Initial Conditions and the Kinetics of the Sodium Conductance in *Myxicola* Giant Axons

II. Relaxation Experiments

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ABSTRACT The time-course of the decay of I_{Na} on resetting the membrane potential to various levels after test steps in potential was studied. The effects of different initial conditions on these Na tail currents were also studied. For postpulse potentials at or negative to −35 mV, these currents may be attributed nearly entirely to the shutdown of the activation process, inactivation being little involved. Several relaxations may be detected in the tail currents. The slower two are well defined exponentials with time constants of ~ 1 ms and 100 μs in the hyperpolarizing potential range. The fastest relaxation is only poorly resolved. Different initial conditions could alter the relative weighting factors on the various exponential terms, but did not affect any of the individual time constants. The activation of the sodium conductance cannot be attributed to any number of independent and identical two-state subunits with first order transitions. The results of this and the previous paper are discussed in terms of the minimum kinetic scheme consistent with the data. Evidence is also presented suggesting that there may exist a small subpopulation of channels with different kinetics and a faster rate of recovery from TTX block than the rest of the population.

INTRODUCTION

In the previous paper (Hahin and Goldman, 1978) we showed that there are at least two distinctly different kinds of processes in the activation of the sodium conductance (G_{Na}). Evidence for this comes primarily from two-pulse experiments in which G_{Na} is partially activated during the first pulse, and the time-course of its further development during a series of more depolarizing probing pulses was studied.

Another way to study the activation process is to examine the way in which it decays away on resetting the membrane potential to less depolarizing values. In this paper we present our observations on these Na tail currents. These experiments, too, indicate that there are at least two and very likely three distinctly different activation processes, and show that the activation machinery

is not built up of any number of independent and identical two-state subunits with first order transitions.

METHODS

All methods and solutions were as described in the preceeding paper (Hahin and Goldman, 1978). As before, series resistance (R_s) compensation was used throughout, and the residual error was further reduced by reducing the currents with added tetrodotoxin (TTX) so that the product of maximum membrane current and the residual uncompensated series resistance during any experiment was always < 3 mV. Holding potentials under voltage clamp were always the natural resting membrane potential (generally −65 to −75 mV). All potentials are reported as absolute membrane potential (inside minus outside) and have been corrected for liquid junction potentials as described in the preceeding paper. Temperature was 5 ± 1°C.

Na currents, (I_{Na}) were extracted by repeating the pulse schedules in artificial sea water (ASW) containing 10^{-6} M TTX, to block completely I_{Na}, and then making a point by point in time subtraction of the two sets of records. Photographic records of the oscilloscope screen were enlarged, traced onto graph paper, and subtracted by hand.

RESULTS

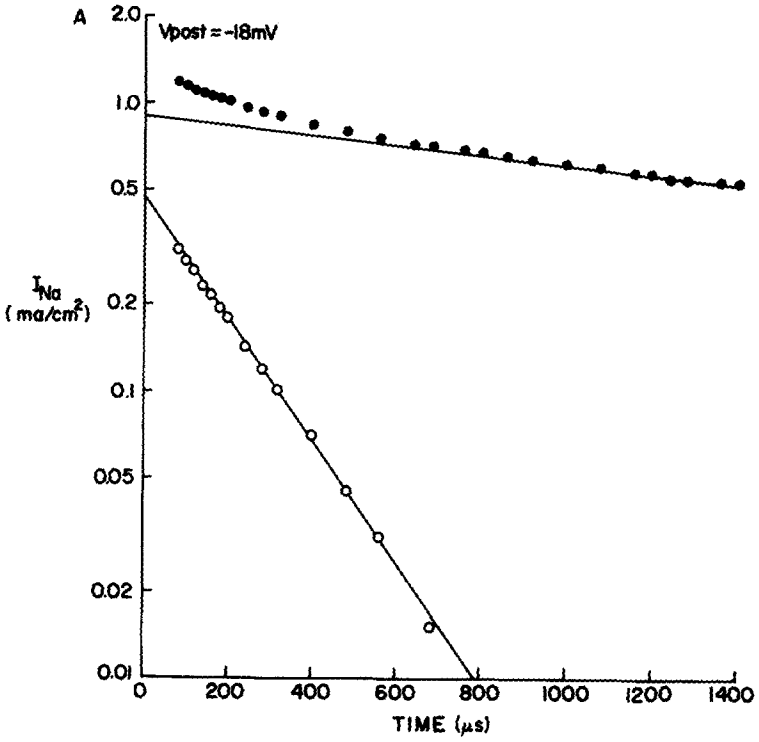
Na Tail Currents

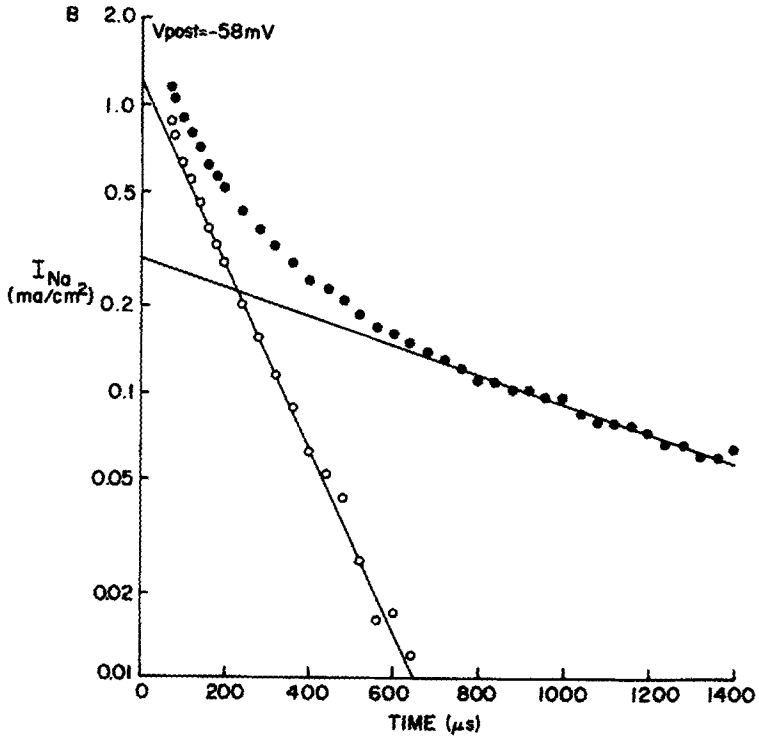
DEPENDENCY ON POSTPULSE POTENTIAL In three axons test potentials of 0–2 mV, always 1 ms in duration, were followed by postpulses to various potentials ranging from ~ −5 to −130 mV, and the decay of I_{Na} during the postpulse was observed. 1 ms was always somewhat longer than the time to peak of I_{Na} during the test pulse. These tail currents were corrected for leak and K currents as described in Methods and plotted semilogarithmically against time. Typical plots are shown in Fig. 1 (●). Tail currents were always monotonically decreasing inward currents.

