A SLOW COMPONENT OF GATING CURRENT IN CRAYFISH GIANT AXONS RESEMBLES INACTIVATION CHARGE MOVEMENT

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ABSTRACT Recent experimental evidence from a number of preparations indicates that sodium channel inactivation may be intrinsically voltage sensitive. Intrinsically voltage sensitive inactivation should produce a charge movement. Crayfish giant axons provide a unique opportunity to reexamine the slower components of gating currents (I_g) for a contribution from inactivation (I_g^h). In reference to other axon preparations, this preparation has relatively rapid inactivation, and steady-state inactivation has a comparatively steep voltage dependence. As predicted by a two-state scheme for voltage-sensitive sodium channel inactivation, I_g in crayfish axons includes a slow component with time constant comparable to the time constant of decay of the sodium current. Allowing for some delay in its onset (60 μs), inactivation as described by this slow component of I_g carries roughly the amount of charge predicted by the voltage dependence of inactivation.

INTRODUCTION

Although the kinetic models describing the activation sequence of sodium channels are in general agreement, no universal picture of the relationship of inactivation with this sequence has developed (see Khodorov, 1979; Armstrong and Gilly, 1979; Nonner, 1980). The original proposition of Hodgkin and Huxley (1952), that sodium inactivation is a process completely independent of the activation process, was disproven by the gating current (I_g) measurements of Armstrong and Bezanilla (1974). The authors demonstrated that activation and inactivation are linked together; however, the degree of voltage sensitivity of inactivation is still undetermined. Inactivation may be coupled to activation and still be independently, or intrinsically, voltage sensitive. Recent experimental evidence from recordings of single sodium channels (Horn et al., 1981) and from kinetic analysis of I_{Na} in crayfish axons (Bean, 1981) suggests some degree of intrinsic voltage sensitivity to inactivation.

The question of the voltage sensitivity of inactivation may be directly addressed through the study of I_g. The simplest case, a two-state inactivation process with intrinsic voltage sensitivity, should produce a I_g (I_g^h) with the same time course as inactivation of the sodium current (T_h). The relative magnitude of I_g^h is defined by the ratio of the voltage dependence of inactivation to activation (see Armstrong, 1981). Although no component of I_g with these characteristics has been demonstrated in squid axons (Armstrong and Bezanilla, 1974), the predicted I_g^h is small and slow in comparison with that derived from channel activation. Thus it may be argued that the failure to isolate I_g^h in squid results from the limitations of resolving such a small signal.

In contrast to squid axons, inactivation in crayfish giant axons is quite rapid, proceeding roughly five times more quickly at similar voltage and temperature (Swenson, 1980), while activation is only about two times faster. Moreover, inactivation in crayfish fibers is 40% more voltage sensitive than in squid axon, changing e-fold in 5 mV. If inactivation has its own voltage sensor, these factors predict a larger, faster I_g^h for the sodium channel of crayfish axons, one well within the limits of resolution of the present equipment.

Measurements of I_g in crayfish axons reveal a slow component of I_g at membrane potentials ≥ -20 mV. The time constant of the exponential fitted to this slow component (T_s) is nearly identical to the time constant of inactivation of the sodium current (T_h). Consideration of the area under the exponential (T_s), suggests that inactivation as defined by I_g^h must develop following a delay. Sufficient charge is carried by this component to indicate that inactivation may be completely, intrinsically voltage dependent in crayfish fibers.

METHODS

Single giant axons from the crayfish, *Procamburus clarki*, ranging in diameter from 140–220 μm were dissected and cleaned until the giant fiber represented roughly one-half the volume of the nerve bundle. The axons were mounted in a lucite chamber with a small, moveable silver block surrounding the fiber on all sides. The block consisted of a central

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region, 1.5 mm in length used for measuring current and a horseshoe-shaped piece with each 2-mm branch connected to circuit ground. Surface impedance of the block was reduced with a coating of platinum black.

The mounted fiber was internally perfused through a glass cannula with a 50-μm tipette in the left end of the axon. Fibers were typically perfused initially with a K^+-containing solution and then with a K^+-free solution (Table I). The external solution exchanged within the silver block was generally either a low Na solution (1/3 SVH) to measure I_{Na} or a 0-Na solution to measure I_g (TMA-SVH) with 100-nM TTX for the measurement of I_g. 1/3 SVH represents a solution made from 1 part SVH and 2 parts TMA-SVH. On a few occasions *n*-methylglucamine (NMG) was used instead of TMA to make 0-Na solutions, and no difference was noted between thee two Na substitutes.

