

# Sodium Channel Activation in the Squid Giant Axon

## *Steady State Properties*

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**ABSTRACT** Treatment of giant axons from the squid, *Loligo pealei*, with pronase removes Na channel inactivation. It was found that the peak Na current is increased, but the activation kinetics are not significantly altered, by pronase. Measurements of the fraction of open channels as a function of voltage ( $F$ - $V$ ) showed an e-folding at 7 mV and a center point near -15 mV. The rate of e-folding implies that a minimum of 4  $e^-$ /channel must cross the membrane field to open the channel. The charge vs. voltage ( $Q$ - $V$ ) curve measured in a pronase-treated axon is not significantly different from that measured when inactivation is intact:  $\sim 1,850 e^-/\mu\text{m}^2$  were measured over the voltage range -150 to 50 mV, and the center point was near -30 mV. Normalizing these two curves ( $F$ - $V$  and  $Q$ - $V$ ) and plotting them together reveals that they cross when inactivation is intact but saturate together when inactivation is removed. This illustrates the error one makes when measuring peak conductance with intact inactivation and interpreting that to be the fraction of open channels. A model is described that was used to interpret these results. In the model, we propose that inactivation must be slightly voltage dependent and that an interaction occurs between the inactivating particle and the gating charge. A linear sequence of seven states (a single open state with six closed states) is sufficient to describe the data presented here for Na channel activation in pronase-treated axons.

### INTRODUCTION

The study of the kinetic behavior of the Na channel has always been complicated by the fact that the current activates and then rapidly inactivates when a depolarizing pulse is applied to the membrane (Hodgkin and Huxley, 1952).

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Until it was shown that inactivation of the Na current ( $I_{Na}$ ) in squid giant axons could be removed by treatment with pronase (Rojas and Armstrong, 1971; Armstrong et al., 1973; Rojas and Rudy, 1976), it was not known if inactivation was a process clearly distinguishable from the process of activation, or if activation and inactivation were both manifestations of a single kinetic process. The model proposed by Hodgkin and Huxley (1952) stated that the two processes were independent. When Armstrong et al. (1973) showed that inactivation could be removed by the internal perfusion of pronase, they lent support to the Hodgkin-Huxley model. However, later work has shown that the two processes, although separable, are not entirely independent (Goldman and Schauf, 1972; Armstrong and Bezanilla, 1977; Bezanilla and Armstrong, 1977; Nonner, 1980). A coupled model was proposed in which inactivation can occur only after the channels have partially or fully activated (Armstrong and Bezanilla, 1977; Armstrong and Gilly, 1979). More recent evidence from work on single channels in GH3 cells has shown that the inactivation process, while coupled to activation, does have some voltage dependence of its own (Horn et al., 1984). It was suggested earlier that inactivation might have a small voltage dependence, although there was no direct evidence to support this suggestion, so no voltage dependence was assumed (Armstrong and Bezanilla, 1977; Armstrong and Gilly, 1979).

In studying the Na channel, we have sought to simplify its behavior by removing inactivation with pronase. This has allowed us to look at the properties of the activation process alone. Oxford (1981) has also attempted this, but his work did not include the measurement of the gating current ( $I_g$ ) associated with the Na channel. The gating current provides more detailed information about the channel, especially transitions between the closed states, and thus provides a more critical test for models of the Na channel. Some of the information obtained from measurements of the gating current cannot be obtained solely from ionic currents, either macroscopic or single channel currents. In particular, this paper will discuss the prediction of Na channel models that the charge-voltage ( $Q$ - $V$ ) curve must always be above the curve of the fraction of open channels as a function of voltage ( $F$ - $V$ ).

We show in this paper the results that were obtained on the steady state properties of the Na channel. By steady state we mean the state reached after 5–10 ms under constant conditions, so that slow inactivation is excluded from consideration in this paper. We found that if care is taken not to overtreat the axon with pronase, the activation process is not altered and the peak  $I_{Na}$  is increased. Furthermore, comparisons of the steady state properties of  $I_{Na}$  with those of  $I_g$  show that virtually all of the recorded gating charge movement ( $Q$ ) is associated with the opening of the Na channels. The data were found to agree well with the predictions of a linear sequential model of six closed states and a single open state. Adding inactivation to the model adds seven more states in parallel with the other seven states but adds only two more rate constants. When including inactivation in the model, we found that it was necessary to give it some voltage dependence; otherwise, the prediction for the peak conductance curve as a function of voltage does not saturate until 1 V, in contrast to the data that saturate near 50 mV. This is also true when the simulation is done using

the Hodgkin-Huxley (H-H) model. This prediction differs from the experimental data so much that it becomes a very sensitive test for the model used to represent the inactivation process. This discrepancy between the H-H model and the actual results is discussed more fully later. A preliminary report of these results has appeared (Stimers et al., 1983).

#### METHODS

Carefully cleaned, internally perfused, voltage-clamped giant axons from the squid, *Loligo pealei*, were treated according to the methods previously described (Bezanilla and Armstrong, 1977; Bezanilla et al., 1982a). All recordings of membrane currents were done at 7.5°C. The only modifications were that the current records were filtered to 50, 100, or 200 kHz by a four-pole filter when the data were digitally sampled at 10, 4, or 2  $\mu$ s, respectively.

#### Solutions

All internal and external solutions used are listed in Table I. In the text, we will refer to the solutions as external solution//internal solution. Membrane potentials are not cor-

TABLE I  
Solutions

Internal solutions*	Cs	TMA <sup>‡</sup>	TEA <sup>§</sup>	F	Gluta- mate	Trizma-7.2 <sup>¶</sup>
300 Cs	300	70	20	100	290	10
160 Cs	160	70	20	100	150	10
External solutions	Na	Ca	Mg	Tris <sup>¶</sup>	Cl	TTX**
1/2 Na-SW, 10 Ca, 50 Mg	440	10	50	10	570	—
1/2 Na-SW, 50 Ca	455	50	—	10	565	—
Tris-SW, 10 Ca, 50 Mg	—	10	50	450	570	0.0003
Tris-SW, 50 Ca	—	50	—	475	575	0.0003

All solutions were adjusted to an osmolality of 970 mosmol/kg. Internal pH, 7.3; external pH, 7.5. Concentrations are in millimolar.

