channel in the absence of Ca²⁺. As shown in the single channel traces of MthK recorded at different intracellular pH without Ca²⁺, the channel open probability increases from pH 7.5 to 8.5 (Fig. 4 C). A plot of P_o vs. pH shows that a significant increase in channel activity occurs at pH \geq 8.0 for MthK, whereas pH has no obvious effect on the open probability of the isolated channel pore (Fig. 4 D). It is likely that raising pH, similar to Ca²⁺ activation, increases the channel open probability by shifting the equilibrium of the gating ring conformations

toward the open state, which effectively reduces the amount of Ca²⁺ required for channel activation. This conclusion is further supported by the pH effect on single channel kinetics discussed below.

Kinetics of MthK–Ca²⁺ activation

The pH effect on MthK gating complicates the kinetic study of Ca²⁺ activation. On one hand, Ca²⁺ activation requires the assembly of the gating ring, which happens at a pH near or above 7.5. On the other hand, at pH \geq 8.0, channel activity increases even in the absence of Ca²⁺. We chose to analyze the Ca²⁺-dependent gating kinetics of MthK at pH 7.5, because at this pH the gating ring is able to form and the channel open probability is very low in the absence of Ca²⁺. Therefore, the increased channel activity at higher [Ca²⁺] can be attributed to the Ca²⁺-induced conformational changes of the gating ring.

Single channel behavior of MthK is characterized by bursts of rapid openings and closings separated by long interburst closings (Fig. 5 A). The dwell time distribution

