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Sodium channel opening as a precursor to inactivation

A route to the inactivated state

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Abstract. The time constant of the process producing the delay in Na inactivation development as determined by the two pulse method (τ_{delay}) was extracted and compared to that of the slowest Na activation process (τ_3) for the I_{Na} during the conditioning pulse of that same determination. τ_{delay} and two pulse inactivation (τ_C) values were computer generated using a non-linear least squares algorithm. τ_3 and single pulse inactivation (τ_h) values were independently generated for each determination also with the aid of the computer using the same non-linear least squares algorithm. In one determination at 2 mV, τ_C was 4.68 and τ_{delay} 0.494 ms while τ_h was 4.70 and τ_3 0.491 ms for a τ_C/τ_h of 0.996 and a τ_{delay}/τ_3 of 1.006. Mean τ_{delay}/τ_3 from five determinations in four axons, both Cs and K perfused, and spanning a potential range of −27 to 2 mV was 1.068. The precursor process to inactivation is channel opening. Some fraction of channels presumably inactivate via another route where prior channel opening is not required.

Key words: Sodium currents, inactivation, kinetics, channel gating

Introduction

A clear delay preceding the development of Na inactivation has been reported in well controlled experiments in the giant axons of *Myxicola* (Goldman and Schauf 1972; Goldman and Kenyon 1982) and crayfish (Bean 1981), and small delays are also evident in the data of Gillespie and Meves (1980) in squid. The delay cannot be attributed to distortion of the time course of inactivation determined with the two pulse method by the development of activation during the conditioning pulse, voltage clamp errors associated with the current magnitude, or contamination by K currents. For some fraction of the channels, inactivation develops only subsequent to a precursor process, i. e. for these channels prior to inactivation gate closing there must first be another process which enables closure. Other fractions of the channel population may reach the inactivated state by a route that requires either no or a different immediately preceding process (Aldrich and Stevens 1983).

Some information is available as to the identity of the precursor process in *Myxicola*. In an extensive series of determinations Goldman and Kenyon (1982) found that the operationally defined inactivation delay (time at which the unconditioned peak current value intersected the fitted τ_C exponential in a two pulse inactivation determination) was about proportional to the time to peak Na conductance (g_{Na}) during the conditioning pulse. If the percursor process is channel opening then both the inactivation delay and the time to peak g_{Na} would be approximate measures of the activation rate, and proportionality between these measures would be expected. These results suggest that the potential dependency of the time constant of the delay process and that of the activation process are similar. In addtion, in 10%–15% of their experiments, amounting to seven determinations, Goldman and Kenyon were able to extract the time constant of the delay process, τ_{delay}. The measured $\tau_{delay}(V)$ values agreed well with activation kinetics, $\tau_m(V)$, previously reported in *Myxicola* (Goldman and Schauf 1973), suggesting that the precursor process to inactivation is channel opening. However, it is not clear from these results wheter inactivation delay and activation kinetics agree quite closely, or are only rather similar. I present here new determinations of inactivation delay and activation time constants obtained from the same axons under the same conditions. Kinetics for the two processes are essentially indistinguishable. A preliminary report of these findings has been presented (Goldman 1987).

Methods

Myxicola infundibulum were obtained from the Laboratory of the Marine Biological Association of the United

Kingdom (Citadel Hill, Plymouth, United Kingdom). Methods for preparing and electrically recording from the giant axons were as described by Binstock and Goldman (1969). Normal artificial sea water (ASW) had the following composition: 440 m*M* Na, 10 m*M* Ca, 50 m*M* Mg, 560 m*M* Cl, 5 m*M* *Tris* (tris[hydroxymethyl] aminomethane; Sigma Chemical, St. Louis, MO), pH 8.0 ± 0.1. Temperature was 5 ± 0.5 °C. Potentials are reported as absolute membrane potential (inside minus outside) and have been corrected for liquid junction potentials as described by Ebert and Goldman (1975).

Internal perfusion was initiated with the KCl-axoplasm dispersal method of Goldman and Kenyon (1979). Standard (K) internal perfusate had the following composition: 410 m*M* K, 50 m*M* F, 360 m*M* glutamate, 1 m*M* HEPES (*N*-2-hydroxyethylpiperazine *N'*-2-ethane sulfonic acid; Calbiochem-Behring, San Diego, CA), 4 m*M* EGTA (ethyleneglycol-*bis*-(β-amino-ethyl ether) *N*,*N'*-tetra acetic acid; Sigma Chemical Co.), 145 m*M* sucrose, pH 7.30 ± 0.05. Cs perfusate was identical except that K glutamate and KF were replaced equivalent per equivalent with Cs glutamate and CsF. All internal perfusates had an osmotic pressure within 5% of that of ASW as determined by osmometer (osmette A; Precision Systems, Sudbury, MA). Internal perfusates were freshly prepared every few days.

