Independent Versus Coupled Inactivation in Sodium Channels
Role of the Domain 2 S4 Segment

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ABSTRACT The voltage sensor of the sodium channel is mainly comprised of four positively charged S4 segments. Depolarization causes an outward movement of S4 segments, and this movement is coupled with opening of the channel. A mutation that substitutes a cysteine for the outermost arginine in the S4 segment of the second domain (D2:R1C) results in a channel with biophysical properties similar to those of wild-type channels. Chemical modification of this cysteine with methanethiosulfonate-ethyltrimethylammonium (MTSET) causes a hyperpolarizing shift of both the peak current–voltage relationship and the kinetics of activation, whereas the time constant of inactivation is not changed substantially. A conventional steady state inactivation protocol surprisingly produces an increase of the peak current at −20 mV when the 300-ms prepulse is depolarized from −190 to −110 mV. Further depolarization reduces the current, as expected for steady state inactivation. Recovery from inactivation in modified channels is also nonmonotonic at voltages more hyperpolarized than −100 mV. At −180 mV, for example, the amplitude of the recovering current is briefly almost twice as large as it was before the channels inactivated. These data can be explained readily if MTSET modification not only shifts the movement of D2/S4 to more hyperpolarized potentials, but also makes the movement sluggish. This behavior allows inactivation to have faster kinetics than activation, as in the HERG potassium channel. Because of the unique properties of the modified mutant, we were able to estimate the voltage dependence and kinetics of the movement of this single S4 segment. The data suggest that movement of modified D2/S4 is a first-order process and that rate constants for outward and inward movement are each exponential functions of membrane potential. Our results show that D2/S4 is intimately involved with activation but plays little role in either inactivation or recovery from inactivation.

KEY WORDS: gating • cysteine modification

INTRODUCTION

Voltage-dependent ion channels have evolved to react rapidly to small changes in membrane potential. In the superfamily of channels selective for sodium, calcium, or potassium ions, this sensitivity to voltage is conferred principally by four positively charged transmembrane segments, known as S4 segments. Each S4 segment has two to eight basic residues, either arginine or lysine, that are typically separated from each other by two hydrophobic residues. In response to a change of membrane potential, the positive residues move with respect to the membrane electric field, and this movement is coupled to the gating process that opens and closes the ion-selective pore of the channel protein (for reviews, see Catterall, 1986; Patlak, 1991; Keynes, 1994; Sigworth, 1994). Depolarization is expected to drive the basic residues of S4 segments outward.

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Mutations of S4 residues usually cause changes in gating, but these changes rarely provide direct insight into either the movement of the S4 segments or the interaction between S4 movement and gating. Furthermore, voltage-dependent gating typically involves at least two distinguishable processes, activation and inactivation. A step depolarization causes channels to open (activate) and then spontaneously close (inactivate). The relationship between S4 movement and each of these processes is purely speculative at the moment.

The most direct evidence for S4 movement comes from experiments that use the cysteine scanning methodology introduced by Falke et al. (1988) and Akabas et al. (1992). In studies of S4 function, a cysteine is substituted for a selected S4 residue by site-directed mutagenesis, and the expressed channel is then exposed to a hydrophilic cysteine reagent while monitoring its biophysical properties (Yang and Horn, 1995; Larsson et al., 1996; Yang et al., 1996, 1997). The consequence of the reaction between the introduced cysteine residue and the reagent is a change in either activation or inactivation of the channel. For some S4 residues, a change of membrane potential causes a change in the

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surface accessibility of the cysteine, which can be monitored as a change in the rate of its modification by a fixed concentration of cysteine reagent in either the extracellular or intracellular solution bathing the channel. The voltage dependence of the modification rate is, therefore, an assay of S4 movement that either exposes or buries individual residues (Horn, 1998).

We have used this technique to explore the voltage-dependent movement of the S4 segment of the fourth homologous domain (D4) of sodium channels (Yang and Horn, 1995; Yang et al., 1996, 1997). In these experiments, we monitored the reaction between specific residues in D4/S4 and hydrophilic reagents by pronounced changes in the kinetics of inactivation. By contrast, modification of D4/S4 cysteines had relatively small effects on activation. Although we interpreted these results as evidence that cysteine modification causes a change in the kinetics and voltage dependence of D4/S4 movement, we had no data to support or reject this idea. Similarly, mutations in other S4 segments of sodium channels affect gating by unknown mechanisms (Stühmer et al., 1989; Chen et al., 1996; Kontis and Goldin, 1997; Kontis et al., 1997).

Here we report that chemical modification of a cysteine introduced into the S4 segment of the second domain (D2/S4) causes a hyperpolarizing shift of activation, but has little effect on inactivation. We further provide evidence that chemical modification of this cysteine causes a marked decrease in the kinetics of D2/S4 movement. These results contrast strongly with those obtained for modification of D4/S4, showing the unique contributions played by these two S4 segments in sodium channel gating.

MATERIALS AND METHODS

Mutagenesis

The mutation R669C (D2:R1C) was constructed in hSkM1 using a single-step polymerase chain reaction mutagenesis strategy. Primers were designed to create the desired mutation and incorporate natural restriction sites for StuI (nucleotide [nt] 2051) and BsteII (nt 2179) in the final product. Amplifications (20 cycles) were performed using 20 ng of hSkM1 cDNA as template and Taq DNA polymerase. Final products were purified by spin-column chromatography (QIAGEN Inc., Chatsworth, CA), digested with StuI and BsteII, and the resulting 128-bp fragment ligated into the corresponding sites in the plasmid pRc/CMV-hSkM1. The amplified region was sequenced entirely in the final construct to verify the mutation and exclude polymerase errors.

Electrophysiology and Data Acquisition

Standard whole cell recording methods were used as previously described (Yang and Horn, 1995). Supercharging reduced the expected charging time constant for the cells to <10 μs. Series resistance errors were <3 mV. Data were filtered at 5–10 kHz and acquired using pCLAMP (Axon Instruments, Burlingame, CA). Patch electrodes contained (mM): 105 CsF, 35 NaCl, 10 EGTA, 10 Cs-HEPES, pH 7.4. The bath contained (mM): 150 NaCl, 2 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 Cs-HEPES, pH 7.4. Corrections were made for liquid junction potentials. Most experiments were done at room temperature (20–22°C). In a few experiments, the temperature was set at 11.1°C by use of feedback-regulated Peltier devices (Dagan Corp., Minneapolis, MN).

Methanethiosulfonate-ethyltrimethylammonium (MTSET),[1] -ethylamine (MTSEA), and -ethylsulfonate (MTSES) were obtained from Toronto Research Chemicals (North York, Ontario, Canada). MTSET covalently attaches ethyltrimethylammonium to the reduced cysteine sulfhydryl via a disulfide bond, MTSEA attaches ethylamine, and MTSES attaches ethylsulfonate. Aqueous stocks of these reagents were kept at 4°C, and diluted in the bath solution immediately before use. The reagent solutions were presented to the cells with a macropipette placed in apposition to the cell (Yang et al., 1997). In a few experiments, MTSET was introduced into the patch pipette solution to expose it to the cytoplasmic face of the channel (Yang et al., 1996).

For modification of D2:R1C by MTS reagents, we used the following voltage protocol. In the presence of a fixed concentration of cysteine reagent (typically 50 μM MTSET), a single 9.7-s depolarization to a selected voltage from −150 mV was followed by a 10-s return to −150 mV. The depolarized voltage was either −60 or −20 mV. These pulses were followed by a 200-ms prepulse to −110 mV and a 20-ms test depolarization to −55 mV to measure the amplitude of the currents. This voltage protocol was repeated over a period of 10 min or until the channels were completely modified. We estimated the first-order modification rate by fitting the time course of the change in peak amplitude of the current to a single exponential relaxation.

