Sodium Channel Inactivation from Closed States: Evidence for an Intrinsic Voltage Dependency

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ABSTRACT The time course of Na channel inactivation from closed states was determined on inside-out excised patches from neuroblastoma N1E 115. Closed-state inactivation develops as a single exponential with mean time constants of 66.4 ms at −80 mV, 29.6 ms at −70 mV, 20.1 ms at −60 mV, and 15.1 ms at −50 mV. Corresponding mean steady-state values of the fitted exponentials were 0.321, 0.098, 0.035, and 0. Closed-state inactivation, in general, should develop either with a delay or as more than one exponential, depending on which closed state(s) directly inactivate. The absence of additional components cannot be attributed to a rate of exchange between closed states too rapid to detect. The time course is simply accounted for if all closed states directly inactivate and do so with the same rate constant for each closed state, suggesting that those conformational changes constituting the transitions between closed states have little effect on the structural components involved in inactivation. Closed to inactivated rate constants ranged from a mean of 0.0108 ms^{-1} at −80 mV to 0.0690 ms^{-1} at −50 mV. This voltage dependency is entirely intrinsic to closed-state inactivation with closed to inactivated rate constants similar for all closed states. Over the potential range studied nearly all the inactivation is from closed states.

INTRODUCTION

On a depolarizing step in potential, Na channels progress to the inactivated state by two different routes. Some channels open before the inactivation gate can close (Goldman and Kenyon, 1982; Aldrich and Stevens, 1983; Goldman, 1989). Channels can also inactivate without having opened (Gillespie and Meves, 1980; Bean, 1981; Horn et al., 1981; Aldrich and Stevens, 1983; Horn and Vandenberg, 1984). However, nearly nothing is known about this process of inactivation from closed states. We have, then, one function of the Na channel protein that is essentially undescribed, and the first task is to characterize its properties.

Aldrich and Stevens (1983) presented a method for studying inactivation from closed states. Using single channel recording, an ordinary two-step (conditioning pulse–test pulse with no intervening gap) determination of the inactivation time course is made. Average currents are constructed from the single-channel records during the test pulses. Peak value of the average test pulse current against conditioning pulse duration traces out the time course of Na channel inactivation that developed during the conditioning pulse. If now only traces with no channel openings during the conditioning pulse are included in the average, inactivation just from closed states is isolated. Aldrich and Stevens (1983) reported just two values for the time constant of inactivation from closed states, τ_{IC}. Two determinations of τ_{IC} at −90 mV were also reported by Lawrence et al. (1991).

τ_{IC} in excised patches from neuroblastoma was determined, using a protocol slightly modified from that of Aldrich and Stevens (1983). The observed closed-state inactivation time course is simply accounted for if all closed states can inactivate and do so with about the same closed to inactivated rate constant for each closed state. In this case, the observed voltage dependency of τ_{IC} would be an intrinsic property of the inactivation process. Over the potential range examined in these experiments, −80 to −50 mV, nearly all of the inactivation is from closed states. Preliminary reports of some of these results have been made (Goldman, 1994, 1995a).

MATERIALS AND METHODS

Methods for cell culture and electrical recording were as described in Goldman (1995b). Briefly, all single channel recordings were from inside-out excised patches from neuroblastoma N1E 115 cells. Seal resistance typically ranged from 20 to 50 GΩ. The external recording (pipette) solution contained 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, and 10 mM HEPES, pH adjusted to 7.30 ± 0.05 with NaOH. The solution bathing the cytoplasmic face of the membrane contained 145 mM CsF, 10 mM CsCl, 5 mM Cs-EGTA, and 10 mM HEPES, pH adjusted to 7.30 ± 0.05 with CsOH. A modification was that the continuously flowing solution inflow to the recording dish was via an electrically controlled DC pump (N-07002-33 and N-07002-39; Cole-Palmer, Niles, IL) rather than by gravity feed. In this way, better control of temperature was achieved, and temperature in these experiments was 7.5 ± 0.5°C.

Data were sampled at 100-μs intervals, except for conditioning pulses longer than 90 ms, where the sampling interval was 200 μs. Records were usually low-pass filtered at 2 kHz (−3 dB), but in a few cases at 1 kHz. Pulses were presented at 1/s except for conditioning pulses longer than 90 ms, where they were presented at 1/2 s.

Data analysis

Leak and capacity currents were reduced by analog subtraction during the experiment and eliminated by digital subtraction of the average of traces with no channel openings or sometimes by an appropriately scaled average from a smaller depolarizing step. Data acquisition and construction of average currents from the single-channel records was performed with

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pCLAMP (Axon Instruments, Foster City, CA). All other data analysis was performed with SigmaPlot (Jandel Scientific, San Rafael, CA).