Na tails never decayed as a single exponential. Over this range there were always at least two and sometimes three exponential components in the tail currents. 1.4–1.6 ms of tail current were analysed for each postpulse. With these durations the taii currents always displayed a well-defined linear region on the semilog plots shown by the solid lines through the filled cirlces of Fig. 1 A and B. The exponentials are, then, well separated.

Collected time constants for this slower tail component are shown as the filled circles in Fig. 2 A and are also given in Table I (τ_3). τ_3 showed no clear dependency on postpulse potential over the range −35 to −130 mV, and was typically ~ 1.0–1.2 ms. For postpulses more positive than −35 mV, the slow tail current component ought to be significantly affected by the inactivation which develops during the postpulse. For example, at a postpulse potential of −10 mV the tail current should be primarily the normal decay due to inactivation seen

FIGURE 1. Absolute value of the Na tail current as a function of postpulse duration (●). The open circles are the differences between the line fitted to the filled circles at long times and the filled circles. A and B are from the same axon with two different postpulses. Prepulse potential was 2 mV and its duration was 1 ms.


A
Vpost = -18mV
I_{Na} (ma/cm²)
2.0
1.0
0.5
0.2
0.1
0.05
0.02
0.01
0
200
400
600
800
1000
1200
1400
TIME (μs)


B
Vpost=-58mV
I_{Na} (ma/cm²)
2.0
1.0
0.5
0.2
0.1
0.05
0.02
0.01
0
200
400
600
800
1000
1200
1400
TIME (μs)

with steps to this potential, the shutting down of activation contributing relatively little to the relaxation. Indeed, the mean value of the slower time constant for postpulses in the vincinity of −10 mV was 2.18 ms which agrees with the value of 1.72 ms for τ_h at −10 mV, reported by Goldman and Schauf (1973).

As the postpulse potential is made less positive, the tail time-course ought to be governed ever more by the shutting down of activation (i.e., the draining of the conducting state into the resting state) and ever less by inactivation. For a

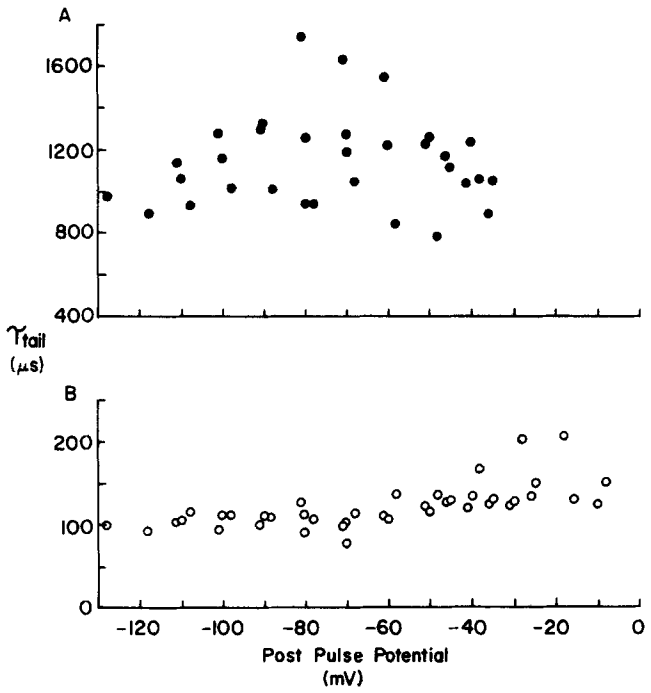


FIGURE 2. Time constants in the Na tail currents as a function of postpulse potential for τ_3 (●) and τ_2 (○) component (see text). Data pooled from three axons.

postpulse potential of −35 mV peak, G_{Na} is only 25% of that during a test pulse to 0 mV. A postpulse to −35 mV after a test step to 0 mV will, then, substantially shut down the activation process, whereas only little inactivation will develop during the postpulse. The conducting state should have drained primarily by a faster route before the inactivated state loads very much. Correspondingly, the slowest time constants seen in I_{Na} tails for postpulse potentials of −35 mV and below are almost never > 1.3 ms. This is much faster than τ_h which is ~ 2.0–2.5 ms for small depolarizations. Moreover, as ever less positive postpulses are applied, inactivation should first develop less, enter a region where it doesn't develop at all, as the loading of the inactivated state during the test pulse matches the steady state occupancy during the postpulse, and then reverse. At very negative postpulse potentials inactivation will not develop further during the postpulse, but will only dissipate and tend to increase the current during the postpulse. However, τ_3 was independent of postpulse potential negative to −35

TABLE I

DEPENDENCY OF THE TIME CONSTANTS IN THE Na TAIL CURRENTS ON POSTPULSE POTENTIAL

Axon	Prepulse	Postpulse	τ_2	τ_3	τ_3/τ_2
	mV	mV	μs	μs	
H77M51	2	−8	148		
	"	−18	205		
	"	−28	202		
	"	−38	166	1,055	6.34
	"	−48	135	785	5.81
	"	−58	135	843	6.23
	"	−68	113	1,047	9.23
	"	−78	106	938	8.85
	"	−88	108	1,010	9.39
	"	−98	111	1,011	9.09
	"	−108	114	930	8.18
	"	−118	93	888	9.55
	"	−128	99	974	9.84
H77M55	0	−10	123		
	"	−25	147		
	"	−30	126		
	"	−35	129	1,051	8.18
	"	−40	132	1,234	9.32
	"	−45	128	1,114	8.71
	"	−50	114	1,263	11.10
	"	−60	106	1,219	11.55
	"	−70	100	1,270	12.70
	"	−80	91	1,257	13.81
	"	−90	109	1,328	12.17
	"	−100	112	1,166	10.41
	"	−110	105	1,066	10.11
H77M56	0	−6			
	"	−16	130		
	"	−26	132		
	"	−31	122	1,025	8.43
	"	−36	123	889	7.25
	"	−41	118	1,029	8.69
	"	−46	125	1,170	9.34
	"	−51	122	1,230	10.08
	"	−61	111	1,551	13.95
	"	−71	97	1,631	16.75
	"	−81	125	1,746	13.98
	"	−91	99	1,296	13.15
	"	−101	94	1,283	13.59
	"	−111	105	1,143	10.93

Prepulse duration was always 1 ms.
Temperature was 5°C.

mV (Fig. 2 A, Table I). Hence, it is not likely that τ_3 is an inactivation process or even significantly contaminated by inactivation. Similarly there can be little contamination from any incompletely subtracted K currents, as K tail currents reverse direction at ~ −80 mV (Binstock and Goldman, 1971). This must be the

shutdown of the additional activation process anticipated from the time shift experiments.