\* Osmolality adjusted with sucrose.

<sup>‡</sup> Tetramethylammonium ion.

<sup>§</sup> Tetraethylammonium ion.

<sup>¶</sup> Tris(hydroxymethyl) aminomethane at pH 7.2 from Sigma Chemical Co. (St. Louis, MO).

<sup>¶</sup> Trizma base (Sigma Chemical Co.).

\*\* Tetrodotoxin (Sigma Chemical Co.).

rected for junction potentials. Inactivation was removed by internally perfusing axons with 0.5–2.0 mg/ml pronase (Calbiochem-Behring Corp., La Jolla, CA, or protease type XIV from Sigma Chemical Co., Saint Louis, MO) at 14°C. The internal perfusion was made as rapid as possible so that the exposure time to pronase was minimized.

#### Data Analysis

The data were collected using either a Nova 3 computer system (Data General Corp., Westboro, MA) or a 16-bit microcomputer (Intel 8086-based Lightning-1 from Lomas Data Products, Westboro, MA). Analysis and modeling were done on the Lightning-1 because of its greater speed and capacity.

### *Fraction of Open Channels vs. Voltage*

The number of open channels at a given potential can be found by measuring the steady state current produced by pulsing the membrane from the holding level to the test potential and dividing this value by the current flowing through a single channel at the same potential. Since in this case the single channel current cannot be measured, we used a relative measure by dividing the steady state current by the current flowing through a constant number of channels and normalizing the results over the potential range  $-70$  to  $+60$  mV. The current flowing through a constant number of channels is estimated by the instantaneous current at the test potential. The instantaneous current-voltage curve was determined by first applying a step to one potential (0 or usually  $+50$  mV) and then measuring the current at very short times following pulses to various test potentials in the range of  $-70$  to  $+60$  mV. The current at times short compared with the kinetics of the gating process and long compared with ion redistribution times as a function of the potential during the second step should be the current-voltage curve of an open channel, provided that the channels are all identical. Typically, the instantaneous current was measured  $\sim 20$   $\mu$ s after the transition to the test potential.

### *Gating Current and Charge vs. Voltage Curve*

When no ionic currents are present, a voltage- and time-dependent capacity current in the membrane can be recorded using the P/4 procedure. This current has been shown to be associated with the opening and closing of the Na channels and has been given the name "gating current" (Armstrong and Bezanilla, 1973). Gating current was measured in the usual way (Bezanilla and Armstrong, 1977). The solutions used were Tris-seawater (SW) with 300 nM tetrodotoxin (TTX)//160 Cs or 300 Cs, so that there were no permeant ions. The K channels were blocked by tetraethylammonium (TEA) and the Na channels were blocked by TTX. Currents were recorded using the P/4 procedure with a subtracting holding potential that was usually  $-150$  but occasionally ranged to  $-130$  mV. Typically, 20 records were averaged to improve the signal-to-noise ratio. To determine the charge at each potential, the stored data were digitally integrated over a period of time, usually 2–3 ms from the start of the pulse. The values obtained were then normalized over the range  $-150$  to  $+60$  mV to obtain the normalized charge vs. voltage ( $Q$ - $V$ ) curve.

## RESULTS

### *Effect of Pronase on Na and Gating Currents*

Internal perfusion of a squid giant axon with pronase removes inactivation of  $I_{Na}$ . This is illustrated in Fig. 1 with two superimposed current records obtained before and after the axon was internally treated with 0.5 mg/ml pronase for  $\sim 5$  min. In both cases, the voltage-clamped axon was stepped from a holding potential of  $-70$  mV to 0 and returned to  $-70$ . Note that both records represent raw data and are plotted on the same scale. This is comparable to Fig. 2 of Bezanilla and Armstrong (1977), except that the two current records in their figure were scaled to make the gating current superimpose. Since this axon was bathed in 1/5 Na-SW, 10 Ca, 50 Mg, the gating current is clearly visible as a brief outward current at the beginning of each record. The large tail of current after pronase treatment corresponds to the inward surge of  $I_{Na}$  on repolarization to the holding potential plus a small contribution from the OFF gating current. As can be seen, neither the ON gating current nor the rising phase of the inward

$I_{Na}$  appears significantly altered by the pronase treatment. The tail currents in both records decay with the same time constant. This suggests that the pronase treatment does not alter the activation kinetic processes of the Na channels.

In this axon, the inactivation was at least 95% removed. The treatment was stopped at this point because of the danger that overtreating the axon with pronase would cause the membrane to become leaky, which would produce problems in recording  $I_g$ . Another problem resulting from overtreatment with pronase is that peak  $I_{Na}$  is decreased. This is paralleled by a decrease in the amplitude of  $I_g$ , which suggests that some of the Na channels are destroyed. In Fig. 1, the peak  $I_{Na}$  was increased by the pronase treatment. We found that when the axon was not overtreated and the treatment was very fast, peak  $I_{Na}$  was increased. However, even in those axons where the peak current was decreased by the pronase treatment, the activation kinetics were not altered.

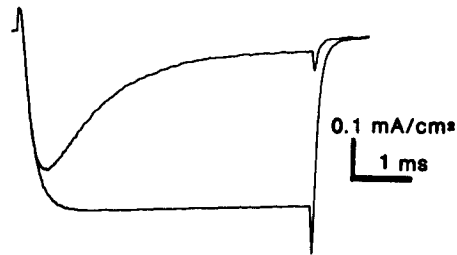


FIGURE 1. Pronase treatment removes inactivation without affecting activation kinetics or gating current. Two current records from the same axon (WJUL272A) for pulses to 0 mV from a holding level of  $-70$  mV are shown before (above) and after (below) perfusion with 0.5 mg/ml pronase. Notice that the gating currents visible at the beginning of each record are superimposable. Peak current was increased in this axon, although this is not always true. The time constants of the two Na tail currents are equal. Solutions: 1/5 Na-SW, 10 Ca, 50 Mg//300 Cs. Subtracting holding potential (SHP) =  $-140$  mV, P/ $-4$ .