RESULTS

Time course of closed-state inactivation

Aldrich and Stevens (1983) reported a τ_{IC} of 65.9 ms at −50 and one of 1.74 ms at −10 mV. There was considerable variance in these determinations. Two sources of this variance are that, first, channel activity always decreases substantially over the lifetime of the patch (Goldman, 1995b), and second, even over periods of several minutes peak value of the unconditioned test pulse current is not very stable, and can either increase or decrease, especially in patches with few active channels. To reduce the variance in these determinations, presentations of the test pulse alone, with no conditioning pulse, were included before and after each conditioning pulse–test pulse pair. Peak values of the conditioned test pulses were then normalized to the mean of these bracketing unconditioned test pulse controls. This did reduce the variance, but now an individual τ_{IC} determination required from 1.5 to 2.5 h of recording from a single patch. Control experiments established that kinetics remained stationary in excised patches for these times (Goldman, 1995b). Determinations were discarded if bracketing unconditioned controls differed by more than 25% or if fewer than 40 single channel traces were available to construct an average current. If the peak value of an unconditioned control current fell below 0.4 pA, that determination and the rest of the experiment were discarded. The holding potential was −120 mV and the test potential was −20 mV throughout.

Fig. 1 presents a plot of the time course of inactivation at each of the four conditioning potentials studied. These plots show the time course of inactivation just from closed states. Traces with channel openings during the conditioning pulse have been excluded. The peak value of the test pulse current relative to that with no conditioning pulse is shown as a function of conditioning pulse duration. Each determination is from a different patch. The solid curves indicate best-fit single exponentials. The closed-state inactivation time course could always be well described by a single exponential.

Collected τ_{IC} values are shown in Fig. 2. Each point is from a different patch. Mean values ranged from 66.4 ms at −80 to 15.1 ms at −50 mV. On average about six patches were excised and data collected for each completed τ_{IC} determination. In most cases channel activity fell to too low a level before a sufficient number of data points had been collected. It is difficult to extend the potential range of these observations. At more positive potentials traces with no openings during the conditioning pulse become very infrequent. At more negative potentials relatively little inactivation develops, and it is necessary to resolve small differences.

Collected steady-state values of the single exponential fits are shown in Fig. 3. Circles indicate means and the brackets the total range of variation. The mean at −80 mV must be taken as biased to the low side. Patches for which relatively little inactivation developed could not be reliably analyzed, and so larger steady-state values were systematically excluded. For this reason no attempt was made to fit a Boltzmann function to the data of Fig. 3.

Which closed state(s) directly inactivate?

A question of interest is, which closed states can directly inactivate? The question is a somewhat difficult one. The

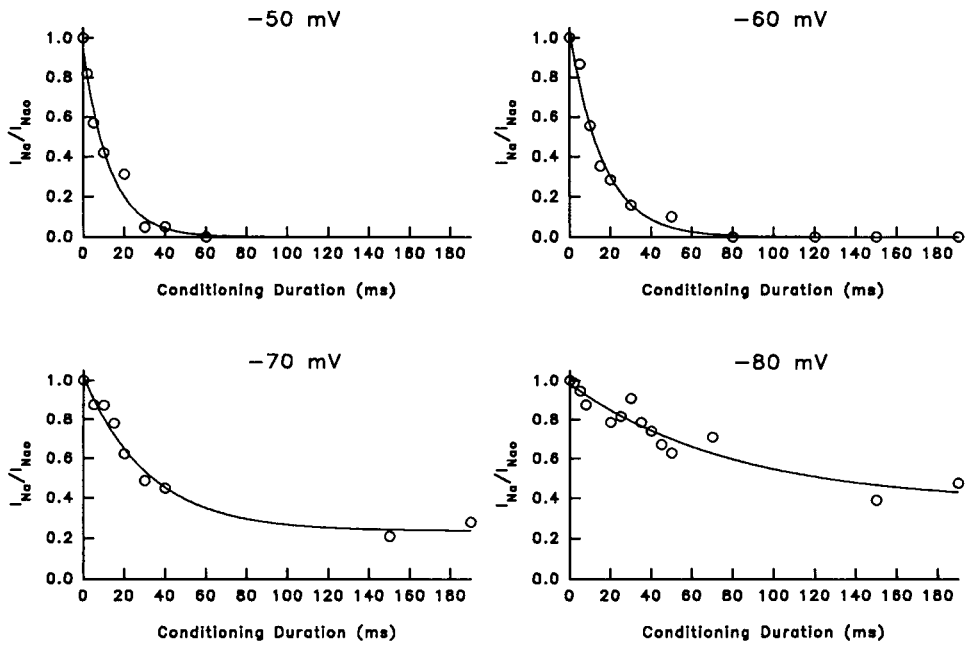


FIGURE 1 Time course of inactivation from closed states at each of the indicated potentials. Peak value of the average current during the test pulse, relative to that with no conditioning pulse, is shown as a function of conditioning pulse duration. Each plot is from a different patch. Solid lines are best-fit single exponentials. For these and all determinations the holding potential was −120 mV, the test pulse potential was −20 mV, and the temperature was 7.5 ± 0.5°C.

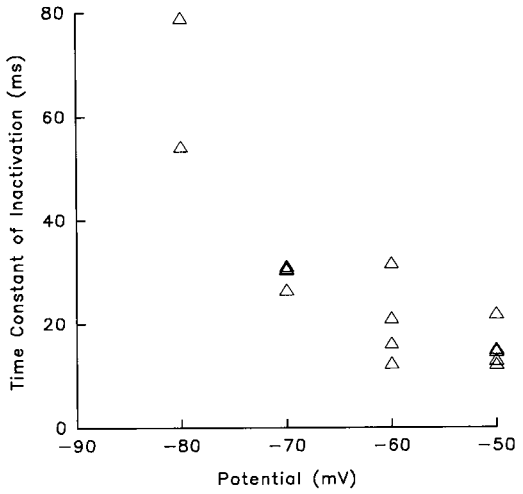


FIGURE 2 Time constant of inactivation from closed states, τ_{IC}, as a function of membrane potential. Each value is from a different patch.

number of closed states is not even known with any certainty. However, some information might be gained. For example, if closed-state inactivation developed with a delay, then it could not be only the closed state furthest from the open state that directly inactivated. The experimental results, however, show no indication of a delay or any other additional relaxation.

