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Transmembrane Movement of the *Shaker* K^+ Channel S4

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Summary

We have probed internal and external accessibility of S4 residues to the membrane-impermeant thiol reagent methanethiosulfonate-ethyltrimethylammonium (MTSET) in both open and closed, cysteine-substituted *Shaker* K^+ channels. Our results indicate that S4 traverses the membrane with no more than 5 amino acids in the closed state, and that the distribution of buried residues changes when channels open. This change argues for a displacement of S4 through the plane of the membrane in which an initially intracellular residue moves to within 3 amino acids of the extracellular solution. These results demonstrate that the putative voltage-sensing charges of S4 actually reside in the membrane and that they move outward when channels open. We consider constraints placed on channel structure by these results.

Introduction

To account for the voltage dependence of the Na^+ and K^+ conductances in the squid giant axon, Hodgkin and Huxley (1952) proposed the existence within the membrane of charged gating particles that move in response to changes in the transmembrane electric field, turning the conductance on or off. The S4 region of voltage-gated channel proteins has been proposed to serve this function (Greenblatt et al., 1985; Noda et al., 1984; Guy and Seetharamulu, 1986) because of the concentration in this region of basic residues in a motif that is conserved among the otherwise divergent families of voltage-gated Na^+, K^+, and Ca^{2+} channels (reviewed in Catterall, 1988).

Although highly charged, S4 is proposed to reside within the hydrophobic membrane-spanning core of voltage-gated channel proteins so that it may experience changes in the transmembrane electric field (Figure 1). This hypothesis has not been directly tested. Epitope tagging of the S3–S4 loop in *Shaker* has confirmed that this loop faces the extracellular solution (T. M. Shih and A. L. Goldin, personal communication). However, while mutagenesis of the S4–S5 loop alters binding by internal N-terminal ball peptide and internal tetraethylammonium (TEA; Isacoff et al., 1991; Slesinger et al., 1993), no direct evidence exists showing that S4–S5 lies on the internal side of the membrane.

Several studies have used mutagenesis to try to establish that S4 charges serve a voltage-sensing function, but not with clear success. It has been shown that neutralizing or reversing specific S4 charges affects the voltage dependence of channel opening (Stühmer et al., 1989; Papazian et al., 1991; Liman et al., 1991; Logothetis et al., 1992). However, other mutations that conserve charge or that substitute one hydrophobic residue for another have been shown to produce similar effects (also see Schoppa et al., 1992).

Two recent studies have attempted to show that S4 moves in the manner expected of a gating particle. Membrane depolarization was found to increase the accessibility to the extracellular solution of an amino acid position in the S4 region of a Na^+ channel (Yang and Horn, 1995) and of several S4 positions of the *Shaker* K^+ channel (Mannuzzu et al., 1996). Moreover, increased outward exposure correlated in time and in voltage dependence with the recorded positive outward flow of the gating current (Mannuzzu et al., 1996). However, neither study could show that S4 residues, when inaccessible, lie in the membrane, or that the increased accessibility originates from a translocation of S4 charges through the plane of the membrane in the manner required of the gating charge.

We set out to obtain such evidence. We investigated the state-dependent intracellular and extracellular solvent exposure of the *Shaker* S4 by substituting cysteines at various positions and assaying their accessibility to a membrane-impermeant thiol reagent. We chose for study 7 residues (Figure 1), including 3 charged ones, defining a window of 26 amino acids both bordering and within the conventional ~20 amino acid S4 region of *Shaker* channels (Tempel et al., 1987; Kamb et al., 1988; Pongs et al., 1988; Durell and Guy, 1992; Sigworth, 1994).

We have established that the *Shaker* S4 spans the membrane and that the distribution of membrane-buried S4 residues depends on the gating state of the channel. In addition to providing information about the structure and movement of S4, these experiments indicate that only a particular subset of the basic S4 residues can contribute to the gating charge, and enable us to predict which charges contribute most.

Results

We substituted cysteines into inactivation ball–removed (Δ6–46; Hoshi et al., 1990) ShH4 K^+ channels (Kamb et al., 1988) and assayed their accessibility with the membrane-impermeant thiol reagent methanethiosulfonate-ethyltrimethylammonium (MTSET; Akabas et al., 1992, 1994a, 1994b; Kurz et al., 1995; Pascual et al., 1995). RNAs encoding the seven cysteine-substituted mutants gave rise to channel currents at wild-type levels in Xenopus laevis oocytes. As observed with other S4 amino acid substitutions (Stühmer et al., 1989; Lopez et al., 1991; Papazian et al., 1991; Liman et al., 1991; Logothetis et al., 1992), these cysteine substitutions altered the voltage dependence of channel opening (Figure 2, top left and top center). The neutralization of consecutive basic S4 residues with cysteines led to

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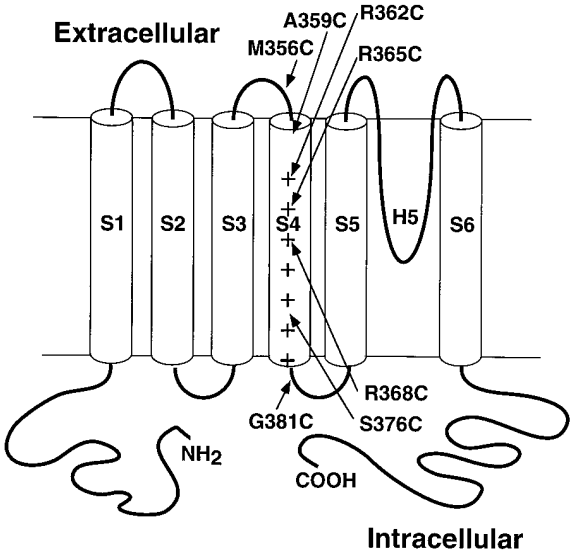

Figure 1. Conventional Membrane Topology Model of the *Shaker* K^+ Channel

Six transmembrane domains (S1–S6) and the pore-lining region (H5). Positions of cysteine substitution bordering and within S4 are marked with arrows. Basic residues in S4 are also marked (+).

shifts in the depolarizing direction, contrary to the pattern observed in Kv1.1 when neutralizations were made with glutamine (Liman et al., 1991). One substitution, S376C, produced channels that exhibited a higher than wild-type rate of rundown upon excision of patches, which caused a depolarizing shift in activation (Figure 2, top left).

MTSET reacts with the sulfhydryl moiety of cysteine, transferring the charged trimethylammonium-ethanesulfide moiety of MTSET to the cysteine sulfide (Akabas et al., 1992). We assayed the sensitivity of channels to external MTSET by superfusion of whole oocytes under two-electrode voltage clamp (TEVC) and assayed sensitivity to internal MTSET by perfusion of excised inside-out patches. ShH4 (Δ6–46) wild-type channels exposed to internal MTSET occasionally exhibited a small (<10%) diminution in conductance, with no effect on voltage dependence or kinetics, and exhibited no sensitivity to external MTSET (data not shown). In channels in which a cysteine had been substituted into S4, however, MTSET caused significant irreversible changes in gating. We assessed these changes by comparing current records acquired before exposure to MTSET with those acquired after MTSET wash-out. MTSET shifted the voltage dependence of channel opening in six of the seven cysteine-substituted channels (Figure 2). In the seventh (G381C), MTSET eliminated current, possibly by shifting activation out of the range of measurable voltages ($\Delta V_{1/2} > +150$ mV; Figure 2). MTSET also affected kinetics: accelerating the activation of R362C (data not shown), slowing the activation of R365C (see Figure 5), slowing the activation and deactivation of R368C (Figure 2), and increasing the sigmoidicity of activation of S376C (Figure 2).