Fig. 2 shows families of  $I_{Na}$  (left) and  $I_g$  (right) before (upper) and after (lower) pronase treatment. Note that all data were obtained from the same axon except for the gating current before pronase (upper right), which for technical reasons had to be taken from another axon. The families of  $I_{Na}$  shown in this figure have many of the same features described above for Fig. 1, but appear over a wide voltage range. The gating current families on the right show that the kinetics of  $I_g$  are not significantly changed by the pronase treatment. Since the two families are not from the same axon, the amplitudes cannot be quantitatively compared.

#### *Fraction of Open Channels*

In general, the number of open channels at a given potential is equal to the steady state current divided by the current through a single channel (see Methods). Fig. 2 shows the raw data obtained from a pronase-treated axon. The current was recorded when the membrane was stepped from a holding potential of  $-70$  mV to the potential indicated in the legend. The gating current seen at

the beginning of each record does not interfere with the measurement of the steady state current, since in the steady state there is no contribution from  $I_g$ . When the membrane was stepped first to +50 mV for 3 ms and then to the indicated potential, we recorded the instantaneous current, which is an estimate of the open channel conductance. Approximately 20  $\mu$ s after the step from +50 mV to the indicated potential, the magnitude of the current was measured. The prepulse to +50 mV opened a constant number of channels that do not close in

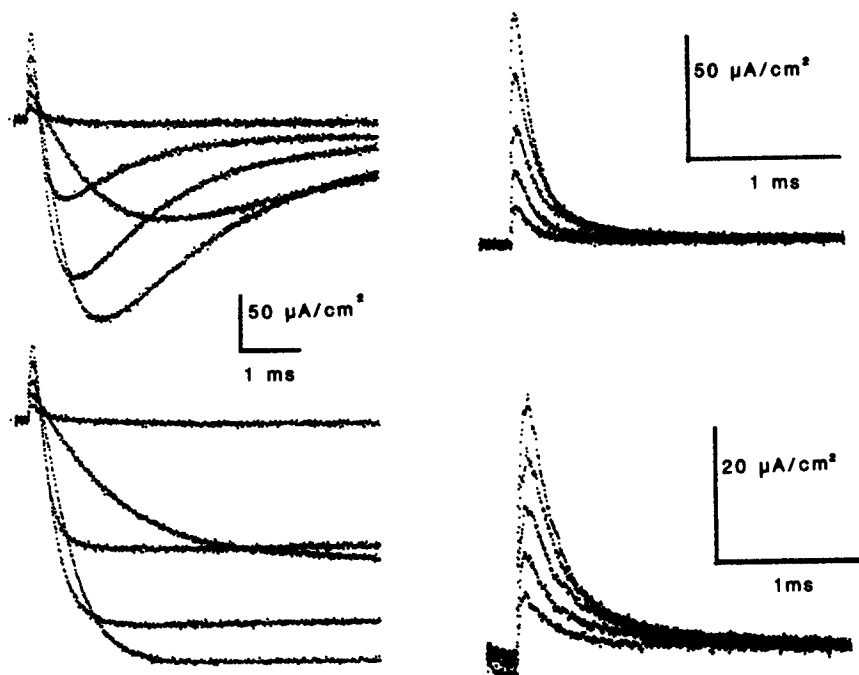


FIGURE 2. Brief families of Na (left) and gating (right) current before (top) and after (bottom) perfusion with 1 mg/ml pronase. In all parts, the holding potential was  $-70$  mV. The currents shown illustrate pulses in the range of  $-40$  to  $40$  mV in steps of  $20$  mV. All the records are from the same axon (WJUN103A), except for the gating currents before pronase (top right), which for technical reasons are from another axon (WMAY243B). Solutions:  $1/5$  Na-SW,  $50$  Ca// $160$  Cs. External solution for gating: Tris-SW,  $50$  Ca +  $300$  nM TTX. SHP =  $-150$  mV, P/ $-4$ . Na currents were sampled at  $10$   $\mu$ s/point, while gating currents were sampled at  $4$   $\mu$ s/point (filtered at  $100$  kHz).

the  $20$   $\mu$ s needed for the electronics to settle so that reliable measurements of the current at the new potential can be made. Fig. 3 shows both the steady state current and the instantaneous current plotted as a function of voltage. Since the instantaneous current-voltage relation is not a straight line, it is a mistake to simply divide the steady state current by the driving force ( $V - V_{Na}$ ) in estimating the  $F$ - $V$  curve. A more serious error arises from using the peak inward current as an estimate of the steady state current (see Fig. 1). Fig. 4A shows the fraction of open channels obtained from an axon not treated with pronase, while Fig. 4B

shows the same curve for a pronase-treated axon. Note that in the untreated axon, the  $F$ - $V$  curve rises more steeply than in the pronased axon; this is most noticeable at the more positive potentials where the curve for a normal axon reaches saturation quite rapidly, while that for the pronase-treated axon slowly approaches saturation. The center point of this curve for normal axons is about  $-20$  mV, while that for pronase-treated axons is  $-12 \pm 3$  mV. This is due at least in part to the error produced by using the peak inward current when inactivation is present (see Discussion).

#### *Number of Charges per Channel*

The rate of rise of the  $F$ - $V$  curve gives an estimate of the minimum number of electronic charges per channel that is necessary for the opening of one channel

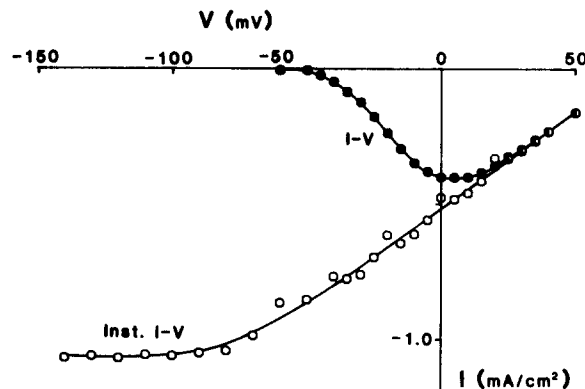


FIGURE 3. Current-voltage ( $I$ - $V$ ) and instantaneous current-voltage (Inst.  $I$ - $V$ ) curves from a pronase-treated axon (WJUL272B). The  $I$ - $V$  curve was measured at the steady state of the Na current. The instantaneous current was measured by pulsing the membrane to 50 mV for 3.5 ms and then stepping to the indicated potential. The peak of the Na current recorded after subtracting the gating current from each record is plotted. The bending of the instantaneous  $I$ - $V$  in the negative region is probably due to a voltage-dependent block of the Na channels by  $\text{Ca}^{2+}$  (see text). Solutions: 1/5 Na-SW, 10 Ca, 50 Mg//300 Cs. SHP =  $-140$  mV, P/ $-4$ .