For postpulse potentials postive to −35 mV, the time constant of the slow component in the tail currents increases. This is most likely due to increasing contamination with inactivation. By −10 mV this component is just τ_h. The slow tail component has therefore been excluded from the analysis for postpulse potentials positive to −35 mV, owing to these uncertainties.

Another, faster, activation process is seen by extrapolating the τ_3 component back towards $t = 0$. This faster component is small in magnitude, relative to the τ_3 component, at more positive postpulses, but increases with more negative postpulse potentials. The differences between the filled circles and the solid line fitted to them at longer times is plotted as the open circles in Fig. 1 A and B. This component decays as a single exponential over the whole (Fig. 1 A) or nearly the whole (Fig. 1 B) of the time-course for which it could be observed. Time constants for this component are shown in Fig. 2 B and in Table I (τ_2). τ_2 is nearly independent of postpulse potential from −130 mV to ~ −60 mV and is ~ 100 μs in this range. For more positive postpulses τ_2 rises by ~ 50%. τ_2 behaves much like τ_{shift} (Hahin and Goldman, 1978). Their magnitudes are similar for hyperpolarized potentials and they both increase with moderately depolarizing potentials. It seems possible that τ_2 is the relaxation underlying the time shift. A more definite identification is not possible at the present time because the ratio between τ_{shift} and the underlying activation time constant is at least somewhat model dependent.

There might be another, even faster, activation process. Fig. 3 shows the instantaneous $I(V)$ for the Na tail currents of axon H77M51 in Table I, constructed in the following way. Na tail currents at the start of the postpulse were determined by extrapolating the exponential terms for the τ_2 and τ_3 relaxations back to $t = 0$ ($I_{Na_{02}}$ and $I_{Na_{03}}$, respectively). The plotted values in Fig. 3 are the sum of the computed $I_{Na_{02}}$ and $I_{Na_{03}}$, corrected for any variation in peak I_{Na} during the preceeding test step. The $I(V)$ so constructed at first declines linearly as the postpulse potential is made less positive, but then deviates from linearity, and beyond ~ −50 mV even shows a negative slope. Similar results were obtained in two other axons.

The presence of the negative slope region indicates that there is an additional, or possibly more than one, faster relaxation which also contributes to channel gating. There is an indication of an additional fast activation process for most postpulse potentials negative to ~ −40 to −50 mV. Fig. 1 B shows one example. At short times the open circles systematically deviate from the solid line fitted to them at longer times, in the direction expected for an additional exponential term. However, this component (τ_1) is far too poorly resolved in these experiments to define time constant values.

There are then three exponential terms identifiable in the Na tail currents, all of which represent activation processes. One way to get three exponential terms in the Na tail currents is to suppose that the activation part of the gating machinery is composed of three identical and independent two-state subunits, i.e., m^3 in Hodgkin-Huxley (1952) kinetics. However, this interpretation may be conclusively ruled out. For such a scheme, the time constants are in the ratio

3:2:1. τ_3/τ_2 would then have a maximum value of 2 or less if there are slower relaxations which have not been resolved, even assuming that time constants differing by so little could be distinguished experimentally, which is questionable. τ_3/τ_2 values are given in Table I. They are never less than ~ 6 and often reach 10 or even higher (see also τ_3 and τ_2 values in Table II). If 1.4–1.6 ms of tail record were too short to resolve τ_3 accurately (as seems unlikely from Fig. 1), the true τ_3:τ_2 ratio would only be greater.

A second argument against the identical and independent two-state subunit interpretation is that the τ_3/τ_2 ratio is a function of the postpulse potential. τ_3/τ_2 rose by ~ 1.5-fold as the postpulse potential was made more negative in each of the three axons examined (Table I). This arises from the dependency of τ_2 on

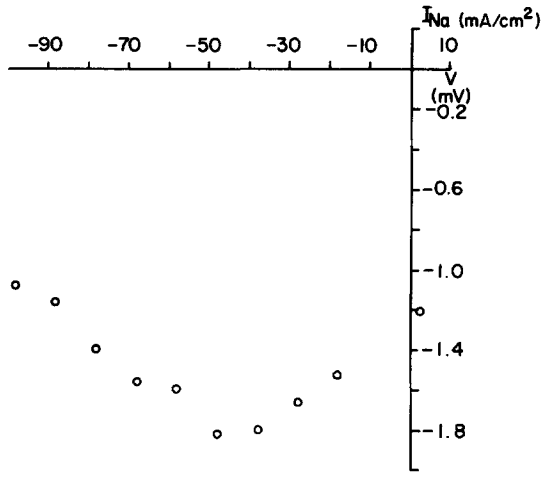


FIGURE 3. Instantaneous Na current-voltage relation. The values are the sums of the zero time intercepts for the τ_3 and τ_2 components in the Na tail current.

the postpulse potential over a range for which τ_3 is independent of potential, and may also be seen from the pooled values in Fig. 2. For such two-state subunits, τ_3/τ_2 should have been independent of potential.

A third argument comes from the effects of different durations of the same test (pre-) pulse currents during a fixed postpulse (see below). Consider the results from axon H77M58 in Table III. In this experiment the most extensive range of test pulse durations were used. $I_{Na_{03}}$ relative to I_{Na} at the end of the test pulse increases dramatically as test pulse conductance develops. Relative $I_{Na_{02}}$ is insensitive to the prepulse duration over the range examined. This is as expected for a sequential process with the faster (τ_2) component filling before the τ_3 component. However, it is in the opposite direction expected for independent two-state subunits. In this case $I_{Na_{02}}$ should have increased much more steeply than $I_{Na_{03}}$ during the period for which G_{Na} during the test pulse was rising, inasmuch as the probability of two simultaneous transitions from the conducting to the rest state should have increased more steeply with occupancy of the conducting state than that for a single transition.