The robust gating effect of MTSET modification for each S4 position studied enabled us to assay functionally whether a particular position was exposed intracellularly or extracellularly, and whether it was exposed in open and/or closed channels. We monitored modification with brief depolarizations at fixed intervals to a test potential, which was chosen for each mutant to maximize the visibility of the particular voltage shift or kinetic effect of MTSET. Arrows in Figure 2 indicate the test potentials used to monitor the steady-state current response of M356C, A359C, R365C, and G381C channels and the specific time point in the current response by which R368C and S376C channels were monitored.

In all of the cysteine-substituted channels, labeling followed a monoexponential time course (see Figures 4–6). In describing these results, we provide the fitted time constant of labeling, τ, as (time [s]) $\times$ (concentration MTSET [mM]). From these values, we calculate the second order rate constants, k_{closed} and k_{open} ($mM^{-1}s^{-1}$), which describe labeling by MTSET and provide a measure of cysteine exposure in the closed and open channel conformations. Since rapid patch perfusion enabled us to deliver MTSET exclusively during the hyperpolarizing or depolarizing epochs of the voltage protocol (Figure 3), we were able to assay internal accessibilities in closed and open channels directly. Owing to the slower rate of whole-oocyte perfusion, it was necessary to deduce external accessibilities in closed and open channels from the relative rates of labeling when channels were depolarized briefly to test potentials corresponding to a relatively low P_{open} for 0.5%–4% of the time (referred to as "closed" channels) and depolarized with long duration steps to a relatively high P_{open} for 50% of the time (referred to as "closed/open" channels). The explicit voltages and pulse durations used in each experiment are given in the figure legends (see Figures 4–6), and the equations for deriving the state-dependent rates of labeling are provided in the legend of Table 1 (see Experimental Procedures for a more detailed discussion of the assessment of state dependence).

In describing our results, taking as an example the mutation M356C, we refer to the modifiable target cysteine as "356C," to the wild-type residue as "M356," to the amino acid position as "356," and to the mutant channel as "M356C."

S4 Crosses the Membrane

If the conventional model of voltage-gated channel topology is correct, then residues at the N-terminal end of S4 should be accessible only from the extracellular solution, whereas residues at the C-terminal end should be accessible only from the intracellular solution. To test this, we examined positions 356 and 381 (see Figure 1).

Externally perfused MTSET labeled 356C with similar time course in both closed ($\tau = 0.075 \pm 0.007$ mM s; $n = 5$) and closed/open channels ($\tau = 0.051 \pm 0.008$ mM s, $n = 3$) (Figure 4). In contrast, perfusion of MTSET onto inside-out patches did not label 356C in either the closed or open state (cumulative application of 90 mM s; $n = 2$; Figure 4).

External MTSET did not modify either closed or open

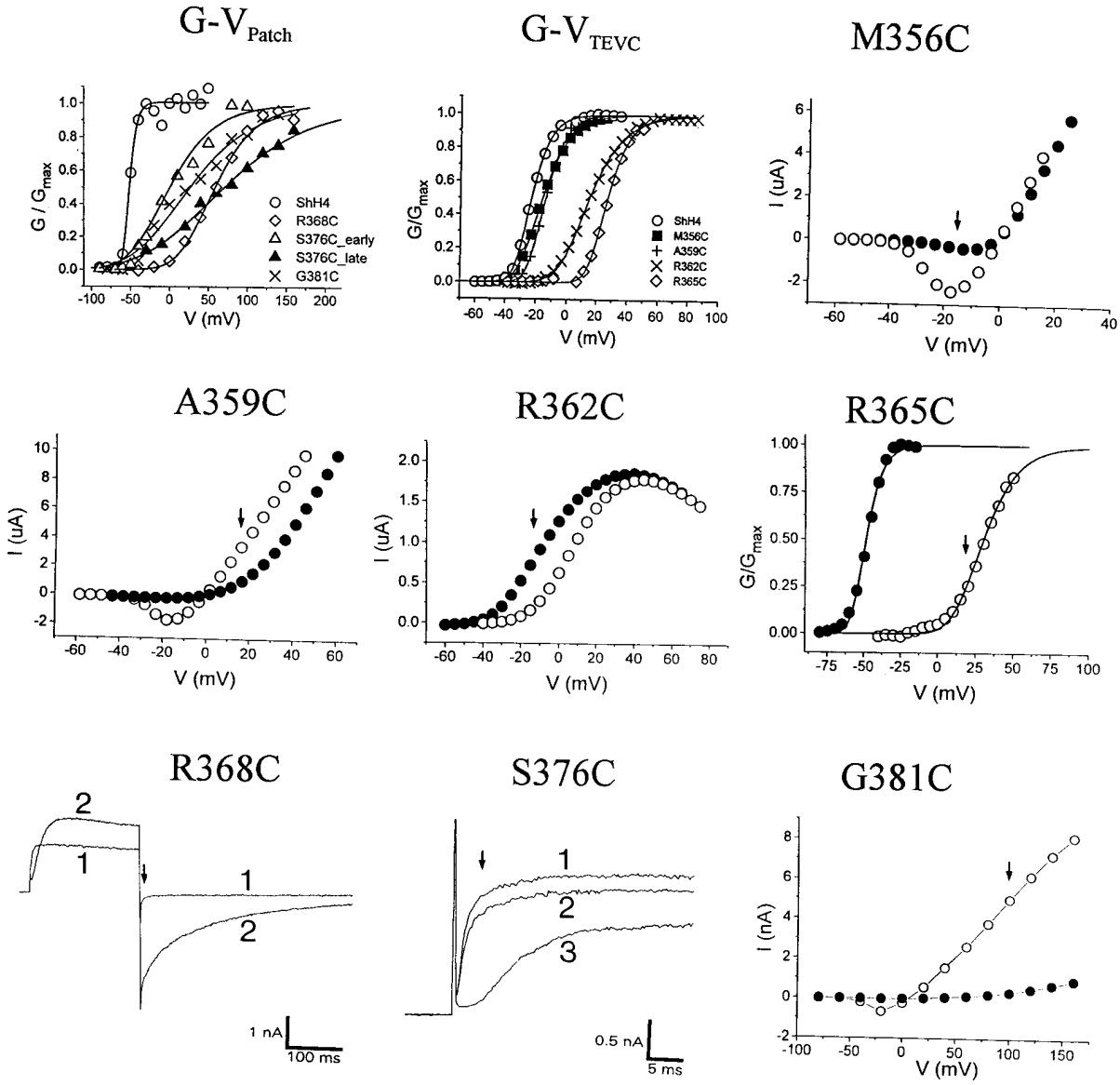

Figure 2. Effect of Cysteine Substitutions and MTSET Modifications in S4

(Top Left and Top Center) Conductance–voltage (G–V) relations determined by measuring isochronal tail current amplitude following voltage-clamp steps applied to excised inside-out patches ($G–V_{patch}$), and from steady-state whole-cell current response to steps acquired using two-electrode voltage clamp ($G–V_{TEVC}$). Results from wild-type ShH4 (Δ6–46) and seven cysteine-substituted channels are shown. Rundown of S376C shifted the $G–V_{patch}$, as revealed by comparison of data acquired immediately after excision (S376C early) and after 6 min of rundown (S376C late).

