Delays in Inactivation
Development and Activation Kinetics
in Myxicola Giant Axons

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ABSTRACT Na inactivation was studied in Myxicola (two-pulse procedure, 6-ms gap between conditioning and test pulses). Inactivation developed with an initial delay (range 130-817 µs) followed by a simple exponential decline (time constant τd). Delays (deviations from a simple exponential) are seen only for brief conditioning pulses where gNa is slightly activated. Hodgkin-Huxley kinetics with series resistance, Rs, predict deviations from a simple exponential only for conditioning pulses that substantially activate gNa. Reducing INa fivefold (Tris substitution) had no effect on either τd or delay. Delay is not generated by Rs or by contamination from activation development. The slowest time constant in Na tails is ~1 ms (Goldman and Hahin, 1978) and the gap was 6 ms. Shortening the gap to 2 ms had no effect on either τd or delay. Delay is a true property of the channel. Delay decreased with more positive conditioning potentials, and also decreased approximately proportionally with time to peak gNa during the conditioning pulse, as expected for sequentially coupled activation and inactivation. In a few cases the difference between Na current values for brief conditioning pulses and the τd exponential could be measured. Difference values decayed exponentially with time constant τm. The inactivation time course is described by a model that assumes a process with the kinetics of gNa activation as a precursor to inactivation.

INTRODUCTION
An issue of some interest in the question of how the Na channel gating machinery is organized is whether activation and inactivation proceed independently, as suggested by a literal interpretation of Hodgkin-Huxley (1952) kinetics, or rather are coupled together into some sort of multistate sequence. One of a number of observations bearing on this issue is the presence of an initial delay in the development of inactivation, as determined with the two-
pulse method, reported for several preparations (see Goldman, 1976, for a review). Delays are in general expected if inactivation develops after a precursor process. Of particular interest for the issue of coupled kinetics is the observation, in *Myxicola* axons (Goldman and Schauf, 1972), that the delay is reduced with a more positive conditioning potential. This is as expected if inactivation is sequentially coupled to activation, due to the shorter time needed for activation to develop at more positive potentials. Correspondingly, Goldman and Schauf did find in a single experiment that the decrease in delay seen with the more positive potential was roughly paralleled by the decreased time to peak $g_{Na}$ during the conditioning pulse.

Recently, Gillespie and Meves (1980), in a careful study, reported small inactivation delays in squid axons. In addition, they criticized some of the earlier reports of inactivation delays because a gap (a step back to the holding potential between conditioning and test pulses) as in the original protocols of Chandler et al. (1965) was not included. A gap that is long relative to the time constant of activation is essential for these experiments so that the time course of inactivation will not be distorted by the activation that develops during brief conditioning pulses. As demonstrated both by simulations with Hodgkin-Huxley kinetics (Gillespie and Meves, 1980) and analytical calculations (Kniffki, 1979), omitting the gap can artificially introduce delays in the time course of inactivation. In their experiments, Goldman and Schauf (1972) always included a gap of 5 ms width in a single experiment conducted at 5°C and 7.5 ms width in experiments at 2°C. These gap widths are long relative to the time constant of activation (Goldman and Schauf, 1973; Goldman and Hahin, 1978), and it seems very unlikely that inactivation delays reported for *Myxicola* were produced by this error. We present experiments below that establish that the inactivation delays reported in this paper are also not generated by an inadequate gap width.

A second criticism raised by Gillespie and Meves (1980) is that in a gap experiment, for conditioning potentials long enough to substantially activate but not inactivate $g_{Na}$, there will be an inward tail of Na current flowing during the gap. This current flowing across any residual uncompensated portion of the series resistance, $R_s$, and that flowing during the conditioning pulse will produce a depolarization and hence additional inactivation. Gillespie and Meves showed with simulations from Hodgkin-Huxley kinetics, including $R_s$, that such effects will produce an inactivation curve with an initial exponential decline of just the Hodgkin-Huxley $\tau_i$ value for conditioning pulses too brief to activate $g_{Na}$ very much, followed by a period of steeper decline for conditioning pulses that terminate when $g_{Na}$ is large, followed by a return to the original exponential for longer conditioning pulses during which $g_{Na}$ is inactivating. They suggested that such distorted curves might have been mistakenly interpreted as demonstrating a delay.