There seems to be little question, then, that the activation portion of the Na

gating machinery is not built up of independent and identical two-state subunits, although two-state subunits of a more general sort are not excluded. This suggests that the delay in the rise of G_{Na} during steps in potential arises at least in part from distinct, sequential activation processes; i.e., there is a state

TABLE II

DEPENDENCY OF THE TIME CONSTANTS IN THE Na TAIL CURRENTS ON INITIAL CONDITIONS

Axon	Prepulse	Postpulse	Prepulse duration	τ_2	τ_3
	mV	*mV*	*μs*	*μs*	*μs*
H77M51	0	−80	500	88	871
	"	"	600	101	1,040
	"	"	700	99	942
	"	"	800	110	943
	"	"	900	101	789
	"	"	1,000	133	913
	"	"	1,000*	106	938
	"	"	1,200	114	749
	"	"	1,500	128	906
	20	−80	400	97	909
	"	"	500	88	1,002
	"	"	600	106	1,119
	"	"	700	112	1,033
	"	"	800	101	844
	"	"	900	119	858
	"	"	1,000	133	1,075
	"	"	1,200	116	977
	"	"	1,500	112	935
H77M58	0	−70	250	73	1,187
	"	"	800	67	1,305
	"	"	1,200	68	1,217
	"	"	1,700	68	1,141
	20	−70	250	88	1,067
	"	"	500		1,247
	"	"	800	78	1,345
	"	"	1,200	72	1,165
	"	"	1,700	73	1,043

* From another run on this axon. Temperature was 5°C.

intermediate between rest and conducting. The activation processes, sequentially coupled together, are in turn sequentially coupled to the inactivation process (Goldman, 1976; Armstrong and Bezanilla, 1977), to produce a multistate gating unit. This is examined more fully in the Discussion. Note that we have made the implicit assumption throughout this analysis that the transitions between states are first order, and our conclusions obtain with any generality only within this restriction.

DEPENDENCY ON INITIAL CONDITIONS In any model of the Na gate in which a structure displays some number of discrete orientations or conforma-

tions and for which the transitions between the several states are themselves first order (i.e., no subunit interactions), all individual time constants are independent of the initial conditions. This is because the probability that any single gating particle will make a transition between two given states is independent of the distribution of all other particles and hence of the initial conditions. For a

TABLE III

EFFECT OF INITIAL CONDITIONS ON THE ZERO TIME INTERCEPT OF THE τ_2 AND τ_3 COMPONENTS OF THE Na TAILS

Axon	Prepulse	Postpulse	$I_{Na_{pre}}/I_{Na_{peak}}$	Prepulse duration	$I_{Na_\infty}/I_{Na_{pre}}$	$I_{Na_\infty}/I_{Na_{pre}}$
	mV	*mV*		*μs*		
H77M51	0	−80	0.852	500	1.086	0.194
	"	"	0.922	600	0.869	0.203
	"	"	0.922	700	0.866	0.217
	"	"	1.00	800	0.840	0.203
	"	"	1.00	900	0.736	0.234
	"	"	0.945	1,000	0.730	0.176
	"	"	0.922	1,200	0.791	0.254
	"	"	0.797	1,500	0.766	0.214
	20	−80	0.863	400	1.626	0.244
	"	"	0.968	500	2.138	0.283
	"	"	1.00	600	1.404	0.284
	"	"	0.968	700	1.332	0.293
	"	"	0.874	800	1.657	0.331
	"	"	0.863	900	1.230	0.339
	"	"	0.747	1,000	1.279	0.277
	"	"	0.621	1,200	1.497	0.381
	"	"	0.516	1,500	1.582	0.476
H77M58	0	−70	0.382	250	0.818	0.027
	"	"	0.982	800	0.633	0.206
	"	"	0.715	1,200	0.763	0.220
	"	"	0.615	1,700	0.714	0.248
	20	"	0.738	250	0.974	0.044
	"	"	0.996	500		0.268
	"	"	0.838	800	0.688	0.282
	"	"	0.515	1,200	0.746	0.418
	"	"	0.309	1,700	0.763	0.525

$I_{Na_{pre}}$ is the current density at the end of the prepulse.

multistate model of this sort, the time-course of some processes may depend on the initial conditions, but this will be due to the mix in differing proportions of the same individual time constants in the relaxation. Different initial conditions will not affect the rate constants of the various exponential terms in the relaxation, but can differentially affect the magnitude of the coefficients of these exponential terms.

It is possible to construct models in which the elementary time constants do depend on the initial conditions. For example, models which include a polymerization process, as in the aggregation model of Baumann and Mueller (1974), can display this property if the total concentration of reacting particles is altered

by different initial conditions. This might be the case if there were a diffusion step in the aggregation process. Another way in which initial conditions could seem to affect a time constant is if what appeared to be a single time constant were actually the weighted mean of several which were not well separated. We have therefore examined the effects of initial conditions on the Na tail currents during fixed postpulse potentials, to see if there were effects on any of the time constants in the relaxation and to determine the effects on the I_{Na_0} terms, the weighting factors.

Test pulses to 0 and 20 mV, each for a number of different durations, were applied and tail currents during a step back to the holding potential were observed. The results are given in Tables II and III.

Over this rather limited range of experimental conditions, there were no systematic effects on any of the time constants in the Na tail current (Table II). The most dramatic effect is on the weighting factors for the exponential terms (Table III). As described above, increasing prepulse duration increased $I_{Na_{03}}$ relative to $I_{Na_{02}}$. This is seen most clearly for axon H77M58, but is also evident for H77M51 with a 20-mV test pulse. Hence, as prepulse duration increases the time-course of the Na tail will appear to change, but the effect will not be visually dramatic. Frankenhaeuser and Hodgkin (1957) were the first to report such effects, best appreciated if tail currents of similar amplitude are compared to allow for the effects of an uncompensated R_s in their experiments. The constancy of $I_{Na_{02}}$, but not $I_{Na_{03}}$, as G_{Na} during the prepulse rises is consistent with the view that it is τ_3 which reflects the unloading of the conducting state itself.

Recently Schauf et al. (1977) reported their observations on Na tail currents in *Myxicola*. Our results differ from theirs in several significant respects. (*a*) They detected two exponentials in the tail currents only for postpulses negative to −40 to −50 mV. For more positive postpulses they reported only a single exponential. (*b*) Their single component at 0 mV, the most positive postpulse they applied, is too slow to be an activation time constant and too fast to be τ_h. Our slowest component at more positive postpulses is just τ_h. (*c*) Their time constant increases as postpulse potential is increased from −40 to 0 mV. If this were the only activation time constant, it should decrease. (*d*) Negative to −40 mV, their slow time constant is only about half ours although the experiments were done at the same temperature. We believe all of these discrepancies can be accounted for by a single difference in method. We analysed tail current records that were 3–4 fold longer than what they used. The short lengths of record they analysed were not sufficient to separate the exponentials at all positive to −40 mV, and not completely negative to −40 mV.

