The Activation Gate of a Voltage-Gated K⁺ Channel Can Be Trapped in the Open State by an Intersubunit Metal Bridge

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Summary

Voltage-activated K⁺ channels are integral membrane proteins containing a potassium-selective transmembrane pore gated by changes in the membrane potential. This activation gating (opening) occurs in milliseconds and involves a gate at the cytoplasmic side of the pore. We found that substituting cysteine at a particular position in the last transmembrane region (S6) of the homotetrameric Shaker K⁺ channel creates metal binding sites at which Cd²⁺ ions can bind with high affinity. The bound Cd²⁺ ions form a bridge between the introduced cysteine in one channel subunit and a native histidine in another subunit, and the bridge traps the gate in the open state. These results suggest that gating involves a rearrangement of the intersubunit contacts at the intracellular end of S6. The recently solved structure of a bacterial K⁺ channel shows that the S6 homologs cross in a bundle, leaving an aperture at the bundle crossing. In the context of this structure, the metal ions form a bridge between a cysteine above the bundle crossing and a histidine below the bundle crossing in a neighboring subunit. Our results suggest that gating occurs at the bundle crossing, possibly through a change in the conformation of the bundle itself.

Introduction

In response to a membrane depolarization, voltage-activated K⁺ channels open a gate at the cytoplasmic entrance of the pore. The idea of such a gate was originally proposed by Armstrong, based on work with intracellularly applied quaternary ammonium (QA) blockers (Armstrong, 1966, 1969, 1971). He found that high affinity QA blockers like nonyltriethylammonium (C9) could block the K⁺ current particularly well after the channels have been opened, as if the access of QA blockers to their binding site is regulated by the gate that opens and closes the pore. The presence of a blocker can also prevent the gate from closing (by a “foot in the door” effect; Yeh and Armstrong, 1978). In some cases, the gate can be forced to close with a QA blocker still bound, trapping the blocking ion inside the pore (Armstrong, 1971; Armstrong and Hille, 1972; Holmgren et al., 1997). Because the QA ion cannot pass through the narrowest part of the pore (the selectivity filter), the trapping suggests that in closed channels there is a cavity located between the gate and the selectivity filter that can accommodate organic pore blockers up to about 12 Å in diameter.

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In our previous investigation of the activation gating mechanism of voltage-activated K⁺ channels, we studied the state-dependent accessibility of cysteines substituted at specific positions in the S6 region (Liu et al., 1997). Several S6 cysteines (470, 474, 476, and 478) are rapidly modified by chemical modification reagents applied from the intracellular side when the channel is open, but modification is much slower when the channel is closed, as if the gate that closes the channel prevents the chemical reagents from reaching these sites. Three of these positions—474, 476, and 478—apparently line the pore. These sites can be effectively protected from chemical modification by the binding of intracellular open channel blockers (e.g., tetrabutylammonium), as judged from the much lower modification rate in the presence of blocker. These results are consistent with the idea that the activation gate is located intracellularly and suggest that part of the S6 transmembrane segment lines the cavity between the gate and the selectivity filter.

Cysteines at some of these positions are also capable of forming high affinity sites at which Cd²⁺ ions bind and block K⁺ current through the channel. For example, at position 474, cysteines from the four different subunits can form a single high affinity binding site (Liu et al., 1997). Once Cd²⁺ is bound, it is trapped inside the pore by closing the activation gate, and the energetics of the transition between the open and closed states of a channel with a Cd²⁺ in the pore were not very different from the energetics of the same transition of a channel without Cd²⁺. Qualitatively similar results were obtained when Cd²⁺ was applied intracellularly to the mutant channel I470C. These results, together with a study of QA trapping in the I470C mutant channels (Holmgren et al., 1997), show that the presence of blockers does not make it substantially more difficult to close the channel, and suggest that even in the absence of blockers the channels close not by a general collapse of the pore but by the closure of a discrete intracellular gate.

