Can Shaker Potassium Channels be Locked in the Deactivated State?

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Abstract For structural studies it would be useful to constrain the voltage sensor of a voltage-gated channel in its deactivated state. Here we consider one Shaker potassium channel mutant and speculate about others that might allow the channel to remain deactivated at zero membrane potential. Ionic and gating currents of F370C Shaker, expressed in Xenopus oocytes, were recorded in patches with internal application of the methanethiosulfonate reagent MTSET. It appears that the voltage dependence of voltage sensor movement is strongly shifted by reaction with internal MTSET, such that the voltage sensors appear to remain deactivated even at positive potentials. A disadvantage of this construct is that the rate of modification of voltage sensors by MTSET is quite low, \( \sim 0.17 \text{mM}^{-1}\text{s}^{-1} \) at \(-80\text{mV}\), and is expected to be much lower at depolarized potentials.

Key words: patch clamp • gating current • MTSET • cysteine • S4

Introduction

The fourth transmembrane segment S4 of Shaker potassium channels has been intensively examined through mutagenesis, fluorescent labeling, and covalent modification. This segment is thought to be a major part of the channel voltage sensor; consistent with this are observations of voltage-dependent changes in accessibility of many S4 residues to the extracellular and intracellular solutions (Bezanilla, 2000; Gandhi and Isacoff, 2002; Jiang et al., 2003). As might be expected, most of the modifications of introduced cysteine residues produce changes in the voltage-dependent gating of the channels as well.

The most striking change in channel activation was observed by Baker et al. (1998) in the mutant F370C. As can be seen in Fig. 1, residue 370 lies near the center of the S4 helix where residues are poorly accessible to reagents like MTSET but nevertheless show state-dependent accessibility to hydrogen ions (Starace et al., 1997). When exposed on the intracellular side to the sulphydryl reagent MTSET at negative membrane potentials, F370C channels altogether stopped conducting ionic current. At more positive potentials, there was no effect of MTSET, consistent with the idea that the accessibility of this residue to covalent modification from the intracellular side is voltage dependent. Also interesting is the observation by Baker et al. (1998) that the loss of intracellular accessibility of the 370C residue occurred over a more negative voltage range than the gain of extracellular accessibility of a second cysteine residue, 365C. The disparate voltage ranges of movements of these residues are not unexpected, since the charge movement in the Shaker voltage sensor is known to occur in two or more components. The voltage dependence of the change in accessibility of 370C corresponds to \( Q_1 \), the first component of charge movement (Bezanilla et al., 1994). It therefore appears that the reaction with MTSET occurs only in the fully deactivated state of the channel.

If the cysteine residue 370C is accessible only in the fully deactivated state, it is possible that the modified cysteine, with the charged ethyltriethylammonium moiety attached, may in turn constrain the voltage sensor to remain in the deactivated state. With the modification of 370C residues in each of the four subunits, the result would then be the trapping of the entire channel in its deactivated state.

For structural studies of voltage-gated channel proteins, it would be very useful to modify channels so that they are locked in the fully activated or fully deactivated state. It is easy to place Shaker channels into the activated state: they are near maximal activation at zero membrane potential, and the presence of an open-channel blocker can ensure that the voltage sensor is constrained in the activated conformation. However, of the many mutations that have been made of the Shaker channel, none have been shown convincingly to result in a channel that is trapped in the deactivated state. The mutations investigated so far that eliminate ionic currents appear either to block ionic conduction through an inactivation process (Perozo et al., 1993; Yang et al., 1997) or prevent assembly and maturation of channel protein (Schulteis et al., 1998; Papazian, 1999). Horn et al. (2000) have demonstrated the immobilization of Shaker voltage sensors using a photo-activatable
cross-linking reagent, but the nature of the photoreaction is such that only 30% of the voltage sensors can be immobilized. Laine et al. (2003) demonstrate that the formation of a disulfide bond between S4 and the pore region impedes channel opening, but does not prevent it entirely. In this context, the F370C mutant channel shows some promise as a variant that can be forced to remain in the deactivated state. The goal of the present work was to determine whether modification of this channel with MTSET yields permanently deactivated channels. In the discussion, we also consider some other Shaker mutants that may have appropriate properties.

MATERIALS AND METHODS

Molecular Biology

The basic wild-type truncated (WT) Shaker construct was Shaker H4 (Schwarz et al., 1988) in which residues 6–46 were deleted to remove fast inactivation. The F370C mutation was introduced into this background and propagated in the Bluescript vector. The constructs F370C/W434F and F373C were made in the background of a Shaker 29-4 construct in pGEM-A (Swanson et al., 1990), in which amino acid residues 2–29 were deleted. Plasmids of ShakerB-H4 and Shaker 29-4 were linearized with HindIII and NotI, respectively, and all cRNAs were transcribed with the MEGAscript T7 RNA polymerase kit (Ambion). The sizes of transcribed cRNAs were verified by gel electrophoresis.