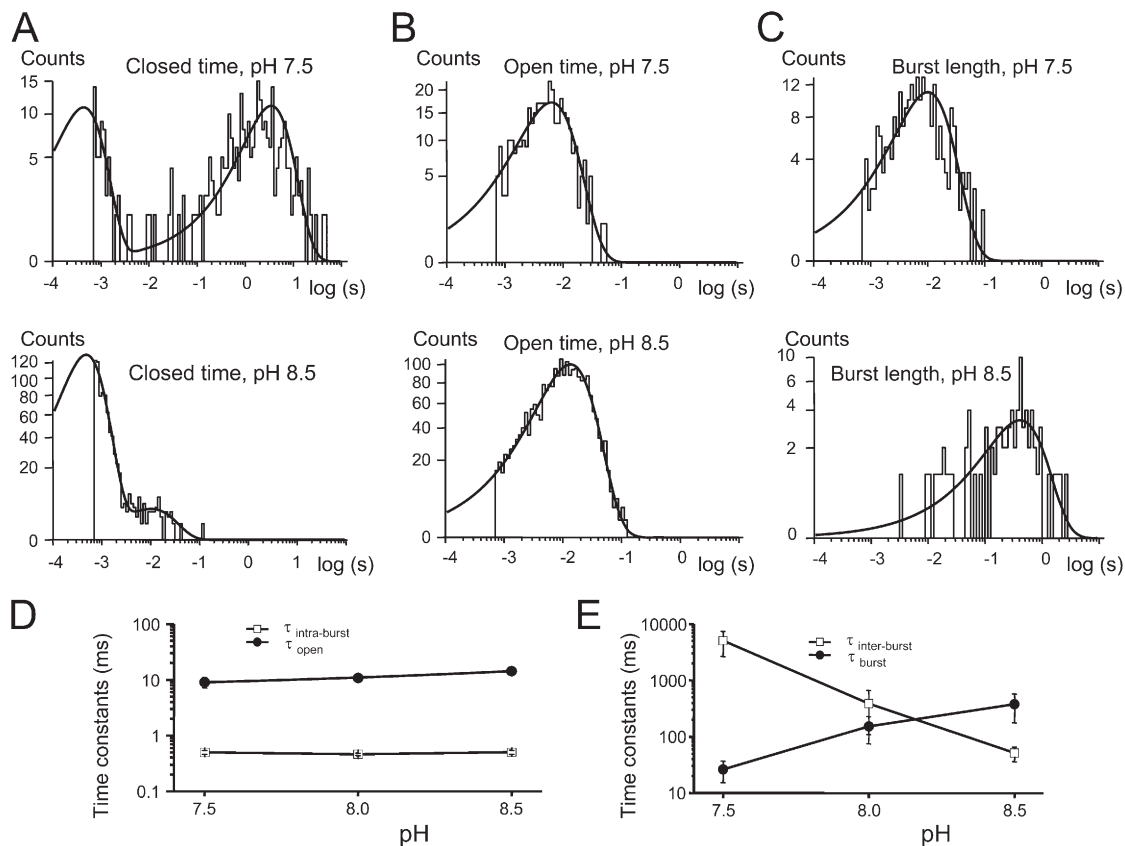


Figure 6. Kinetics analysis of pH activation. (A–C) Dwell time histogram of closed (A), open (B), and burst states (C) at pH 7.5 and 8.5. (D) Time constants of intraburst opening (τ_{open}) and closing ($\tau_{\text{intra-burst}}$) at various intracellular pHs. (E) Time constants of burst length (τ_{burst}) and interburst closing ($\tau_{\text{inter-burst}}$) at various intracellular pHs. All data in D and E are mean \pm SEM from three separate single channel bilayers.

of the closed states can be fit with two or three exponential components (Fig. 5 B). For simplicity, we chose to fit the data with two exponential components and only consider the short and long components. The short component corresponds to rapid closings within a burst (intra-burst closing) and is insensitive to changes in calcium concentration. The second component corresponds to the interburst intervals and has a long time constant that becomes shorter with higher $[\text{Ca}^{2+}]$. The dwell time distributions of open and burst states were fit with one exponential component as shown in Fig. 5, C and D, respectively. While the duration of bursts (burst length) increases with increasing Ca^{2+} concentration, individual open times (intra-burst openings) exhibit weak $[\text{Ca}^{2+}]$ dependence.

Fig. 5 (E and F) shows plots of time constants obtained from the analyses of dwell time distribution of the closed, open, and burst states at various Ca^{2+} concentrations. From 0 to 10 mM Ca^{2+} , the time constant of intra-burst closing remains unchanged ($\tau_{\text{intra-burst}} \sim 0.5$ ms) while the time constant of intra-burst openings (τ_{open}) has about a twofold increase, from 8 ms at 0 mM Ca^{2+} to 18 ms at 10 mM Ca^{2+} (Fig. 5 E). The time constants of the interburst closing ($\tau_{\text{inter-burst}}$) and burst length (τ_{burst})

show strong Ca^{2+} dependence. From 0 to 10 mM Ca^{2+} , $\tau_{\text{inter-burst}}$ decreases ~ 40 -fold while τ_{burst} increases ~ 5 -fold (Fig. 5 D), indicating that the rate out of the long interburst closed state has a stronger $[\text{Ca}^{2+}]$ dependence than the rate out of the burst state.

Kinetics of MthK–pH activation

To analyze the pH effect on MthK gating, we performed a similar kinetic analysis on the pH-activated channel in the absence of Ca^{2+} . Similar to Ca^{2+} activation, pH has little or no effect on the rates of rapid intra-burst closings and openings, but has strong effects on the rates of the burst and interburst closing events (Fig. 6, A–C). Fig. 6 (E and F) shows plots of time constants obtained from the analyses of dwell time distributions of the closed, open, and burst states at three pHs (7.5, 8.0, and 8.5). The time constant of rapid intra-burst closing remains at ~ 0.5 ms and the time constant of rapid intra-burst opening has a less than twofold change (τ_{open} is ~ 9 ms at pH 7.5 and 14 ms at pH 8.5; Fig. 6 D). From pH 7.5 to 8.5, the time constant of interburst closing is shortened by ~ 100 -fold while the burst length is increased by ~ 15 -fold (Fig. 6 E). The similarity of the gating kinetics between pH activation and Ca^{2+} activation