The axon was voltage clamped using a straight piggy-back style axial electrode similar to that previously described (Swenson and Narahashi, 1980). The currents were corrected for linear leakage and capacitive currents using the P/4 technique reported by Bezanilla and Armstrong (1977) and stored on the same PDP-8f computer for later analysis. Series resistance was compensated to 3 Ω cm^2 when I_{Na} was recorded and for 1 Ω cm^2 in the case of I_g. Temperature was maintained at 8°C.

Procedure for Approximating dh/dt

I_{Na} traces from the same experiments as I_g were fitted with the Hodgkin-Huxley formulations at 0, 20, and 40 mV (m^3h). Next inactivation was removed from the model by fixing h = 1 and another 3 traces were simulated (m^3). The pair of simulated traces at each voltage (m^3h and m^3) were subtracted, producing a representation of the closing of the inactivation gates (m^3h-m^3). This trace was given a fixed delay of 60 μs. Taking the derivative with respect to time gave the approximation of dh/dt shown in Fig. 3. dh/dt was scaled arbitrarily by a constant to match the slow phase of I_g. In all cases base lines for I_g represent a straight line through the final points of the experimental and simulated traces.

RESULTS

Fig. 1 illustrates the temporal relationships between I_g and I_{Na} in crayfish giant axons. Panel *A* shows a typical I_g recorded at +20 mV using the $P/4$ correction paradigm. The decay of I_g was two clear components: a fast phase (T_f = 0.093 ms) and a slow phase (T_s = 0.48 ms). In *B* the dotted trace presents I_{Na} recorded at the same potential in the same fiber. I_g has been subtracted. The solid line through the I_{Na} record is the fit obtained using the Hodgkin-Huxley formulations (1952) with the appropriate time constants and arbitrary scaling (see legend to Fig. 1). The Hodgkin-Huxley equations provide an excellent fit of I_{Na} for membrane potentials from −30 to +60 , but do not

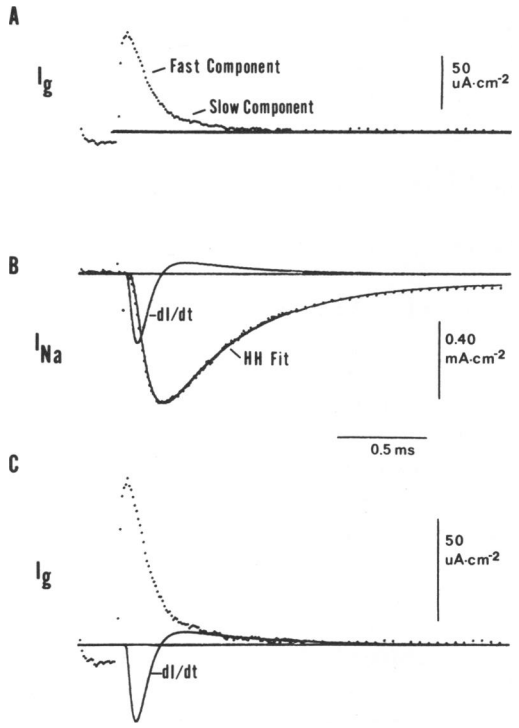

FIGURE 1 Comparison of the slow component of I_g with inactivation of I_{Na} at +20 mV. I_g, at the top and bottom, is the average of fifty cycles after the subtraction of linear capacitance and leakage currents using the $P/4$ technique. Control pulses originated from −160 mV. External solution consisted of TMA-SVH and 100 nM TTX. Center panel shows I_{Na} recorded in 1/3 TMA-SVH. The solid trace through I_{Na} was generated using the Hodgkin-Huxley formulations with T_m = 0.061 ms and T_h = 0.49 ms. The solid trace in the center and bottom panels labeled dI/dt is the first time derivative of the fitted trace and is scaled arbitrarily or to follow the time course of the slow component of I_g. Axon NO280z. Holding potential −80 mV. 8°C.

TABLE I
SOLUTIONS*

Internal solutions	KF	K_3 citrate	NaCl	CsF	Mannitol	HEPES
	mM	*mM*	*mM*	*mM*	*mM*	*mM*
Shrager's‖	100	25	15	—	140	10
K-free	—	—	—	120	240	10
External solutions	**NaCl**	**TMACl**	**NMGCl‡**	**$CaCl_2$**	**Trizma 7.5§**	
	mM	*mM*	*mM*	*mM*	*mM*	
SVH	200	—	—	20	5	
TMA-SVH	—	200	—	20	5	
NMG-SVH	—	—	200	20	5	