Table 1 presents collected results for the single exponential fits to the closed-state inactivation time course. Values of the zero time intercepts are shown in the middle column. Means at each potential are never far from unity, and the overall mean is 0.993. The experimental result, then, is a zero time intercept at or near unity.

TABLE 1 Time course of Na channel inactivation from closed states in inside-out excised patches from neuroblastoma N1E 115

Patch	Conditioning potential (mV)	Zero time value	Time constant (ms)	Steady-state value
50317	−50	—	—	0
50322	−50	1.0180	21.70	0
50324	−50	0.9259	11.91	0
50329	−50	0.9574	12.76	0
50331A	−50	0.9590	14.85	0
50331B	−50	1.0048	14.46	0
Mean		0.9730	15.14	0
60D29A	−60	—	—	0.1029
60D29B	−60	1.0077	31.48	0.1479
60107	−60	—	—	0
60121A	−60	—	—	0
60121B	−60	0.9529	12.14	0
60122	−60	1.0452	16.01	0
60330	−60	0.9959	20.84	0.0259
60408	−60	—	—	0
Mean		1.0004	20.12	0.0346
70415	−70	1.0520	26.35	0
70510	−70	1.0238	30.90	0.2360
70511	−70	0.9279	30.47	0.1553
70818	−70	1.0210	30.53	0
Mean		1.0062	29.56	0.0978
80720	−80	0.9824	78.77	0.3742
80N12	−80	1.0204	54.07	0.2669
Mean		1.0014	66.42	0.3206

Holding potential was −120 mV, and test potential was −20 mV throughout. Temperature was 7.5 ± 0.5°C. Steady-state values were determined either from the single exponential fits or independently measured.

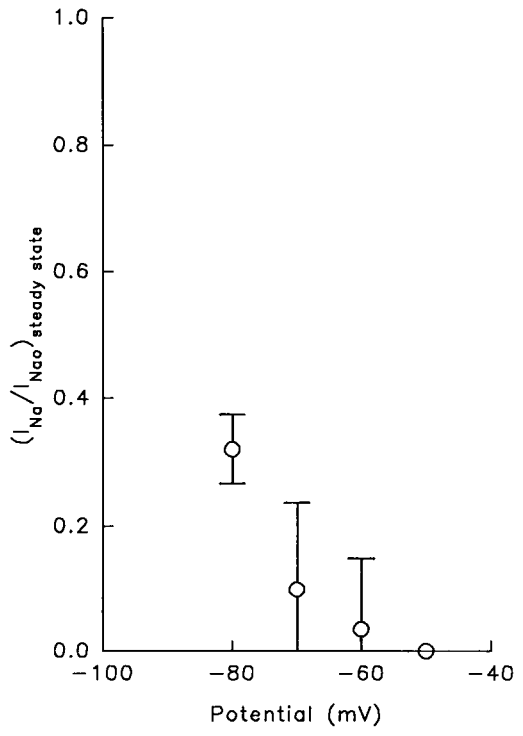
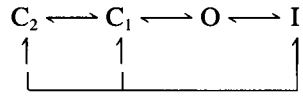


FIGURE 3 Steady-state value of the average test pulse current relative to that with no conditioning pulse. Values are taken from the single exponential fits to the closed-state inactivation time course or independently measured. Circles indicate mean values and the brackets the total range of variation.

Several experiments were examined to see if any additional exponential component could be reliably identified. Fig. 4 shows one example at a conditioning potential of −70 mV. This experiment was selected because it had a large number of data points, a well-defined steady-state value, and relatively low variance. An additional exponential component with reliable parameters could not be defined in these data, in agreement with the zero time intercept values. Only a single exponential component is identifiable in the closed-state inactivation time course.

To assess what sort of behavior might be expected, computations were performed on a two closed, one open, one inactivated state scheme (Scheme 1).