Electrophysiology

Xenopus oocytes were injected with 50 nl of cRNA of F370C, F370C/W434F, or F373C. Patch clamp recordings were made 3–7 d after RNA injection using an EPC-9 amplifier and the Pulse acquisition program (HEKA Electronic). Patch pipettes were pulled from PG150T glass (Warner Instruments) to tip diameters of 2–5 μm after heat polishing. Both bath and pipette solutions for patch clamp recording were (in mM) 160 KCl, 1 MgCl₂, 1 EGTA, 10 HEPES, and pH 7.4, titrated with KOH for all experiments.

The MTSET reagent (Toronto Research Chemicals Inc.) was dissolved in standard bath solution at concentrations ranging from 0.2 to 1.2 mM just before each experiment and maintained at room temperature in the perfusion system. The elapsed time after preparation of the MTSET solution was noted and the effective concentration was corrected, assuming an exponential decay of active MTSET concentration (Swanson et al., 1990).

To quantify the MTSET decay time course, four successive inside-out patch recordings were made of the modification of F373C channels with the 1.2 mM MTSET solution (Fig. 2 A). In each recording, the rate of modification of the channels was evaluated by fitting the decay of the current (Fig. 2 B). The MTSET activity decreased during the experiment with a time constant of 26 min, or a half-time of 18 min. This time constant was used in Eq. 1 to correct the loss of MTSET activity due to hydrolysis.
timed to occur only at the holding potential, which was −80 mV in all experiments. A few experiments were done with MTSET continuously present in the bath solution, with no apparent change in the time course of modifications.

The isochronal voltage dependence of channel conductance from tail currents and the voltage dependence of the integrated gating charge were fitted with a Boltzmann function of the membrane potential $V$, where $z$ is the effective valence, $F$ is the Faraday constant, $R$ is the gas constant, and $T$ is absolute temperature. Statistical quantities are expressed as mean ± SD with the number of determinations $n = 4$ in all cases.

**RESULTS**

**Mixed Channel Populations During MTSET Modification**

We recorded ionic currents from WTT and F370C channels in inside-out patches (Fig. 3). As evaluated from tail current amplitudes, we found the voltage dependence of channel activation to be almost identical in the two channel types. Exposure to MTSET had no effect on the WTT channels, but exposure to 0.5 mM MTSET for 24 s reduced the F370C ionic current to unmeasurable levels, <0.5% of control (Fig. 3 A). During the exposure to MTSET, the F370C current decreased gradually in amplitude (Fig. 3, B and C). The activation time course was slowed considerably, but the deactivation time course remained unchanged during MTSET exposure (Fig. 3, D and E).

The change in activation rate can be explained if we assume that partially modified channels can be opened by depolarization to −80 mV, but they open more slowly and less completely than unmodified channels. The simplest model of this kind assumes that if one of the four subunits is modified, the channel has the slowed activation time course $u(t)$, while if two or more subunits are modified, the channel does not open at all. Letting $u(t)$ be the unmodified activation time course, the time course of activation of a population of channels will be

$$I_{\text{total}}(t) = I_0 u(t) + I_1 m(t),$$

where $I_0$ and $I_1$ are the initial currents for unmodified and modified channels, respectively.
Locking Shaker Channels Shut

where $I_0/H_11005$ and $I_1/H_11005$ are constants—the maximum current through unmodified and modified channels, respectively. The probabilities of having zero or one modified subunit are

$$p_0 = (1 - p)^4$$

$$p_1 = 4p(1 - p)^3,$$  \hspace{1cm} (3)

$$p = 1 - e^{-kx}$$  \hspace{1cm} (5)

is the probability that a given individual subunit is modified by MTSET at the cumulative exposure time $x$.