*Osmolarity of solutions was adjusted to 460–480 mosM. PH of external solutions was 7.55 and that of internal solutions 7.40.
‡*n*-methylglucamine
§Tris (hydroxymethyl) aminomethane, Sigma Chemical Co., St. Louis, Mo.
‖Shrager, Macey, and Strickholm, 1969.

adequately describe I_g due to the delay in inactivation described below. dI/dt is the first time derivative of the Hodgkin-Huxley fit of I_{Na} scaled arbitrarily. The two components of dI/dt correspond to the activation (downward) and inactivation (upward) of I_{Na}, with some overlap in between. The correlation between the component of dI/dt corresponding to inactivation (dh/dt) and the slow component of I_g is illustrated in the bottom panel of the figure. The time constant of decay of dh/dt is mathematically equivalent to the time constant of decay of I_{Na} (T_h). dI/dt has been scaled to demonstrate the similarity in the time courses of the slow phase of I_g and dh/dt.

The slow component of I_g in crayfish axons is not analogous to the intermediate component of I_g described by Armstrong and Gilly (1979), which arises from activation of the sodium channel (downward deflection of dI/dt). However, this slow component may be similar to the slow component of I_g not accounted for by their model, as seen in their Fig. 13.

T_s Agrees With T_h

Inactivation of I_{Na} in crayfish axons is well described by a single time constant (T_h) over a wide range of membrane potentials, indicative of a two-state process (Shrager, 1977; Swenson, 1980; Bean, 1981). A similarity in the time constant of the slow phase of I_g (T_s) with T_h was observed over a wide range of potentials where the slow phase was of sufficient magnitude to measure reliably ($V_m \geq -20$). Fig. 2 *A* shows the fit of the slow phase of I_g by a single exponential over a voltage range of 0 to +40 mV. In a few other fibers an exponential could be reliably fit at more negative potentials (−10, −20 mV). As with T_h, T_s decreases as the depolarization eliciting the I_g becomes more positive.

Fig. 2 *B* further illustrates the correlation between T_s and T_h. The solid points (●) represent measurements of T_h made in the same fiber by fitting a single exponential to the falling phase of I_{Na}. In crayfish axons measurements of T_h made in this manner are identical to those measured either from tail currents or with a two pulse technique (Swenson, 1981; Bean, 1981). T_s (0) is similar to T_h at all voltages evaluated, and this correlation was consistently observed. Table II lists the time constants found in the manner described above in several fibers.

I_g^h Must Develop With Some Delay

If inactivation is completely and intrinsically voltage sensitive, in a two-state scheme the movement of charge from the not inactivated state (h_0) to the inactivated state (h) may be described for each channel as

$$I_g^h = Q_h \cdot dh/dt, \qquad (1)$$

where dh/dt is proportional to dh/dt ($=T_h$) under conditions of complete inactivation. Q_h represents the product of the number of charges associated with the h-gate and the

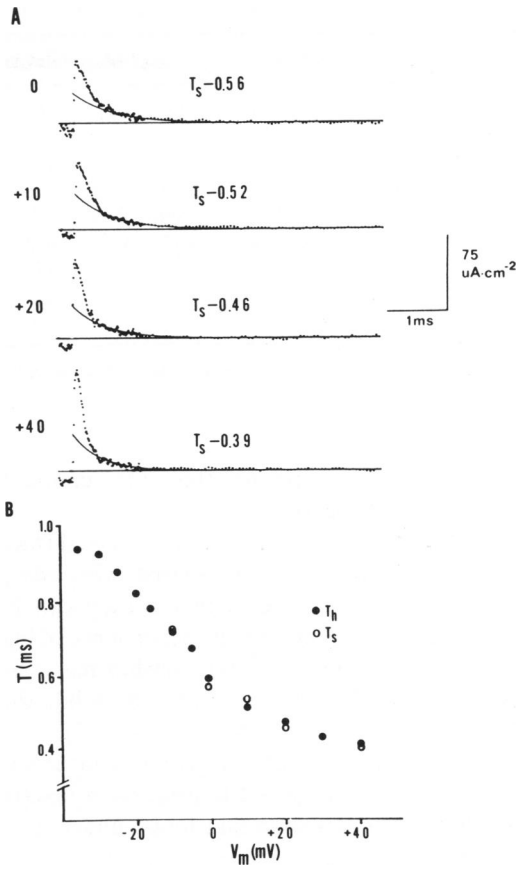

FIGURE 2 Illustrates the fit of the slow phase of I_g by a single exponential (T_s) and the comparison T_s with T_h of I_{Na}. (*A*) Gating currents recorded at 0, +10, +20, and +40 mV are the average of 10 cycles. The thin trace through the slow component represents the best fit by eye of a single exponential, and the values of the T_s appear with each trace. External solution used for these measurements was NMG-SVH with 100 nM TTX. (*B*) Time constants of decay of I_{Na} (T_h) and the slow component of I_g (T_s) vs. membrane potential from the same fiber. T_s values are those shown at the top with the addition of a measurement at −10 mV (0.71 ms). I_{Na} was recorded in 1/5 normal external sodium. Axon DEO40Z. Holding potential −80 mV. 8°C.