Scheme 1

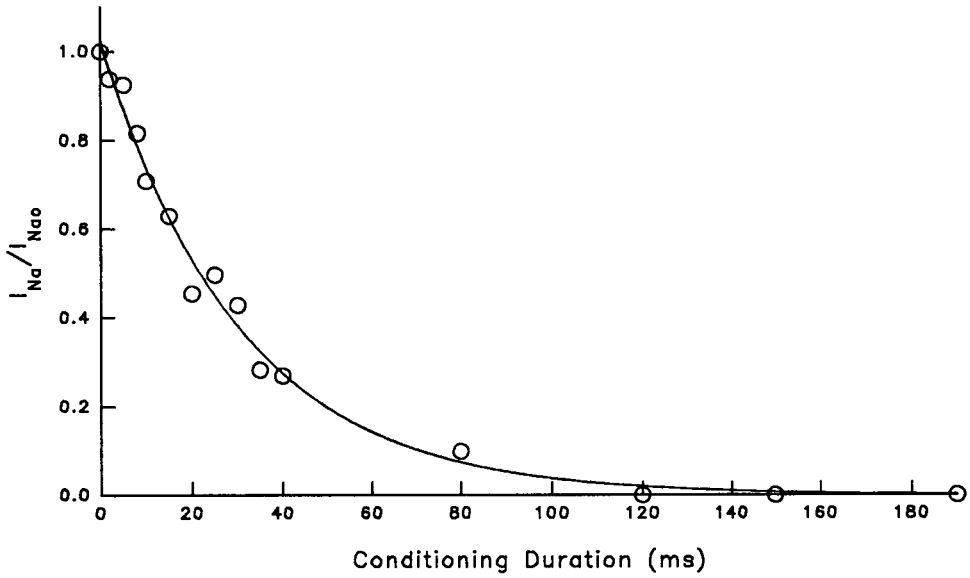


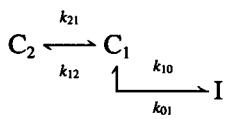
FIGURE 4 Time course of inactivation from closed states in a single patch at −70 mV. The solid curve is a best-fit single exponential. It was not possible to define a second exponential with reliable parameters in these data.

Dashed lines are used in Scheme 1 because it is not clear which closed state(s) directly inactivate. There must be at least two closed states, as there is a delay in the development of activation, but the exact number is not known. Scheme 1 is then the simplest possible scheme that can be considered and is used to illustrate the behavior expected. The final conclusions that will be reached, however, are independent of the number of closed states assumed and apply equally well to schemes with any number of closed states.

If it is only C_1 that directly inactivates (Scheme 2), then the probability of occupancy of the inactivated state, $x(t)$, during development of inactivation just from closed states is given by (see, e.g., Goldman, 1976)

$$x(t) = x(\infty) + \frac{bx(\infty)}{a-b}\exp(-at) - \frac{ax(\infty)}{a-b}\exp(-bt), \quad (1)$$

where $x(\infty)$ is the steady-state occupancy of the inactivated state, a and b are relaxation rate constants, and t is time. Equation 1 is written for the condition that all channels are in C_2 initially. The −120 mV holding potential used in these experiments should be a reasonable approximation to that condition.



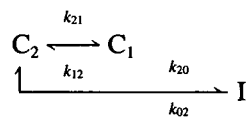
Scheme 2

By inspection, Eq. 1 is the difference of exponentials, and inactivation develops with a delay. In this case the zero time intercepts for the single exponential fits would be greater than unity.

If it is only C_2 that directly inactivates (Scheme 3), then $x(t)$ is given by (again assuming all channels are in C_2 initially)

$$x(t) = x(\infty) - \frac{k_{20} - bx(\infty)}{a-b}\exp(-at) + \frac{k_{20} - ax(\infty)}{a-b}\exp(-bt). \quad (2)$$

$ax(\infty)$ is always greater than k_{20}, while $bx(\infty)$ is always less. Equation 2 is then the sum of two exponentials. Closed-state inactivation develops as two relaxations as channels can transit to C_1 before returning to C_2 and then inactivating. In this case the zero time intercepts for the single exponential fits would be less than unity.



Scheme 3

If either closed state directly inactivates there should be an additional component in the closed-state inactivation time course, while the experimental finding is a single exponential with a zero time intercept at or near unity. One possibility is that any additional component is too small to resolve. The relative amplitudes of the two predicted exponential components are determined in whole or in part by the relative values of the two predicted time constants (Eqs. 1 and 2). In the case of closed-state inactivation only from C_1 the ratio of the amplitude of the two exponential components is just the ratio of the two time constants. For this to be the explanation, the exchange between closed states should be very fast at these potentials, of the range of about one hundred to a few hundred microseconds.

To estimate a value for the rate of exchange between closed states, average currents were constructed from the

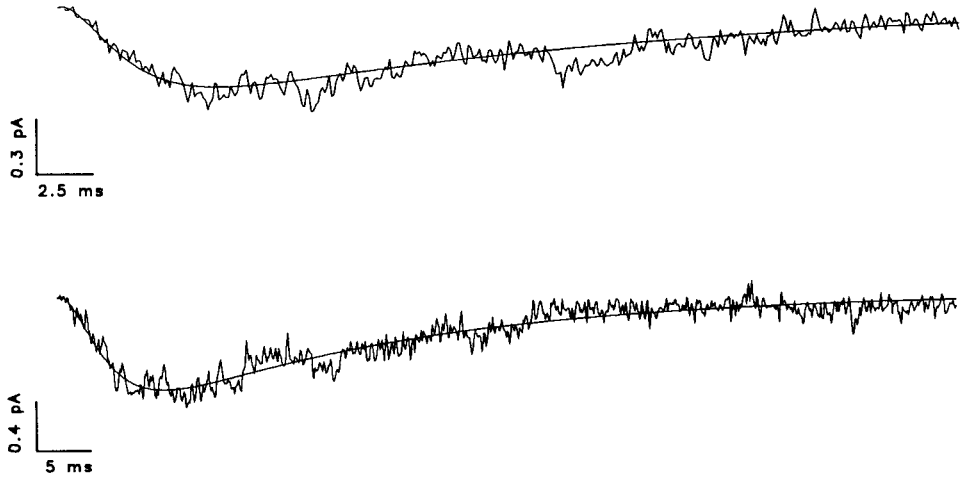


FIGURE 5 Average currents during a conditioning potential of −50 (*top*) and −60 mV (*bottom*). Note the different time bases for the two current records. Smooth curves are three exponential best fits to the average currents. Note the clear delay in the development of activation. The first 300 μs in the top experimental trace and the first 100 μs in the bottom have been blanked.