when the membrane potential is very negative (Almers, 1978). It is difficult to see the difference between the  $F$ - $V$  curves with and without pronase treatment in the region between  $-30$  and  $-50$  mV, but when the data are plotted on a semilogarithmic scale (Fig. 4 C, data from eight pronase-treated axons), it is found that the fraction of open channels increases exponentially in this region with an e-fold change in 7 mV for pronase-treated axons, as compared with 4–5 mV for normal axons. The difference is probably related to the effect of inactivation on  $I_{\text{Na}}$  even in this range of small depolarizations. Others have found values ranging from 4 to 6.5 mV (Hodgkin and Huxley, 1952; Keynes and Rojas, 1976; Oxford, 1981; see also Almers, 1978). From the initial rate of rise of the  $F$ - $V$  curve reported here, we can calculate from a Boltzman distribution that a minimum of

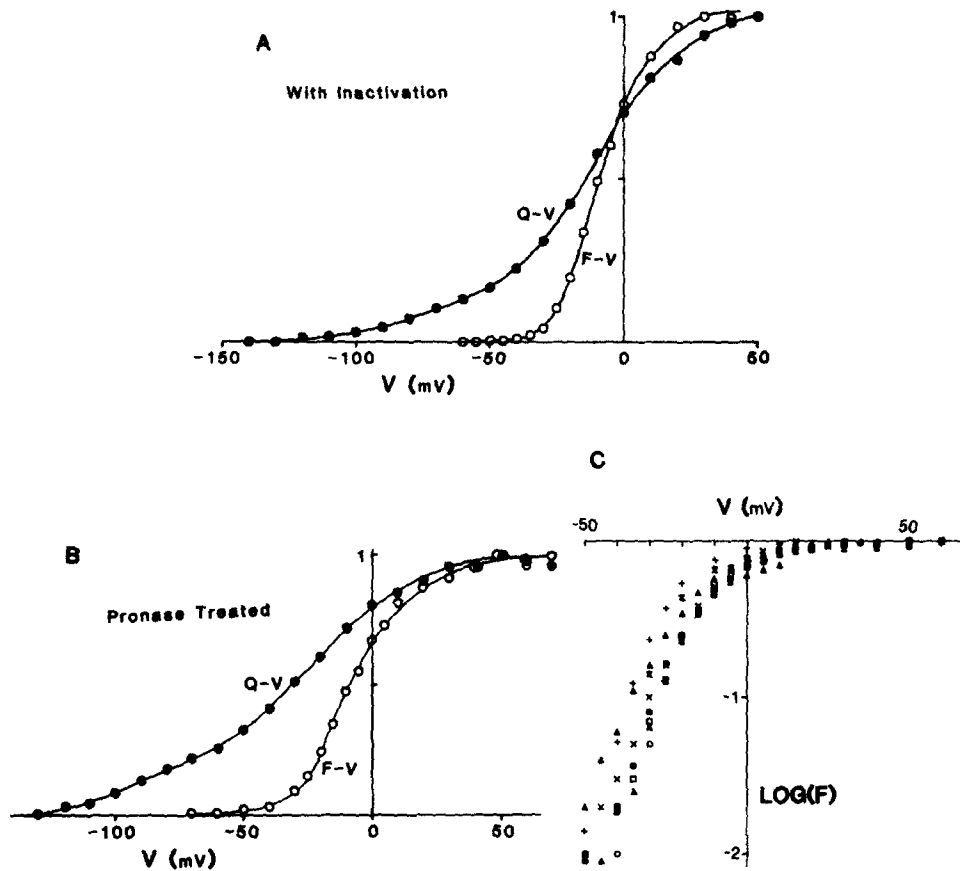


FIGURE 4.  $F$ - $V$  and  $Q$ - $V$  curves from a normal axon, inactivation intact (A), and a pronase-treated axon (B). The  $F$ - $V$  curve was estimated as the normalized ratio of the steady state Na current at each potential to the instantaneous current at the same potential (see Methods). For the  $Q$ - $V$  curve, individual gating currents were integrated digitally for 2.18 ms in A and 3.5 ms in B. (A) Normal axon (WMAY243B). In this case, the  $F$ - $V$  curve was determined using the peak Na current as an approximation of the steady state current. Notice that the  $F$ - $V$  and  $Q$ - $V$  curves cross near 0 mV and that the  $F$ - $V$  curve saturates at a lower potential than does the  $Q$ - $V$  curve. This is not expected if the charge being recorded is associated with the opening of the Na channels (see text). We believe this is due to the poor estimate of the fraction of open channels using the peak of the Na current as an approximation of the steady state current. (B) Pronase-treated axon (WJUL272B). The two curves do not cross and they saturate together near 50 mV. This is expected if all the charge is associated with the Na channels. This lends further support to the hypothesis that all the charge recorded is associated with the Na channels. The  $F$ - $V$  curves from 10 axons had a center point of  $-12 \pm 3$  mV and the initial slope increased  $e$ -fold in 7 mV, which indicates a minimum of  $4 e^-/\text{channel}$ . The  $Q$ - $V$  curve in the same 10 axons had a center point of  $-26 \pm 5$  mV and the total charge averaged  $1,850 \pm 300 e^-/\mu\text{m}^2$ . (C)  $F$ - $V$  curves for eight pronase-treated axons. Each symbol represents a different axon. This semilogarithmic plot demonstrates the exponential nature of the voltage dependence of the fraction of open channels. One measures an  $e$ -folding in 7 mV, which indicates a minimum of  $4 e^-/\text{channel}$  crossing the membrane field to open each channel.