$R_s$ errors are also not likely to be the origin of the inactivation delays reported in *Myxicola*. Fig. 10 of Goldman and Schauf (1972) presented a typical result from that series. Peak $I_{Na}$ during the test pulse vs. conditioning pulse duration displayed an initial plateau followed by a decline rather than
an initial exponential decline. This is qualitatively different behavior from that expected for an $R_s$ error. Moreover, a second determination on this same axon with a more positive conditioning potential resulted in a decreased delay, even though the current during the more positive pulse rose more rapidly to a larger peak value (Goldman and Schauf, 1972). Making the conditioning pulse current density larger made the delay shorter. This is the opposite direction expected for $R_s$ to be the basis of the delay. We present experiments below that establish that the delays reported in this paper are also not generated by $R_s$ errors.

We present here new observations on the delay in inactivation development in *Myxicola* that were designed to reveal something about the process generating it. We first establish that the delay is a genuine property of the Na channel. We then show that the delay arises from a process whose kinetics are identical to those of the activation process. All of our results are consistent with the view that in *Myxicola* Na channels inactivation is sequentially coupled to inactivation.

A preliminary report of some of these results has been presented (Goldman and Kenyon, 1981).

**METHODS**

*Myxicola* were obtained from Marine Research Associates, St. Andrews, New Brunswick, Canada. Methods for preparing and voltage clamping the axons were as in Binstock and Goldman (1969). Artificial sea water (ASW) had the following composition: 440 mM Na, 10 mM Ca, 50 mM Mg, 560 mM Cl, 5 mM Tris (Tris (hydroxymethyl) aminomethane), pH 8.0 ± 0.1. The temperature was 5 ± 0.5°C. All potentials are reported as absolute membrane potentials (inside minus outside) and have been corrected for liquid junction potentials according to the values of Cole and Moore (1960), which are suitable for *Myxicola* (Binstock and Goldman, 1971). Compensated feedback to reduce $R_s$ was used throughout. To further reduce errors produced by any residual uncompensated portion of $R_s$, all voltage-clamp observations were made in bathing media with the Na concentration reduced by substitution with Tris to either 1/2, 1/3, or 1/4 of that in ASW (referred to as 1/2 Na ASW, 1/3 Na ASW, and 1/4 Na ASW, respectively). In nearly all experiments 2 mM 3,4-diaminopyridine (Aldrich Chemical Co., Milwaukee, WI) was added to the bathing medium to reduce $g_K$ (Kirsch and Narahashi, 1978). The 3,4-diaminopyridine solutions were made fresh each day and the pH was checked. In a few cases the K-channel blocker was omitted without any obvious effect on the results (compare Figs. 1 and 2).

For each experiment, the holding potential was set equal to the natural resting potential at the start of the first clamp run and held constant thereafter. Each voltage-clamp run consisted of a series of conditioning pulses of fixed amplitude and variable duration each followed by a gap (6 ms in duration unless otherwise noted) and a fixed-amplitude test pulse. Each conditioning pulse-gap-test pulse sequence was both preceded and followed by an unconditioned test pulse, and each conditioned determination was normalized to the mean of the bracketing unconditioned values. 15 s were allowed between each voltage-clamp step whether conditioned or unconditioned to minimize the effects of slow inactivation (Rudy, 1981). Pulses sent to the voltage clamp were formed by a PDP 11/34 computer (Digital Equipment Corp., Maynard, MA) in a programmed sequence for each experiment. $I_{Na}$ was extracted by repeating
for each axon, the entire voltage-clamp protocol in the presence of 1 μM tetrodotoxin (TTX; Calbiochem-Behring Corp., San Diego, CA), and subtracting the two sets of current records. For those experiments in which determinations were made in more than one Na concentration, TTX controls were run in each of the bathing media used. No differences in the current records were found with any of the TTX-containing media.