single-channel traces during the conditioning pulses. The time constant of the delay in the development of activation in these average currents should be determined by the same exchange between closed states that would generate an additional component. Average currents sufficiently large in amplitude and low in noise to permit kinetic analysis were obtained in four of 18 patches at −50 and one of 13 patches at −60 mV. In each of these cases a clear delay was seen in the activation of the average current. Fig. 5 presents two of these current traces. The top trace at −50 mV is shown with a time base expanded twofold over that for the lower trace at −60 mV.

The smooth curves superimposed on the average currents indicate best-fit three exponential descriptions of the current time course. Collected results for the fitted delay time constant, τ_D, activation time constant, τ_A, inactivation time constant, τ_h, as well as steady-state values are shown in Table 2. Mean τ_D at −50 mV is 1.98 ms, and the single value at −60 mV is 1.97 ms, both well slower than what would be required to make any additional component undetectable in the closed-state inactivation determinations. A τ_{IC} value is available for only one of the patches in Table 2. However, these experiments were done at the same time and under the same conditions as for the τ_{IC} determinations and should, then, be representative.

Table 3 presents predicted zero time intercepts based on the measured τ_{IC} and τ_D values. The reciprocals of these experimental values are the predicted relaxation rate constants, b and a respectively, of Eqs. 1 and 2, and the predicted intercepts are given by the coefficients to the $\exp(-bt)$ terms. If only C_1 directly inactivates, then mean values for τ_{IC} and τ_D at −50 mV predict a zero time intercept of 1.151. If only C_2 directly inactivates, an exact prediction cannot be made, as k_{20} is not known. If it is assumed that the ratio of amplitudes of the two predicted exponential components is the same as that given by Eq. 1, then the predicted zero time intercept is 0.849. Numerical computations on Eq. 2 with a broad range of values for the rate constants suggest that the correct prediction would be this far or even further from unity. These predicted values of 1.151 and 0.849 are clearly different from the experimental mean of 0.973. For −60 mV, the mean τ_{IC} and single τ_D value predict a zero time intercept of 1.108 if only C_1 directly inactivates and 0.892 if only C_2 directly inactivates, while the experimental value is 1.0004. At both potentials, then, predicted values for the zero time intercepts, based on the measured τ_D values, are clearly different from unity, while the experimental results are approximately unity. The lack of any indication of an additional component in the closed-state inactivation time course cannot, then, be attributed to a rate of exchange between closed states that is too rapid to detect.

TABLE 2 Kinetics of average currents at −60 and −50 mV

Patch	Potential (mV)	τ_D (ms)	τ_A (ms)	τ_h (ms)	Steady-state value
50127	−50	5.118	6.018	23.67	0
50317	−50	0.649	0.853	14.40	0
50324	−50	0.769	2.200	16.55	0
50329	−50	1.395	1.830	21.92	0
Mean		1.983	2.725	19.14	
60121	−60	1.965	2.564	24.35	0

Holding potential was −120 mV throughout. Temperature was 7.5 ± 0.5°C.

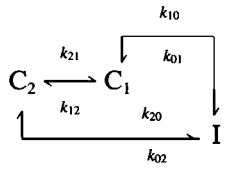
TABLE 3 Time constants of the delay in activation (τ_D) and inactivation from closed states (τ_{IC})

			Zero time intercept		
Potential (mV)	τ_D (ms)	τ_{IC} (ms)	Delay (predicted)	Two-phase* inactivation (predicted)	Experimental
−50	1.983	15.14	1.1507	0.8493	0.9730
−60	1.965‡	20.12	1.1082	0.8918	1.0004

*Correct prediction not known. Computed assuming coefficient on fast inactivation relaxation is the same as that for the delay process, which is likely to be an underestimate (see text).

‡Single determination. All other values are the means of four or five determinations.

There is another way to account for these results. If both C_1 and C_2 can directly inactivate, then closed-state inactivation develops with a delay if $k_{10} > k_{20}$ and in two phases if $k_{20} > k_{10}$ (Scheme 4).



Scheme 4

However, the relative amplitudes of the two exponential components are no longer determined just by the ratio of the two time constants. The amplitude of the additional component is now determined in part by the net result of the transitions through the two pathways, and as k_{10} and k_{20} approach each other this amplitude can be arbitrarily small. In the limiting case where $k_{10} = k_{20}$, the amplitude becomes zero, and the zero time intercept is just unity. A simple way to account for the closed-state inactivation time course, then, is for all closed states to directly inactivate and to do so with about the same rate constant. This conclusion is independent of the number of closed states assumed and applies equally well to schemes including any number of closed states.