Experiments with Low Na Currents

One experimental observation, not reconcilable with Hodgkin-Huxley kinetics is the τ_c-τ_h difference discussed in the Introduction to the preceding paper. This result is particularly interesting in that the effect is dramatic. τ_c may be up to 7–8-fold greater than τ_h in *Myxicola* (Goldman and Schauf, 1973) and lobster (Oxford and Pooler, 1975). It seemed important to be sure that this result could not be attributed to some technical artifact.

One possible source of error is in the TTX subtraction method for extracting the Na currents. If the K currents do not hold stable, some errors might result. However, this does not seem to have been a significant problem. Schauf et al. (1976) repeated the τ_c-τ_h determinations in *Myxicola* axons injected with tetraethylammonium ions so as to reduce substantially the K current (Armstrong and Binstock, 1965), and assumed a linear leak correction. Subtractions were then not needed to extract the I_{Na}. Their results were identical to those of Goldman and Schauf (1973). This was never a likely basis for the τ_c-τ_h difference in *Myxicola*. G_K in this preparation is activated over a more positive range of potentials than is G_{Na} (Binstock and Goldman, 1969), and the G_{Na} may be turned on substantially at potentials where the G_K is only little activated. Hence, the K error is least where the τ_c-τ_h difference is greatest.

A second possible source of error is in the residual R_s left after compensation. However, this error should not only be small, but seems also to be in the wrong direction. At small depolarizations the magnitude of I_{Na} is low and residual R_s effects on τ_h should be minimal. Similarly a τ_c determination with small depolarizing conditioning steps produces a relatively small steady-state decrease in the peak I_{Na} during the test step. It is the amount of the peak current decrease during the course of the conditioning that determines the R_s error which acts to slow the rate at which the peak current declines. Again this error should be least where the τ_c-τ_h difference is greatest. A third possible source of error would arise if good spatial stability under voltage clamp were not achieved (Taylor et al., 1960). This also does not seem to be a likely basis for the τ_c-τ_h difference, because the measurements are highly reproducible quantitatively (Goldman and Schauf, 1973; Schauf, 1973; Schauf and Davis, 1975; Schauf et al., 1976), whereas lack of spatial stability should result in widely varying behavior. Moreover, the *Myxicola* results were obtained with an internal wire clamp whereas the lobster results were done with a sucrose gap clamp, and a τ_c-τ_h difference was even reported for G_{Ca} in isolated snail nerve cell bodies (Akaike et al., 1978). It is not at all clear that the different sorts of errors that the various methods are prone to should result in such similar results, if they are artifactual. However, there is a test which can resolve these questions. The R_s and spatial stability errors both depend on the current magnitude. Hence, if τ_c and τ_h values, with very low Na currents, agree with previous values, then neither of these errors can be the basis for the τ_c-τ_h difference. We have tested this by using TTX to reduce the I_{Na} magnitude.

In Fig. 4 A, parts 1 and 2 show current records as a response to a potential step to −35 mV, shortly after exposing the axon to ASW containing 1×10^{-6} M TTX. In part 1, after 7–8 min in TTX, the peak net inward current is 0.3 mA/cm^2. In part 2, taken 3 and 5 min later, there is no net inward current in either record, yet all three τ_h values were found to be the same. I_{Na} was extracted by waiting until the TTX had abolished the I_{Na} entirely and subtracting in the usual way. R_s compensation was used throughout (see Methods). In Fig. 4 B, the decay of I_{Na} from the top record of part 2 in Fig. 4 A is plotted semilogarithmically against time. The points are well fitted by a straight line from the peak current density of 70 μA/cm^2 down to the smallest observed value, 13 μA/cm^2. τ_h is 2.2 ms in close agreement with the Goldman and Schauf (1973) value of

about 2.15 ms at this potential. Similar results were obtained on two other axons. Neither R_s nor spatial instability errors, therefore, could have significantly affected the τ_h values. −35 mV is well into the negative conductance region, and τ_c here is about 8–10 ms. The insensitivity of τ_h to the current magnitude also confirms the lack of any significant contamination with incompletely subtracted K currents, because such errors would only be exacerbated with low I_{Na}.

τ_c is also not dependent on the current magnitude. For their τ_c determinations Goldman and Schauf (1972) used test steps with a peak net inward current of the unconditioned test step ranging from 0.75 to > 1 mA/cm². On one axon in

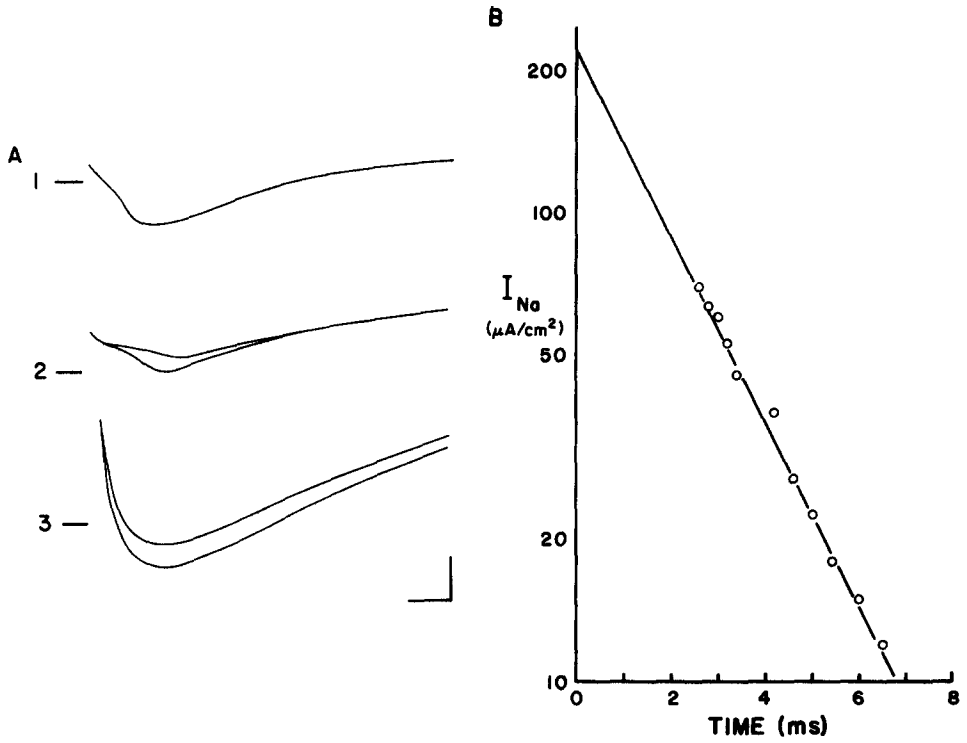


FIGURE 4. (A) Tracings of current records for step to −35 mV at 7 min (part 1) and 10 and 12 min (part 2) after introducing 1 × 10^{-6} M TTX into the bathing medium. The records in part 3 are for the same axon and potential step but at ~ 20 and 25 min after return to TTX-free ASW. Scale: 300 (part 1), 150 (part 2), and 60 μA/cm² (part 3), 1 ms. (B) Na current density as a function of test pulse duration for the top curve in A, part 2.