(M356C, A359C, R362C, R365C, and G381C) Current–voltage or G–V relations before (open circles) and after (closed circles) application of MTSET until saturation of effect.

(R368C) Current in response to $V_{step} = +40$ mV, $V_{tail} = -40$ mV before (1) and after (2) application of MTSET.

(S376C) Current in response to $V_{step} = +40$ mV. Three traces at 30 s intervals show the difference between the effect of 30 s of rundown (from 1 to 2) and the effect of 30 s of rundown and perfusion of MTSET (from 2 to 3).

MTSET modification was monitored from steady-state currents at $V_{test} = -15$, $+15$, -15, $+20$, and $+100$ mV for M356C, A359C, R362C, R365C, and G381C, respectively; from instantaneous currents 2 ms after step to $V_{tail} = -40$ mV following clamp to $V_{step} = +40$ mV for R368C; and from 5 ms after step to $V_{step} = +40$ mV for S376C (arrows). Recordings were performed in K^+_o (M356C, A359C, R365C, R368C, S376C, and G381C) or Na^+_o (R362C).

G381C channels (closed/open protocol, cumulative application of 9 mM s; n = 2; Figure 4), but internal perfusion of MTSET onto inside-out patches modified G381C channels in both the open and closed states (twice as readily in the open state [$\tau = 0.66 \pm 0.03$ mM s; n = 4] as in the closed state [$\tau = 1.47 \pm 0.01$ mM s; n = 4]; Figure 4).

These results show that in both open and closed channels, residue 356 faces outside and residue 381 faces inside, providing direct evidence that S4 crosses the

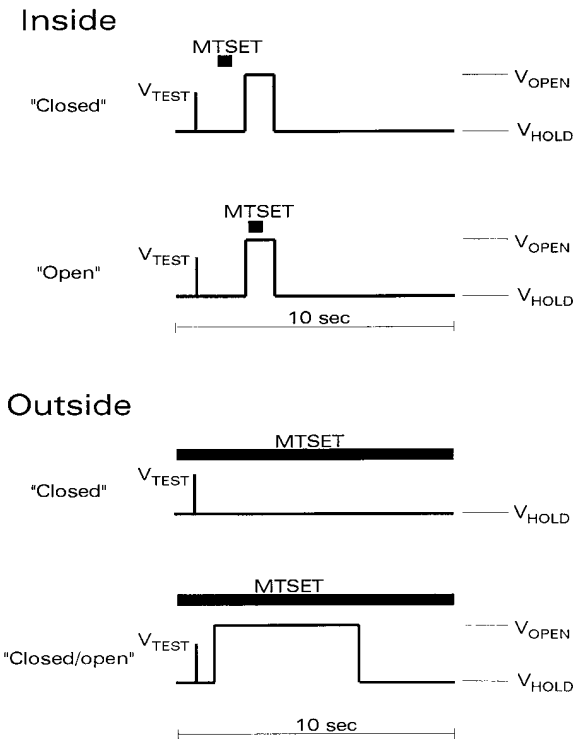

Figure 3. Voltage/Perfusion Protocols for the Assay of State-Dependent Modification

(Inside) Excised inside-out patches clamped to $V_{hold} = -100$ mV were depolarized for 50–200 ms to a monitoring voltage, V_{test}, and for 1 s to V_{open}, a voltage that opened channels maximally, once every 10 s. A computer-driven valve confined the flow of MTSET to a 500 ms interval during either the V_{open} (open protocol) or V_{hold} (closed protocol) epochs of each cycle. Bath solution flowed onto the patch at all other times.

(Outside) MTSET was superfused continuously in the assay of extracellular accessibility during whole-oocyte voltage clamping. Oocytes were depolarized briefly (25–400 ms) to a monitoring voltage, V_{test}, once every 10 s from $V_{hold} = -80$ or -100 mV (closed protocol). From the same V_{hold}, oocytes were depolarized briefly to V_{test} and for a period of 5 s (i.e., 50% of each cycle) to V_{open}, a potential corresponding to a large fractional P_{open} (closed/open protocol).

membrane and indicating that a segment of transmembrane residues lies somewhere between the N- and C-termini of the conventionally defined S4.

S4 Appears to Move Outward When Channels Open

Having established that S4 crosses the membrane, we assayed whether the transmembrane portion of S4 is constant or depends on channel state. Therefore, we investigated the state-dependent accessibility of 2 putative voltage-sensing residues, R365 and R368, lying midway between M356 and G381.

External MTSET did not modify closed R365C channels (cumulative application of 0.6–0.9 mM s; n = 4; Figure 5) but rapidly modified R365C channels under the closed/open protocol ($\tau = 0.25 \pm 0.06$ mM s; n = 3; Figure 5). Internally perfused MTSET produced no effect on closed or open R365C channels (cumulative application of 8.5–130 mM s in each state; n = 3; Figure 5). These results demonstrate that 365C is inaccessible from both sides of the membrane in closed channels but is made accessible to the external solution by depolarization.

Position 368, only 3 residues away from 365, exhibited the opposite exposure profile. External MTSET had no effect on either closed or open R368C channels (closed/open protocol, cumulative application of 0.9–15 mM s; n = 3; Figure 5). However, these channels were modified by internal MTSET. Internal MTSET labeled 368C in the closed state ($\tau = 1.6 \pm 0.3$ mM s; n = 5) but not in the open state (cumulative application of 3.6–16 mM s; n = 5; Figure 5). These results demonstrate that 368C faces the internal solution in closed channels and that depolarization renders this residue inaccessible from both sides of the membrane.

The appearance of 365C on the outside and the disappearance of 368C from the inside when channels open strongly argues for an outward movement of S4. The fact that these residues are accessible from opposite sides of the membrane and are separated by only 2 amino acids indicates that, were S4 not to move, it would have to span the membrane in only 2 residues. These results imply that when *Shaker* channels open, movement of S4 translocates the basic residue R368 outward from the cytosolic side of the membrane to within 3 positions of the external solution, and the basic residue R365 from within 3 positions of the cytosol to the external solution.

State-Dependent Window of Buried Residues

To gain a broader view of the accessibility of S4 in each state, we next assayed the internal accessibility of a position between 368 and the always internally exposed position 381; and the external accessibility of 2 positions between 365 and the always externally exposed position 356.