For Scheme 4, with $k_{10} = k_{20}$, $x(t)$ is given by (assuming all channels are in C_2 initially)

$$x(t) = x(\infty)\,(1.0 - \exp(-(k_{20} + k_{02} + k_{01})t)) \qquad (3)$$

where

$$x(\infty) = k_{20}/(k_{20} + k_{02} + k_{01}). \qquad (4)$$

$x(t)$ does not depend on k_{21} or k_{12}, as now the rate of filling the inactivated state is independent of the relative occupancies of C_1 and C_2, or of the relative occupancies of any number of closed states. If the inactivated state is absorbing, $x(\infty) = 1.0$, $k_{01} = k_{02} = 0$, $x(t)$ is given by

$$x(t) = 1.0 - \exp(-k_{20}t), \qquad (5)$$

and τ_{IC} is just $1/k_{20}$. Equation 5 is identical whatever the number of closed states, and analogous expressions can be obtained for Eqs. 3 and 4 by adding a k_{0j} term for each additional closed state.

k_{20} is determined directly from τ_{IC} or from τ_{IC} and the steady state value. Note that the value computed for k_{20} is the same no matter what the actual number of closed states. k_{20} as a function of potential is shown in Fig. 6. Values range from a mean of 0.01076 ms^{-1} at −80 to 0.0690 ms^{-1} at −50 mV. With the same rate constants for all closed states, the voltage dependency of k_{20} is an entirely intrinsic property of the closed-state inactivation process.

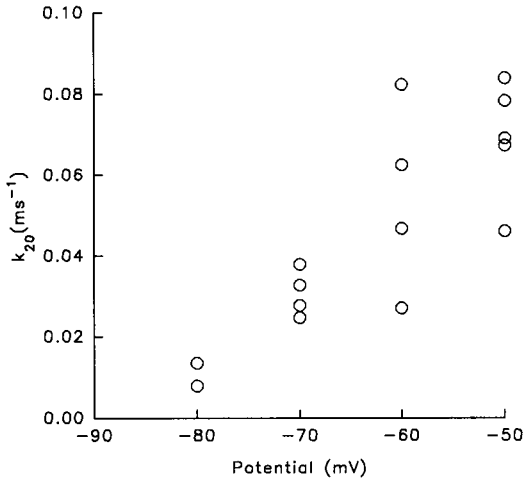


FIGURE 6 Closed to inactivated transition rate constant, k_{20}, as a function of potential. Each value is from a different patch. k_{20} increases e-fold for 22 mV.

Inactivation from both open and closed states

To try and compare the rates of inactivation from open and closed states, the inactivation time course in those same patches was again determined, now including only traces with channel openings during the conditioning pulse. These determinations necessarily include inactivation from both open and closed states. Three of the closed-state inactivation determinations at −50 and two at −60 mV yielded a sufficient number of traces with openings during the conditioning pulse to describe the inactivation time course.

Fig. 7 shows one example at a conditioning potential of −60 mV. The open circles indicate the inactivation time course just from closed states and the filled triangles the values only from traces with conditioning pulse openings. Inactivation time courses determined by the two methods are indistinguishable. This same result was obtained in each of the five patches for which comparisons were possible. The solid curve in Fig. 7 is a single exponential fitted just to the closed-state inactivation data. It describes the data from both open and closed states about equally well.

One interpretation of these results is that the rates of inactivation from open and closed states are the same. The closed to inactivated rate constants seem to be the same for all closed states. If the open to inactivated rate constant also had the same value, then Na channel activation and inactivation would be strictly independent processes. There is compelling evidence that activation and inactivation are not independent (Goldman, 1976; Armstrong and Bezanilla, 1977). The presence of a delay in the macroscopic inactivation time course (Gillespie and Meves, 1980; Bean, 1981; Goldman and Kenyon, 1982; Goldman, 1989) is itself sufficient to rule out the possibility that inactivation rate constants are the same for all states, as in that case macroscopic inactivation would develop as a simple exponential.

A more likely interpretation of the results of Fig. 7 is that even when only traces with conditioning pulse openings are

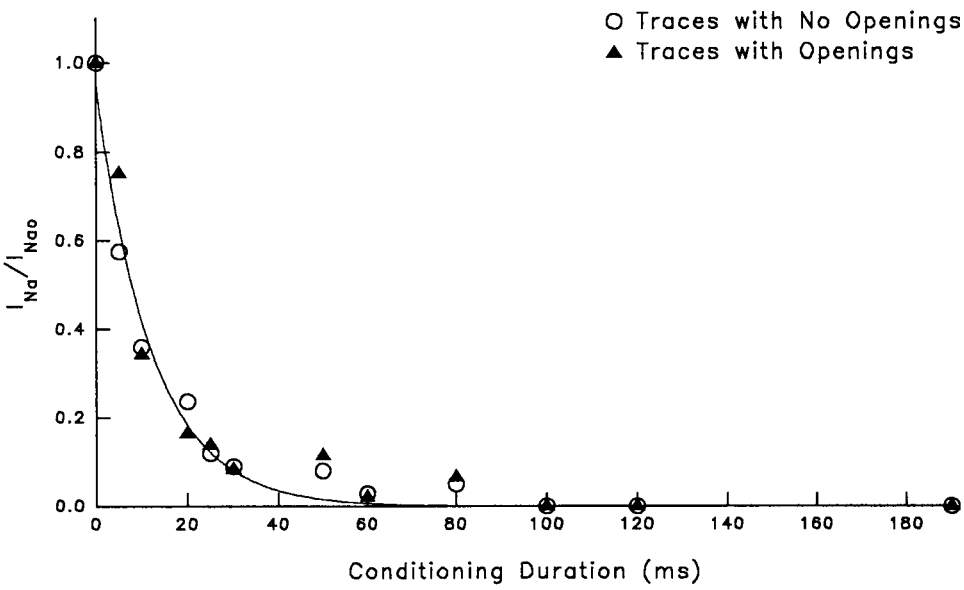


FIGURE 7 Inactivation time course in a single patch at −60 mV. Open circles indicate the closed-state inactivation time course. Filled triangles indicate the values when only traces with channel openings during the conditioning pulse are included in the average. The solid curve is a single exponential fitted to just the closed-state inactivation data.