RESULTS

Demonstration of a Delay in Inactivation

TIME COURSE OF INACTIVATION

Fig. 1 presents a typical determination. The peak value of $I_{Na}$ during the test pulse is shown as a function of the duration of the conditioning potential. Inactivation developed with a relatively brief initial delay of 285 μs followed by a simple exponential decay of time constant, $\tau_c$, of 5.71 ms (solid curve). This same pattern, an initial delay followed by a simple exponential decline, was seen for each of the 57 determinations on 27 axons reported here. Again, this is a qualitatively different pattern than expected for $R_s$ errors. The delays (i.e., deviations from a simple exponential) are seen only for brief conditioning potentials where $g_{Na}$ is little activated. Hodgkin-Huxley kinetics in the presence of some $R_s$ predict
deviations from a simple exponential only for conditioning pulse durations long enough to activate $g_{Na}$ substantially (Gillespie and Meves, 1980, Fig. 8).

Note that to demonstrate a delay, an initial plateau (i.e., initial slope of zero) in the inactivation curve is not required. The initial slope can be positive, zero, or even negative, depending on the relative values of the time constants for any precursor (delay producing) and inactivation processes and on the relative values of the coefficients on these two exponential terms. To demonstrate a delay one only need show that the unconditioned value falls below the exponential.

The inset in Fig. 1 shows the early part of the curve on an expanded time scale to illustrate how the inactivation delays were operationally determined. They are taken as the time at which the unconditioned $I_{Na}$ value intersects the exponential.

**DELAY IS NOT A SERIES RESISTANCE ARTIFACT**

Several arguments already presented strongly suggest that the delay is not an $R_s$ artifact. We have also put the issue to direct experimental test. Fig. 2 illustrates one such experiment.

![Figure 2](image)

**Figure 2.** Two inactivation determinations on the same axon. Conditioning and test potentials were $-29$ and $1$ mV, respectively, for both determinations. $\tau_e$ was 7.49 ms and delay 403 $\mu$s for both curves. Upper curve in 1/2 Na ASW and lower curve in 1/4 Na. Holding potential was $-74$ mV and gap width was 6 ms throughout. 2 mM 3,4-diaminopyridine. Axon 81M11.

In this experiment two determinations were made on the same axon with the same holding potential, gap width, and conditioning and test-pulse amplitudes. As indicated, one determination (upper curve in Fig. 2) was made in 1/2 Na ASW and the other (lower curve) was made in 1/4 Na ASW. Again for both curves we see an initial delay followed by a simple exponential decline. The two solid curves are drawn according to the same exponential function ($\tau_e$ of 7.49 ms). In fact the upper curve is just the lower scaled up by
the ratio of the two unconditioned $I_{Na}$ values. The delays computed for both determinations, then, are also the same (0.40 ms), and reducing the current density by nearly fivefold has no effect on either $\tau_{o}$ or the delay. Identical results were obtained on three other axons. Inactivation delays are therefore not produced by voltage-clamp artifacts dependent on the current magnitude.

The experiment of Fig. 2 was done in the presence of 2 mM 3,4-diaminopyridine, whereas that of Fig. 1 was done without any K-channel blocker, which suggests that these data have not been affected by K currents. As the data of this section indicate that identical results are obtained with either 1/2 Na or 1/4 Na ASW, the rest of the determinations of this paper have been made in 1/3 Na ASW and in the presence of 2 mM 3,4-diaminopyridine.