4  $e^-$ /channel must be moved through the membrane field to open a single Na channel.

#### *Charge vs. Voltage Curve*

Gating current was measured in pronase-treated axons bathed in  $\text{Na}^+$ -free solution ( $\text{Tris}^+$  replaces  $\text{Na}^+$ ) with 300 nM TTX, using the P/-4 procedure to eliminate the voltage-independent components of the membrane current. Examples of  $I_g$  recorded in this manner are shown in Fig. 2. When these records are digitally integrated over a period of time (usually 2–3 ms), we obtain the charge moved through the membrane at each potential. The normalized  $Q$ - $V$  curve for a normal axon is shown in Fig. 4A, and that for a pronase-treated axon is shown in Fig. 4B.

In the normal axon, the  $F$ - $V$  curve is above the  $Q$ - $V$  curve for potentials more positive than 0 mV, and the  $F$ - $V$  curve saturates at a more negative potential than does the  $Q$ - $V$  curve. Since the charge must move before the channels open, neither of these findings is expected. If all the charge being recorded in  $I_g$  is associated with the opening of the Na channels, then the two curves must saturate together and not cross. In the case of the normal axon, we would be forced to conclude either that not all of the charge measured is associated with the gating of the Na channels or that there was an error in some or all of the measurements.

Fig. 4B shows the data obtained from a pronase-treated axon. In this case, the  $Q$ - $V$  curve is always above the  $F$ - $V$  curve and the two curves saturate together at +50 mV. From the arguments presented above, we find no reason to exclude any of the recorded charge from being associated with the opening of the Na channels. It is important to recognize, however, that under the conditions of these experiments, the gating current due to the K channels is not recordable, being at the level of the noise because of its slower kinetics (Bezanilla et al., 1982b). In axons treated with pronase, the total charge moved over the entire range of potentials, -150 to +50 mV, averaged  $1,850 \pm 300 e^-/\mu\text{m}^2$ . This value is not significantly different from that obtained in normal axons. If one assumes a channel density of 360 channels/ $\mu\text{m}^2$  (the average of values from Levinson and Meves, 1975, and Strichartz et al., 1979), then a value of 5  $e^-$ /channel is obtained. This value meets the minimum requirement established by the initial slope of the  $F$ - $V$  curve described previously.

## DISCUSSION

### *Effects of Pronase on Na Current*

Treatment of the squid giant axon with internally perfused pronase removes fast inactivation of  $I_{\text{Na}}$  (Fig. 1). If the treatment time is not too long and the perfusion is rapid, we find that the peak inward current is increased and  $I_g$  is not changed in amplitude. Activation kinetics are also not changed. Similar results have been reported when inactivation was removed by pronase (Rojas and Rudy, 1976) or by *N*-bromoacetamide (Oxford et al., 1978), although, more typically,  $I_{\text{Na}}$  decreases while activation kinetics remain unaltered (Armstrong et al., 1973; Oxford et al., 1978). We also find that when the pronase treatment is overdone, the peak  $I_{\text{Na}}$  decreases. This is usually accompanied by a rapid deterioration of

the axon, with an increase in the leakage current, which usually has both a time and a voltage dependence. In those cases where the leakage did not increase noticeably, we found that the activation kinetics still were not affected and that these axons gave results similar to those in which  $I_{Na}$  increased. It was only the magnitude of the currents (ionic and gating) that was decreased. Under these conditions, the steady state properties can be measured.

#### *Fraction of Open Channels*

We report here a measurement for the steady state fraction of open channels as a function of the membrane potential. This measurement is important in understanding the behavior of the Na channel, since this is one of the predictions that comes out of any model of the kinetic properties of the Na channel. The  $F$ - $V$  curve has been measured by others (Hodgkin and Huxley, 1952; Keynes and Rojas, 1976; Oxford, 1981), although in all cases the driving force ( $V - V_{Na}$ ) was used, and in several the peak  $I_{Na}$  (with inactivation intact) was used in estimating the fraction of open channels. Our results indicate that these procedures lead to errors in estimating the  $F$ - $V$  curve. We and others (Gilly and Armstrong, 1982; Yamamoto et al., 1984; Cachelin et al., 1983) have found that the instantaneous current-voltage curve is not linear. Therefore, using the linear equation ( $V - V_{Na}$ ) as an estimate of the current through a constant number of channels produces an error in the  $F$ - $V$  curve. The second major source of error comes from what is used to estimate the steady state Na current. Using the peak  $I_{Na}$  with inactivation intact underestimates the steady state current because significant inactivation has already occurred at the time of peak  $I_{Na}$ . An alternative method for determining the steady state  $I_{Na}$  is to subtract a single-exponential component for inactivation (Oxford, 1981). This method may yield a good approximation of the  $F$ - $V$  curve, but it requires the assumption that inactivation proceeds with a single time constant, which is not the case.

We find in axons with inactivation removed an initial slope of 7 mV/e-fold change in  $Na^+$  conductance at negative potentials where the activation of the Na channels is just detectable. Thus, a minimum of 4  $e^-$ /channel must cross the membrane field to open one channel. We stress that this is a minimum estimate and that the charge recorded in  $I_g$  might be greater than this. More charge per channel is possible since this estimate does not necessarily reflect all the charge movement that is possible as the channels evolve through the closed states at very negative potentials. Although it is customary to represent the charges per channel as an integer, physically this is not a necessary constraint for two reasons: (a) dipoles need not be equal to one electronic charge, and (b) the measure of charges per channel assumes that the charge moves completely through the membrane field, which is also not necessarily true.