the present experiments we made a τ_c determination with the peak net inward current during the unconditioned test step reduced to 100 μA/cm² with TTX. The conditioning potential was −45.5 mV and the measured τ_c was 19.9 ms. This is in good agreement with the value of ~ 18 ms reported by Goldman and Schauf (1972) for this potential. In this same axon when the net I_{Na} was some

sixfold greater, τ_c differed by only 15% which is within the normal variance in such determinations. τ_h in this potential range is ~ 2.5 ms (Goldman and Schauf, 1973) or some seven- to eightfold less. As neither τ_h nor τ_c depend on the current magnitude, the τ_c-τ_h difference cannot be attributed to any of the technical errors discussed, and seems then to be a true property of the gating machinery.

There May Be Two Types of TTX Sensitive Channels

In three axons after I_{Na} was eliminated completely in TTX, the preparations were returned to ASW and the redevelopment of I_{Na} was followed under voltage clamp. The first inward currents to appear always looked like the top record in Fig. 4 A part 3, which is from the same axon and same test step as in parts 1 and 2 above. The current rose to a peak more rapidly and declined more slowly than before exposure to TTX. These records were, then, very similar to the current records presented by Hodgkin and Huxley (1952) for very small depolarizing steps. The inactivation time constant was neither τ_h nor τ_c. As recovery proceeded in ASW, a current very similar to those seen in parts 1 and 2 appeared superimposed on these more slowly inactivating currents (not illustrated), and eventually grew sufficiently large to obscure the more slowly inactivating currents completely. Note that the more slowly inactivating currents in part 3 of Fig. 4 A all are larger inward currents than in part 2 where the current has the same τ_h value seen for large current magnitudes. The kinetics do not, therefore, depend on the current magnitude, but only on whether the axon is entering into or recovering from TTX block. This dependency clearly cannot be attributed to technical errors associated with the voltage clamp. A possible explanation for these results is that there is a small population of Na channels with a relatively fast rate of recovery from TTX block and which also have somewhat different kinetics than the main body of channels. Sevcik (1976) from observations on the kinetics of TTX block of I_{Na} in squid axons, has also suggested that there may be a small subpopulation of sites with different TTX binding characteristics.

DISCUSSION

There Are At Least Two Distinct Activation Processes

The results of this and the preceding paper suggest that there are at least two processes in the activation of G_{Na}. The time shift experiments indicated that there is a process in the activation that is both too fast at small depolarizations and shows the wrong dependency on membrane potential to account for the times to peak of G_{Na}. This suggested that there must be an additional process present to provide the correct behavior. Additionally, experiments on the Na tail currents provided direct evidence for this second process. The various relaxations in the tail currents, all activation processes under our experimental conditions, cannot be attributed to any number of identical and independent two-state subunits, and sequential activation processes are then needed to account for the delay in the rise of G_{Na}. Note that, although the physical pictures

called to mind by these alternative means of producing the delay in the rise of G_{Na} are quite different, mathematically they amount to no more than relaxing the requirements of fixed ratios between the time constants and weighting factors.

There is evidence consistent with more than one activation process available from experiments on the gating current in squid. Meves (1974) found that exposing axons to D_20 slowed G_{Na} kinetics without affecting the gating current kinetics. This suggested that the gating current reflects a precursor process to the actual channel opening. Also, two exponential components have been directly observed in the gating current (Armstrong and Bezanilla, 1975; Bezanilla and Armstrong, 1975; Meves and Vogel, 1977). Neumcke et al. (1976) concluded that there is multistate activation in the Na channels of myelinated nerve fibers also, because the time shifts needed to make Hodgkin-Huxley kinetics fit their experimental $G_{Na}(t)$ records could not be eliminated with any single power on m over the whole potential range. They were also unable to obtain the proper correspondence between the gating current tails at off and the Na tail currents with any power on m.

If one considers only schemes in which the transitions between states are themselves first order, then the minimum organization suggested for the gating unit would be to imagine four states: resting, activated (i.e., a state between rest and conducting), conducting, and inactivated, all sequentially coupled. This is consistent with the computations on the three-state model, from which one possible conclusion was that the gating unit displayed more than three states.

There May Be Only One Gating Unit per Channel

There is an additional, fast relaxation in the tail currents, with a time constant τ_1, which is only poorly resolved, but definitely present (Fig. 3). This is most simply accounted for with yet another activation process. The gating unit would then consist of five states sequentially coupled. Only one such unit per channel would be required; i.e., there would be no necessity to assume that the gating machinery was built of several identical and independent (now multistate) subunits. Three activation processes are sufficient to account for the delay in the rise of G_{Na} (see below), and there would be no experimental observations which required subunits.

The possibility that the gate is built of multistate subunits cannot be excluded, however. With a four-state subunit, the shutdown of G_{Na} in a tails experiment will be describable by the sum of two exponentials raised to some power. If there were just two subunits, the time constants would be: τ_a, $\tau_a/2$, τ_b, $\tau_b/2$, and $\tau_a\tau_b/(\tau_a+\tau_b)$, where τ_a is faster than τ_b. The slowest two time constants are still in the ratio 2:1. However, problems of interpretation arise if τ_a is very much faster than τ_b, as τ_b and $\tau_b/2$ may well not be distinguishable experimentally. Hence, τ_3/τ_2 may actually be the ratio of some weighted mean of τ_b and $\tau_b/2$ to τ_a. Note that $\tau_a\tau_b/(\tau_a+\tau_b)\approx\tau_a$ for $\tau_b >> \tau_a$.