fraction of the electric field through which they move. With complete inactivation Q_h may be estimated experimentally from the steepness of the steady-state inactivation curve. Rearranging the familiar two-state h-equation of Hodgkin and Huxley (1952),

$$Q_h = \ln\left[\frac{1 - h_\infty}{h_\infty} \cdot \frac{kT}{e(V_m - \bar{V}_m)}\right] \qquad (2)$$

where $\bar{V}_m$ is the midpoint of the steady-state inactivation curve, and k, T, and e have their usual meaning.

Experimentally, if inactivation begins immediately upon initiation of a voltage step, then the total charge transferred by a completely intrinsic voltage-sensitive inactivation process should be described by the integral of the exponential fitted to the slow phase of I_g extended back in time to the beginning of the pulse. It can be seen in Fig. 2 *A* that the areas beneath the exponentials are a large fraction

TABLE II

Vm	N0280Z		DE040Z		DE170Z		DE180W*	
	T_h	T_s	T_h	T_s	T_h	T_s	T_h	T_s
mv	*ms*	*ms*	*ms*	*ms*	*ms*	*ms*	*ms*	*ms*
−20	—	—	—	—	0.73	0.78	—	—
0	0.54	0.53	0.58	0.56	0.60	0.58	0.77	0.79
10	0.50	0.47	0.50	0.52	0.54	0.57	0.59	0.65
20	0.49	0.45	0.46	0.45	0.48	0.43	0.64	0.62
30	0.43	0.38	—	—	—	—	—	—
40	—	—	0.40	0.39	—	—	0.51	0.50

*DE180W was performed at 5°C, while all other measurements were at 8°C.

of the total area. At +10 mV this area represents 927 $e-/\mu m^2$, or 70% of the total.

As noted by Armstrong (1981) the expected fraction of the total charge movement associated with completely intrinsic voltage dependence of inactivation can be estimated from the ratio of the voltage dependence of inactivation to activation. The g_{Na}–V relationship has a limiting steepness of e-fold/3.3 mV ± 0.8 (n = 6), while the h_∞-V relation changes e-fold/5.1 mV ± 1.1 (n = 5). At potentials ≥ +5 mV, where the Q-V distribution saturates, 40% of the total charge is expected to arise from inactivation. This is significantly less than that found under the curves in Fig. 2 *A*.

A large body of evidence exists in this (Bean, 1981) and in other preparations (Armstrong and Bezanilla, 1977; Nonner, 1980; Schauf, 1976) suggesting the inactivation develops following a delay. Fig. 3 demonstrates that the slow component of I_g carries roughly the expected amount of charge for intrinsically voltage sensitive inactivation if inactivation is given some delay. The figure shows that I_g records of Fig. 2 *A* together with approximations of dh/dt. dh/dt was approximated by a slight modification of the procedure of Bezanilla and Armstrong (1977), and is detailed in the Methods section. The closing of the inactivation gates [$h = f(t)$] was simulated at each potential from experimental traces of I_{Na} (m^3h-m^3). I_{Na} records from the same experiment as the I_g records (DEO40Z) were used. As noted by Bezanilla and Armstrong (1977) the progress of inactivation simulated from the Hodgkin-Huxley formulations shows insufficient delay to describe the experimental records (see their Fig. 2 *C*). Therefore, an appropriate delay was estimated (60 μs) and was added to all the simulations (m^3h-m^3) to allow a better approximation of the entire time course of inactivation. Finally, differentiating the simulations with respect to time yielded dh/dt. When dh/dt is scaled, it matches the slow phase of I_g over its entire range, and the area under dh/dt correlates reasonably with the area predicted from the voltage dependence of inactivation (40%). The cross-hatched areas in Fig. 3 represent 46, 47, and 48% of the total charge movement, respectively.