All voltage clamp observations were made using compensated feedback to reduce errors produced by the series resistance, R_s, as described by Goldman (1986). To reduce R_s errors further, observations were made in bathing media with the Na concentration reduced by substitution with *TRIS* to 1/2 of that in ASW. With the current densities encountered in these measurements, displacements in membrane potential produced by any residual, uncompensated R_s, will generally be well less than 1 mV.

Pulses sent to the voltage clamp were formed by a PDP 11/34 computer (Digital Equipment, Maynard, MA). Membrane currents were filtered at 100 kHz with a four pole Bessel filter (model 4302; ITHACO, Ithaca, NY) digitized at a 20 μs sampling interval with a 12-bit analog to digital converter (model ADC-EH 12 B 3; Datel Systems, Canton, MA) and stored on floppy disks for later analysis. Na currents were extracted by repeating the entire voltage clamp protocol in 1 μ*M* tetrodotoxin (TTX; Calbiochem-Behring) and subtracting the two sets of records with the aid of the PDP-11/34. All experiments were done in the presence of 2 m*M* 3,4-diaminopyridine (Aldrich Chemical, Milwaukee, WI) in the external bathing medium to suppress the K conductance. 3,4-diaminopyridine solutions were freshly prepared each day and the pH carefully checked.

Two pulse inactivation determinations were made as described by Goldman and Kenyon (1982), an included a 5 ms gap (step back to the holding potential) between conditioning and test pulses. This gap width was sufficient to allow Na tail currents to decay to zero well before the start of the test pulse (Goldman 1986). Each conditioning pulse, gap, test pulse sequence was both preceded and followed by an unconditioned test pulse. The means of the peak I_{Na} values from these bracketing unconditioned test pulses were used to normalize each conditioned determination. Holding potential was always near −100 mV. All axons were held at the holding potential for 2 min before the start of each voltage clamp run, and 15 s were allowed between each voltage clamp pulse to minimize any effects of slow inactivation (Rudy 1981).

Peak currents were read with the aid of the PDP 11/34 after digitally filtering as described by Goldman and Chandler (1986). Time constants as determined both with one and two pulse methods were also determined with the aid of the PDP 11/34 using pattern-search, a non-linear least squares minimization procedure (see Colquhoun 1971). As I have configured it, the pattern-search procedure will fit optionally from one to four exponentials to any selected region of a current record or any selected region of a two pulse inactivation determination, providing an objective determination of time constants. These are simultaneous multi-exponential fits, and so computing time for single pulse current records increases substantially with each additional exponential fitted.

Results

In all cases multi-exponential fits to two pulse inactivation time courses and those to the I_{Na} during the conditioning pulses were generated independently by the PDP 11/34.

Both the results of the two pulse determinations and the single pulse current time course, $I_{Na}(t)$, are described as a sum of exponentials. For a complete description of $I_{Na}(t)$ a minimum of three relaxations are needed: an inactivation time constant (τ_h) and at least two activation time constants as there is a delay in the rise of g_{Na}. There is some experimental evidence for yet an addtional activation process in *Myxicola* (Goldman and Hahin 1978). A possible description for the entire $I_{Na}(t)$, then, is

$$I_{Na}(t) = -I_1 \exp(-t/\tau_1) + I_2 \exp(-t/\tau_2) - I_3 \exp(-t/\tau_3) + I_h \exp(-t/\tau_h) + I_{Na\infty}. \quad (1)$$

In none of the two pulse determinations analyzed here was there more than one inactivation delay process detected, and these results have all been described as the sum of just two exponentials, with time constants

τ_C and τ_{delay}, plus a steady state term. Accordingly, for $I_{Na}(t)$ no attempt was made to accurately define more than two time constants, τ_h and τ_3. Those governing the delay in the rise of g_{Na} are either partially or totally unresolved. Single pulse fits were never extended to times shorter than 100 μs into the voltage step, and in no case were fits attempted with more than three exponentials.