To understand more about the molecular mechanism by which K⁺ channels close, we have pursued a remarkable effect of Cd²⁺ ion on a cysteine in a nearby position. With a cysteine at position 476, Cd²⁺ binding does not block K⁺ current through the channel. Instead, intracellularly applied Cd²⁺ traps the V476C mutant channels in the open state even at negative voltages. We have identified another amino acid residue that participates in the high affinity binding of Cd²⁺ and established that the Cd²⁺ effect involves an intersubunit bridge that stabilizes the open configuration of the channel. The results imply that the four S6 regions must intertwine to some extent to allow the two positions to coordinate Cd²⁺. Our results fit nicely with the recently published crystal structure of a related bacterial potassium channel, Kcsa, from Streptomyces lividans (Doyle et al., 1998). In that structure, the homologs of S6 cross in a bundle, like the four supporting rods of a teepee. Our results suggest that gating occurs at the bundle crossing, possibly through a change in the conformation of the bundle itself.
shown). The effect of Cd\textsuperscript{2+} on V476C mutant channels opened normally, but they could not close. This result suggests that the high affinity state with high affinity binding of Cd\textsuperscript{2+}—it slowed down activation and speeded up deactivation (thinner traces in Figures 2A and 2B)—but this effect was also observed in the wild-type channels (data not shown). Because DEPC is not completely specific for the modification of imidazole groups and can also modify amino groups, we also tried the amino-specific modification reagent trinitrobenzenesulfonic acid (TNBS; Means and Feeney, 1971) (Figures 2C and 2D). TNBS treatment did alter the kinetics of gating (thin traces), but it did not alter the ability of Cd\textsuperscript{2+} to trap V476C mutant channels in the open state (thick traces).

The results with DEPC treatment suggest that some histidine in the channel protein may collaborate with the 476C residue in coordinating Cd\textsuperscript{2+}. There are 13 histidines in the Shaker K\textsuperscript{+} channel, and twelve of them are located intracellularly (Figures 3A and 3B). Initially, our attention was drawn to histidine 378, located at the intracellular side of the S4 transmembrane region that forms part of the voltage sensor of the channel (Papazian et al., 1991; Aggarwal and MacKinnon, 1996; Seoh et al., 1996). However, eliminating this histidine (in V476C H378Y double mutant channels) did not prevent 20 \mu M Cd\textsuperscript{2+} from trapping channels in the open state (Figure 3B). The remaining 11 histidines were substituted, one at a time, with tyrosine, and the effect of Cd\textsuperscript{2+} was tested in each mutant.

Only the histidine at position 486 appears to play a role in the Cd\textsuperscript{2+} effect. The V476C H486Y double mutant channels were no longer trapped in the open state by 20 \mu M Cd\textsuperscript{2+} (Figure 3C); Cd\textsuperscript{2+} produced only a small reduction in current with no appreciable effect on the activation gating. This result suggests that the high affinity binding of Cd\textsuperscript{2+} in the V476C mutant channels is due to coordination between the introduced cysteine at 476 and the native histidine at 486. An alternative explanation is that the additional mutation H486Y somehow changed the structure of the V476C mutant channels and indirectly altered the ability of the channel to be trapped at all in the open state. We had previously found that V476C channels could also be trapped in the open state by chemical modification of the cysteine residue, for instance with trimethylaminoethyl-methanethiosulfonate (MTSET) (Liu et al., 1997). This effect can still be seen in the V476C H486Y double mutant channels (Figure 3D), showing that these channels can indeed still be trapped open. Apparently, the effect of chemical modification is independent of H486, but the high affinity effect of Cd\textsuperscript{2+} depends on the presence of both 476C and 486.

**Results and Discussion**

**Cd\textsuperscript{2+} Traps V476C Mutant Channels in the Open State with High Affinity**

Figure 1 shows the effect of 100 nM intracellular Cd\textsuperscript{2+} on the V476C mutant channels. In the absence of Cd\textsuperscript{2+}, the V476C mutant channels opened and closed normally in response to voltage. In the presence of Cd\textsuperscript{2+}, V476C mutant channels opened normally, but they could not be closed upon repolarization to −110 mV. The slow decline in current was probably due to inactivation rather than closing, because the nonconducting channels could not be activated by depolarization (Liu et al., 1997).