Internal MTSET modified closed S376C channels ($\tau = 0.019 \pm 0.005$ mM s; n = 5; Figure 6). Modification increased the sigmoidicity of activation of S376C (see Figure 2), and the rate of onset of this effect was rapid and readily distinguishable from the relatively high rate of rundown in these channels (Figure 6). Internal MTSET had no effect on open channels (cumulative application of 1–2 mM s; n = 5; Figure 6, bottom left). These results demonstrate that 376C, like 368C, faces the internal solution in the closed state and that depolarization renders this residue inaccessible. The state dependence of internal 376C exposure, therefore, supports the picture of outward S4 movement with channel opening.

External MTSET labeled 362C in closed channels ($\tau = 1.18 \pm 0.13$ mM s; n = 10) and labeled closed/open channels more rapidly ($\tau = 0.43 \pm 0.06$ mM s; n = 7; Figure 6). Channels labeled only partially when closed: labeling that saturated when channels were held closed renewed with a more rapid exponential time course upon switching to the closed/open protocol. External MTSET labeled 359C under the closed protocol ($\tau = 1.1 \pm 0.1$ mM s; n = 7) and more rapidly labeled under the closed/open protocol ($\tau = 0.15 \pm 0.04$ mM s; n = 3; Figure 6). These results show that positions 359 and 362

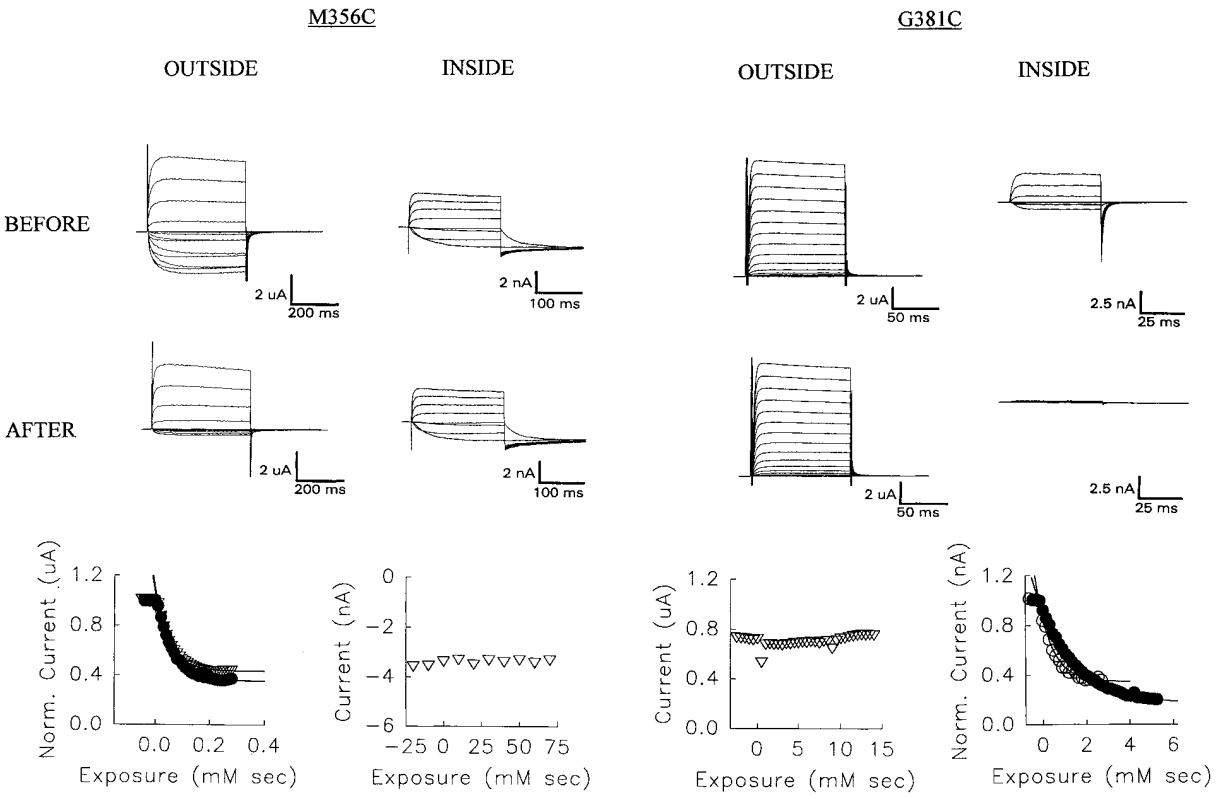

Figure 4. Internal and External Accessibility of 356C and 381C

(Top and Middle Rows) Voltage-clamp currents before and after saturating MTSET. Voltage protocols are given as V_{hold}, $V_{step}/V_{increment}$ × number of episodes, V_{tail}. (M356C Outside) Before and after: −80 mV, −60 mV/5 mV × 16, −55 mV. (M356C Inside) Before and after: −100 mV, −60 mV/20 mV × 8, −40 mV. (G381C Outside) Recordings in Na^+_o. Before and after: −80 mV, -60 mV/5 mV × 18, −45 mV. (G381C Inside) Before and after: −100 mV, −80 mV/20 mV × 8, −70 mV.

(Bottom Row) Time course of MTSET modification. Voltage protocols are given as V_{test}, V_{hold}, V_{open} (see Figure 3). (M356C Outside) Current in response to 400 ms test pulses during closed/open (open triangles) or closed (closed circles) protocol with continuous perfusion of 1 μM MTSET (−15 mV, −80 mV, +15 mV). (M356C Inside) Steady-state current in response to 200 ms test pulses during continuous perfusion of 1 mM MTSET on closed and open channels depolarized for 1 s of every 10 s cycle (open triangles; −40 mV, −100 mV, +100 mV). (G381C Outside) Steady-state current in response to 100 ms test pulses on closed/open channels (open triangles) during continuous perfusion of 50 μM MTSET (+20 mV, −80 mV, +20 mV). (G381C Inside) Steady-state current in response to 100 ms test pulses with perfusion of 300 μM MTSET, for 500 ms once every 10 s, on closed (closed circles) or open (open circles) channels (+100 mV, −100 mV, +100 mV). Abscissa, application time (s) × [MTSET] (mM). Monoexponential fits are superimposed.

are externally exposed in both open and closed channels but that their relative accessibilities to MTSET are state dependent.

Table 1 summarizes the accessibilities, expressed as second-order rate constants of modification by MTSET, of the 7 S4 cysteines. Table 2 summarizes these results qualitatively, illustrating a straightforward pattern of S4 exposure. When *Shaker* channels are closed, positions 356, 359, and 362 are exposed externally; 368, 376, and 381 are exposed internally; and position 365 in between is inaccessible, and therefore probably lies buried in the plane of the membrane. This defines a maximal window of 5 S4 residues (V363–V367) around 365 that apparently lies buried in the membrane in the closed state of *Shaker* channels.

When *Shaker* channels are open, positions 356, 359, 362, and 365 are exposed externally; 381 is exposed internally; and 368 and 376 in between are inaccessible, and therefore probably lie buried in the plane of the membrane. This defines a window of at least 9 (R368–S376) and perhaps as many as 15 residues in length (L366–K380) that apparently lies buried in the membrane in the open channel state.