**ADEQUACY OF THE GAP WIDTH** The width of the gap between conditioning and test pulses (6 ms) is long relative to the slowest time constant seen in Na tail currents in *Myxicola* at these same potentials and temperature (typically ~0.8-1.2 ms; Goldman and Hahin, 1978). Correspondingly, for the experiments reported here, in each case the tail currents flowing during the gap were seen to decay to zero well before the start of the conditioning pulse. A typical record for a conditioning pulse terminating at peak $I_{Na}$ is shown in Fig. 3. Whatever the conditioning pulse duration, then, the occupancy of the

![Current record from an inactivation delay experiment](image)

**FIGURE 3.** Current records from an inactivation delay experiment. Conditioning and test potentials were 13 and 8 mV, respectively. Conditioning pulse duration was 0.75 ms, which was the time of peak $g_{Na}$. Current tails following the conditioning pulse decay to baseline during the 6-ms gap well before the start of the test pulse. 1/3 Na ASW. 2 mM 3,4-diaminopyridine. Holding potential -67 mV. Axon 81M35. Scale: 0.15 mA/cm², 2 ms.

conducting state has always declined to negligible values by the start of the test pulse, and contamination of this sort cannot be the basis of the delay.

For an activation process describable by Hodgkin-Huxley $m^3$ kinetics, Na tail currents should relax as the sum of three exponentials of time constants in the ratio 3:2:1 with the slowest time constant being just $\tau_{m}$. Na tail currents in *Myxicola* relax as the sum of at least three exponentials (Goldman and Hahin, 1978), although with time constants not in the ratios required by $m^3$ kinetics. Three processes are sufficient to account for the observed delay in the rise of $g_{Na}$ during depolarizing steps (Goldman and Hahin, 1978), and the
slowest activation time constant seen in the tail currents is about $\tau_m$. Hence it is unlikely that there is any slower relaxation not resolved in the current records as the activation processes seem to be accounted for. Correspondingly, conditioning potentials too small to noticeably activate $g_{\text{Na}}$ still produced clear inactivation delays, and a 6-ms gap seems sufficient to distinguish the time course of inactivation from activation development during the conditioning pulse.

We have put this issue to direct experimental test also. Two determinations were made in each of four axons, with holding potential, conditioning and test-pulse amplitudes, and solution composition held constant. For one determination the gap width was the usual 6 ms, whereas for the other it was reduced to 4 ms. In three of these axons the gap was further reduced to 2 ms. In each case for every gap width the results were the same.

A typical experiment is illustrated in Fig. 4. As indicated, the upper curve was determined with a 6-ms gap and the lower curve was determined with a 2-ms gap. The two solid curves are drawn according to the same exponential function ($\tau_c$ of 7.32 ms). The lower curve decays to a lower steady state value as less inactivation has dissipated during a 2-ms gap as compared with a 6-ms gap. The delays are therefore the same (0.48 ms). Hence even a 2-ms gap is sufficient to avoid any significant distortion of the inactivation time course by activation development. If the delay seen for the 6-ms gap had been produced by such distortions, then the delay would necessarily have increased with the

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**Figure 4.** Two inactivation determinations from the same axon. Conditioning and test potentials were $-30$ and $0$ mV, respectively, for both determinations. $\tau_c$ was 7.32 ms and delay 478 $\mu$s for both curves. The upper curve had a 6-ms gap and the lower curve had a 2-ms gap. Holding potential was $-75$ mV throughout. 1/3 Na ASW and 2 mM 3,4-diaminopyridine. Axon 81M39.
2-ms gap and the data points for durations long relative to $\tau_m$ must then fall above the curve in the lower part of Fig. 4 (see simulations with Hodgkin-Huxley kinetics in Gillespie and Meves, 1980). However, no such effects are seen. For squid at 2.6°C, Gillespie and Meves found that a 1-ms gap is sufficient to avoid distortion of the inactivation time course.

The results presented so far establish that there is a process preceding the development of inactivation (i.e., inactivation is not a simple two-state process). Similar results have been reported in well-controlled experiments in crayfish axons (Bean, 1981) and squid (Gillespie and Meves, 1980). However, these experiments provide little information about the nature of the precursor process. The experiments described in the following section address this issue.

Properties of the Inactivation Delay

POTENTIAL DEPENDENCY OF THE DELAY  Fig. 5A shows the results of two determinations on the same axon. In this experiment the holding potential, gap width, test-pulse amplitude, and solution composition were all held constant. As indicated, the conditioning potential was $-26 \text{ mV}$ for the
determination shown in the upper and -11 mV for that shown in the lower part of the figure. \( \tau_c \) decreased with a more positive conditioning potential in the usual way (5.51 ms for the upper curve and 3.37 ms for the lower curve). However, as shown in Fig. 5B, which is the early portion of both parts of Fig. 5A on an expanded time scale, the delay also decreased with a more positive conditioning potential (525 \( \mu s \) for -26 mV and 309 \( \mu s \) for -11 mV).