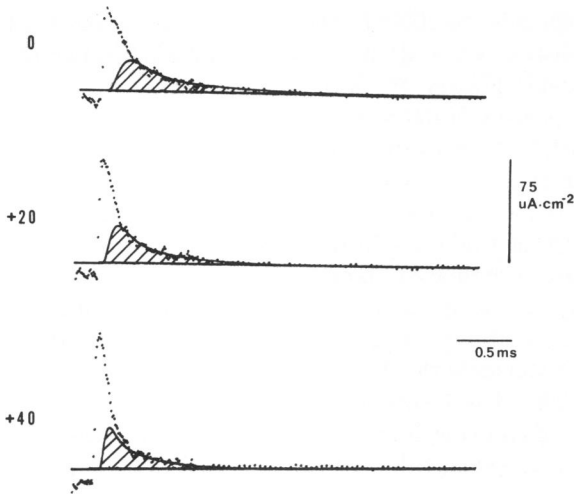

FIGURE 3 Comparison of I_g with dh/dt. I_g records at 0, +20, and +40 mV are the same as those shown in Fig. 2. dh/dt was calculated by the procedure described in the Methods. The shaded areas under dh/dt represent 564, 532, and 592 $e-/\mu m$, respectively.

DISCUSSION

The results demonstrate the existence of a slow component of I_g in crayfish whose time course parallels the time course of inactivation of I_{Na} at the voltages examined. A component of I_g with these characteristics is predicted by an intrinsically voltage-sensitive inactivation process. Experimental support of intrinsically voltage sensitive inactivation is not limited to the measurements described herein. Nonner (1980) resolved that inactivation must have some voltage sensitivity, and the works of Bean (1981) and Horn et al. (1981) reach a similar conclusion. Although the most comprehensive kinetic model of sodium channel gating (Armstrong and Gilly, 1979) argues that inactivation is voltage insensitive, they admit that their model is unable to account for the slow component of I_g persistent in their records (see their Figs. 12 *c*, and 13 *a*, *b*). Thus it is not surprising to find a component of I_g with the characteristics of $I_g{}^h$ in crayfish where it should be most readily visible.

The excess charge described by the exponential fitted to the slow phase of I_g extrapolated to the initiation of the pulse suggests that inactivation develops with a delay. Linking inactivation to the open state of the channel would produce a delay in the onset of inactivation; however, Bean (1981) has shown that inactivation in crayfish axons cannot be strictly coupled to the open state of the channel. Thus a delay must be hypothesized to arise through coupling of inactivation to an intermediate state in the activation sequence. Armstrong and Gilly's model (1979) shows this step. Preliminary modeling of I_{Na} and I_g suggests a similar activation sequence of the Na channel in crayfish axons.

The possibility that the slow component of I_g represents charge movement related to another voltage-dependent event cannot be ignored. Under certain conditions in a

linear sequential reaction a non–voltage-sensitive transition may elicit a current. For example, in the sequence $A \rightleftharpoons B \rightleftharpoons C$ where the $A \rightleftharpoons B$ transition involves the movement of charge while the $B \rightleftharpoons C$ transition does not, a current with the time constant of $B \rightleftharpoons C$ may arise, when (*a*) the $B \rightleftharpoons C$ transition is rate-limiting, and (*b*) A and B have reached equilibrium. Each transition of $B \rightleftharpoons C$ will drain $A \rightleftharpoons B$ producing a current. An upper limit of 15% of the slow component of I_g in crayfish axons could develop from draining the closed to open ($X_2 \rightleftharpoons X_1$) transition by the inactivation step. This estimate was made with the Armstrong-Gilly model (1979) at 0 mV with the rate constants adjusted to fit I_{Na} and I_g, with and without charge associated with the inactivation step.

It is unlikely that the slow component of I_g arises from potassium channel activation. While initial conditions (i.e., holding potential) have been shown to strongly influence the kinetics of potassium gating current (Bezanilla et al., 1982), examination of the effect of holding potential on the slow component of I_g revealed no change in the kinetics. As previously reported, the magnitude of the slow component is modulated by the holding potential (Swenson, 1981; Starkus et al., 1981). The more negative the holding potential the greater the magnitude of the slow component becomes. As the magnitude of the peak of the I_g does not diminish with more negative holding potentials, it is difficult to reconcile the increase in the magnitude of the slow phase with a delay in kinetics. Secondly, g_K is only ~0.65 of its maximum at 0 mV and is fully saturated at +40 mV (Shrager, 1974). Thus, if the slow component of I_g was contributed solely by g_K, it would be expected to increase in magnitude over the voltage range inspected herein, but the slow component of I_g measured either from the integral of T_s or by dh/dt does not appear to do so. Other possible contributors cannot be simply ruled out, but the parallel change in T_s and T_h with membrane potential strongly suggests inactivation is the process underlying the slow component of I_g.

In summary, the present results suggest that (*a*) inactivation is intrinsically voltage sensitive, producing a slow component of I_g (I_g^h), and (*b*) inactivation develops with a delay perhaps through the coupling to an intermediate step in the inactivation sequence.

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