One observation not consistent with this interpretation is seen in the data of Tables II and III on the effects of initial conditions on the τ_3 component. As the duration of the prepulse increases through the rise and decline of G_{Na}, the

occupancy of the conducting state will also rise and decline again. At short times when the conducting state is little loaded τ_3 should be nearly τ_b, but at longer times when the state occupancy is high the probability of two simultaneous transitions out of the state has increased considerably and τ_3 should shift towards $\tau_b/2$. At still longer times τ_3 should again increase as it approaches τ_b. None of these effects are seen (Table II). For example, at a test potential of 20 mV peak G_{Na} will be at 500–600 μs, but there is no sign of a fall in τ_3 near such durations for either axon. Similarly, τ_3 might be expected to be relatively large at more positive postpulse potentials where again the τ_b term would be more significant. This effect is also not seen (Table I). However, it might be argued that there is a potential dependency of τ_3 which acts to just balance such effects.

Another effect that may be relevant here is the τ_c-τ_h difference. For a three-state model, or any multistate model with a single inactivation step, τ_h and τ_c are generated by the same elementary relaxation, given by the right hand term in Eq. 4 of the preceding paper. The τ_c-τ_h difference computed from the ν^5 model (Goldman, 1975; see preceding paper) arises from the assumption of subunits. Both the τ_h and τ_c relaxations are then governed by a spectrum of time constants, in the ratio 5:4:3:2:1. During single steps in potential, the fastest time constant dominates inasmuch as the steady-state occupancy of the conducting state is low, and τ_h will reflect the rate at which entire channels inactivate, not the rate of draining of the conducting state. For τ_c the slowest exponential dominates at small conditioning depolarizations and the fastest at more positive potentials. This is because the course of changes in the initial conditions goes as $\nu(t)$ (and $u(t)$) not $\nu^5(t)$. Hence, τ_c and τ_h will be most different at small and converge at large conditioning potentials. The maximum τ_c:τ_h ratio is equal to the number of subunits, five for the three-state model. For a four-state subunit with two activation processes, the number of subunits must be less as now some of the delay in G_{Na} rise has been provided by the two-step activation. Hence, the maximum τ_c:τ_h ratio should be no more than four whereas up to seven or eight is found experimentally. Subunits cannot provide for the observed τ_c-τ_h differences. There must be another mechanism. However, it might be argued that the observed differences are generated by two quite different mechanisms operating simultaneously.

There is very little other evidence bearing on the question of subunits. Fox et al. (1976) compared the blocking effects of ultraviolet irradiation on I_{Na} and the gating current in myelinated nerve fibers. I_{Na} was two to three times more sensitive than the gating current. This is consistent with the idea of two to three gating subunits per channel. However, this same result might also be obtained with only a single gating unit per channel, if the activation is multistep and if the transition generating the gating current precedes the channel opening, as seems to be the case (Meves, 1974). The two or three sites showing similar sensitivities to irradiation need not be on independent structures.

The possibility that there are multistate subunits cannot be entirely excluded. However, the view that there are not subunits is the more attractive, because it is far simpler in that there are fewer states of the channel overall. Because there is no kinetic evidence which directly requires subunits, we see no reason for introducing this additional complexity.

A Minimum Kinetic Scheme

Consider the following kinetic scheme:

$$R \rightleftharpoons A_1 \rightleftharpoons A_2 \rightleftharpoons C \rightleftharpoons I,$$

with R the resting, C conducting, I inactivated, and A_1 and A_2 activated but nonconducting states. There would be only one such unit per channel. Moore and Cox (1976) proposed a basically similar multistate single unit scheme for the Na gate, and this seems to be the view favored by Neumcke et al. (1976) for myelinated nerve fibers. It is very likely that this scheme will have to be modified as more kinetic data on the gating current is developed. It should be viewed only as one convenient way to summarize most of the experimental results now available.

Some properties of the scheme may be briefly discussed. It can provide a delay in the development of inactivation which is dependent on the rate at which activation develops, owing to the sequential nature of the model. The four states R–C provide the three relaxations seen in the tail current, and different initial conditions can change the relative weighting of each of these terms. These same relaxations should appear in the gating current tails at off, but with a different distribution of weighting factors, as now one is observing the occupancy of a different state(s). Different combinations of initial and final conditions, then, can cause the Na tail current and gating tail current time-courses either to converge or diverge depending on how the two distributions of weighting factors are affected. This is seen experimentally (Keynes and Rojas, 1976; Neumcke et al., 1976).

Only those channels that conduct can be inactivated. Therefore, even if all the channels activated during a small depolarizing step are inactivated, reducing the steady-state G_{Na} to zero, a second larger depolarizing step immediately after the first can still produce a substantial peak G_{Na}. The greater the number of channels activated during the second step, the less the effect of the few channels inactivated during the first, producing the h_∞ shift. The nonmonotonic h_∞ curve of Chandler and Meves (1970) is accounted for by noting that the steady-state occupancy of the C state can continue to increase even at potentials where h_∞ as determined with two pulses seems minimal. One can no longer transiently overfill the C state during the test pulse beyond its equilibrium level by any appreciable amount. Hence, h_∞ has fallen to low levels. However, the C and I states may still not be at their maximum occupancy.

The time-course of G_{Na} during steps in potential is also accounted for. $G_{Na}(t)$ will be proportional to the occupancy of the C state, Y. Under voltage clamp conditions Y is given by

$$Y(t) = Y_\infty + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} + A_3 e^{-t/\tau_3} + A_4 e^{-t/\tau_4}. \quad (1)$$

The fit of Eq. 1 to clamp data is shown as the solid curves in Fig. 5, and the open circles indicate the experimental values for each of the potentials indicated. The experimental results, including the delay in the rise of G_{Na}, are reasonably well accounted for by Eq. 1. Each step was preceded by an 80-ms conditioning pulse to −110 mV, so that the delay ought to have been maximal. This fit is not

surprising, as Moore and Cox (1976) have fitted a similar scheme to clamp data from squid, and these same data were also well fitted by m^3h kinetics (Goldman and Schauf, 1973, Fig. 9) which is a more restrictive case.