The decrease in delay with potential provides further evidence that the delay is not generated by \( R_s \). Records of the time course of the current during the -26- and -11-mV conditioning pulses for this experiment are shown in Fig. 5C (upper and lower traces, respectively). The current for the -11-mV
pulse rises more rapidly (time to peak, 1.05 ms) and to a larger peak value (214 µA/cm$^2$) than does that for the -26-mV pulse (1.50 ms and 82 µA/cm$^2$). For the entire period for which the delays are seen, the inward current at -11 mV is larger than that at -26 mV, but the delay is less at -11 mV. This is in the opposite direction for the delay to be caused in any way by $R_s$. However, it is just as expected if activation and inactivation are sequentially coupled owing to the reduced time for activation to develop at -11 mV.

Collected values of delay as a function of conditioning potential are shown in Fig. 6. At negative potentials the delay decreases steeply with less negative potential and tends to saturate at positive potentials. These delay values are about half those reported by Schauf and Davis (1975).

Somewhat more information about the delay process is gained by plotting inactivation delay as a function of the time to peak $g_{Na}$ during the conditioning
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The collected results are shown in Fig. 7. Delay increases with time to peak $g_{Na}$, and the two variables can be described as roughly proportional. If activation and inactivation are sequentially coupled, then both the inactivation delay as well as time to peak $g_{Na}$ will vary with the activation time constant. If both variables remain equally good measures of the activation time constant over their whole range, then proportionality between the two is expected for the simplest sort of sequentially coupled process. Considering the operational way in which the delay is determined, the agreement between the data of Fig. 7 and the expectations for a sequentially coupled process is quite reasonable.

**Figure 7.** Inactivation delay as a function of time to peak $g_{Na}$ during the conditioning pulse. Data pooled from 24 axons. Same determinations as in Fig. 6.

**TIME CONSTANT OF THE DELAY** In seven of the determinations from six different axons, the difference between the experimental Na currents for brief conditioning pulses and the extrapolated $\tau_c$ exponential were both sufficiently large in magnitude and small in variance to measure reliably. These difference values could also be described as an exponential and in these few cases the time constant of the delay process, $\tau_{delay}$, could then be determined. $\tau_{delay}$ values are plotted as a function of conditioning potential as the filled triangles in Fig. 8. Also included for comparison (circles) are the *Myxicola* $\tau_m (V)$ values for this potential range (Goldman and Schauf, 1973). $\tau_{delay} (V)$ is in reasonable agreement with $\tau_m (V)$, again as expected if activation and inactivation are sequential.
\( \tau_m \) is the activation time constant for an independent kinetic model. However, no difficulties are raised by the agreement between \( \tau_m (V) \) and \( \tau_{\text{delay}} (V) \). Fig. 8 also includes (open triangles) activation time constants determined by fitting a sequentially coupled scheme to *Mynicola* voltage-clamp data (Goldman and Hahin, 1978, Fig. 5). The scheme used was:

\[
A \rightleftharpoons B \rightleftharpoons C \rightleftharpoons D \rightleftharpoons E
\]

where \( A \) is the resting state, \( B \) and \( C \) are activated but not conducting states, \( D \) is the conducting state, and \( E \) is the inactivated state. This scheme displays three activation time constants consistent with the three relaxations seen in Na tail currents, and the slowest of these (\( \tau_3 \) from Goldman and Hahin, 1978) is plotted in Fig. 8. Goldman and Hahin presented evidence from initial conditions experiments that it is the \( \tau_3 \) relaxation that is most closely associated with the filling of the conducting state. \( \tau_3 (V) \) also agrees with \( \tau_m (V) \), and the agreement between \( \tau_{\text{delay}} (V) \) and \( \tau_m (V) \) is fully consistent with a sequentially coupled process.