The high apparent affinity of Cd\textsuperscript{2+} for these V476C channels suggests that Cd\textsuperscript{2+} is coordinated by several ligands on the protein. We first tested whether native cysteines in the Shaker channel contribute to the binding site for Cd\textsuperscript{2+}. We removed each of the native intracellular cysteines at positions 96 and 505 by substitution mutagenesis (Boland et al., 1994), and there was no change in the effect of Cd\textsuperscript{2+} on the V476C mutant channels; removal of C462 (located toward the extracellular end of the S6) also did not alter the Cd\textsuperscript{2+} effect (data not shown).

**Cd\textsuperscript{2+} Is Coordinated by 476C and a Native Histidine**

In some naturally occurring metal binding sites in proteins, Cd\textsuperscript{2+} or Zn\textsuperscript{2+} ions are coordinated by imidazole groups from histidines (Krizek et al., 1993). To learn whether histidines were involved at all in the effect of Cd\textsuperscript{2+} on V476C mutant channels, we used diethyl pyrocarbonate (DEPC), a reagent that modifies histidine residues (Means and Feeney, 1971). Treatment with DEPC almost completely abolished the effect of Cd\textsuperscript{2+} on V476C channels (Figure 2B). For the treated channels, Cd\textsuperscript{2+} had only a very small remnant effect on the rate of deactivation and certainly did not prevent channels from closing as observed before DEPC treatment (Figure 2A). DEPC treatment also had some effect on the gating of channels in the absence of Cd\textsuperscript{2+}—it slowed down activation and speeded up deactivation (thinner traces in Figures 2A and 2B)—but this effect was also observed in the wild-type channels (data not shown). Because DEPC is not completely specific for the modification of imidazole groups and can also modify amino groups, we also tried the amino-specific modification reagent trinitrobenzenesulfonic acid (TNBS; Means and Feeney, 1971) (Figures 2C and 2D). TNBS treatment did alter the kinetics of gating (thin traces), but it did not alter the ability of Cd\textsuperscript{2+} to trap V476C mutant channels in the open state (thick traces).

Intersubunit Interaction between 476C, Cd\textsuperscript{2+}, and H486

Is Cd\textsuperscript{2+} coordinated by a 476C and an H486 from the same subunit, or by a 476C from one subunit and an H486 from a different subunit? To answer this question, we designed a tandem dimer with two different protomers, one of which contains only the 476 cysteine (but no histidine at 486) and the other of which contains only the 486 histidine (but no 476 cysteine) (Figure 4A). If the
interaction between 476C, Cd$^{2+}$, and H486 is exclusively within the same subunit, these channels should be insensitive to Cd$^{2+}$, because no single subunit contains both 476C and H486. Contrary to this hypothesis, however, Cd$^{2+}$ was still able to hold these channels open (Figure 4B).

Figure 3. Cd$^{2+}$ Is Coordinated in V476C Mutant Channels by Cysteine at 476 and Histidine at 486
(A) Positions of the native histidines (open pentagons) in the Shaker K$^{+}$ channel. The positions are 77, 85, 91, 93, 254, 378, 486, 501, 516, 546, 580, 622, and 644. Cysteine at 476 is shown by a closed circle.
(B) Tyrosine substitution of histidine 378 located at the C terminus of the voltage sensor of the channel (S4 segment) did not prevent Cd$^{2+}$ from trapping the V476C H378Y double mutant channels in the open state.
(C) Tyrosine substitution of histidine 486, the closest histidine to 476C, completely abolished the effect of Cd$^{2+}$. Intracellular application of 20 μM Cd$^{2+}$ to the V476C H486Y double mutant channels produced only a small current reduction with no appreciable effect on the activation gating.
(D) Modification with MTSET (200 μM for 2 s in the open state) of the V476C H486Y double mutant channels produced the same effect as in the V476C mutant channels; i.e., it held the channels open. Recordings in (C) and (D) are from the same inside-out patch.
Figure 4. Cd\(^{2+}\) Binding Is Coordinated by Residues 476C and H486 of Different Subunits

(A) Tandem dimer. Protomer A: V476C, H486Y; Protomer B: Wild Type. In this tandem dimer, the subunit containing the mutated cysteine at 476 does not contain the native histidine at 486.