included in the average, inactivation is nearly entirely from closed states over this potential range. To test this interpretation, data from the 190-ms conditioning pulse of the experiment of Fig. 7 were examined in detail.

To estimate the relative amounts of inactivation from open as compared to closed states, the total number of channel openings was determined. Eighty traces had one or more openings, for a total of 132. There were at least seven channels in this patch, as estimated from the maximum number of simultaneous openings during the two bracketing −20 mV unconditioned test pulses. Inactivation went to completion in this experiment, and 190 ms is more than 15 times longer than τ_{IC}. Hence it is very unlikely that any channels remained in resting states by the end of the conditioning pulse, and at the end of each conditioning pulse all of the seven channels must have inactivated, for a total of 560 entries into the inactivated state during the 80 traces with openings. If we assume each of the 132 openings terminated in a transition to the inactivated state, then a maximum of 23.6% of the total entries into the inactivated state were from the open state, even when including only traces with openings. This figure must be an overestimate, as some open channels will first close and then inactivate, and some of the counted openings will be of the same channel. Moreover, seven channels is likely to be an underestimate determined in this way (Horn, 1991), and inactivation from closed states must account for nearly all of the inactivation that occurred during these 80 traces. The open circles and filled triangles of Fig. 7 trace out the same time course because they are reporting the same process.

An overall estimate of the relative amounts of inactivation from open as compared to closed states is obtained from the fraction of conditioning pulse traces with no openings. The time independent probability of an individual channel inactivating without having opened, P_I, is given by

$$\text{Fraction of traces with no openings} = P_I^n \qquad (6)$$

where n is the number of channels in the patch. n was again estimated from the maximum number of simultaneous openings during the −20 mV unconditioned test pulse. Patches that satisfied the conditions that inactivation went to completion and conditioning pulses at least five times longer than τ_{IC} were available were selected for analysis. With these conditions it is likely that all channels in the patch had inactivated by the end of each conditioning pulse. Six patches satisfied these conditions, and collected values for P_I are shown in Table 4. P_I ranged from 0.803 for one patch at −50 mV to 0.980 for one at −70. Inactivation from closed states is not just an alternative route to the inactivated state. It is the dominant route over this potential range. Scanley et al. (1990) and Lawrence et al. (1991) also found that P_I reached similar large values over similar negative potential ranges. The estimated values for P_I are approximations for several reasons. However, the greatest source of error is likely to be an underestimate of the number of

TABLE 4 Probability of a sodium channel inactivating without having opened (P_I) at relatively negative potentials

Patch	Potential (mV)	Conditioning pulse duration/τ_{IC}	Estimated* number of channels in patch	P_I
50324	−50	5.04	4	0.803
60121A	−60	>11.40‡	4	0.805
60121B	−60	15.65	7	0.903
60122	−60	11.87	4	0.938
70415	−70	5.69	5	0.887
70818	−70	6.22	4	0.980

Inactivation went to completion (test pulse current was zero at steady state) in each case.

*Estimated from the maximum number of simultaneous openings seen during the −20 mV test pulse.

‡τ_{IC} not determined, but conditioning pulse durations of 50, 80, 120, 150 and 190 ms all produced test pulse currents of zero. Analysis on 190 ms conditioning pulse. Value computed assuming that 50 ms is 3 times τ_{IC}.

channels in the patch (Horn, 1991), and the reported P_I values are very likely biased to the low side.

DISCUSSION

All closed states can directly inactivate

Na channel inactivation from closed states develops as a single exponential with a zero time intercept at or near unity. The absence of any additional components in the closed-state inactivation time course is not because the rate of channel transition between closed states is too rapid to detect. The observed time course is simply accounted for if all closed states inactivate with about the same transition rate constant. This suggests that those conformational changes that constitute the transitions between closed states have little effect on the structural components involved in inactivation.

Closed to inactivated rate constants do not have to be identical to account for the observed time course. Just how different they can be depends on the values of the other rate constants of the scheme. One computation on Scheme 4 illustrates the effect. Computations were performed assuming a k_{21} of 1.30 ms^{-1}, a k_{12} of 0.70 ms^{-1}, and an absorbing inactivated state (i.e., $k_{01} = k_{02} = 0$). One closed to inactivated rate constant, k_{20}, was set to 0.05 ms^{-1} and the other, k_{10}, was half that value or 0.025 ms^{-1}. Expressions relating the transition rate constant, k_{ij}, terms to the relaxation rate constants, a and b, are given in Goldman (1976). The computed τ_{IC} was 29.7 ms, and the amplitude of the fast component was less than 1% of that of the slow component and so would not be experimentally detectable. Increasing the C_2 to C_1 rate constant threefold to 3.90 ms^{-1} changed the computed τ_{IC} by less than 15%, in approximate agreement with Eq. 5. Closed to inactivated rate constants need only be similar for each closed state in order to account for the experimental results. In general, the slower the time constant of exchange between closed states (τ_D) is relative to τ_{IC}, the more similar k_{10} and k_{20} must be to yield a zero time intercept at or near unity.