#### *Q-V Curve Compared with the F-V Curve*

In measuring the  $Q$ - $V$  curve, one is measuring the voltage dependence of the gating process. It is clear that if all the charge that moved is involved in the gating of Na channels, then the normalized  $Q$ - $V$  curve should always be above the normalized  $F$ - $V$  curve, since the gating charge must move before the channel

can conduct. Furthermore, the two curves should saturate at the same potential, since when all the charge has been moved, all the channels must be open. These two conditions must be true if the process is voltage dependent. We had been perplexed that this was not the case when the  $F$ - $V$  curve was measured in a normal axon (see Fig. 4A). However, we now believe that this results from the fact that the  $F$ - $V$  curve was not being measured accurately when inactivation was present. Our data on pronase-treated axons (Fig. 5) show that these two requirements are met; therefore, there is no reason to exclude any of the recorded charge from being associated with the gating of the Na channels. It has been suggested (Greff et al., 1982; Keynes et al., 1982) that the charge moved in the voltage region negative to  $-70$  mV is not associated with the Na channels. We find no reason to exclude this charge from consideration, and Taylor and Bezanilla (1983) show clear evidence for including it. They found that the charge moved in this region is responsible for a Cole-Moore-type shift in the activation

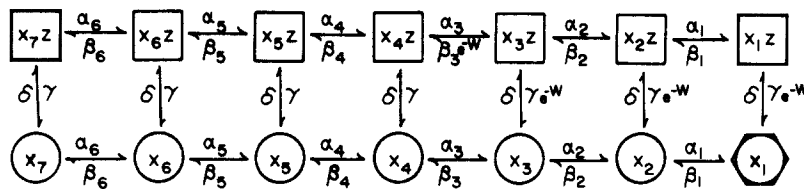


FIGURE 5. State diagram for the kinetic model of the Na channel. States  $X_1$ – $X_7$  represent the noninactivated states of the channel (lower row), where  $X_1$  is the single conducting state, and  $X_1Z$ – $X_7Z$  represent the same states now with the inactivating particle in position to inactivate the channel. The calculation of the rate constants is described in the text.  $\gamma$  is the only voltage-independent rate constant, but it is multiplied by  $\exp[-W]$  in some states, which is similar to the proposal of Bezanilla et al. (1982a). This extra term results from the proposed interaction between the inactivating particle and the gating particle moved in the transition between states  $X_4$  and  $X_3$ . This interaction deepens the wells for both particles when they are together. See the text for discussion.

of the Na channel kinetics. Cahalan and Almers (1979) succeeded in blocking 90% of  $I_g$  using *N*-methylstrychnine, while Armstrong and Croop (1982) found that virtually all of  $I_g$  could be blocked by the introduction of thiazin dyes into the Na channel, which suggests that  $\geq 90\%$  of the charge recorded is associated with the gating of the Na channels. Keynes (1983) has recently reinterpreted his results assuming that all the charge is associated with Na channels, since the total charge displaced parallels the increase in  $\text{Na}^+$  conductance.

#### *A Kinetic Model for the Na Channel in the Absence of Inactivation*

We consider here a model for the Na channel similar to that proposed by Bezanilla et al. (1982a), excluding slow inactivation and extending it to include multiple closed states as shown in Fig. 5. In the figure, states  $X_2$ – $X_7$  are closed,  $X_1$  is the single open state,  $X_1Z$  is open-inactivated, and  $X_2Z$ – $X_7Z$  are closed-inactivated. Using an Eyring rate model, we consider the states to be energy

wells, or conformations of the channel where the energy is minimized, and each state is separated by energy barriers from adjacent states. This model differs from that presented by Armstrong and Gilly (1979) (see also Armstrong and Bezanilla, 1977) in that an interaction is proposed between the activation gating particles and the inactivation particle, the energy of which we represent as  $W$  in units of  $kT$ . This interaction deepens the wells for those states where the particles interact by an energy  $W$ ; this energy appears as  $\exp[-W]$  in the rate constants leaving the states where the interaction is present. Bezanilla et al. (1982a) were restricted to putting this interaction only in the open-inactivated state, while we represent the interaction as existing in inactivated states  $X_1Z$ ,  $X_2Z$ , and  $X_3Z$ . We feel that this is a better representation since it can easily account for the fact that one-third of the gating charge is still able to move when the conductance is completely inactivated (Armstrong and Bezanilla, 1977). In this model, the charge that is not immobilized by inactivation moves as the channels evolve between states  $X_1Z$ ,  $X_2Z$ , and  $X_3Z$ . This requires that these two transitions account for one-third of the total recorded gating charge. The  $\alpha$ 's and  $\beta$ 's (activation rate constants) and  $\delta$  (rate constant for entering the inactivated states) for each transition,  $i$ , are all voltage dependent and have been calculated from the expressions (e.g., Woodbury, 1971):

$$\alpha_i = \frac{kT}{h} \exp[-W_{\alpha_i} + z_i e d_i V / kT];$$

$$\beta_i = \frac{kT}{h} \exp[-W_{\beta_i} - z_i e (1 - d_i) V / kT];$$

$$\delta = \frac{kT}{h} \exp[-W_{\delta} + z e d V / kT],$$

where  $W$  is the difference in units of  $kT$  from the well to the barrier peak,  $e$  is the electronic charge,  $z$  is the valence,  $d$  is the fraction of the distance through the field where the peak of the barrier is located,  $V$  is the membrane potential,  $k$  is the Boltzmann constant,  $T$  is the absolute temperature, and  $h$  is the Planck constant.  $\gamma$ , the rate constant for leaving the inactivated states, is not voltage dependent and is selected so that most of the channels are in the noninactivated states at hyperpolarized potentials. By having  $\delta$  slightly voltage dependent and  $\gamma$  constant, we can satisfy the experimental finding that no component of the gating current has the time course of inactivation, since the contribution of  $\delta$  to the gating current would be too small to measure. There are several reasons for giving any voltage dependence to the inactivation process, and they will be discussed in detail below.

Using this model to simulate an axon with inactivation intact, we can solve for the  $F$ - $V$  and  $Q$ - $V$  curves. The results of such an analysis are shown in Fig. 6A. Similar to the experimental results for intact axons, the two curves cross with the  $F$ - $V$  curve saturating to the left of the  $Q$ - $V$  curve and declining slightly at large depolarizations. Again, this is not what is expected if all of  $I_g$  is associated with the opening of the Na channels.

