SLOW INACTIVATION OF THE SODIUM CONDUCTANCE IN SQUID GIANT AXONS. PRONASE RESISTANCE

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SUMMARY

1. Squid giant axons internally perfused with CsF have their Na conductance inactivated due to the low value of the resting potential. When hyperpolarized with voltage clamp to normal values of resting potential, the Na conductance recovers with an exponential time course. The time constant of recovery is of the order of 30 sec at a membrane potential of -70 mV and at 5 °C. The recovery from slow inactivation has a Q_{10} of about 3.

2. The development of inactivation during depolarization is also slow. The time constant varies between 10 and 20 sec at 5 °C, depending upon the value of the membrane potential.

3. Slow inactivation is also observed in NaF perfused axons and in intact axons with a low resting potential.

4. Although internal perfusion with pronase (or a purified fraction of this enzymic complex) blocks the fast (h) inactivation of the Na conductance, the slow inactivation remains. The recovery is similar before and after the proteolytic treatment. However, slow inactivation appears to develop faster after enzymic perfusion.

5. Slow inactivation develops without any apparent change in distributed or local membrane surface charge.

6. The experiments suggest that slow inactivation is a general property of the Na conductance as in many other conductance channels in excitable membranes. The experiments can be interpreted by proposing that slow inactivation is a phenomenon independent of fast inactivation, and that pronase somehow accelerates the onset of slow inactivation.

7. An alternative model, in which slow inactivation is coupled to fast inactivation, is proposed. This model is consistent with the results presented here and is very similar to one proposed to explain the frequency response of the sodium currents in *Myxicola* giant axons (Rudy, 1975, 1978).

INTRODUCTION

During depolarization of a nerve membrane the voltage dependent Na conductance behaves differently from the voltage dependent K conductance. While the K conductance is maintained at a constant level during a depolarization of several milliseconds,

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the increase in Na conductance is transient. The process by which Na conductance decays to a very low value is known as inactivation. The inactivation process is partly responsible for the decline of the action potential and for the refractory period (Hodgkin & Huxley, 1952a, b).

Ehrenstein & Gilbert (1966) have shown that the K conductance in squid giant axons also decreases if the depolarization is maintained for very long periods of time (several seconds). This slow decay is known as 'slow inactivation.' Other voltage-dependent conductances in excitable tissues also exhibit slow inactivation (Baker, Meves & Ridgway, 1973; Baker & Rink, 1975; Beeler & Reuter, 1970; Weight & Vatava, 1970; Standen, 1974; Nakajima, Iwasaki & Obata, 1962; Luttgau, 1960; Schwarz & Vogel, 1971). Slow inactivation of the Na conductance has also been demonstrated by Chandler & Meves (1970b) in squid axons internally perfused with NaF. It is not clear, however, whether the phenomenon was induced by the perfusate or whether it is a normal property of the membrane. Armstrong & Bezanilla (1974) have reported the presence of a slow inactivation of the Na conductance in squid giant axons, but the characteristics of such inactivation were not studied. Therefore it is not possible to compare the observations of Armstrong & Bezanilla with those reported by Chandler & Meves or those described by Ehrenstein & Gilbert.

The purpose of this work is to explore further the characteristics of the slow inactivation in squid giant axons. In particular, we study the effect of internal perfusion with pronase, which is known to block fast inactivation (Armstrong, Bezanilla & Rojas, 1973). We examine the possibility that slow inactivation is produced by a change in membrane surface charge due to phospholipid transmembrane movements.

METHODS

Material

Giant axons with diameters between 600 and 1000 μm were dissected from the hindmost stellar nerve in mantles of *Loligo forbesi* and occasionally, *Loligo vulgaris*. Most of the experiments were done with mantles obtained from freshly killed squid, but some were performed with refrigerated mantles. The axons were cleaned of surrounding small fibres.

Experimental procedure

Most of the axons were perfused intracellularly by a modification of the Tasaki technique (Tasaki, Watanabe & Takenaka, 1962), as described by Rojas & Ehrenstein (1965). The chamber arrangement, including temperature measurement and control, was similar to that described by Armstrong *et al.* (1973) and by Rojas, Taylor, Atwater & Bezanilla (1969). The 'piggyback' assembly described by Chandler & Meves (1965) was used as an internal current-supplying and potential-recording electrode. For intact axons the same procedure was used, except for the perfusion procedures. Both the external and the internal solutions were perfused continuously throughout the experiment. Solutions could be changed during perfusion.

Proteolytic treatment

For the experiments presented in section 7 of Results the proteolytic enzymes were always internally perfused. The 1974–75 experiments were done with purified alkaline proteinase b prepared by a modification of the procedure reported by Narahashi (1970) (see Rojas & Rudy, 1976). The 1975–76 experiments were done with pronase that had been dialysed for 48 hr against perfusion solution containing 10 mM-EGTA. After dialysis only three enzymatic activities remain (out of eleven present in the mixture), one of which is the alkaline proteinase b. Before use, the solution is diluted 10 times, so that the concentration of EGTA in the perfusate is only 1 mM, and the total concentration of protein 0·5 mg/ml. This solution must be used shortly after preparation, since the alkaline proteinase b is unstable at pH above 5.

Voltage clamp

The point control system for voltage clamping developed by Moore & Cole (1963) was used to control the membrane potential. Current measurement, current supply and voltage measurement across the membrane employed the electrode arrangement described by Keynes & Rojas (1974). Compensated feed-back was used. In general, 65–70% of the series resistance could be compensated.