**RECONSTRUCTION OF THE TIME COURSE OF INACTIVATION** The circles in Fig. 9A indicate experimental inactivation values for the first 5 ms of the determination on a somewhat expanded time scale so that the delay can be seen clearly. This is the same experiment as the upper curve of Fig. 5A. The solid curve has been computed assuming that the gate can be described simply as a three-state scheme, i.e.,

\[
\text{Rest} \rightleftharpoons \text{Conducting} \rightleftharpoons \text{Inactivated}
\]

using the \( \tau_{\text{delay}} \) and \( \tau_c \) values determined experimentally for this axon. Three states are known to be inadequate (Goldman and Hahin, 1979). However, the fit to the data is quite good, and the point of the calculation is to illustrate
that the experimentally observed time course of inactivation is consistent with a sequential activation-inactivation process. Another reconstruction with a more depolarized conditioning potential (−8.5 mV as compared with −26 mV) and a shorter delay is given in Fig. 9B. The whole inactivation time course for this experiment is shown in Fig. 9C.

![Diagram](image)

**Figure 9.** A. Early time course of inactivation development. Circles are experimental values and the solid curve is computed assuming sequential activation and inactivation with \( \tau_{\text{delay}} \) of 583 μs and \( \tau_c \) of 5.51 ms. Same experiment as Fig. 5, upper curves. B. Early time course of inactivation development from another axon. Solid curve computed with a \( \tau_{\text{delay}} \) of 290 μs and a \( \tau_c \) of 3.45 ms. C. The whole inactivation time course for the experiment of part B. Conditioning and test pulses were −8.5 and −3.5 mV, respectively. Holding potential was −78.5 mV. \( \tau_c \) is the same 3.45 ms as for part B. Gap width, 6 ms. 1/3 Na ASW. 2 mM 3,4-diaminopyridine. Axon 81M31.

A difficulty with the reconstructions of Figs. 9A and B is that they do not allow for the additional activation processes reported for *Myxicola* (Hahin and Goldman, 1978; Goldman and Hahin, 1978), which ought to appear as a residual delay. Possibly these more rapid activation processes produce effects too small to be detected on this time scale.
**The \( \tau_c - \tau_h \) Difference**

For each inactivation delay determination in these experiments, a \( \tau_c \) value was first obtained. These collected \( \tau_c \) values (half-filled triangles) are plotted as a function of membrane potential in Fig. 10. Also included in Fig. 10 are the original \( \tau_c (V) \) values of Goldman and Schauf (1973) (half-filled circles). Goldman and Schauf did their experiments in full-Na ASW without a K-channel blocker, whereas the present experiments were done in the presence of 2 mM 3,4-diaminopyridine and with 1/3 Na ASW or, in a few cases, 1/4 Na ASW. However, both series of experiments produce the same \( \tau_c (V) \). This is consistent with the results of Fig. 2, which show directly that \( \tau_c \) does not depend on the current density. The open, inverted triangle in Fig. 10 indicates a \( \tau_c \) value determined with the peak \( I_{Na} \) during the unconditioned test step.

![Figure 10](image-url)
reduced to $\sim 100 \mu A/cm^2$ now with TTX rather than reduced Na (Goldman and Hahin, 1978). It too lies on the same $\tau_c (V)$ function.

$\tau_h$ values (inactivation time constant determined from the $I_{Na}$ decay during a single potential step) were also obtained during the course of these experiments and they are plotted as the open triangles in Fig. 10. The open circles are the original $\tau_h$ values of Goldman and Schauf. Again the two series of experiments produce the same $\tau_h (V)$.