Data Analysis and Modeling

Whole cell data were displayed and analyzed by a combination of pCLAMP programs, ORIGIN (MicroCal Software, Inc., Northampton, MA), and our own FORTRAN programs. Data from at least three cells for each measurement are presented as mean ± SEM. We fit data from individual cells to theoretical functions of choice, and the reported values are the means and standard errors of the estimated parameters from these fits. Boltzmann functions (steady state inactivation and peak conductance–voltage [G-V] relationship) were fit by use of a variable metric algorithm. The midpoint ($V_{0.5}$) and slope (q, the valence in units of the number of elementary charges; Yang et al., 1997) were estimated from these fits. The shift (ΔV) of the fitted G-V curve caused by MTSET modification was used to estimate the stabilization of the open state (French et al., 1996). Specifically, the change in free energy ($\Delta G°$) is given by

$$\Delta G^{o} = q \cdot \Delta V \cdot N_{\text{Avog}} \cdot e_0 , \qquad (1)$$

where q is the slope of the G-V curve, $N_{\text{Avog}} = 6.022 \times 10^{23}$ charges/mol and $e_0 = 1.602 \times 10^{-19}$ coulomb.

After modification by MTSET, the effect of 300-ms prepulses on peak current at −20 mV was fit by a product of two Boltzmann functions (see Fig. 3 *B*), one corresponding to steady state inactivation at relatively depolarized potentials, and the other corresponding to the probability of D2/S4 being in an outward position. Because of the nonzero asymptote at hyperpolarized potentials, the Boltzmann function describing D2/S4 position also has a nonzero asymptote. The slope, midpoint, and limits of each Boltzmann function were estimated simultaneously by least squares minimization

[1] *Abbreviations used in this paper:* D2:R1C, R669C mutant of hSkM1; D2:R1C-SET, MTSET-modified D2:R1C; G-V, conductance–voltage; I-V, current–voltage; MTS, methanethiosulfonate; SET, S-ethyltrimethylammonium; WT, wild type.

using a variable metric algorithm. For accuracy and speed, the analytic derivatives of the fitted function with respect to each parameter were used in the estimation. A variable metric algorithm was also used to fit the kinetics of recovery from inactivation in Fig. 4.

RESULTS

Voltage-dependent Modification of D2:R1C by MTSET

Substitution of the outermost arginine of the S4 segment of domain 2 (D2) by cysteine has a rather modest effect on the gating of the skeletal muscle sodium channel hSkM1, when expressed transiently in tsA201 cells. Whole cell currents of this mutant, D2:R1C, are compared with those of the wild-type (WT) channel in Fig. 1. Families of currents in response to a series of depolarizations are shown in Fig. 1, *A* and *B*, and the corresponding peak current–voltage (I-V) and conductance–voltage relationships are shown in Fig. 1, *C* and *D*. Boltzmann fits to the G-V data (theory curves in Fig. 1 *D*) show that the cysteine substitution produces a 5.7-mV depolarizing shift and a decrease in slope equivalent to a reduction of 1.4 elementary charges (e_0). These results are consistent with a role of this WT arginine of D2/S4 in sensing the membrane potential. By contrast with a cysteine substitution for the outermost arginine of the S4 segment of domain 4 (Chahine et al., 1994), the D2:R1C mutation has little effect on the inactivation kinetics (Fig. 1, *A* and *B*).

Modification of D2:R1C by the hydrophilic cysteine reagent MTSET (Fig. 2 *A*) mainly affects activation. MTSET causes a hyperpolarizing shift of both the peak current–voltage relationship (Fig. 2 *B*) and the kinetics of activation (Fig. 2 *C*), without affecting the time constant of inactivation (Fig. 2 *D*). Note: although the kinetics of inactivation appear slower after MTSET modification (Fig. 2 *A* vs. Fig. 1 *B*), this is primarily a consequence of the fact that currents at more hyperpolarized voltages are larger after MTSET modification, due to the shift in activation gating (Fig. 2 *B*), and to the fact that inactivation is slower at more negative voltages.

Some S4 residues in D4 are externally exposed only upon depolarization (Yang and Horn, 1995; Yang et al., 1996). To examine the possibility that D2:R1C is also accessible only when depolarized, we determined the rate of modification by extracellular 50 μM MTSET at three voltages, −150, −60, and −20 mV. To do this, we exploited the fact that the current at the foot of the activation curve increases dramatically after modification (Fig. 2 *B*). We therefore monitored the modification by measuring the peak current induced by a brief test pulse to −55 mV every 20 s, while exposing the cell to MTSET. If the membrane potential is maintained at −150 mV between these test pulses, there is very little modification of the channels (data not shown). A 9.7-s depolarization to −20 mV between the test pulses (see MATERIALS AND METHODS for details) exposes this cysteine residue to MTSET, causing a progressive increase in the amplitude of the current at −55 mV (Fig. 2 *E*; trace 0 represents the current before exposure to MTSET). As expected for an outward movement of D2/S4 when depolarized, the rate of modification by MTSET increases with depolarization (Fig. 2 *F*).

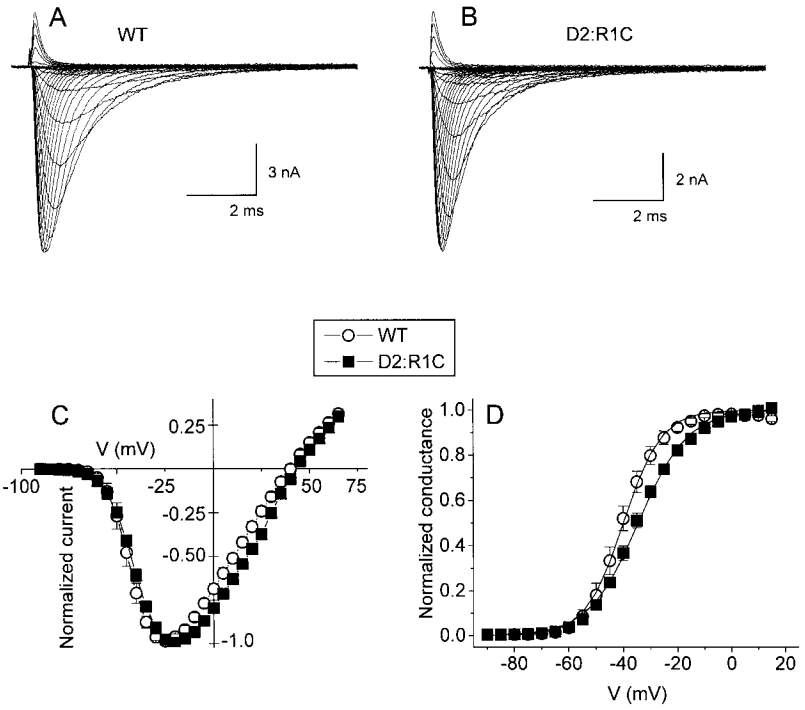

FIGURE 1. WT and D2:R1C sodium currents. (*A* and *B*) Sodium currents from the tsA cells transiently transfected with WT and D2:R1C cDNA elicited by a family of depolarizing pulses from a −140-mV holding potential to voltages ranging from −80 to +70 mV in 5-mV steps. (*C*) Current-voltage relationships normalized to the maximum peak inward current in WT (○, $n = 10$) and D2:R1C (■, $n = 14$) channels. (*D*) Conductance–voltage plots normalized to the maximum conductance and fit to the Boltzmann equation. The midpoint ($V_{0.5}$) and the slope (q) were −41.7 ± 1.3 mV, 4.37 ± 0.32 e_0 ($n = 10$) for WT and −36.0 ± 0.9 mV, 2.99 ± 0.08 e_0 ($n = 14$) for D2:R1C, respectively.