Discussion

Our results, summarized in Table 2, show that a segment of up to 5 S4 residues about position 365 lies buried in the transmembrane core of the protein when *Shaker* channels are closed. Furthermore, they suggest that when channels open, this segment is displaced outward through the plane of the membrane, exposing 365 to the outside and drawing a segment including 368 and 376 into the membrane. The uniform direction of motion implied by 365, 368, and 376, spanning over half of S4, as well as the straightforward topology implied by the accessibility of these and 4 other positions outside this span, suggest that the ~20 residues of the conventionally defined S4 can indeed be regarded as part of a single functional domain. The deduced motion is one

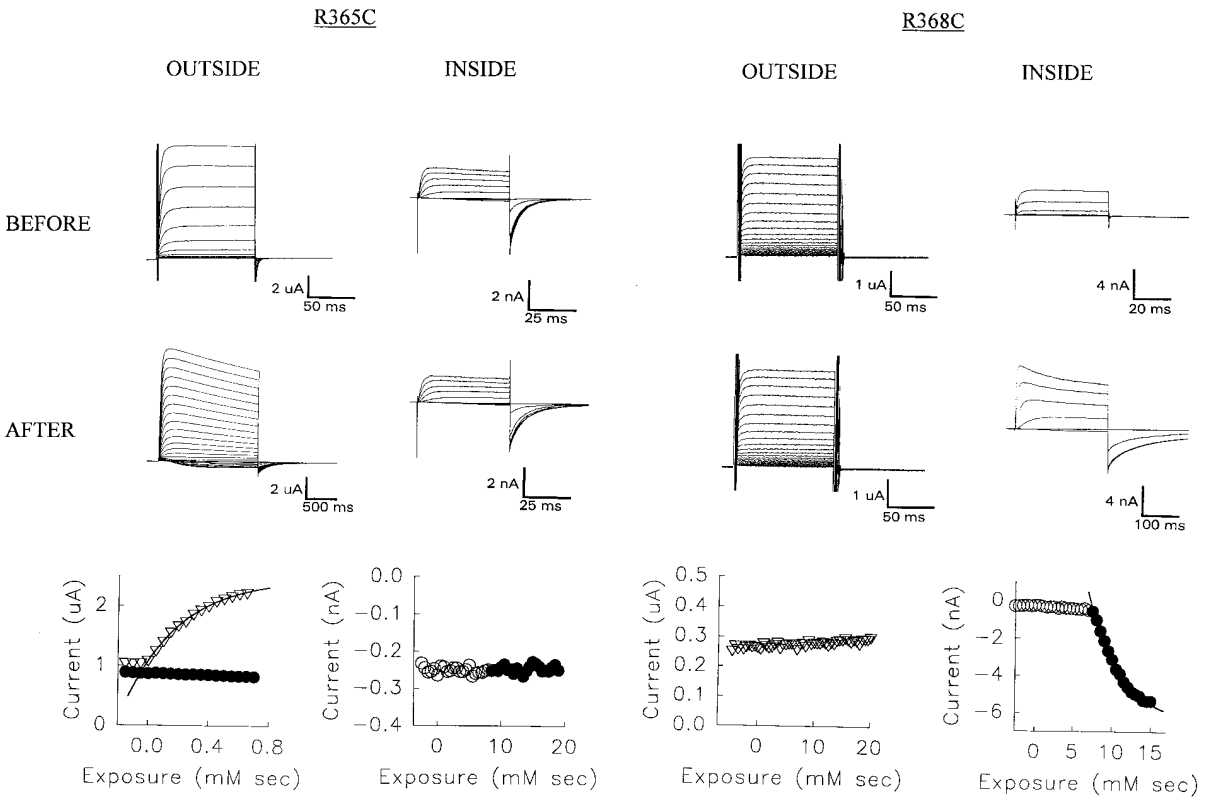

Figure 5. Internal and External Accessibility of 365C and 368C

(Top and Middle Rows) Voltage-clamp currents before and after saturating MTSET. Voltage protocols given as in Figure 4. (R365C Outside) Before: −80 mV, −40 mV/5 mV × 18, −20 mV; after: -80 mV, −80 mV/5 mV × 30, −70 mV. (R365C Inside) Before and after: −100 mV, −40 mV/20 mV × 8, −80 mV. (R368C Outside) Before and after: −80 mV, −40 mV/5 mV × 26, −40 mV. (R368C Inside) Before and after: −100 mV, −60 mV/20 mV × 8, −40 mV.

(Bottom Row) Time course of MTSET modification. Voltage protocols are given as in Figure 4. (R365C Outside) Steady-state current in response to 25 ms test pulses during closed/open (open triangles) or closed (closed circles) protocol with continuous perfusion of 5 mM MTSET (+20 mV, −80 mV, +70 mV). (R365C Inside) Steady-state current in response to 200 ms test pulses with perfusion of 1 mM MTSET for 500 ms once every 10 s on closed (closed circles) or open (open circles) channels (−20 mV, −100 mV, +100 mV). (R368C Outside) Steady-state current in response to 100 ms test pulses on closed/open channels (open triangles) during continuous perfusion of 50 μM MTSET (+15 mV, −80 mV, +15 mV). (R368C Inside) Tail current at −40 mV 2 ms after end of 200 ms test pulses with perfusion of 1 mM MTSET, for 500 ms once every 10 s, on closed (closed circles) or open (open circles) channels (+40 mV, −100 mV, +100 mV). Abscissa, application time (s) × [MTSET] (mM). Monoexponential fits are superimposed.

that translocates basic residues outward across a large fraction of the thickness of the transmembrane core of the protein, consistent with the idea that this domain functions as the gating particle or voltage sensor of *Shaker* and other voltage-gated channels. We examine this idea further below.

S4 Structure and Movement

To reduce the energetic cost of placing charges in a low dielectric environment, it has been hypothesized that basic residues in S4 form salt bridges with acidic residues in other transmembrane domains (Catterall, 1986; Durell and Guy, 1992). However, only three negatively charged groups are found in the other putative transmembrane domains of *Shaker*: two in S2 and one in S3. Our results argue that only a subset of the 7 basic residues of S4 are buried in the membrane-spanning core of the protein at one time, indicating that the acidic residues of S2 and S3 may be sufficient to stabilize S4 in the membrane.

Papazian et al. (1995) have obtained evidence that acidic residues E293 in S2 and D316 in S3 interact electrostatically with K374 and R377 in S4, and have proposed that E293 and D316 pair with K374 in the closed state and with R377 in the open state. However, our results indicate that neither K374 nor R377 lies in the membrane when channels are closed. On the other hand, the interactions proposed by Papazian et al. could take place, according to our results, in the open state. While this conclusion runs counter to their proposed model, it does not necessarily contradict their central findings: recent studies report a very high permeability to Na^+, K^+, and Cl^- in endoplasmic reticulum (Hoffer et al., 1995), indicating that its transmembrane potential is probably close to zero. Therefore, the improper folding that inhibits processing in K374Q and R377Q mutations, and which is prevented by neutralization of S2 and S3 acidic residues, might take place when channel proteins are in the open conformation.