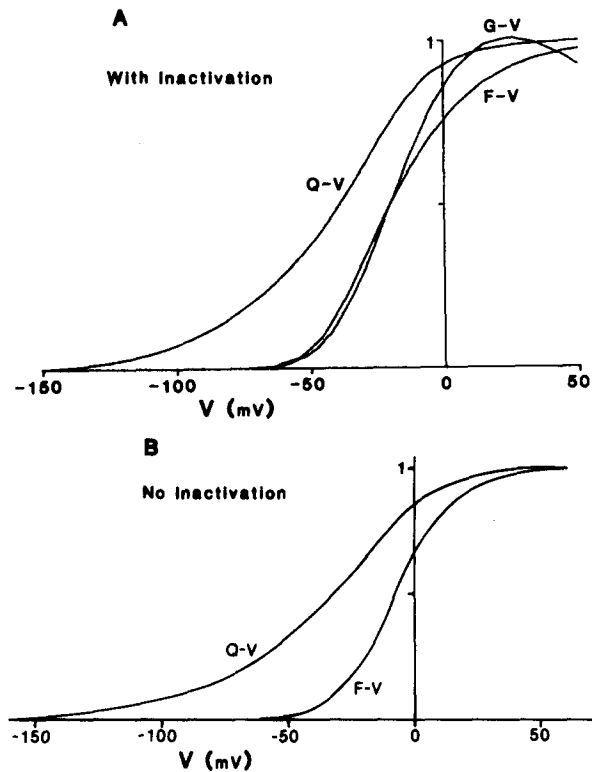


FIGURE 6. Steady state predictions of the model with inactivation (A) and without inactivation (B). The parameters for calculating the rate constants were chosen to produce a qualitatively good fit to the data from pronase-treated axons and then the parameters for inactivation ( $\gamma$  and  $\delta$ ) were simply added to produce the inactivated case. The parameters used for calculating the  $\alpha$ 's and  $\beta$ 's for each transition were:

Transition	$d$	$z$	$W_\alpha$	$W_\beta$
1	0.5	1.2	19.00	20.20
2	0.5	1.0	21.00	22.39
3	0.5	0.5	19.50	20.37
4	0.5	0.5	19.50	20.37
5	0.5	0.5	19.50	20.37
6	0.5	1.0	19.50	22.37
	1.0	0.6	22.23	20.44 (inactivation parameters)

and  $W$  (the interaction energy) was 4 (see text for description of parameters and their units). (A) As expected for this model, the  $Q-V$  and  $F-V$  curves do not cross when inactivation is present, where the  $F-V$  curve is determined as the fraction of channels in the open ( $X_1$ ) and the open-inactivated ( $X_1Z$ ) states. However, as was found in the data, when the peak conductance is calculated, the peak conductance-voltage ( $G-V$ ) curve does cross with the  $Q-V$  curve and in fact declines at very positive potentials, which is sometimes observed in the data. However, because of the small magnitude of the currents at these potentials, the possibility of errors is high experimentally. (B) Without inactivation, the  $Q-V$  and  $F-V$  curves do not cross and are qualitatively very much like the data shown in Fig. 4B.

To simulate the action of pronase, we simply leave out all of the inactivated states and their associated rate constants, leaving a simple linear sequence of states. Notice that the interaction ( $\exp[-W]$ ) is also eliminated when this is done. This should be the case, since removal of inactivation is paralleled by the removal of gating charge immobilization. Furthermore, we can deduce that few or none of the channels are trapped in the inactivation sequence (splitting the model into two independent parallel pathways and two populations of channels), since this would be detectable in the gating tails as a slow component that was not observed experimentally. The simulation of a pronased axon is shown in Fig. 6B. In this case, to obtain the  $F$ - $V$  curve, we need only solve for the fraction of channels that populate the single open state as a function of voltage. Note that the agreement between the model and the data is quite satisfactory. We believe that this model can account for the observed behavior of the Na channel, although further work must be done to verify that it can accurately reproduce the kinetics of both  $I_{Na}$  and  $I_g$  under all conditions.

#### *Comparisons with Previous Results and Implications for the Inactivation Process*

Our interpretations of the data may not be directly comparable to others in this field because their interpretations were made with respect to the Hodgkin-Huxley model for the Na channel. While the H-H model was a great success for the prediction of a propagated action potential using their voltage-clamp data, the model does not fit the existing data in several important areas, at least in part because of its phenomenological nature and incomplete data (i.e., no gating current and no agent such as pronase or *N*-bromoacetamide to remove inactivation). Several important discrepancies of the H-H model are discussed here.

The H-H model predicts that the  $Na^+$  tail current should decay three times faster than  $I_g$ . This has been shown not to be the case (Armstrong and Bezanilla, 1974, 1975; see also Armstrong, 1981). It has even been suggested (Oxford, 1981) that the  $Na^+$  tail current is not well fitted by the H-H model. A premise of the H-H model is that inactivation develops with an exponential time course and is independent of the activation process. Neither of these assumptions has been found to be entirely true (Armstrong and Bezanilla, 1977; Bezanilla and Armstrong, 1977; Armstrong and Gilly, 1979; Oxford, 1981; Goldman and Schauf, 1972; Goldman and Kenyon, 1982; Armstrong, 1981; see also Gillespie and Meves, 1980, for an opposing view). The lag seen in  $I_{Na}$  during activation is much more than can be produced by the H-H model (Armstrong and Bezanilla, 1974; Keynes and Rojas, 1976; Hahn and Goldman, 1978; Taylor and Bezanilla, 1983).