Certain D4/S4 residues are translocated completely from an internally accessible to an externally accessible position upon depolarization (Yang et al., 1996). To test whether this also occurs for D2:R1C, we introduced 400 μM MTSET into the patch pipette solution and, while maintaining the cells at a −140-mV holding potential, looked for shifts in gating equivalent to those observed for external application. Over a period of 20 min, no such effects were observed (data not shown). Furthermore, these cells remained susceptible to modification by extracellular 50 μM MTSET, showing that at hyperpolarized voltages D2:R1C is inaccessible on either side of the channel, whereas at depolarized voltages it is exposed externally. This is exactly equivalent to experiments involving the outermost basic residue of D4/S4 (Yang et al., 1996).

The above results can be interpreted as follows. Modification of D2:R1C by MTSET alters the conformation of D2/S4 to favor an open state of the channel. This could be explained if the probability of being in an outward position of D2/S4 is enhanced by the attachment of the SET adduct. In other words, the voltage-dependent probability of D2/S4 being in its outward conformation is shifted to more hyperpolarized voltages by MTSET modification. This shows, not remarkably, that D2/S4 plays a role as one of the four voltage sensors underlying activation. We were surprised, however, to see pronounced consequences of MTSET on what appeared to be inactivation at more hyperpolarized potentials.

Fig. 3 *A* shows the effects of a series of 300-ms prepulses on the currents elicited by a test pulse to −20 mV after modification by MTSET. This relatively standard procedure, used to quantify steady state inactivation, produced a nonmonotonic effect of prepulse potential on the peak current of the test pulse. Depolarization of the prepulse from −190 to ∼−110 mV caused an anomalous increase in current; further depolarization reduced the current strongly, as expected for steady state inactivation. Other characteristics of the currents are also affected by the prepulses, notably the kinetics of activation and inactivation (Fig. 3 *A*). The rates of these processes are both increased monotonically by prepulse depolarization, an effect not observed for un-

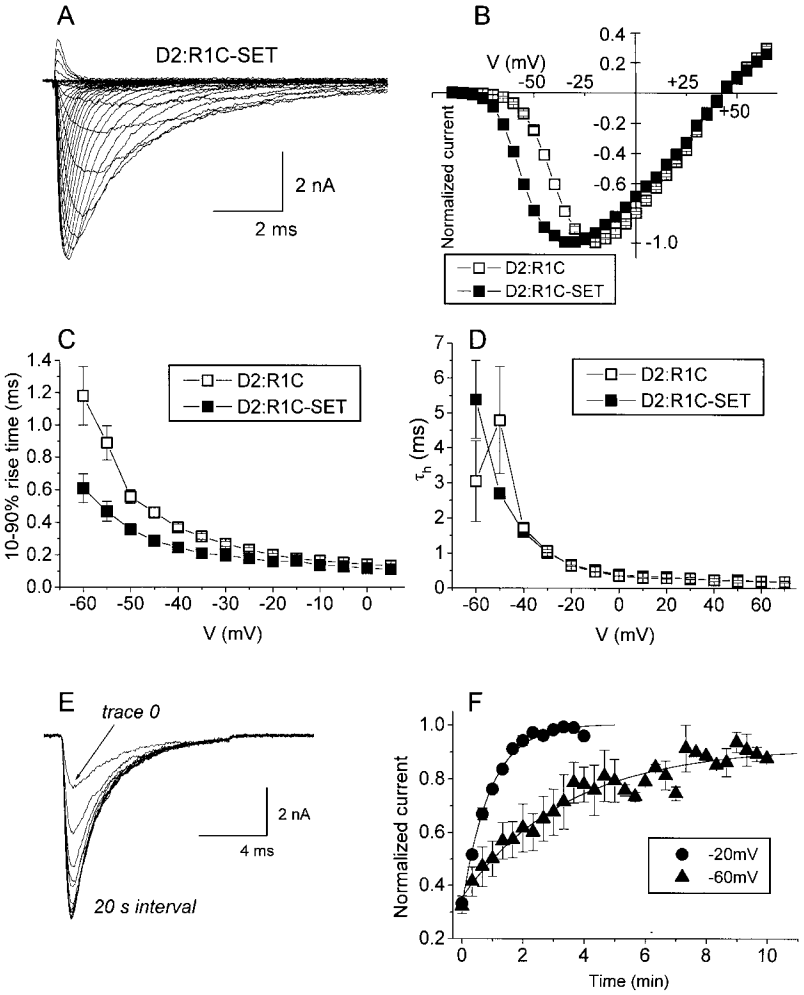

FIGURE 2. Effects of 50 μM MTSET on D2:R1C. (*A*) Currents activated after MTSET modification as described in Fig. 1 *A*. (*B*) Normalized I-V plots for D2:R1C before (n = 14) and after (n = 7) modification with MTSET. The G-V data derived from these I-V relationships were well fit by a Boltzmann relationship with $V_{0.5}$ and q estimates of −36.0 ± 0.9 mV, 2.99 ± 0.08 e_0 (unmodified, see Fig. 1 *D*) and −50.4 ± 1.0 mV, 2.35 ± 0.10 e_0 (modified, not shown). (*C*) Rise time between 10 and 90% of the peak amplitude vs. voltage before and after MTSET modification as a measure of activation kinetics (n = 6–10). (*D*) Inactivation time constants (τ_h) vs. voltage for D2:R1C and D2:R1C-SET (n = 3–4). The current decay was fit to a single exponential function. (*E*) Modification of D2:R1C by MTSET monitored as an increase in current amplitude after a voltage step to −55 mV every 20 s. Holding potential, −150 mV. A single 9.7-s depolarization to −20 mV was applied between test pulses to expose D2:R1C to extracellular MTSET (50 μM) (see MATERIALS AND METHODS for details). Trace 0 represents the current before exposure to MTSET. No effect of MTSET was observed in the absence of depolarization. (*F*) Normalized increase of peak current of D2:R1C by 50 μM MTSET after a 9.7-s depolarization to −20 mV (n = 3) or −60 mV (n = 3). The same modification procedure as described in Fig. 2 *E* was used. The theoretical curves are single exponential relaxations with time constants of 0.83 (−20 mV) and 3.86 (−60 mV) min.

modified channels (see below). Fig. 3 *B* plots the normalized peak current during the test pulse as a function of prepulse voltage, showing a typical steady state inactivation curve for D2:R1C before modification (□), and the pronounced alteration after MTSET modification (■).