(B) In the absence of Cd\(^{2+}\), the channels opened and closed normally in response to a voltage pulse from \(-110\) mV to \(+60\) mV. However, in the presence of Cd\(^{2+}\) (2 \(\mu\)M), most of the channels remained open upon repolarization to \(-110\) mV.

For instance, multiple cysteines at position 474 approach closely within the pore to form a single high affinity binding site for a Cd\(^{2+}\) ion (Liu et al., 1997). If 476C and H486 can interact in the open state, then the four S6 regions must intertwine to some extent, tilting or bending to allow the H486 at the C-terminal end to approach the 476C located 10 amino acids toward the N terminus of a different subunit (Figure 5). The Cd\(^{2+}\) bridge between these residues may stabilize this bundle of S6s in the open conformation.

This conclusion fits beautifully with the recently solved structure of a related bacterial potassium channel, Kcsa, from Streptomyces lividans (Doyle et al., 1998). Kcsa lacks the first four membrane-spanning regions of the Shaker class channels, and within the membrane has only an M1 (corresponding to S5), P, and M2 (corresponding to S6) segment. The M2 regions of Kcsa are nearly straight helical rods that are spread apart at the top (extracellular side) of the channel to accommodate the selectivity filter formed by the P regions, but they cross as a bundle near the bottom of the channel, leaving an aperture at the bundle crossing.

In spite of the limited sequence homology between Kcsa and Shaker (and the virtually absent homology in the region corresponding to Shaker 470–486), there is remarkable concordance between our studies of gating in the Shaker S6 and the structure of Kcsa M2. The pore-lining residues (470, 474, and 478) of Shaker face the pore of Kcsa, while the 476 residue faces away from the pore, outside the bundle. All Shaker residues whose accessibility is strongly coupled to gating (470–477) lie above the bundle crossing of the Kcsa M2, while those that are rapidly modified in either open or closed states lie below (toward the cytoplasm). Thus, based on the evidence from Shaker interpreted in the context of the Kcsa structure, gating occurs at the bundle crossing, either by obstruction of the aperture by another segment of the protein or by closing of the aperture through a change in the conformation of the bundle itself. The Cd\(^{2+}\) bridge between 476C of one subunit and H486 of another subunit may trap the channel in the open state, either by disrupting the interaction between the S6 and a neighboring voltage sensor or gate or by “freezing” the S6 bundle in the open conformation.

Experimental Procedures

Mutagenesis and Expression

Mutations were introduced in the cDNA for the Shaker H4 channel (Kamb et al., 1988) with three additional modifications: a deletion between residues 6 and 26 to eliminate N-type inactivation (Hoshi et al., 1990), substitution of internal cysteines 301 and 308 by serines to diminish the wild-type effect of chemical modification (Holmgren et al., 1996), and mutation of position 449 to valine, which greatly reduced C-type inactivation (López-Barneo et al., 1993). Mutations V476C and all of the H—Y substitutions were performed by PCR mutagenesis (Nelson and Long, 1989). Channels were transiently expressed in HEK 293 cells and were used 1–3 days after transfection. Methods for transfection and identification of transfected cells
have been described previously (Jurman et al., 1994). The tandem dimers were subcloned into a construct prepared elsewhere (Heginbotham and MacKinnon, 1992).

**Physiological Recording**

All experiments were performed with excised inside-out patches (Hamill et al., 1981) from identified transfected cells. The standard external solutions contained (in mM): 150 NaCl, 10 KCl, 3 CaCl₂, 1 MgCl₂, and 10 HEPES, at pH 7.4 (pipette). In the experiments shown in Figures 1 and 4, pipette solution contained 100 KCl and 60 NaCl. The internal (bath) solution contained (in mM): 160 KCl, 0.5 MgCl₂, 1 EGTA, and 10 HEPES, at pH 7.4. Solutions with Cd²⁺ contained no EGTA. The experiment in Figure 1 was done with the divalent chelator nitrilotriacetic acid (10 mM) instead of EGTA. Mg²⁺ and Cd²⁺ were added to the nitrilotriacetic acid buffer to achieve final free concentrations of 0.5 mM and 100 nM, respectively, at the adjusted pH of 7.4. The methods for electrophysiological recordings and rapid perfusion switches have been previously described in detail (Holmgren et al., 1996; Liu et al., 1997).

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**References**