Our results also have broader implications for channel

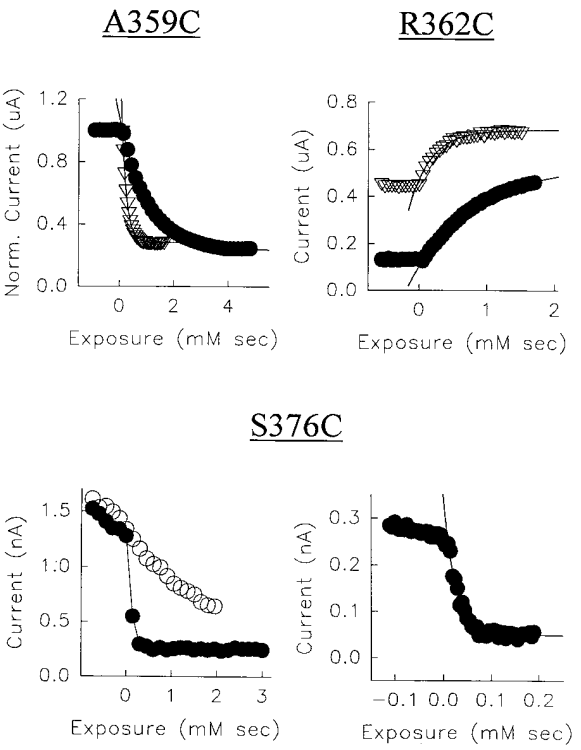

Figure 6. External Accessibility of 359C and 362C and Internal Accessibility of 376C

Time course of modification by MTSET. Voltage protocols given as in Figure 4.

(A359C) Steady-state current in response to 250 ms test pulses with continuous perfusion of closed channels with 15 μM MTSET (closed circles) and closed/open channels with 5 μM MTSET (open triangles) (+15 mV, −80 mV, +30 mV).

(R362C) Steady-state current in response to 50 ms test pulses during closed/open (open triangles) or closed (closed circles) protocol with continuous perfusion of 5 μM MTSET (−15 mV, −100 mV, +15 mV).

(S376C) Left: current response 5 ms after onset of 50 ms test pulses with perfusion of 300 μM MTSET, for 500 ms once every 10 s, on closed (closed circles) or open (open circles) channels (+40 mV, −100 mV, +140 mV). Right: current response 5 ms after onset of 50 ms test pulses to +40 mV from a holding potential of −100 mV at 1 s intervals during continuous perfusion of 5 μM MTSET. Abscissa, application time (s) × [MTSET] (mM). Monoexponential fits are superimposed on A359C, R362C, and S376C (right).

structure. They argue that not more than 5 residues in S4 lie buried in the membrane when channels are closed, and that as few as 9 may lie buried in the open state. Both numbers are less than the 18–20 residues required to span the core of the lipid bilayer as an α helix (Stryer, 1988). Therefore, apparently S4 is not α-helical and/or the distance between intracellular and extracellular S4 boundaries is less than the thickness of the bilayer hydrocarbon core. The latter possibility supports the models of Durrel and Guy (1992) and Kubo et al. (1993), in which S4 is insulated from the bilayer by a surrounding region of the channel protein.

Both 359C and 362C were about 10-fold more accessible in the open state than in the closed state. Although these differences are small and may be attributed to differences in local chemistry, they correlate with the pattern of accessibility changes exhibited by positions

Table 1. State-Dependent Exposure of S4 Amino Acid Positions

Residue	Closed		Open	
	Inside	Outside	Inside	Outside
M356C	<0.001	13	<0.01	27
A359C	ND	0.91	ND	12
R362C	ND	0.85	ND	6.8
R365C	<0.01	<0.1	<0.01	8.0
R368C	0.63	<0.01	<0.01	<0.01
S376C	50	ND	<0.05	ND
G381C	0.67	<0.02	1.4	<0.02

Second-order rate constants for modification by internal and external MTSET of substituted S4 cysteines in open and closed channels ($s^{-1}mM^{-1}$). Internal open and closed rates were calculated as the inverse of the time constants of modification under open channel or closed channel perfusion, respectively. External closed rates were calculated according to:

$$k_{closed} \approx (1/\tau_{closed}) \quad (1)$$

$$k_{open} \approx 2/(p\tau_{closed/open}) - [(2/p) - 1]k_{closed} \quad (2)$$

where τ_{closed} and $\tau_{closed/open}$ are the time constants of modification under the closed and closed/open protocols, respectively, and p is the open state probability of the mutant channel at V_{open}. Equation 2 neglects the contribution of channels opened during the test pulse phase of the closed/open protocol, and equation 1 is a valid approximation of the true expression:

$$k_{closed} = (1 - p'f)^{-1}(1/\tau_{closed} - p'fk_{open}) \quad (3)$$

in the case where $p'f << 1$ and k_{open} is not much greater than k_{closed}, which applies to all the cases where labeling during the closed protocol was observed (equations 1 and 2 assume modification to be slow compared with the rate of equilibration between open and closed channel states). Here p' is the open state probability of the mutant channel at V_{test}, and f = (duration test pulse)/10 s is the fractional time of depolarization. Upper limit values for rates (i.e., table entries expressed as "<#") were calculated for cases where no labeling effect was observed. We assumed that, to have escaped observation, any labeling that did occur could not have accumulated to >10% of that observed at saturation, and calculated the maximum possible rate according to $k < 10\% \times$ (total cummulative exposure)$^{-1}$. ND, not determined.

365, 368, and 376 and could be explained if positions 359 and 362 move from a recess, or crevice, into the bulk solution when channels open. Access to 359C and 362C by the MTSET molecule in the closed state would then be possible, but sterically constrained. Whether

Table 2. Summary of State-Dependence of S4 Exposure

Residue	Closed	Open
M356C	Out	Out
A359C	Out[a]	Out
R362C	Out[a]	Out
R365C	Inaccessible	Out
R368C	In	Inaccessible
S376C	In	Inaccessible
G381C	In	In

Qualitative Summary of Data in Table 1. Positions were inferred to be either accessible from the external side (out), the internal side (in), or found inaccessible (inaccessible).

[a] The external accessibility of 359C and 362C was ~10-fold less in the closed state than in the open state (see Table 1).