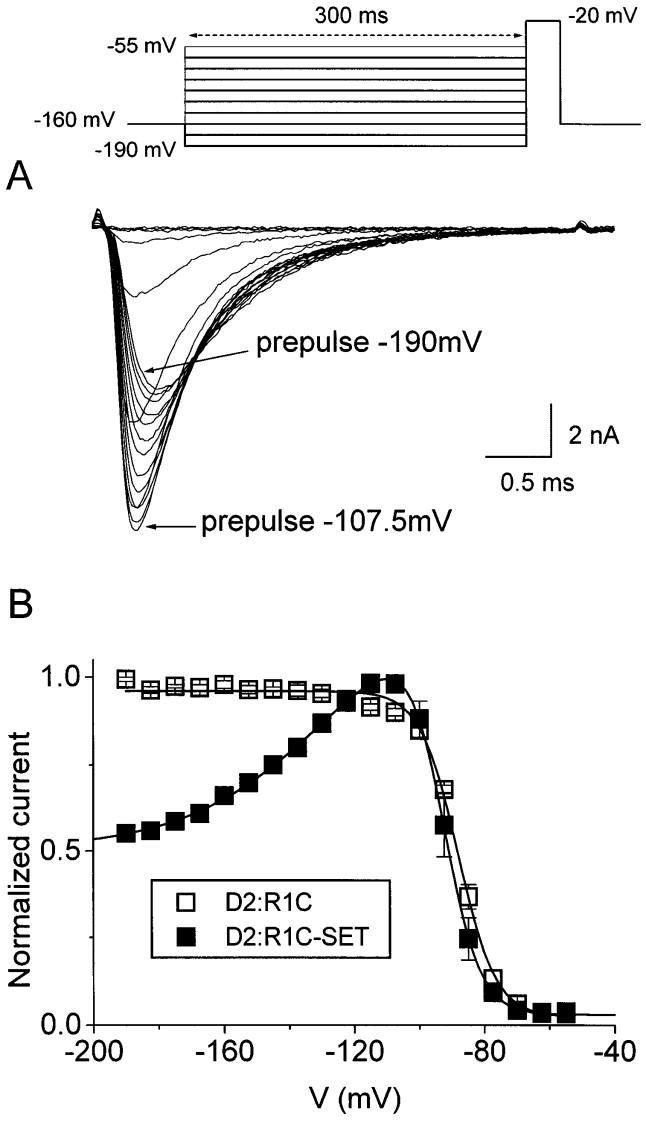

FIGURE 3. Steady state inactivation. (*A*) D2:R1C-SET currents elicited by a 3.7-ms test pulse to −20 mV after a series of 300-ms prepulses in 7.5-mV increments from −190 to −55 mV (see *inset*). Holding potential, −160 mV. Note the change of activation and inactivation kinetics as a function of prepulse potential. (*B*) Peak current vs. prepulse voltage, as in *A*, before and after modification with MTSET. The peak current is plotted as a fraction of maximal current. The data points are fit to a Boltzmann function (D2:R1C, $n = 3$) and a product of two Boltzmann equations (D2:R1C-SET, $n = 5$). The half inactivation ($V_{0.5}$) was −88 ± 1 and −94 ± 2 mV and the number of elementary charges moved across the membrane (q), was 5.7 ± 0.4 and 5.6 ± 0.2 e_0 for D2:R1C and D2:R1C-SET, respectively. Boltzmann parameters describing the probability of modified D2/S4 being inward or outward, $V_{0.5}$ and q, were −128 ± 3 mV and 1.19 ± 0.07 e_0.

Sluggish Movement of D2/S4 Induced by MTSET Modification of D2:R1C

What could be responsible for the unusual effects of prepulse potential on the currents during the test pulse? We decided to explore the following hypothesis. Suppose that cysteine modification causes not only a hyperpolarizing shift in the steady state conformation of D2/S4, but also a drastic reduction in the rate of its movement in response to changes of membrane potential. This could account for the effects observed in Fig. 3 *A* as follows.[2] A long-duration prepulse to −190 mV would drive all the S4 segments of a channel into their inward positions. A subsequent depolarization to −20 mV would elicit a current only after all S4 segments moved outward. The sluggish response of modified D2/S4 to the depolarization is expected to have three consequences on the macroscopic current elicited by a step to −20 mV. First, the kinetics of activation would be slow because the movement of D2/S4 would limit the rate of opening. Second, the kinetics of inactivation at −20 mV would be slow, because of coupling between the rate of channel opening and the rate of macroscopic inactivation (Aldrich et al., 1983). Finally, the amplitude of the current would be small because some closed channels would inactivate before they had a chance to open. A more depolarized prepulse, say to −110 mV, would set the modified D2/S4 in an outward conformation. A subsequent depolarization to −20 mV would drive the other S4 segments outward, allowing the rapid and efficient opening of the channels, because only three S4 segments have to move. More depolarized prepulses would have the usual effect of producing inactivation, and thus reducing the current amplitude. Note that this model assumes that outward movement of D2/S4 has a greater effect on activation than on inactivation, consistent with previous mutagenic studies (Chen et al., 1996; Kontis and Goldin, 1997; Kontis et al., 1997).

We fit the normalized data of Fig. 3 *B* with a product of two Boltzmann functions, one accounting for the probability of D2/S4 being in an inward versus an outward conformation, and the other for the probability of a channel being noninactivated. This makes the apparently extreme assumption that the conformation of D2/S4 after modification by MTSET is independent of the process of inactivation. However, in the voltage range of most relevance to the D2/S4 movement (more negative than −110 mV), there is little contami-

[2]For simplicity, we will describe the conformations of the S4 segments as "inward" at hyperpolarized voltages and "outward" at depolarized voltages. We also assume that each of the four S4 segments must be in a fully outward conformation for the channel to be open (Hodgkin and Huxley, 1952; Patlak, 1991; Keynes, 1994; Sigworth, 1994; Zagotta et al., 1994; Aggarwal and MacKinnon, 1996; Seoh et al., 1996).

nation from steady state inactivation. The fit of the double Boltzmann model to the data for modified channels (theory curve in Fig. 3 *B*) suggests that 50% of the D2/S4 segments are in outward conformations at a membrane potential of −128 ± 3 mV, and that this outward movement has a voltage dependence equivalent to a translocation of 1.19 ± 0.07 e_0 across the membrane electric field.

Our working hypothesis for the sluggish and shifted voltage dependence of D2/S4 movement after modification produces a number of kinetic predictions, which we explore here. One prediction, as discussed above, is that for certain voltages the kinetics of D2/S4 movement will be slower than those of inactivation. An example is shown in Fig. 4 *A*. In this experiment, all channels were inactivated by a 100-ms depolarization to −20 mV from a holding potential of −180 mV. Subsequently, a variable-duration return to −180 mV induced recovery from inactivation (see Fig. 4 *A, top,* for voltage protocol). A test pulse to −20 mV tracks the process of recovery (Fig. 4 *B,* ◆). During recovery at −180 mV, the amplitude of the current was briefly almost twice as large as it was before the channels were originally inactivated. This nonmonotonic recovery is expected if the recovery from inactivation at −180 mV is faster than the inward movement of D2/S4. For a recovery pulse of 2 ms, for example, D2/S4 has not had enough time to move inward, whereas recovery from inactivation is nearly complete. Longer duration recovery pulses cause the inward movement of D2/S4, which reduces the peak current in the same fashion as described for the pulse protocols of Fig. 3. This nonmonotonic recovery behavior is less pronounced for more depolarized recovery potentials, where there is