Figure 1 presents two Na current records from different axons spanning the range of potentials examined. The top trace was obtained from a Cs perfused axon at −27 mV. Superimposed on the current trace (thin line) is a three exponential fit constructed with τ_h and two activation time constants, τ_3 and τ_2. The fitted curve and the current record agree quite closely. The rise in g_{Na} is determined nearly entirely by a single exponential process (τ_3). A two exponential fit to this same record now starting at 300 μs into the voltage step produced a τ_3 only 1% faster and an identical τ_h. The lower trace is a current record at 2 mV also from a Cs perfused axon and again with a superimposed three exponential fit. A two exponential fit starting at 300 μs into the voltage step yielded a τ_3 3% faster and again an identical τ_h. The faster activation time constants (τ_2) in the three exponential fits can only be approximate values. These fits were started at 100 μs into the step. The accuracy of the τ_2 values depends both on their relative magnitude and on the relative magnitudes of the coefficients on these exponential terms as well as the relative magnitudes and relative magnitudes of the coefficients on the exponentials of any additional activation relaxations, which are totally unresolved in these computations. The point of the three exponential fits is to demonstrate that τ_3 can be determined reliably without accurately resolving the activation delay process(es).

The two pulse inactivation time course for the same determination as the lower trace of Fig. 1 is shown in Fig. 2. For this experiment both the test pulse and the conditioning pulse were at 2 mV. Open circles indicate peak values of I_{Na} during the test pulse, normalized to that with no conditioning pulse, as a function of conditioning pulse duration. The solid curve is a two exponential fit to these data, and the dotted curve indicates the extrapolation of the slower fitted τ_C exponential to zero time. The inset presents the first two ms of this determination on an expanded time scale. The delay in inactivation development is quite prominent. Following very brief conditioning pulses I_{Na} during the test pulse actually increases somewhat, indicating that states preceding the conducting state have not all returned to their resting occupancies by the end of the gap. In fact, such depolarizing conditioning potentials do shift the $I_{Na}(t)$ to the left along the time axis (Hahin and Goldman 1978). A three exponential fit to these same data did not produce any indication of a process that was itself a precursor to this delay process.

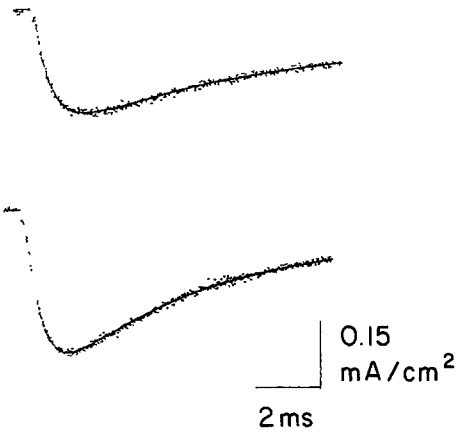


Fig. 1. Na current records from two different Cs perfused axons. The zero current level is indicated by the horizonal line at the left in each case. *Top*: Trace at a step to −27 mV from a holding potential of −102 mV. Superimposed thin line indicates a three exponential fit to the record using a τ_3 of 0.581 ms and a τ_h of 6.70 ms. Axon 4MP25. *Bottom*: Trace at a step to 2 mV from a holding potential of −103 mV. Superimposed three exponential fit computed with a τ_3 of 0.491 and a τ_h of 4.70 ms. Axon 4MP16. Inactivation was well described by single exponential in both cases. Measured current at 30 ms for the record at 2 mV was 0.068 mA/cm² while the computed value was 0.071 mA/cm². For the record at −27 mV the corresponding values at 30 ms were 0.049 and 0.055 mA/cm². Scale: 0.15 mA/cm², 2 ms

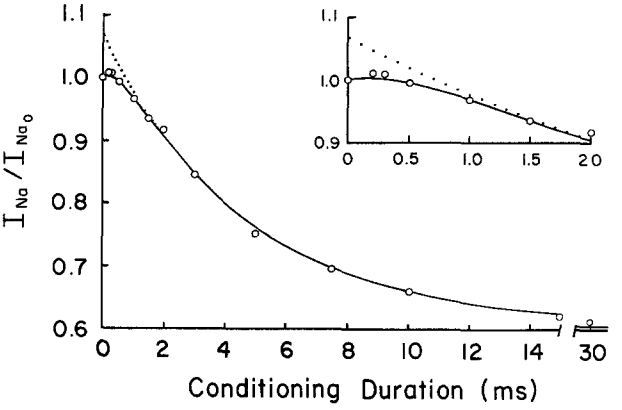


Fig. 2. Two pulse inactivation time course for the same determination as that for the lower trace of Fig. 1. Points indicate peak I_{Na} during a 2 mV test pulse, relative to that with no conditioning pulse, as a function of the duration of a 2 mV conditioning pulse. There was always a 5 ms step back to the holding potential between conditioning and test pulses. *Solid curve* is a two exponential fit computed with a τ_{delay} of 0.494 and a τ_C of 4.68 ms. *Dotted line* indicates the extrapolation of the τ_C relaxation to zero time. *Inset*: First 2 ms of the determination on an expanded time scale