These experiments provide no quantitative information on the relative rates of inactivation from open as compared to closed states. Macroscopic inactivation develops with a delay (Gillespie and Meves, 1980; Bean, 1981; Goldman and Kenyon, 1982) that has the same time constant as Na current activation during the conditioning pulse (Goldman, 1989). These results together with those on closed-state inactivation are sufficient to establish that the open to inactivated rate constant differs appreciably from those from the closed to the inactivated state. Aldrich and Stevens (1983) came to similar conclusions.

Closed to inactivated rate constants are intrinsically voltage dependent

Inactivation from both open and closed states goes to completion at some potentials and not at others. Hence there is at least some intrinsic voltage dependency for both open- and closed-state inactivation (see also Horn and Vandenberg, 1984; Aldrich and Stevens, 1987). A question is, how much of the observed voltage dependency of inactivation time constants is intrinsic to the inactivation process and how much arises from coupling to other transitions that are voltage dependent? In general, each macroscopic time constant depends to some degree on all the rate constants of the state diagram, and observed voltage dependencies of macroscopic time constants generally will arise in part from all voltage-dependent rate constants.

For closed-state inactivation the rate of filling the inactivated state is independent of the relative occupancies of the various closed states, owing to the similar values for all closed to inactivated rate constants, and hence is also independent of the rate constants of exchange between these states. The observed voltage dependency of τ_{IC} and the extracted k_{20} values are then intrinsic to the closed-state inactivation process. The available k_{20} data extend over too narrow a voltage range to reliably define the voltage dependency. However, assuming the voltage dependent work terms are simply linear in potential, the data of Fig. 6 are consistent with an e-fold change in k_{20} for 22 mV.

How many inactivated states are there?

As channels can inactivate from both open and closed states, there may be two inactivated states, one with activation gates open and the other with them closed. Direct evidence for more than one inactivated state is provided by the presence of a delay in the recovery from inactivation seen in squid (Chandler and Meves, 1970) and *Myxicola* axons (Schauf, 1974) and in rat hippocampal CA1 neurons (Kuo and Bean, 1994). Kuo and Bean (1994) found that inactivated channels nearly always recovered to closed rather than the open state, and so the recovery process they studied must reflect the same transitions studied here, albeit over a different range of gating behaviors. Closed-state inactivation develops as a single exponential with no indication of a second inactivated state. This is because the experimental closed-state inactivation time course would, with two inactivated states, be determined by the sum of the probabilities of occupancy of the two putative inactivated states, which is a single exponential when closed to inactivated rate constants are the same for all closed states. If, in addition, the summed inactivated states are absorbing, with free exchange between the two, then closed-state inactivation is exactly defined by Eq. 5.

The detailed nature of the two inactivated states is unclear. Recovery from gating charge immobilization proceeds with the time course of recovery from inactivation (Armstrong and Bezanilla, 1977), not with that of the delay in recovery from inactivation, as might be expected if the

delay in recovery reflected activation gates returning to their normal closed state. The putative inactivated-closed state, then, may not have activation gates closed in the same way as for resting, noninactivated channels, but may reflect an intermediate or partially closed configuration. This would imply that the closed to inactivated transition necessarily involves changes in activation as well as inactivation gates. Possibly activation and inactivation gates cannot both be fully closed at the same time.

At moderately negative potentials nearly all of the inactivation is from closed states

Over the potential range examined nearly all of the inactivation is from closed states, and inactivation from open states makes no detectable contribution to the overall inactivation time course. The probability that a Na channel will inactivate without having opened, over this range, is considerably greater than the probability that it will open. The closed to inactivated rate constants must, then, be well greater than the closed to open. However, τ_{IC} is substantially slower than the Na current activation time constant, τ_A. The relatively rapid τ_A very likely arises from a large open to closed back rate constant, and, over this range, open channels would be more likely to return to a closed resting state than to inactivate.

τ_{IC} could have been determined just from the macroscopic two-step inactivation time course, and it may be possible to do this over the appropriate potential range. The presence or absence of a delay in the development of inactivation studied in this way might serve to define the potential range over which inactivation from open states does or does not contribute significantly to the inactivation time course.

Closed-state inactivation has implications for the information processing properties of nerve cells. Information is encoded at the action potential initiating locus in a number of cases (e.g., French and Korenberg, 1989; French and Torkkeli, 1994). The past history of the membrane potential at this locus can significantly affect the probability of an action potential firing in response to, for example, a synaptic input, and closed-state inactivation is likely to be a primary mechanism for mediating such effects. A moderate synaptic potential lasting only a few tens of milliseconds can completely inactivate the action potential initiating site without itself having produced any appreciable Na channel opening, owing to the existence of the closed-state inactivation pathway. Similarly, this pathway can be of some significance for the problem of cardiac arrhythmias.

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