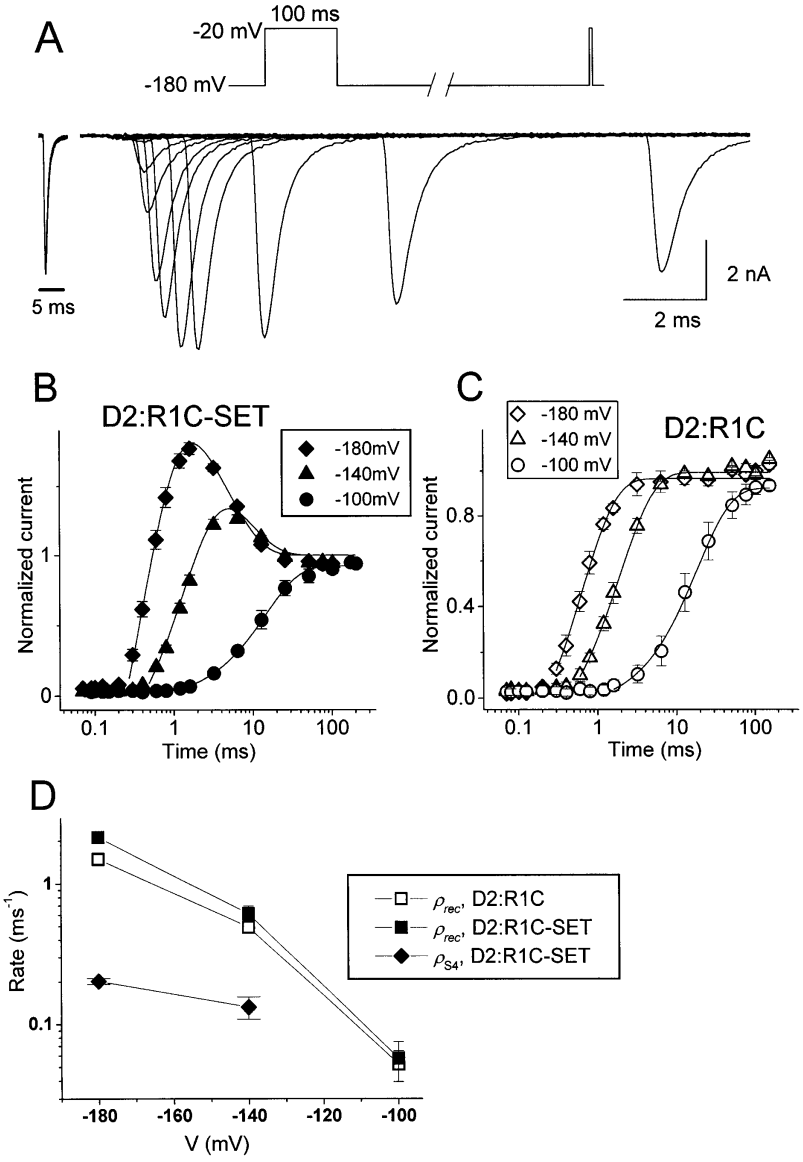

FIGURE 4. Recovery from inactivation. (*A*) Recovery from inactivation for D2:R1C-SET at −180 mV in the time interval between 0.06 and 300 ms. Inactivation of sodium channels was induced by a 100-ms prepulse to −20 mV (*left*) from a holding potential of −180 mV (see *inset*). The superimposed traces show, from left to right, currents elicited after increasing durations at −180 mV. Note the expanded time scale for currents elicited by the test pulse to −20 mV. Recovery from inactivation for D2:R1C-SET (*B*) and D2:R1C (*C*) at −100, −140, and −180 mV (n = 3–4), using voltage protocols as in *A*. The data points were normalized to the peak current of the 100-ms prepulse. Theoretical curves are fits either to a single exponential relaxation plus a voltage-dependent delay, or to Eq. 2. The estimates of $R_{out/in}$ are 2.52 ± 0.06 (−180 mV) and 3.33 ± 0.96 (−140 mV). These values are not significantly different. (*D*) Rate of recovery (ρ_{rec}) and inward movement of D2/S4 (ρ_{S4}) obtained from fits in *B* and *C*. The time course of recovery at −100 mV for modified channels is expected to be dominated by ρ_{rec}, because only 21% of D2/S4 segments are expected to move inward at −100 mV (see Eq. 2). Therefore, the estimate of ρ_{rec} at −100 mV was obtained from a fit to a single exponential.

less inward movement of D2/S4. For example, at −100 mV, only ~20% of the modified D2/S4 segments are expected to move inward (Fig. 3 *B*). Notice that recovery is preceded by a voltage-dependent delay of 200–1,000 μs, as observed in previous studies of sodium channels (Kuo and Bean, 1994; Ji et al., 1996). As expected for our working hypothesis, recovery from inactivation is monotonic for unmodified D2:R1C channels (Fig. 4 *C*).

We fit the recovery data for modified channels with a model in which D2/S4 movement has first-order kinetics, and recovery from inactivation is independent of the conformation of D2/S4. The ratio of peak currents of the second to the first depolarizations ($I_{rec}(t)/I_1$) is represented in this model as

$$I_{rec}(t)/I_1 = (1 - e^{-\rho_{rec}(t - t_{lag})}) \times \left(1 - \frac{1 - R_{out/in}}{1 + \frac{p_{out}}{p_{in}} R_{out/in}} e^{-\rho_{S4} t}\right), \quad (2)$$

where recovery has a single exponential relaxation with rate ρ_{rec} after a voltage-dependent lag t_{lag}, D2/S4 kinetics after modification are first order with rate ρ_{S4}, p_{out} is the steady state probability of D2/S4 being in an outward conformation, and $p_{in} = 1 - p_{out}$. Channels that had recovered from inactivation, and therefore were responsible for the currents during the test depolarization, were assumed to be in one of two kinetic states, corresponding to outward or inward conformations of D2/S4. The relative amplitude of the current elicited from these two conformations is designated in Eq. 2 as $R_{out/in}$. Because of the postulated independence of recovery and D2/S4 movement, the fraction of recovered current appears in Eq. 2 as a product of two probabilities.

The fit of the data in Fig. 4 *B* requires estimation of four free parameters, ρ_{rec}, ρ_{S4}, t_{lag}, and $R_{out/in}$. The values of p_{out} and p_{in} were obtained from the fits of prepulse inactivation in Fig. 3 *B*. Specifically, at −180 mV $p_{out} = 0.0761$, and at −140 mV $p_{out} = 0.360$. The best-fit theory curves for this model are shown as the solid lines for the nonmonotonic data at −180 and −140 mV (Fig. 4 *B*). The fit produces estimates of the rate of recovery from inactivation (ρ_{rec}) and the rate of inward movement of D2/S4 (ρ_{S4}). As expected, $\rho_{rec} > \rho_{S4}$ (Fig. 4 *D*). Furthermore, ρ_{rec} is not affected strongly by MTSET modification (compare Fig. 4 D, ■ and □), further supporting the assumption that the conformation of D2/S4 has little influence on recovery from inactivation.

Our working hypothesis also predicts that activation kinetics will depend on prepulse potential after D2:R1C channels are modified by MTSET. To measure these kinetics more accurately, we did a number of experiments at a reduced temperature of 11.1°C. We used a holding potential of −150 mV, and applied a series of depolarizations from −90 to +65 mV, preceded by a 300-ms prepulse to either −180 mV (Fig. 5, *A* and *B*, △) or −110 mV (▲). For unmodified D2:R1C, the more depolarized prepulse reduced the currents by a factor of 0.70[3] with no effect on activation kinetics (Fig. 5 *A*). After modification, the more depolarized prepulse increased the peak current by a factor of 1.70, consistent with the results of Fig. 3, and also increased the rate of activation (Fig. 5 *B*). This is the expected consequence on activation kinetics, as discussed above, because from the more hyperpolarized potential the outward D2/S4 movement is the rate-limiting step in activation in response to a depolarization.