The current magnitudes encountered in these experiments deserve comment. For potentials $\leq -20$ mV where the $\tau_c - \tau_h$ difference is substantial, we have, in the present series of experiments, 17 determinations of $\tau_h$. For these 17 determinations the net peak inward current ranged from 0.22 mA/cm$^2$ down to 18 $\mu A/cm^2$ (with no net inward current in one case) and with a mean in this potential range ($-20$ to $-39.5$ mV) of 87 $\mu A/cm^2$. Any displacement in the membrane potential caused by the residual uncompensated $R_e$, then, will generally be $< 0.5$ mV and $\leq 1$ mV in the worst case. Hence the relative insensitivity of $\tau_h$ to membrane potential in this range cannot be attributed to voltage-clamp errors, which depend on the current magnitude. The inverted, filled triangle in Fig. 10 indicates a $\tau_h$ determination made with the peak $I_{Na}$ reduced to 70 $\mu A/cm^2$ with TTX (Goldman and Hahin, 1978). It too lies on the same $\tau_h (V)$ function. The $\tau_c - \tau_h$ difference, then, is also a genuine property of the Na channel in *Myxicola*.

**DISCUSSION**

The central result of this paper is that Na channels in *Myxicola* display a delay in the development of inactivation whose time course parallels activation kinetics. Therefore, either inactivation, at least in part, sequentially follows the activation process, or inactivation sequentially follows a process whose kinetics are identical to the activation process (Meves, 1978). There are other data available from *Myxicola* that favor coupled kinetics. The steady state inactivation curve, $h_w (V)$, shifts to the right along the $V$ axis as $g_{Na}$ of the test pulse increases (Goldman and Schauf, 1972). These experiments were done in a way that obviated the effects of any residual uncompensated $R_e$, and correspondingly, the shifts did not correlate with residual $R_e$. This effect was first predicted by a specific coupled model (Hoyt, 1968), but is expected in general for sequential activation and inactivation. If those channels that have activated are primarily those that inactivate, then the fraction inactivated during the test pulse will decrease as test pulse $g_{Na}$ increases. In both these respects, then, Na channels in *Myxicola* behave just as expected if activation and inactivation are sequentially coupled.

The $\tau_c - \tau_h$ difference is also difficult to reconcile with independent, parallel activation and inactivation. The observation is that $I_{Na}$ during moderate depolarizing steps in potential declines, with a time constant $\tau_h$, to negligible values. However, when probed with strongly depolarizing test steps during and after the decline, inactivation is found to be far from complete, as if the inactivation that developed during the moderate step had been overcome by
the test step. This effect cannot be reconciled with any model in which activation and inactivation are strictly independent (i.e., described by monotonically increasing or monotonically decreasing variables, respectively), and appear as a product, no matter what the number of states assumed for either process. However, it is not in principle inconsistent with a model in which only those channels that activate can inactivate, although additional assumptions are also needed. For *Myxicola* Na channels, therefore, the available kinetic data are most simply accounted for if activation and inactivation are not independent but are coupled together such that inactivation is, at least in part, sequential to activation.

Results from other preparations that suggest coupling are reports of \( \tau_c - \tau_h \) differences in lobster (Oxford and Pooler, 1975), crab axons (Connor, 1976), and cultured heart cells (Ebihara and Johnson, 1980), and inactivation delays in careful studies in squid (Gillespie and Meves, 1980) and crayfish (Bean, 1981). Bezanilla and Armstrong (1977) also found an inactivation delay in squid by comparing \( I_{Na} \) before and after removing inactivation. Conti et al. (1980) found that fluctuation power spectra from myelinated fibers were fitted somewhat better by coupled than by Hodgkin-Huxley kinetics. In squid (Armstrong and Bezanilla, 1974, 1977; Meves and Vogel, 1977), myelinated fibers (Neumcke et al., 1976; Nonner et al., 1978), *Myxicola* axons (Rudy, 1976; Bullock and Schauf, 1979), and crayfish axons (Swenson, 1980; Starkus et al., 1981), the asymmetrical displacement current is reduced by depolarizations that produce inactivation, so that charge displacements with activation kinetics (Armstrong and Gilly, 1979) are affected by inactivation. Ca currents in *Helix* neurons show both the \( h \) (\( V \) shift and a \( \tau_c - \tau_h \) difference (Akaike et al., 1978), and Ca channel inactivation in snail neurons seems to be an inherently coupled process (Eckert and Tillotson, 1981) at least in part for *Helix* (Brown et al., 1980), in that Ca inactivation is dependent on Ca entry through the conducting channel. These observations do not all suggest that inactivation is obligatorily sequential to activation, but they all do suggest coupling to some degree.