For the two exponential fits of Fig. 2 τ_C and τ_{delay} are 4.68 and 0.494 ms respectively, while for the time course of the I_{Na} during the conditioning pulse of this same experiment the three exponential fit illustrated in

Table 1. Comparison of Na activation and inactivation kinetics. τ_3 values were generated from three exponential fits starting at 100–200 μs into the step. τ_{delay} values were all from two exponential fits

Axon	Potential [mV]	Perfusate	τ_3 [ms]	τ_{delay} [ms]	τ_{delay}/τ_3
4MP25	−27	Cs	0.581	0.568	0.978
4MP26	−23	K	0.444	0.523	1.178
4MP24	−22	Cs	0.641	0.763	1.190
4MP24	− 2	Cs	0.571	0.563	0.986
4MP16	2	Cs	0.491	0.494	1.006
				Mean	1.068

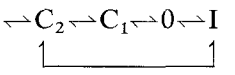
Fig. 1 (lower trace) was generated with a τ_h of 4.70 and a τ_3 of 0.491 ms, yielding a τ_C/τ_h of 0.996 an a τ_{delay}/τ_3 of 1.006. The close agreement between both pairs of time constants indicates that these data have not been significantly affected by voltage clamp errors or any residual unblocked g_K effects as these will not affect the two measurements in just the same way. The near identity of τ_3 and τ_{delay} also indicates that the inactivation delay and Na activation processes have the same kinetics.

τ_C determinations were available from 17 axons in this series. Of these, seven determinations from six different axons had inactivation delays sufficiently clear and extensive to attempt multi-exponential fits to. Of these attempts, pattern-search generated five τ_{delay} values from four different axons. These are presented in Table 1 along with the τ_3 values from these same determinations. Mean τ_{delay}/τ_3 is 1.068 over a range of potentials from −27 to 2 mV and including both Cs and a K perfused preparation. Inactivation delay and Na activation kinetics, then, agree quite closely.

Discussion

The rise of g_{Na} during a step in potential is determined nearly entirely by the τ_3 relaxation. The other activation processes primarily determine the delay in the g_{Na} rise. Hence τ_3 must be the time constant most closely associated with the transition into the conducting state. The precursor process to inactivation development, then, is channel opening. In a series of exponentials description, delays are generated by a difference of exponentials, indicating sequential processes. For fully coupled schemes (as in Scheme I, below) only transitions leading to both activation and inactivation can produce an inactivation delay. Any states in a sequence leading exclusively to the final inactivated state and which do not yield a net transition towards the conducting state are themselves inactivated states in that their occupancy contributes to the fraction of channels unavailable for carrying current. The final precursor process to inactivation gate closure, for some fraction of the channels, is in fact channel opening. It is not now clear whether the remaining fraction require other precursor processes or whether inactivation gates can close in some cases with no delaying process. The delaying processes to channel opening ought themselves also to contribute to the inactivation delay, but are apparently not within resolution in these experiments.

One sample of a class of models often proposed for the Na channel gate (see e.g. Bezanilla and Armstrong 1977) is presented in Scheme I:



Three closed states are sequentially coupled to a single conducting state (0) which is in turn sequentially coupled to an inactivated state. In addition, the second closed state directly couples to the inactivated state. This Scheme is described by Eq. (1). The designation of C_2 as the only closed state directly coupling to I is arbitrary. Other closed states instead of or in addition to C_2 may directly couple to I. However, it is well established that Na channels can inactivate without opening (Gillespie and Meves 1980; Bean 1981; Horn et al. 1981; Aldrich and Stevens 1983; Horn and Vandenberg 1984; Aldrich and Stevens 1987), and so some such loop between closed and inactivated states is needed. Note that any additional states between C_2 and I are also inactivated states.

A requirement of Scheme I is that some channels reach the inactivated state by a pathway in which they must first open. In contrast to the impressive array of evidence establishing that channels need not open before inactivating, there is relatively little experimental evidence indicating that Na channels also utilize a route in which they can reach the inactivated state only after opening. An alternative would be some degree of parallel activation and inactivation in which some channels conduct before inactivating, but are not required to do so. Aldrich and Stevens (1983) on studies of single Na channels found that inactivation is in fact more probable from conducting than from closed states. An independent line of evidence in support of models of the class of Scheme I is a delay in inactivation development generated by channel opening.

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