We showed in Fig. 3 *A* that, after modification, prepulse voltage affects inactivation kinetics during a depolarizing test pulse in experiments intended to measure steady state inactivation. These kinetics were measured at 11.1°C, and inactivation time constants at −20 mV are plotted in Fig. 5 *C*. The rate of inactivation during the test pulse increases with prepulse depolarization, an effect seen only after modification of D2:R1C by MTSET (Fig. 5 *C*, ■). Q_{10} values of ~2.1 are obtained for inactivation kinetics by comparing the data in Figs. 2 *D* and 5 *C*. By contrast with inactivation kinetics, deactivation kinetics are not affected by MTSET modification (Fig. 5 *D*). The latter result indicates that D2/S4 of the mutant channel plays little role in deactivation, suggesting that one or more of the other three S4 segments is responsible for the rapid closing of the activation gate in response to a hyperpolarization.

First-Order Kinetics for Modified D2/S4

Movement of D4/S4 is first order and voltage dependent in the unmodified cysteine mutant D4:R1C (Yang and Horn, 1995). The data of Fig. 4 are also consistent with this idea for MTSET-modified D2/S4. Here we provide even stronger evidence that the movement of modified D2/S4 is a first-order process. A stringent requirement for such a process is that the kinetics at any voltage must relax as a single exponential with a time constant independent of initial condition. We estimated the kinetics of D2/S4 movement at room temperature by working in a voltage range more negative than that of inactivation (i.e., between −190 and −110 mV; Fig. 3 *B*). Fig. 6 *A* shows the normalized peak current at −20 mV for D2:R1C-SET in response to variable duration prepulses to either −150 mV (Fig. 6 *A*, □ and

[3]This is a greater reduction than depicted in Fig. 3 *B* (*open symbols*) for experiments performed at room temperature. The cooling to 11.1°C caused a hyperpolarizing shift in the steady state inactivation of unmodified channels (data not shown).

■; see inset) or −135 mV (△ and ▲). In each case, the initial position of D2/S4 was set by a 500-ms conditioning pulse to either a highly negative voltage (Fig. 6 *A*, D2/S4 inward, open symbols) or to −110 mV (D2/S4 outward, closed symbols). The data, plotted against a logarithmic time axis, are well fit by single-exponential relaxations for all voltage protocols. Although the time constants are smaller at −150 than at −135 mV (7.7 ± 0.1 vs. 9.4 ± 0.1 ms), they are independent of the initial conditions at each voltage (see legend to Fig. 6 for details), consistent with a first-order process. These results indicate that the rate of inward D2/S4 movement is the same as the rate of outward movement at a given membrane potential.

We combined the time constants measured at −190, −150, −135, and −110 mV (Fig. 6 *B*, ●) with steady state estimates of the probability of D2/S4 being in an outward position (Fig. 3 *B*), to obtain estimates of the voltage-dependent rate constants for outward (α) and inward (β) movement of this modified S4 segment. These two rate constants are plotted in Fig. 6 *C*, where it is also shown that each is an exponential function of membrane potential (*solid lines*). The time constants for D2/S4 movement can be predicted from the theoretical values of α and β over a wide voltage range ($\tau_{S4} = 1/[\alpha+\beta]$), as shown by the solid line in Fig. 6 *B*. These time constants may be compared with the estimates of $\tau_{S4} = 1/\rho_{S4}$ that we obtained from a very different measurement, recovery from inactivation (Fig. 4). These estimates (Fig. 6 *B*, □) show a good correspondence with the results of experiments like those in Fig. 6 *A*, supporting both that D2/S4 movement is first order and that it is independent of recovery from inactivation.

Our data are therefore consistent with a very simple model for the voltage-dependent movement of D2/S4 after modification. The process is not only first order, but the rate constants are also exponential functions of membrane potential. The time constants in Fig. 6 *B* are also larger than those observed for both deactivation (Fig. 5 *D*) and recovery from inactivation (Fig. 4 *D*),

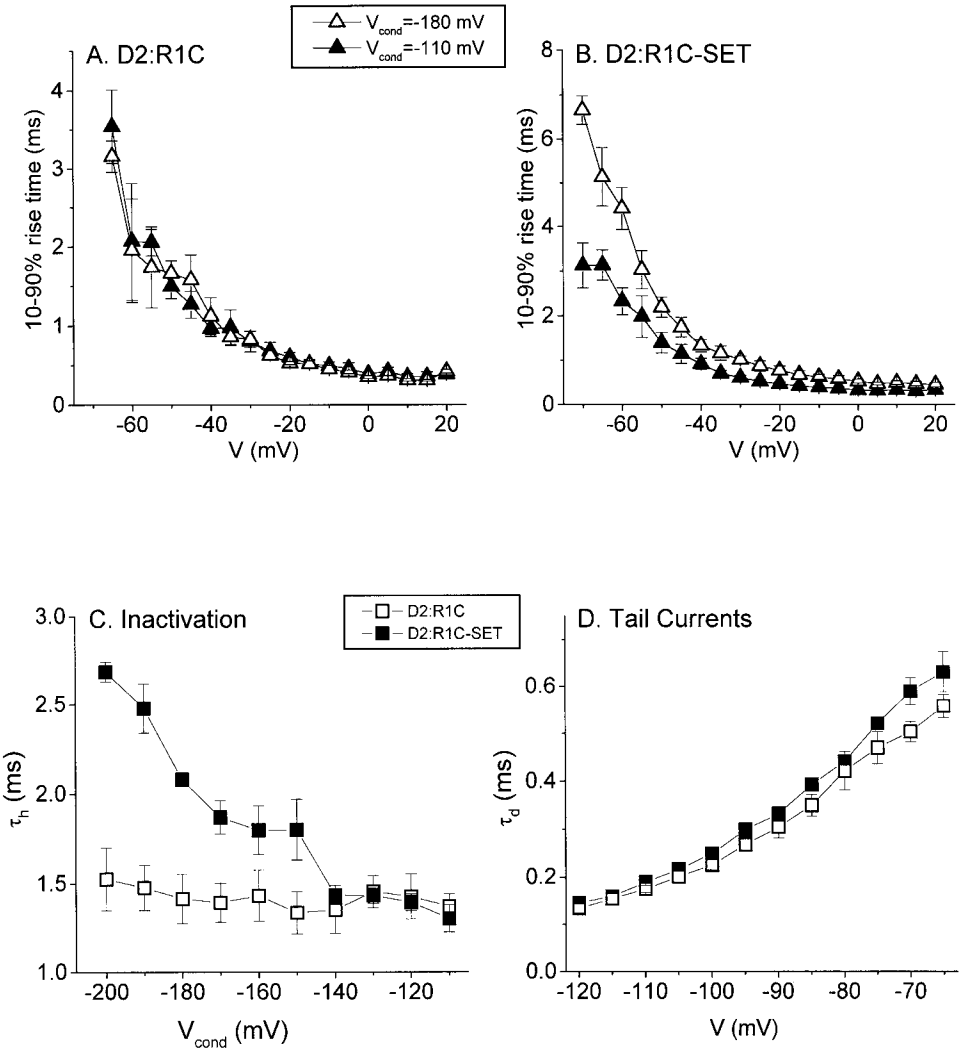

FIGURE 5. Effects of a conditioning prepulse on steady state activation and kinetics of channel gating. For better resolution, recordings were done at 11.1°C. (*A* and *B*) 10–90% rise time vs. voltage depending on the conditioning pulse. A 300-ms conditioning pulse to either −180 or −110 mV was applied before the steady state activation voltage protocol. (*C*) Inactivation time constants (τ_h) at −20 mV vs. prepulse voltage from recordings as shown in Fig. 3 *A*. (*D*) Deactivation time constants (τ_d) vs. voltage. Channels were activated by a 700-s depolarization to +20 mV. The tail current decay was fit by a single exponential relaxation.

consistent with our hypothesis that modification of D2:R1C by MTSET makes the D2/S4 movement sluggish.