Figure 4 demonstrates the fitting of this model to data obtained from depolarizations to $+80$ mV (Fig. 4 A). The function $u(t)$ was obtained from the activation time course of the F370C current before exposure to MTSET. The time course $m(t)$ was then obtained as a fit to the small current remaining after substantial modification (Fig. 4 B). With these functions, each of the 24 sweeps recorded in this experiment could be decomposed into the current amplitudes $I_0$ and $I_1$, reflecting unmodified and singly modified channels, respectively (Fig. 4 C). A simultaneous fit of Eqs. 2–5 was then performed with the free parameters $A$, $B$, and $k$. The resulting values were $A = 366 \pm 9$ pA, $B = 136 \pm 0.4$ pA. The total current amplitude (triangles) can alternatively be fitted by a single exponential with a decay rate of $0.46 \pm 0.02$ mM$^{-1}$s$^{-1}$ (dashed curve).

besides having slow kinetics, the partially modified channels have a strongly shifted voltage dependence of activation. Partial modification yields a new component of current with an activation midpoint of roughly $+100$ mV (Fig. 5). The weak voltage dependence of this activation (a Boltzmann fit yields the apparent valence $z = 0.8$) is consistent with the idea that this component reflects the activation of a single modified subunit in an otherwise intact channel.

MTSET Greatly Reduces Gating Currents

Recording the gating currents from MTSET-modified channels turned out to be much more difficult than recording the ionic currents. Due to channel clustering, only a small fraction of inside-out patches contained

Figure 5. Two components of activation in partially modified F370C channels. Before modification, a Boltzmann fit yields $V_{1/2} = -63.8 \pm 0.8$ mV and $z = 4.27 \pm 0.5$. After partial MTSET modification (7.7 mM-s), there is an additional, right-shifted component of activation, with $z = 0.8 \pm 0.2$ and $V_{1/2}$ estimated to be $\sim +100$ mV.
sufficient channels to yield substantial gating currents (tens of picoamperes in amplitude). We found that the recording baseline would invariably become unstable after \( \sim 10 \) mM\( s \) of MTSET exposure. Noisy picoampere-sized currents would appear and increase with time, making the measurement of small gating currents impossible. Inside-out patches from other cell types (HEK-293 and COS cells) also showed an MTSET-induced instability. Thus, our recordings were limited to a maximum of \( \sim 10 \) mM\( s \) of exposure.

For gating current measurements we used the double mutant F370C/W434F. The W434F mutation has little effect on gating charge movement (Perozo et al., 1993) but eliminates ionic currents (Yang et al., 1997). The voltage dependence of charge movement of F370C/W434F channels is essentially the same as that of the background W434F channels (Fig. 6 A). However, as the double mutant channels are exposed to MTSET, the gating currents become smaller (Fig. 6 B). The total charge, as computed from the on gating currents, decreased by 80% during an 80-s exposure to \( \sim 0.1 \) mM MTSET (Fig. 6 C). The time course was truncated by the limited lifetime of the patch, but it could be well fitted by an exponential decay with a modification rate of 0.21 mM\( ^{-1} \)s\(^{-1} \).

As the gating current is reduced by MTSET, the decay of the on gating current is gradually accelerated (Fig. 6 D). This suggests that the gating charge might not be entirely immobilized by the modification. However, because of the limited lifetime of the patch recordings, we were never able to observe gating currents in an exhaustively modified patch.

**Comparing the Decay of Ionic Currents and Gating Charge**

If there were four independent voltage sensors, all of which had to be fully activated for the channel to open,
then the immobilization of one sensor would render the channel nonconducting. Thus the rate of decay of ionic current during MTSET modification would proceed four times as quickly as the rate of decay of gating current. A factor of about four was found between the decay rates of ionic current and gating charge during photochemical cross-linking of Shaker channels by Horn et al. (2000). A ratio of less than four is however to be expected in Shaker channels, where /H11601/20% of the charge movement appears to occur in one or more concerted steps associated with channel opening (Zagotta et al., 1994; Schoppa and Sigworth, 1998b). A comparison of decay rates in our experiments was consistent with factors of three or four.

Fig. 7 compares ionic and gating current decays in F370C channels under exposure to MTSET. A total of four ionic current recordings made at +40 mV from oocytes expressing F370C channels, the modification rate was 0.63 ± 0.18 mM⁻¹·s⁻¹. From four other experiments, the rate of decay of gating charge movement was seen to be 0.17 ± 0.1 mM⁻¹·s⁻¹ (Fig. 7 B). There is much scatter in the decay rates, such that the comparison we can make with some certainty (P < 0.02, Fisher’s Exact Test) is simply that the gating current decay rate is slower than that of the ionic current. The gating current decay rate is however indistinguishable from the rate k = 0.165 mM⁻¹·s⁻¹ of single subunit modification obtained from the analysis shown in Fig. 4.