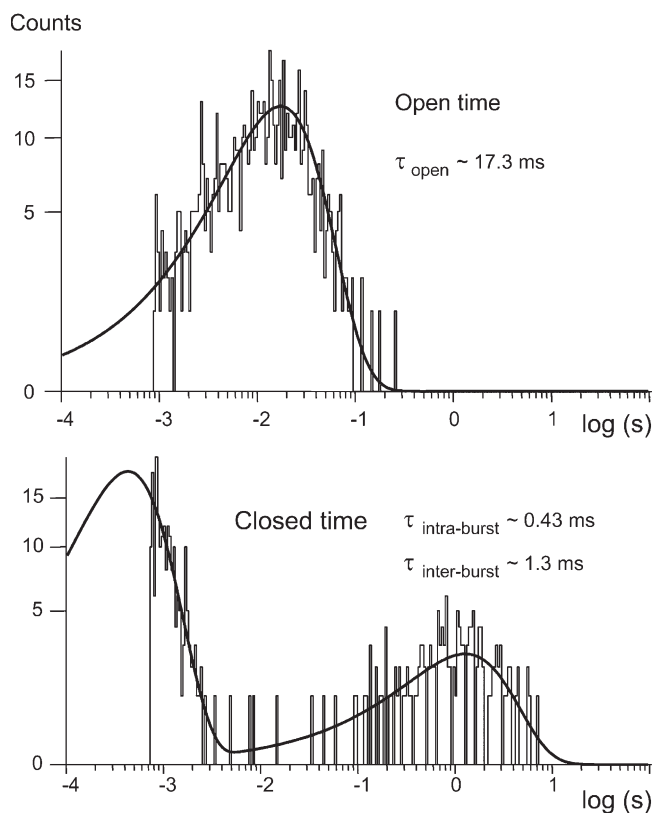


Figure 7. Dwell time histogram of open and closed states of the MthK pore. High protein-to-lipid ratio was used in the reconstitution in order to record sufficient amount of opening events for the analysis.

suggests that both Ca^{2+} and pH activate the channel by changing the equilibrium of the gating ring between open and closed states.

Rapid Intra-burst Opening and Closing Is an Intrinsic Property of the Ion Conduction Pore

Insensitivity of the intra-burst opening and closing to Ca^{2+} and pH suggests that this fast gating process is an intrinsic property of the pore and is not coupled to the gating ring conformational change. To test this possibility, we performed a kinetic analysis on the isolated MthK pore. Due to the extremely low open probability of the pore, high protein-to-lipid ratio was used in the reconstitution in order to have multiple channels in the bilayer. This allowed us to record a sufficient number of opening events for kinetic analysis. As in the wild-type channel, activity of the MthK pore is also marked by bursts of openings, but with short burst lengths and long interburst closings. Analysis of the dwell time distribution of intra-burst openings and closings gives rise to a τ_{open} of 17.3 ms and $\tau_{\text{intra-burst}}$ 0.43 ms (Fig. 7). Data recorded at different pHs, with and without Ca^{2+} give rise to the same time constants. The rates of the intra-burst openings and closings of the pore are similar to those of wild-type MthK ($\tau_{\text{open}} \sim 8\text{--}18$ ms and $\tau_{\text{intra-burst}}$

~ 0.5 ms), which suggests that the rapid intra-burst opening and closing is indeed an intrinsic property of the ion conduction pore. Due to the existence of multiple pores in the bilayer, we are unable to determine the time constant of interburst closing for a single channel pore, which should be much longer than the value (1.3 s) obtained in our measurements.