DISCUSSION

The S4 segments of voltage-gated ion channels are now known to be the principal voltage sensors for gating. This has been demonstrated primarily by two classes of experiments, the effects of S4 mutations on both ionic and gating currents, and the voltage-dependent accessibilities of specific residues using cysteine scanning methods. The cysteine scanning studies provide evidence that S4 segments move when the membrane potential changes over an appropriate range (Yang and Horn, 1995; Larsson et al., 1996; Yang et al., 1996, 1997). Corroborative evidence using fluorescently tagged S4 segments also supports the idea of voltage-dependent S4 movement (Mannuzzu et al., 1996). Furthermore, the kinetics of S4 movement occur on the same time scale as gating kinetics (Yang and Horn, 1995; Mannuzzu et al., 1996). It is important to note, however, that "movement" in all of these studies is defined operationally. Some relative movement certainly occurs between S4 segments and the membrane electric field, the lipid bilayer, and/or other proteinaceous regions of the channel. There has been some speculation how this relative movement translocates charge (Armstrong, 1981; Catterall, 1986; Guy and Conti, 1990; Sigworth, 1994; Aggarwal and MacKinnon, 1996; Larsson et al., 1996; Seoh et al., 1996; Yang et al., 1996). However, the molecular details remain a mystery.

How does modification of S4 segments, either by mutagenesis or by reaction with cysteine reagents, alter gating, and what do such experiments tell us about S4 function? Two obvious consequences of S4 modification could be a change in the equilibrium conformation of the transmembrane segment with respect to the electric field and a change in the kinetics of S4 movement. Our data with D2:R1C suggest that the SET adduct affects both. It energetically biases the S4 segment to be in an outward position, by comparison with an unmodified cysteine. This is seen most clearly from the hyperpolarizing shift of the peak current–voltage relationship caused by MTSET modification (Fig. 2 *B*). The 14.4-mV shift is equivalent to a 3.7-kJ/mol stabilization of the open state (Eq. 1). Note that this calculation underestimates the effect of the modification on the steady state conformation of D2/S4, because this is only one of four S4 segments contributing to the opening of the channel.

We have also claimed that the kinetics of D2/S4 movement are markedly slowed by modification of D2:

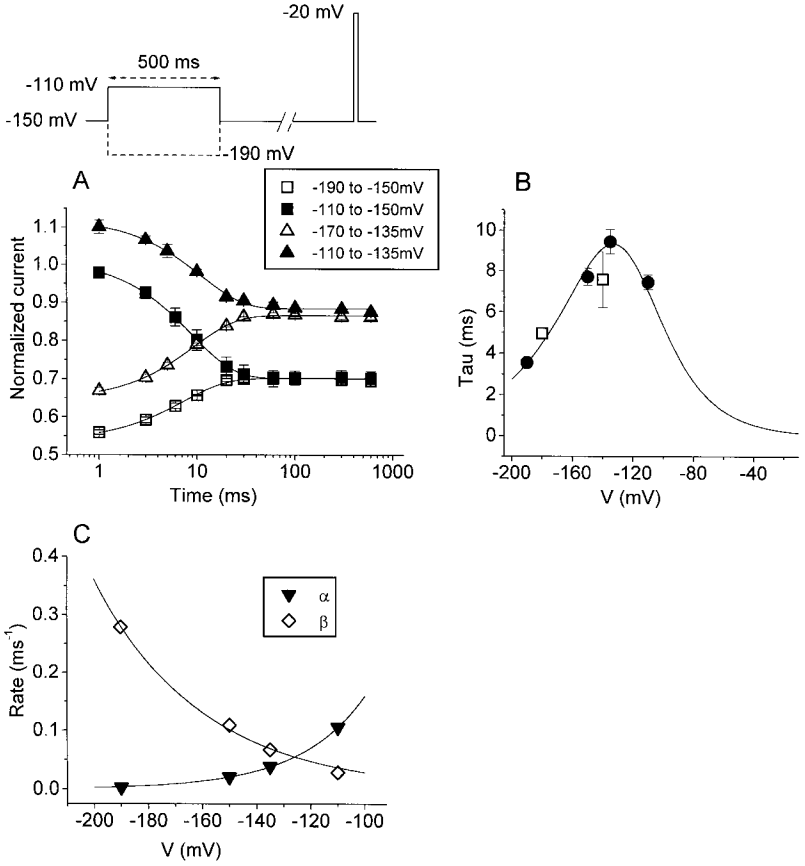

FIGURE 6. Estimation of kinetics of D2/S4 movement. (*A*) Normalized peak current for D2:R1C-SET at −20 mV after a variable duration prepulse to −150 mV (*squares*, n = 3–4). A conditioning pulse either to −190 or −110 mV was used to set D2/S4 in an inward or outward position, respectively (see *inset*). A similar procedure was used for a prepulse to −135 mV (*triangles*, n = 4). In these recordings, conditioning pulses were set to −170 and −110 mV. Holding potential was −150 mV in all cases. The change in the peak amplitude was fit to a single exponential relaxation. Time constants for −150 mV were 7.31 ± 0.75 ms (−190-mV conditioning pulse), and 7.71 ± 0.42 ms (−110-mV conditioning pulse). Time constants for −135 mV were 9.45 ± 0.60 ms (−170-mV conditioning pulse), and 11.18 ± 0.60 ms (−110-mV conditioning pulse). (*B*) Time constants of D2/S4 movement measured at four voltages as in *A* (●). Estimates of τ_{S4} obtained from recovery time course (Fig. 4 *D*) are plotted as □. Time constant of D2/S4 kinetics (*solid line*) predicted from the theoretical fits of α and β in *C*, where $\tau_{S4} = 1/(\alpha+\beta)$. (*C*) α and β are the rate constants for outward and inward movement of D2/S4, respectively. Estimates of these rate constants at each voltage were obtained from the following relationships. $\alpha = p_{out}/\tau_{S4}$ and $\beta = \tau_{S4}^{-1} - \alpha$. p_{out} was estimated from the double Boltzmann fit in Fig. 3 *B*. Lines represent single exponential fits to $Ae^{\kappa V}$, with A = 9.51 ms^{-1} (α) and 0.0022 ms^{-1} (β), and κ = 0.0409 mV^{-1} (α) and −0.0254 mV^{-1} (β).

R1C, based on the results of Figs. 3–6. Before considering the molecular implications of this interpretation, we must first consider the alternative hypothesis, that the sluggish kinetics induced by modification occur downstream from actual S4 movement. How do we know, for instance, that the slow kinetics are due specifically to a retardation of D2/S4 movement, rather than to a slower conformational change that occurs after S4 movement? In fact, we cannot exclude this possibility, although it is likely that the MTSET-modified S4 segment is capable of moving and thereby translocating charge. We previously showed that another S4 segment, D4/S4, is capable of translocating a SET adduct from the extracellular to intracellular face of the protein, where it can be cleaved off by a reducing agent (Yang et al., 1996). Therefore, we will assume for discussion that the slowed activation kinetics caused by MTSET are due to slowed S4 movement.