#### *Peak Conductance and the Voltage Dependence of Inactivation*

A final prediction of the H-H model we would like to discuss in more detail is the peak conductance. We have found that this prediction varies greatly from the data and that this single prediction is very sensitive to the mechanism proposed for inactivation. This inconsistency is illustrated in Fig. 7. We show here the usual curves generated from the H-H model for  $m_\infty$  and  $m_\infty^3$ . These curves are typically used for comparing data for gating charge and peak con-

ductance of the membrane; however, as we will show,  $m_{\infty}^3$  is not the prediction of the H-H model for the peak conductance. The comparison that should be made for this is the mimicking of the measurements made from the data. We have used the H-H model to generate a family of Na currents with inactivation. From these, we measure the peak conductance as a function of voltage, similar to the experimental measurements; in the model, the open channel conductance is known (i.e., linear), so it is not necessary to generate the tail currents. The result of this is also shown in Fig. 7 by the curve labeled  $G-V$ . Comparing this curve with experimental data (Fig. 4A), it is obvious that the prediction of the H-H model does not fit the data. Note that the modeled curve does not saturate

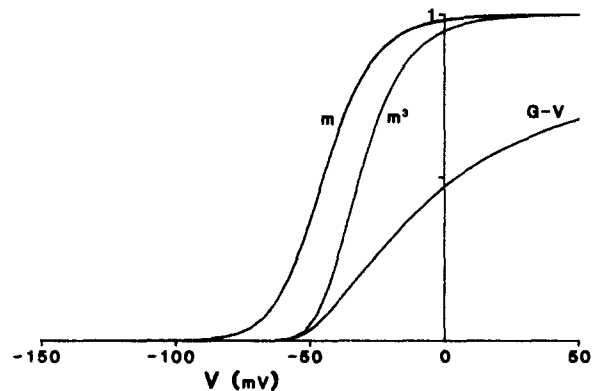


FIGURE 7. Steady state predictions for the Hodgkin-Huxley model. The H-H parameters  $m_{\infty}$  and  $m_{\infty}^3$  are calculated from the H-H equations and plotted. The curve labeled  $G-V$  was determined by generating a family of Na currents at each potential and the value of the peak conductance was determined. It is this curve, not  $m_{\infty}^3$ , which is the prediction of the H-H model for the value of the conductance at the peak of the Na current as a function of voltage. It can be calculated that this curve does not saturate until the membrane potential nears 1,000 mV. We believe this is due to a lack of voltage dependence for the inactivation time constant at potentials more positive than 0 mV. When a slight voltage dependence is added to the model in this region, the curve can be made to saturate at more reasonable potentials.

in this figure and in fact does not saturate until 1 V; even normalizing to the value at 100 mV, it is far to the right of the  $m_{\infty}^3$  curve and the data in Fig. 4A. The implications of this calculation are twofold: (a) measurement of the peak Na conductance is a poor estimate of the fraction of open channels, and (b) the H-H formulation does not predict the peak conductance-voltage curve obtained experimentally. Further modeling has led us to believe that this discrepancy is due at least in part to the fact that Hodgkin and Huxley modeled inactivation as being virtually voltage independent at voltages more positive than  $-20$  mV.

As the voltage is made more positive, beyond the potential at which  $m_{\infty}^3$

saturates, the activation process gets faster. This means that in this range of potentials, all the channels open, but they open faster at higher potentials. If the inactivation process is voltage independent, then at these positive potentials, the rate of decay of the Na current reaches a value that is rate-limited by the inactivation process. Thus, as activation speeds up, the peak of the Na current occurs at earlier times after a step depolarization. The consequence of this is that the peak conductance [ $I_{\text{Na peak}}/(V - V_{\text{Na}})$ ] continues to grow with depolarization even though all the channels have been activated (fraction of open channels is one). We have found that the actual shape of the peak conductance curve is extremely sensitive to the voltage dependence of the rates of the activation and inactivation processes at positive potentials (in both the H-H model and the model presented above). Using a version of the model presented here, where the only difference is that  $\delta$  (the rate constant for entering the inactivated states) is constant, we find that the predicted  $G$ - $V$  curve is identical to that for the H-H model. By modifying the H-H equation for  $\tau_h$  so that it is voltage dependent in the region positive to  $-20$  mV, the predicted peak  $G$ - $V$  curve agrees more closely with the data. Our simulations indicate that the experimentally measured peak conductance-voltage curve cannot be obtained with a voltage-independent inactivation process, which forces us to consider that the inactivation process is at least slightly voltage dependent.

Previously, inactivation was considered voltage independent, although the possibility of a slight voltage dependence was acknowledged (Armstrong and Bezanilla, 1977; Armstrong and Gilly, 1979). Recent evidence from single channel recordings in GH3 cells has shown that inactivation does have a measurable voltage dependence (Horn et al., 1984), although in another preparation (neuroblastoma), inactivation was found to be not very voltage dependent (Aldrich et al., 1983). There are several possible reasons why the results from these two groups differ. If Aldrich et al. missed brief closing events, then they would have underestimated the closing rate constant, again reducing the apparent voltage dependence of the inactivating rate constant. The data from Horn et al. were taken in a more hyperpolarized range of potentials and it is possible that inactivation is more voltage dependent in that range. Also, Horn et al. used detached membrane patches, while Aldrich et al. used cell-attached patches. It is possible that channel kinetics may change when separated from the cell. These two preparations differ from squid axon Na channels in that there is appreciable steady state current in squid axon. This means that the inactivation states are not absorbing as in GH3 and neuroblastoma cells, so the Na channels will evolve into the closed states in significant numbers. This may be due simply to a lower energy barrier for the return to the closed state and not to a significant mechanistic difference between the Na channels in the various preparations.

In summary, the Na and gating currents obtained in pronase-treated axons allowed us to determine the true voltage dependence of the activation process and the voltage dependence of the fraction of open channels. It is now clear that the fraction of open channels cannot be obtained from the peak Na current because the measurement depends on the kinetics of the inactivation process. Also, we have now reconciled the prediction that the  $Q$ - $V$  and  $F$ - $V$  curves should not cross if the charge recorded is associated with Na channel gating. Further-



more, the peak current vs. voltage relation stands now as a sensitive test for the voltage dependence of the rates of activation and inactivation; in particular, it has shown us that the inactivation process is voltage dependent. The exact voltage dependence of the rates governing both processes will only be obtained after extensive fitting of the kinetics of both gating and Na currents with and without inactivation.

This work was supported by fellowships to J.R.S. from the American Heart Association, Greater Los Angeles Affiliate, the Grass Foundation, and the National Institutes of Health, and by U. S. Public Health Service grant GM-30376.

*Original version received 8 March 1984 and accepted version received 17 September 1984.*

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