Parameters used in the construction of the curves of Fig. 5 are listed in Table IV. The tendency for τ_3/τ_2 to decrease with potential, seen in the data of Table I, is continued. Beyond ~ -20 mV this ratio is about two which is why reasonable fits with m^3h were possible. For large depolarizations where the delay

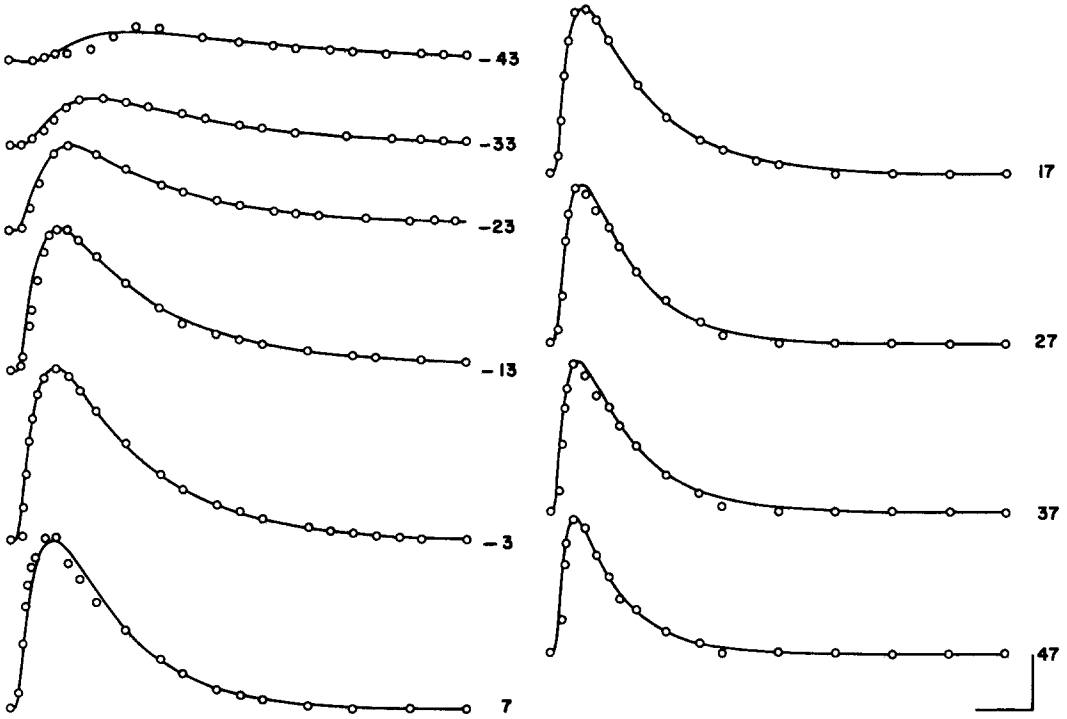


FIGURE 5. G_{Na} as a function of time for each of the potentials indicated. The open circles are experimental values and the solid curves have been computed from Eq. 1 in the text. Axon 71M33, Goldman and Schauf (1973). There was an 80-ms conditioning pulse to -110 mV. R_s compensation was used and temperature was 5°C.

is short, these fits are not very sensitive to τ_1 and these values are therefore not very reliable.

The τ_c-τ_h Difference

For the τ_c-τ_h difference something additional is needed. The experimental result is that G_{Na} during small steps in potential declines, over a time course given by τ_h, to a negligible level. If one then probes with a strong depolarizing test pulse, inactivation is found to be far from complete. The resting state continues to drain into the inactivated state without filling the conducting state

to any appreciable extent, and this further development of inactivation proceeds over a different time course given by τ_c. No addition of pathways between the states of the above scheme will account for this behavior. The simplest way to account for this effect is to suppose that there is yet another nonconducting state. For example, an additional parallel pathway out of the C state to another inactivated state (in addition to I), which loaded rapidly but could never fill to a very great extent, could produce the effect. The rapid filling of the low capacity state would dominate during small depolarizations where the C state never loads very much. The slower but more extensive draining of R into I would primarily determine the changes in peak G_{Na} seen with the large probing depolarizations. Other, quite different arrangements would also work. The important point is that something in addition to the five states of the above scheme is needed. Note that this implies that most of the loading of the I state proceeds after G_{NA} has already declined to its steady negligible level.

TABLE IV

CONSTANTS USED TO COMPUTE THE G_{Na} CURVES OF FIGURE 5

Test potential	A_1	A_2	A_3	A_4	τ_1	τ_2	τ_3	τ_4	Y_∞
mV					*μs*	*μs*	*μs*	*μs*	
−43	−0.093	0.250	−0.300	0.143	123	260	765	3,096	0
−33	−0.113	0.280	−0.387	0.220	76	155	570	2,315	0
−23	−0.150	1.053	−1.400	0.477	60	166	315	1,790	0.02
−13	−0.165	1.618	−2.200	0.727	31	131	243	1,724	0.02
−3	−0.171	1.184	−1.947	0.934	29	103	257	1,613	0
7	−0.092	0.526	−1.724	1.289	42	84	350	1,161	0
17	−0.066	0.658	−1.526	0.934	14	68	214	1,156	0
27	−0.139	0.921	−1.829	1.047	30	75	206	907	0
37	−0.032	1.079	−1.842	0.795	46	86	152	1,026	0
47	−0.329	2.211	−2.632	0.750	25	67	115	803	0

$\overline{G_{Na}}$ was 38 mmho/cm^2.

Meves and Vogel (1977) have recently presented comparisons of the time-course of the gating charge immobilization with G_{Na} inactivation in squid. They found that G_{Na} during single steps in potential decreased more rapidly and to a greater extent than did the loss of the gating charge. When the time-course of recovery from inactivation was studied, using the two pulse method, the gating current and I_{Na} showed similar rates of recovery if the holding potential was −95 mV, but agreement was less good with a holding potential of −70 mV. These results are, then, much like the τ_c-τ_h difference and may be accounted for in a similar way if the gating charge immobilization is identified with the occupancy of the I state.

τ_c-τ_h differences were found in intact squid axons (J. W. Moore cited in Goldman, 1976), but not by Bezanilla and Armstrong (1977) under their internal perfusion conditions. However, it should be possible to abolish the τ_c-τ_h difference simply by an adjustment of rate constants. The results on intact vs. perfused squid are then not very difficult to reconcile, especially as the internal perfusion medium can affect G_{Na} kinetics (see e.g., Chandler and Meves, 1970; Yeh and Narahashi, 1977).

A τ_c-τ_h difference allows a nerve cell to have a long refractory period without unduly increasing the duration of the action potential. Goldman and Schauf (1973) simulated reasonably normal-looking action potentials with m^3h kinetics using their experimental τ_h values. Computations using the τ_c values produced a very prolonged action potential, extending in a plateau. Computations made using τ_c at potentials of −45 mV and below, and τ_h above produced normal-looking action potentials with a refractory period similar to that found experimentally. This was another reason for thinking that the τ_c-τ_h difference is real, and possibly of relevance for information processing properties of nerve cells.

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