Sequential coupling was not seen, however, by Horn et al. (1981) in currents from single Na channels in cultured rat myotubes where inactivation occurred at the same rate whether channels opened early or late, which suggests that channel opening and inactivation proceed in parallel. These results do not indicate independent activation and inactivation but only that these channels need not open before inactivating. Also, not all preparations display the phenomena reported for *Myxicola* (Bezanilla and Armstrong, 1977; Chiu, 1977; Bean, 1981), and there is a range of Na channel properties among different preparations including inactivation at least to some degree sequential to channel opening (e.g., *Myxicola*), inactivation parallel to channel opening (e.g., cultured rat muscle), and possibly intermediate cases (e.g., crayfish axons, Swenson, 1980; Bean, 1981).

A scheme representing a way to summarize these diverse behaviors is presented below.
Various states of the channel are shown. $m_0 h_1$ is the resting state, and depolarization favors transitions to the right along both rows so that $m_0 h_1$ is the conducting state. Depolarization also favors transitions from top to bottom, and all the states in the bottom row are inactivated. As shown, with fixed ratios between the $\alpha_m$ and $\beta_m$ terms, the $\beta_h$ terms all identical, and the $\alpha_h$ terms all identical, this is just $m^3 h$. Activation and inactivation are then strictly independent as the probability of an $h$ particle transition is independent of the $m$ state, and similarly $m$ particle transitions are independent of the $h$ state. This scheme also illustrates the special case nature of $m^3 h$ kinetics. Independence arises from the constraint that each of the $\alpha_c$ and $\beta_c$ terms are identical. Relaxing this constraint by, for instance, making one or more of the $\beta_h$ terms different from the others produces a process that is coupled to some degree.

Another special case of interest is obtained by setting the three left-hand $\alpha_h$ and $\beta_h$ terms to zero. If the $m_2 h_0 \rightleftharpoons m_3 h_0$ transition is also forbidden, as is consistent with gating charge immobilization, we are left with the five states enclosed within the dashed lines. Further relaxing the requirement of fixed ratios between the activation rate constant produces the strictly sequentially coupled scheme:

$$
A \overset{k_{AB}}{\rightleftharpoons} B \overset{k_{BC}}{\rightleftharpoons} C \overset{k_{CD}}{\rightleftharpoons} D \overset{k_{DE}}{\rightleftharpoons} E.
$$

Hence one can obtain the two limiting cases of strictly independent, parallel activation and inactivation, and fully coupled, sequential activation and inactivation by the adjustment of rate constants. Clearly, any variety of intermediate cases with a mix of parallel and sequential processes can also be obtained by a suitable adjustment of rate constants. Such pictures provide a simple way to summarize a diverse body of experimental results. However, they imply a considerable complexity in the molecular organization of the gate, and of course it remains fully possible that Na channels in different preparations differ from one another more than quantitatively.

What seems fairly clear is that, considered separately, the activation process is multistate. This is based on observations of Na tail currents including initial conditions effects (Goldman and Hahin, 1978; Oxford, 1981), other initial conditions effects (Neumcke et al., 1976; Hahin and Goldman, 1978), multiple
activation relaxations in the gating current (Armstrong and Gilly, 1979; Nonner, 1980; Starkus et al., 1981), and differential D$_2$O effects on I$_{Na}$ and gating current time courses (Meves, 1974; Schauf and Bullock, 1979). Similarly, considered separately, the inactivation process is also multistate, based on the observations of multiple inactivation relaxations (Chiu, 1977; Kniffki et al., 1981; Oxford and Pooler, 1975; Connor, 1976) and the delay in its development. Also suggested in a number of cases is that these two multistate processes are coupled together into a single gating structure, but the tightness of the coupling may vary widely from preparation to preparation.

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