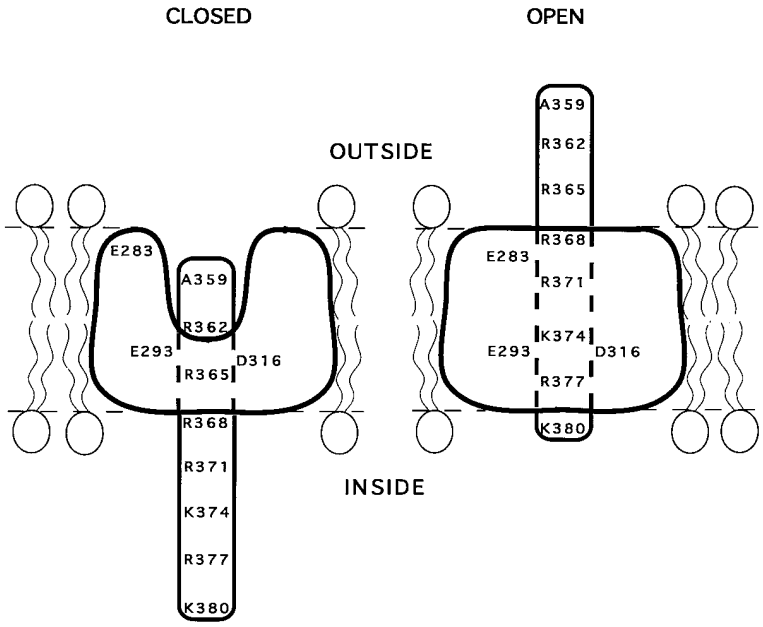

Figure 7. Transmembrane Movement of the *Shaker* S4

The diagrams depict a region of protein around the S4 region of a single subunit of the *Shaker* channel in the conformations associated with the channel closed and open states. Five residues (363–367) span the distance between the intracellular and extracellular solutions in closed channels, leaving only 1 basic residue buried (R365). This distance is shown as 50% of the thickness of the bilayer hydrocarbon core (~25–30 Å), or intermediate between that spannible by an α helix (8 Å) and a β sheet (18 Å) (Stryer, 1988). A crevice permits but restricts access to 359 and 362 from the external solution in the closed state. When channels open, S4 moves outward as a unit. This movement of S4, or a subsequent conformational change, closes the crevice, leaving a total of 12 residues buried (experiments show the number to be between 9 and 15). The basic residues buried in the open state form salt bridges with acidic residues in S2 and S3 (R368 and R371 are shown interacting with E283, and K374 and R377 interacting with E293 and D316), as implied by the findings of Papazian et al. (1995; S. K. Tiwari-Woodruff and D. M. Papazian, personal communication).

the membrane-spanning core of the protein thins precipitously, as in the case of a crevice, or more gradually, our results argue that in the closed state it must approach a "thickness" of 5 residues or less in the vicinity of S4.

Figure 7 shows a model of S4 movement that is consistent with our observations and takes into consideration the counter-charge pairing data of Papazian et al. (1995; S. K. Tiwari-Woodruff and D. M. Papazian, personal communication). The 5 S4 residues that are buried in the closed state are depicted spanning approximately half the thickness of the hydrocarbon core of the bilayer, a distance intermediate between that which would be spanned as an α helix (8 Å) or a β sheet (18 Å) (Stryer, 1988). A crevice causes solvent accessibility to residues A359 and R362 in the closed state to be limited (if this crevice were the channel outer vestibule, the partial modification of closed R362C channels might be explained as being due to the crowding in this state of the 362C cysteines of different subunits at the central axis of the pore; e.g., see model of Durell and Guy, 1992). In the closed conformation, R365 is the only buried charged S4 residue. Upon depolarization, S4 moves as a unit between 9 and 15 amino acids outwards (a 12 amino acid movement has been shown). This conformational change, or a subsequent one (perhaps the same step that opens the channel), closes off the crevice to the solvent so that in the open state a larger portion of S4 is buried. (Alternatively, the observed increase in the number of buried S4 residues may be due to a tilt or to a compaction of secondary structure in S4, e.g., through adoption of an α-helical structure.) Electrostatic interactions occur in the open state, pairing S4 basic residues and acidic residues in S2 and S3, according to the findings of Papazian et al. (1995; S. K. Tiwari-Woodruff and D. M. Papazian, personal communication).

Gating Charge

Our results indicate that 368C moves from the cytosol to within 3 amino acid positions of the extracellular solution, suggesting that it crosses most of the thickness of the transmembrane core of the protein. This implies that R368 crosses almost the entire transmembrane electric field and could contribute almost 1 equivalent gating charge per channel subunit. The large contribution deduced for R368 agrees with the conclusions of Perozo et al. (1994) that the neutralization R368Q reduces the total gating charge by 3 and that R368 traverses 75% of the transmembrane electric field.

We can form an idea of the contribution made by the entire S4 toward the total gating charge of 12–14 per *Shaker* channel (Schoppa et al., 1992; S. K. Aggarwal and R. MacKinnon, personal communiction) if we make a few simplifying assumptions, which have also been incorporated into Figure 7. We assume that the buried S4 residues are uniformly spaced (e.g., as in the case of an α helix, β sheet, or other orderly structure), that this spacing does not change when channels change conformation, and that the membrane electric field falls linearly across the membrane-spanning core of the protein. From 9 to 15 amino acids of S4 appear to be buried in the open state, and for the purpose of calculation, we take here the number to be 12. Under these assumptions, the total contributions made by R368 (1), R365 (1/2), R371 (3/4), K374 (1/2), and R377 (1/4) add up to 3 charges per subunit. For the tetrameric *Shaker* channel, this amounts to 12 charges per channel carried by S4, equal to the lower estimate for the total gating charge of these channels.

Mannuzzu et al. (1996), monitoring external accessibility changes in real time, showed that accessibility changes in the *Shaker* K^+ channel S4, including positions 359 and 365, correlate with gating currents in time

course and voltage dependence. Our results argue that the accessibility changes of these and other S4 residues result from the movement of S4 across a large fractional width of the transmembrane core of the *Shaker* channel protein. This movement is correct in orientation and may be sufficient in extent to account for the positive outward displacement of gating charge. Therefore, we conclude that S4 movement likely underlies the voltage-dependent transitions that generate the gating current and trigger opening of the conduction pathway in *Shaker* K^+ channels.

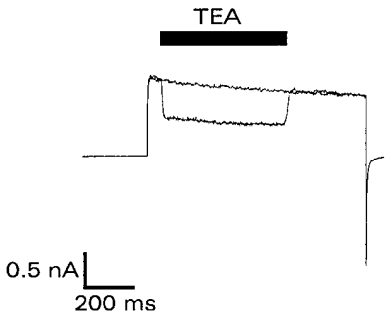

Figure 8. Rapid Perfusion of the Cytosolic Face of Excised Patches

TEA, at a concentration equal to its K_D for internal blockade of *Shaker* (400 μM), was perfused onto inside-out patches prior to each experiment to test for the delivery of perfusate undiluted and well-confined to the desired 500 ms interval. Shown superimposed are two consecutive 1 s duration current responses to voltage steps to $V_{open} = +100$ mV, as applied during assay of intracellular accessibility to MTSET at intervals of 10 s. One current response was acquired during perfusion with bath solution containing no TEA. In acquiring the other, TEA was perfused in the same interval as MTSET later was in the experiment that followed. A sharp decrement in current by ~50% can be seen to overlap with the interval over which an electronically driven valve that controls the flow of TEA is open.

Experimental Procedures

Molecular Biology

Site-directed mutagenesis, cRNA synthesis, and injection into Xenopus laevis oocytes were as described previously (Isacoff et al., 1990). Experiments were conducted 2–10 days after injection.