DISCUSSION

The MthK channel consists of two modules: a cytoplasmic signal-sensing module (the gating ring) responsible for binding calcium ions, and a membrane-spanning pore that facilitates diffusion of ions down their electrochemical gradient. The pore determines the channel conductance and conveys upon the channel the property of inward rectification that is caused by voltage-dependent divalent cation (Ca^{2+} and Mg^{2+}) blockage from the intracellular side. The cytoplasmic ligand-binding domain regulates channel gating by converting the chemical energy of Ca^{2+} binding to the mechanical force of pore opening. The octameric architecture of the MthK gating ring and its eight Ca^{2+} binding sites suggests a cooperative gating ring conformational change upon Ca^{2+} binding. Indeed, the open probability of MthK shows a sigmoidal relationship with $[\text{Ca}^{2+}]$. The magnitude of cooperativity, however, is pH dependent. This is because pH also plays an important role in channel activation. On one hand, Ca^{2+} activation of MthK requires a stably assembled gating ring that occurs near or above physiological pH (~ 7.5). On the other hand, higher pH (≥ 8.0) can by itself activate the channel even in the absence of Ca^{2+} . The pH activation is observed only in the full channel and not in its isolated pore. This, along with the similarity between the effects of pH and $[\text{Ca}^{2+}]$ on channel kinetics suggests that alkaline pH, like Ca^{2+} , regulates channel gating by changing the open/closed equilibrium of the gating ring conformations. Higher pH shifts the equilibrium toward the open conformation, which effectively reduces the amount of Ca^{2+} required for channel activation and leads to a reduction in the apparent cooperativity of Ca^{2+} binding. In the recent study of Ca^{2+} activation of MthK performed by Zadek and Nimigean (2006), an unusually high Hill coefficient ($n \sim 8$) was obtained. In their study, all single channel recordings were performed at pH 7.0, where the gating ring assembly is not stable. At this pH, most of the channel closing events are caused by the disruption of the gating ring instead of Ca^{2+} -dependent gating ring conformational changes between open and closed states. From our functional study presented here, along with our recent structural study (Ye et al., 2006), it is clear that the stabilization effect of pH on gating ring assembly has to be taken into account in studying Ca^{2+} activation.

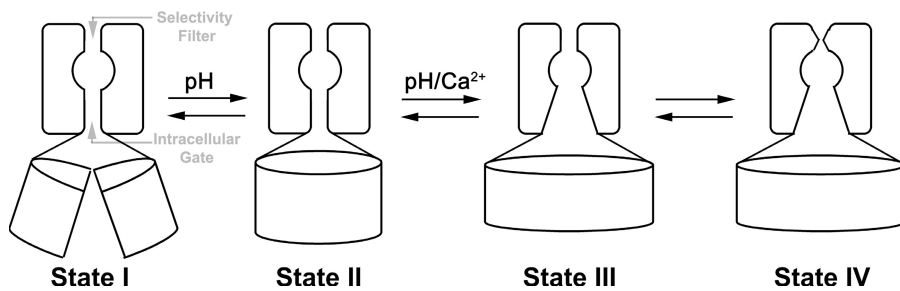


Figure 8. Proposed gating model of MthK.

The molecular basis of pH activation in MthK is unclear. However, comparison of the open and closed gating ring structures provides one possibility (Ye et al., 2006). In the closed gating ring, two neighboring RCK subunits establish an interfacial protein–protein contact that is not present in the open state. This interaction mainly involves salt bridges between basic and acidic residues. It is possible that elevated pH might weaken these salt bridges and destabilize the closed gating ring relative to its open conformation. Further studies are required to validate this speculation. Intracellular pH effects on channel activity have also been studied in BK channels (Laurido et al., 1991; Avdonin et al., 2003), whose C-terminal ligand binding domains were suggested to form a gating ring similar to MthK. Certain BK channels (i.e., Slo3) have been found to be regulated by intracellular pH instead of Ca²⁺, and their activation also occurs around pH 7.5–8.0 (Schreiber et al., 1998). Whether the pH activation of MthK and Slo3 share the same molecular mechanisms requires further investigation.

Under our experimental conditions, millimolar concentrations of Ca²⁺ are required for the activation of the MthK channel, significantly higher than physiological [Ca²⁺]. However, we believe that Ca²⁺ can serve as the physiological ligand for MthK based on a number of observations. First, pH can enhance the channel's sensitivity to Ca²⁺; K_{1/2} of Ca²⁺ activation decreases from 4.1 mM at pH 7.5 to 1.4 mM at pH 8.0. Second, as shown in a recent study of MthK, elevated temperatures significantly increase MthK sensitivity to [Ca²⁺]. At 37°C, channel open probability reaches ~0.7 in the presence of 0.1 mM Ca²⁺, close to physiological Ca²⁺ concentration (Parfenova et al., 2006). Considering this pH and temperature dependence of Ca²⁺ sensitivity and the optimal growth temperature of ~65°C for *M. thermoautotrophicum*, it is indeed possible that Ca²⁺ could be the physiological ligand for MthK.