The modification rates are slow, which make for considerable difficulties in recording from the inside-out patches. One aspect that we did not investigate would be a possible pH dependence of the modification rate. A higher pH might accelerate the modification, as the deprotonated S⁻ is the reactive species (Pascual and Karlin, 1998). Also, all of the modification rates were measured at the holding potential of −80 mV. The modification rate is greatly reduced by depolarization (Baker et al., 1998); exactly how much it is reduced would be a good subject for future study, once the problem of patch instability is solved.

**DISCUSSION**

The potassium current carried by Shaker F370C channels is abolished by treatment with the sulfhydryl reagent MTSET. The effect has the characteristics expected of a modification that traps the channel voltage sensors in the deactivated state: the modification proceeds at negative potentials (Baker et al., 1998) and gating currents are strongly suppressed. During exposure to MTSET, the ionic current decays more quickly than the gating current. This is consistent with the idea that the modification of a single voltage sensor has a large effect in reducing the ionic current but has little effect on the charge movement carried by other voltage sensors.

It appears that Shaker channels require the full charge movement and therefore the activation of all four voltage sensors to enter the main open state (Islas
Mechanism of Charge Immobilization

The residue F370 is in the center of the S4 helix, a region where accessibility to MTS reagents is very limited (Bezanilla, 2000). Scanning histidine mutagenesis (Starr and Bezanilla, 2001), however, shows that both residues R368 and R371 are accessible to protonation from the intracellular or extracellular solutions, depending on the membrane potential. These authors conclude that upon depolarization, these residues move from an intracellularly disposed, aqueous cavity into a very narrow crevice, presumably formed by other parts of the channel protein. Our results are consistent with this view. The additional positive charge introduced by the ethyltrimethylammonium moiety on modification of this residue likely causes the entry of this residue into the crevice to be highly unfavorable. In addition, the extra positive charge inserted between R368 and R371 could greatly increase the energy of transition states when the charge passes through the di-electric barrier between intracellular and extracellular solutions.

Proposals for Locking Shaker Channels in the Deactivated State

It is very difficult to trap the Shaker voltage sensor in its deactivated state; the fact that no mutations have been demonstrated to do this implies that the voltage sensor is a remarkably mobile structure. For structural studies on solubilized channel proteins it would be sufficient to modify the channel so that the voltage dependence of voltage sensor activation is shifted to positive potentials. Unfortunately, the best-studied mutations that produce large shifts in channel activation have only minor effects on the main voltage sensor movements. These mutations, the V2 mutation of Schoppa and Sigworth (1998a) and the ILT mutation of Ledwell and Aldrich (1999), produce their effects by interfering with the final concerted step of channel opening, rather than constraining the movements of the voltage sensors. A characteristic of these mutations is that the positive shift in the activation curve is accompanied by a large decrease in its steepness. There are however some other candidates for a shift in the voltage sensor equi-
librium. Scanning mutagenesis studies have identified mutations that produce positive shifts in activation gating while steepness is maintained, as would be expected from a simple shift in the voltage dependence of voltage sensor movement. These mutations in Shaker channels include I241W in the S1 segment (Hong and Miller, 2000), T284W in S2 (Monks et al., 1999), and S412W in S5 (Li-Smerin et al., 2000a); in Kv2.1, the mutation F236A also has this characteristic (Li-Smerin et al., 2000b). However, gating current measurements have not been reported for these channels, so that the hypothesis of a shift in voltage sensor movements remains untested.

The modification considered here of F370C channels with MTSET appears at present to be the most promising way to keep Shaker channels in the deactivated state. We find that gating currents are abolished along with the ionic currents during modification; this is highly suggestive but does not prove that the voltage sensors are trapped near their resting position. Nevertheless, were one to exploit this effect for structural studies, conditions would have to be found for the MTSET modification of the channel protein either as it is solubilized in detergent or reconstituted into membranes with nominally zero membrane potential. Because the modification proceeds only when the voltage sensor is in the resting state, the modification will occur only very slowly. Indeed, in a nonactivating Shaker channel at 0 mV, the probability of finding a voltage sensor in the resting state is expected to be $\sim10^{-4}$, as estimated from the model of Schoppa and Sigworth (1998b). However, this probability can be increased by disturbing the equilibrium of the final concerted step toward channel opening. With the V2 (L382V) mutation for example, the final transition is less biased toward channel opening and the voltage sensors will behave more independently (Schoppa and Sigworth, 1998b). Thus we predict that a good construct would be a combination of one of these mutations with F370C, yielding voltage sensors that would have a substantial probability of being in the deactivated state at zero membrane potential. In the case of the V2 mutant, this probability is predicted to be $\sim7\%$, and exposure to $\sim1$ M s of MTSET would be sufficient to modify the 370C residues at 0 mV.

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