Electrophysiology

Two-Electrode Voltage Clamp

TEVC recording was performed with 0.5–2 MΩ electrodes (filled with 3 M KCl) using an Axoclamp 2B amplifier (Axon Instruments, Burlingame, CA). Recordings were done in high K^+ solution (K^+_o) containing 89 mM KCl, 0.8 mM $MgCl_2$, 0.4 mM $CaCl_2$, and 10 mM HEPES (pH 7.5), except in cases where 88 mM NaCl replaced 88 mM KCl, leaving 1 mM KCl (noted in the legends as Na^+_o). Data were filtered at 1–4 kHz and acquired using pCLAMP (Axon Instruments). Oocytes were injected with 50 nl of cRNA at a concentration of ~1 ng/μl. The solutions were perfused on the oocyte with a gravity-driven perfusion system ($\tau_{exchange} \approx 8$ s).

Patch

Macropatch recordings were done using a List EPC-7 amplifier (Medical Instruments, Greenvale, NY). Pipets contained 100 mM KCl, 1.5 mM $MgCl_2$, 0.3 mM $CaCl_2$, and 10 mM HEPES (pH 7.1) and had a resistance of <1 MΩ. The bath solution contained 100 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM EGTA, and 10 mM HEPES (pH 7.1). Bath solution was perfused continuously. Data were filtered at 4 kHz and acquired using pCLAMP (Axon Instruments). Oocytes were injected with 50 nl of cRNA at a concentration of ~200 ng/μl. A gravity-driven perfusion system ($\tau_{exchange} < 100$ ms) with a computer-controlled solenoid valve was used to deliver 500 ms long applications of MTSET coincident with specified epochs of the voltage-pulse protocol.

The delivery interval and rate of wash-in and wash-out were checked individually for each excised patch prior to application of MTSET with the perfusion of 400 μM TEA. Since MTSET and TEA have similar molecular weight and structure, delivery and wash-out of MTSET were assumed to follow the onset and recovery from block by TEA (Figure 8). Delivery of perfusate undiluted to the surface of the patch was verified with observation of 50% block, i.e., the fraction expected given a TEA concentration in perfusate approximately equal to its K_D for internal blockade of *Shaker*. Wash-in and wash-out rates varied from patch to patch (probably reflecting varying geometries of patch), but only patches for which wash-in and wash-out were achieved within 100 ms were used for experiments.

MTSET Labeling and State Dependence

Solvent exposure of the inserted cysteines was assayed using irreversible covalent modification by the membrane-impermeant thiol reagent MTSET (Toronto Research Chemicals, Downsview, Ontario, Canada). A 1–100 mM stock solution of MTSET dissolved in recording solution was made, stored on ice, and used to provide aliquots that were freshly diluted ~30 s prior to perfusion. A new stock was made approximately every 3 hr. The robust effect of MTSET modification for each S4 position studied enabled us to assay functionally whether a particular position was exposed intracellularly versus extracellularly and whether it was exposed in open channels and/or closed channels. We assayed external accessibility with bath perfusion of oocytes under TEVC, and internal accessibility by perfusing the cytosolic face of excised inside-out patches. We followed the time course of channel modification by recording ionic current responses to brief (25–400 ms) depolarizations at 10 s intervals to a constant test potential, chosen according to the specific residue and MTSET effect being characterized (see Figure 2). In all of the cysteine-substituted channels, labeling followed a monoexponential time course. In describing these results, we provide the fitted time constant of labeling, τ, (time [s]) × (concentration MTSET [mM]). From these values, we calculate the second order rate constants, k_{closed} and k_{open} ($mM^{-1}s^{-1}$; see legend of Table 1), which describe labeling by MTSET and provide a measure of cysteine exposure in the closed and the open channel conformations. Results are presented as mean ± SEM (n = number of measurements).

Patch

In assaying internal accessibility, we perfused MTSET for 500 ms durations, once every 10 s, either at a point during the 9 s period when channels were held at −100 mV (closed channels) or during a 1 s depolarizing step to +100 mV, except for S376C where steps were to +140 mV (mainly open channels) (see Figure 3). The long periods of hyperpolarization and short depolarizations minimized C-type inactivation, which occurs during long depolarizations. Concentrations of MTSET were chosen that modified channels to completion within ~4 min.

TEVC

Since it was not possible to exchange the bath solution on the time scale of channel C-type inactivation, we assessed the state dependence of MTSET labeling by comparing the rate of labeling for two different voltage protocols during continuous MTSET exposure. In the closed channel protocol, channels were closed 96%–99.5% of the time and only depolarized during 25–400 ms test pulses, once every 10 s. In the closed/open protocol, 5 s depolarizing steps were given every 10 s (see Figure 3), so channels were closed 50% of the time and depolarized 50% of the time. Concentrations of MTSET were found for each cysteine substitution studied that modified channels at a rate that was slow compared with the wash-in and wash-out of perfusate but fast compared to spontaneous degradation of MTSET by hydrolysis ($t_{1/2} = 15$ min; Stauffer and Karlin, 1994).

Voltage Dependence versus State Dependence

Since MTSET is charged, its access to a cysteine in the transmembrane electric field would be voltage dependent. Hyperpolarization would favor access of positively charged MTSET from the extracellular side and disfavor access from the intracellular side; depolarization would do the opposite. In principle, an ambiguity could exist

between dependency on channel conformation, which is controlled by voltage, and dependency on voltage itself. However, since increases of accessibility were observed only for voltage changes that would disfavor MTSET access, this concern does not apply to the results of this paper.

Open-State Dependence versus Activation-State Dependence

Since the state dependence of S4 might be expected to follow the charge–voltage (Q–V) relation rather than the conductance–voltage relation (G–V) of channels, consideration was paid to the relationship between holding potential (hp) and the region of gating charge activation. Q–V relations for nonconducting M356C, A359C, R365C (Mannuzzu et al., 1996), and "S4 wild-type" ShH4 (Δ6–46)/W434F channels (Perozo et al., 1993) have been reported previously. In these, the standard hp of −80 mV for TEVC or −100 mV for patch-clamp recording activates a negligible fraction of the gating charge. Although the Q–V relations for R368C, S376C, and G381C channels were not known, all exhibited G–V relations that were considerably depolarized-shifted with respect to the S4 wild-type. Therefore, the −100 mV hp used in patch experiments was considered sufficiently negative. Since these channels did not label externally even when depolarized for long periods, use of an hp more negative than −80 mV in TEVC experiments was deemed unnecessary. However, since external labeling was observed in R362C channels (the Q–V relation of which was also unknown, but which, like the others, exhibited a depolarized-shifted G–V), in TEVC experiments these were clamped to an hp of −100 mV.

Inaccessibility of Residues

For each of the cysteine-substituted channels studied, at least one set of conditions was obtained under which the cysteine was found to be accessible. Under other conditions (e.g., perfusion of the opposite membrane face), inaccessibility was concluded for these cysteines if a many-fold cumulative exposure to MTSET (duration × concentration of MTSET) produced no observable effect. An upper limit was calculated for the maximum rate of modification that could have escaped observation (see Table 1). It was assumed that no more than 10% of the saturating effect could have accumulated without detection, so that the limiting rate was calculated as:

$$k < (10\%) \times (\text{total cumulative MTSET exposure})^{-1}$$

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