How does the SET adduct slow S4 movement? One possibility is that the bulk of cysteine-SET, with a volume nearly twice that of the WT arginine, increases the height of the activation barrier for translocation due to steric hindrance. This is apparently not the case, however, because modification by the smaller cationic reagent MTSEA had a qualitatively similar effect as MTSET on prepulse inactivation, whereas the intermediate-sized anionic reagent MTSES did not (data not shown). Charge by itself cannot be responsible for the sluggish movement, however, because the WT arginine, like MTSET, is cationic. Our data suggest, therefore, that D2/S4 movement is sensitive to the structure of the residue at this position of the S4 segment. Note that if modification prevented S4 movement altogether, we would expect a very different consequence, assuming standard models for activation. If, for example, the adduct trapped the S4 segment in an inward position, the channel would presumably be incapable of opening. By contrast, if it were stuck outward, activation would be shifted in a hyperpolarizing direction and channel opening would be easier, albeit less voltage dependent. This prediction is consistent with our results. However, a frozen S4 segment is inconsistent with the peculiar effects of hyperpolarizing prepulses.

Contrast between Channels and S4 Segments

Whereas many potassium channels are believed to be homotetramers with four identical S4 segments, the α subunits of sodium channels and the α_1 subunits of calcium channels are monomers, each of which contains four different S4 segments. The difference in primary structure among the S4 segments of sodium channels is highlighted by the fact that the number of basic residues ranges from four to eight in the four domains. Some differences in the function of the individual S4 segments of both sodium and calcium channels have been revealed by systematic mutagenesis (Chen et al., 1996; Garcia et al., 1997; Kontis and Goldin, 1997; Kontis et al., 1997).

One of the most surprising results of this study is that activation is so strongly affected by MTSET modification, whereas both steady state inactivation (Fig. 3 *B*) and the voltage-dependent kinetics of inactivation (Figs. 2 *D* and 4) are relatively insensitive to this treatment. Although the earliest voltage-clamp study of sodium channel gating proposed a model in which activation and inactivation were independent processes (Hodgkin and Huxley, 1952), a myriad of later experiments showed that these processes are strongly coupled in the sense that inactivation gets much of its voltage dependence from activation (for reviews, see Armstrong, 1981; French and Horn, 1983; Bezanilla, 1985; Patlak, 1991; Keynes, 1994). However, some aspects of sodium channel inactivation are inherently voltage dependent (Swenson, 1983; Vandenberg and Horn, 1984; Greeff and Forster, 1991; Sheets and Hanck, 1995). Furthermore, activation gating can be shifted substantially by mutations in the S4 segments of domains 1–3 without affecting inactivation kinetics (Stühmer et al., 1989; Chen et al., 1996; Kontis and Goldin, 1997; Kontis et al., 1997).

The coupling between activation and inactivation is not completely oblivious to the conformation of D2/S4, because if opening is slowed by a hyperpolarized prepulse, macroscopic inactivation is also slowed (Figs. 3 *A* and 5 *C*). This is consistent with the fact, as demonstrated by single channel studies (Aldrich et al., 1983; Horn and Vandenberg, 1984), that open channels tend to inactivate more rapidly than closed channels. However, conformational changes of D2/S4 that bypass the open state have no apparent effect on either the voltage dependence or kinetics of inactivation (Figs. 3 *B*, 4, and 6 *B*). Overall, the data support the idea that some voltage sensors, especially D4/S4, strongly affect inactivation, whereas others, like D2/S4, play a relatively minor role in the inactivation process. Our results indicate that, as in the Hodgkin-Huxley model (see also Keynes, 1994), certain aspects of activation gating are poorly coupled to inactivation. This component of the activation machinery, which apparently includes D2/S4, is likely to contribute to the asymmetrical gating charge that is not immobilized by inactivation (Armstrong and Bezanilla, 1977). Mutagenic studies are beginning to elucidate which voltage sensors are dedicated primarily to activation and which serve double duty for both gating mechanisms.

After modification, the activation kinetics are slowed by hyperpolarized prepulses (Figs. 3 and 5). A similar phenomenon is seen for potassium current (Cole and Moore, 1960); however, the effect is typically much

smaller in sodium currents (Armstrong and Bezanilla, 1974). A partial explanation of this phenomenon in potassium channels is that there is substantial slow gating charge movement at very hyperpolarized potentials (Bezanilla et al., 1994). Our results suggest that charge movement in sodium channels in this voltage range is typically fast. Modification of D2/S4 shifts its voltage dependence and slows its kinetics, sufficient to introduce a substantial Cole-Moore shift into these sodium channels.

Another contrast between our studies and those involving potassium channels is that we have been able to describe our data for both D2/S4 and D4/S4 by a first-order kinetic process. Detailed studies of potassium channels suggest, however, that S4 movement occurs in at least two sequential steps upon a change of membrane potential (Schoppa et al., 1992; Bezanilla et al., 1994; Sigg et al., 1994; Zagotta et al., 1994); similar arguments have been advanced for sodium channels, based largely on gating current measurements in squid axon (Keynes, 1994). The most likely explanation for the discrepancies in these studies is that the charge translocation in sodium channels is also sequential but has a predominant rate-limiting step. As evidence of this possibility, the rate-limiting kinetic process revealed by D2/S4 modification does not have the full voltage dependence expected for an S4 segment, only translocating $\sim$1.2 e_0. In fact, the sequential nature of charge movement in potassium channels can be rather subtle in WT channels (e.g., Schoppa et al., 1992; Seoh et al., 1996). In the case of sodium channels, the data presented here and previously (Yang and Horn, 1995) show that S4 movement can be represented as a first-order process, but the supporting evidence always involves mutated S4 segments, begging the question whether WT S4 segments behave in this manner. In spite of this ambiguity, the modification employed in this paper allowed us the unique opportunity to examine the voltage-dependent movement of a single S4 segment and also the relationship between the D2/S4 conformation and the gating behavior of the channels.

Clearly the translocation of S4 charges in potassium, calcium, and sodium channels involves a delicately orchestrated procession of steps that probably includes hydration and dehydration of charged residues as they traverse the core of the protein. The local environment at each end of the S4 segment undoubtedly plays a role in the energetics of S4 movement. Our data with D4/S4, for example, suggest the presence of a negatively charged vestibule at the outer mouth of the S4 channel (Yang et al., 1997).

HERG-like Behavior of a Sodium Channel

A class of cardiac arrhythmias has been linked to the potassium channel gene HERG (Curran et al., 1995). The currents of the HERG channel are inwardly rectifying, although the channel has the structure of a typical outward rectifier (Sanguinetti et al., 1995; Trudeau et al., 1995; Schönherr and Heinemann, 1996; Smith et al., 1996; Wang et al., 1997). The bizarre biophysical properties of HERG are neatly explained by the fact that inactivation is more rapid than activation upon depolarization, and recovery from inactivation is more rapid than deactivation upon hyperpolarization (Sanguinetti et al., 1995; Trudeau et al., 1995; Schönherr and Heinemann, 1996; Smith et al., 1996; Wang et al., 1997). Our results with modified D2:R1C sodium channels are similar to those obtained with HERG, although the kinetic discrepancies between activation and inactivation are not as severe in the sodium channel. Specifically, prepulse inactivation and recovery from inactivation are nonmonotonic in both cases. As in HERG, the biophysical abnormality of modified D2:R1C sodium channels is due primarily to unusually slow activation kinetics. The data in both cases show that a delicate balance between the kinetics of these two processes is crucial for normal function.

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