The kinetic analysis of MthK indicates two gating processes: a slower process represented by bursts of opening and long interburst closings, and a faster process represented by the rapid opening and closing events within a burst. Rapid intraburst opening and closing is an intrinsic property of the pore, and [Ca²⁺] or pH has a negligible effect on its kinetics. The length of the burst and interburst closing, however, is [Ca²⁺] and pH

dependent. What is the physical location of these two gates? In a closed K⁺ channel, four pore-lining inner helices form a bundle crossing at the intracellular surface of the membrane as exemplified in the KcsA structure (Doyle et al., 1998; Zhou et al., 2001). This bundle crossing is believed to be the gate of the channel (Liu et al., 1997; Perozo et al., 1999; del Camino et al., 2000; del Camino and Yellen, 2001; Liu et al., 2001). Without external force, this intracellular gate of MthK is predominately in its closed state, as indicated by the extremely low open probability of the isolated MthK pore. The structures of MthK and its gating ring reveal that Ca²⁺-induced conformational changes result in a change in diameter of the gating rings (Jiang et al., 2002a,b). The expansion of the ring upon Ca²⁺ binding can exert a lateral force on the pore that disrupts the bundle crossing of the inner helices, bending them and opening the gate. A closed gating ring in the absence of Ca²⁺ has a smaller diameter, exerting less or no mechanical stress on the pore, and the channel remains closed. From the structural information, it is reasonable to suggest that the opening of this intracellular gate is tightly coupled to the Ca²⁺/pH-regulated conformational change of the gating ring. We therefore conclude that the observed Ca²⁺/pH-dependent burst and interburst periods in MthK activity correlate to the opening and closing of this intracellular gate. Where is the possible location of the fast gate that controls the rapid intraburst openings and closings? The fast gating is an intrinsic property of the pore and is insensitive to [Ca²⁺] or pH, indicating that this gating process is independent of the gating ring movement. The kinetics of the fast gating are sensitive to voltage (Zadek and Nimigean, 2006) and K⁺ concentrations (unpublished data), which suggests that the fast gating is related to the permeation process. The most likely region along the ion conduction pathway within the pore that can serve as the fast gate is the selectivity filter. Similar fast and slow gating kinetics have also been observed in inward rectifier K⁺ channels. Mutagenesis studies on Kir6.2 have shown that mutations around the bundle crossing primarily affect the slow gating kinetics (Trapp et al., 1998; Tucker et al., 1998), whereas mutations around the selectivity filter region primarily affect the fast gating kinetics (Proks et al., 2001). From these studies,

the bundle crossing and the filter were also proposed to be the slow and fast gates, respectively, in Kir channels.

Based on our kinetic study and the structures of MthK, we propose a minimal model to account for the gating processes in MthK (Fig. 8). In this model, MthK exists in four states and its gating processes involve three conformational equilibria. The equilibrium between states I and II involves the pH-dependent assembly and disruption of the gating ring. The gating ring is able to form at $\text{pH} \geq 7.5$, and the channel pore stays predominately closed in both states. Once the gating ring assembles, it undergoes a Ca^{2+} /pH-dependent conformational change between open and closed states (state II and III). Increasing pH ($\text{pH} \geq 8.0$) or Ca^{2+} concentration can both shift the gating ring conformation toward the open state. This conformational change is coupled to the opening and closing of the intracellular gate at the bundle crossing. The open gating ring stabilizes the intracellular gate in the open conformation, and the channel is in a conducting state (state III) represented by a burst event in a single channel trace. The equilibrium between states II and III reflects the slow gating process that is strongly influenced by $[\text{Ca}^{2+}]$ and pH. When the intracellular gate is open, the channel pore can still undergo a fast Ca^{2+} /pH-independent open/close equilibrium (states III and IV) as indicated by the rapid intraburst openings and closings. This fast gating process most likely occurs at the selectivity filter and is independent from the gating ring conformational change.

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