Junction potentials between the electrodes or imbalance between the external and internal potential recording amplifiers were corrected at the beginning of each experiment by placing the internal electrode in the external solution and subtracting any difference at the difference amplifier. Drifts were checked at the end of each experiment by the same procedure; none greater than 5 mV occurred in the experiments presented in this paper, and are therefore neglected. The values of membrane potential reported throughout the paper were corrected for junction potentials between external and internal solutions. The pulse timing was controlled by a Digitimer Type 3290. Membrane potential and current were measured with a Tektronix 502–A oscilloscope, and records were stored on film.

When explicitly mentioned in the text, Na currents were corrected by subtracting leak current. Leak currents were obtained by (1) repeating the pulse sequence used during the experiment in the presence of 300 nM-tetrodotoxin (TTX), or (2) from values obtained from hyperpolarizing pulses, assuming that the voltage dependence of the leak current is linear.

Potentials are referred to the external solution as ground, so that the resting potential is negative. Potential changes in the positive direction are referred to as depolarizations; negative changes are referred to as hyperpolarizations. Inward currents are shown as downward deflexions upward deflexions represent outward currents.

Solutions

The compositions of the solutions used are given in Table 1. All were prepared with distilled water and stored at 4 °C.

TABLE 1. Composition of solutions

A, Internal F solutions (m-mole l.$^{-1}$)

	NaF	CsF	Tris-Cl	Sucrose	pH
High Cs	—	550	5	10	7·3
Low Cs	—	350	5	400	7·3
Na	350	—	5	400	7·3

B, External Cl solutions (m-mole l.$^{-1}$)

	K	Na	Ca	Mg	Tris-Cl	pH
K free ASW	—	430	10	50	10	7·2–7·5

In addition, enzymes or inhibitors were added to the corresponding solution. Na permeability was inhibited with 300 nM-TTX in the bathing solution.

RESULTS

1. *Na currents in Cs perfused axons*

Before the main results are presented, it may be useful to outline the most important features of a voltage-clamped Cs perfused axon. A detailed description can be found in Chandler & Meves (1965, 1970a) and in Adelman & Senft (1966).

When a squid giant axon is perfused with Cs, the resting potential decays from its initial value of about −70 mV to a very low value, between −15 and 0 mV in my experiments. The rate of decay of the resting potential can be varied by changing the rate of perfusion. After several minutes, at standard rates, the resting potential

has reached its low value, and the membrane remains in a depolarized state. If the membrane potential is restored by voltage clamp to normal values (holding potential, V_H) and depolarizing pulses are then applied to the membrane (V_p), inward Na currents can be recorded. Except for a small effect on the Na inactivation, character-ized by the presence of a small fraction of inward current that does not inactivate during a pulse of standard length (several milliseconds), the inward Na currents are similar to those recorded in an intact axon or in an axon perfused with K. Since Cs is partly permeable through the Na conductance pathway (Na channel), small out-ward Cs currents are observed. These are similar to the early outward K currents (through the Na channel) in a K perfused axon. The early outward current can be increased by adding Na to the perfusate. The Na channel is probably not greatly altered in a Cs perfused axon. Cs is impermeant through the late K channel, so late outward currents are absent in a Cs perfused axon.

2. *Slow inactivation of the Na conductance in CsF perfused axons*

The work presented here originated in the observation shown in Fig. 1*A*. In this experiment, a CsF internally perfused axon was clamped at a membrane potential of − 73 mV from a resting potential of − 8 mV; 5 sec later, depolarizing pulses of 93 mV were applied to the membrane at 5 sec intervals. The recorded Na currents increase from a low initial value for the first depolarization to a steady value as the pulse is repeated several times. There is no apparent change in the time course of the Na currents.

In some records it appears that the time required for the inward current to peak becomes progressively greater as slow inactivation is removed. The effect is small and varies from one axon to another depending on the current density; it is therefore very probably related to un-compensated series resistance.

The final record in the series (Fig. 1*A*) is the same as that obtained when the same axon had been under clamp for 15 min and is then pulsed to the same depolarization. This means that the final value obtained in the series shown in Fig. 1*A* corresponds to a steady value for that axon when the membrane was hyperpolarized for a suffi-ciently long time. In fact, the series of Fig. 1*A* can be repeated several times with identical results by switching off the voltage clamp and allowing the membrane to sit at its resting potential for a few minutes and then hyperpolarizing again. Fig. 1*B* compares the recovery of the peak Na current obtained from the experiment shown in Fig. 1*A* (filled symbols) with that obtained from another experiment. In the second experiment, the length of the interval at the hyperpolarizing V_H was increased, and the Na current was tested at the end of this interval with a single depolarizing test pulse. Only small differences are observed, supporting the hypothesis that the increase in the magnitude of the sodium currents observed in Fig. 1*A* is produced by the increase in the length of time that the membrane spends at V_H.

In another experiment, the time at resting potential was varied, and we recorded the currents produced by a depolarizing pulse at a fixed time after a − 73 mV holding pulse. The magnitude of the current decreased with increased time at the depolarized resting potential. This indicates that the inhibition of the currents is induced by the depolarized resting potential. Also, in two experiments the membrane was clamped at − 10 mV for 2 min; then the membrane potential was changed to − 70 mV, and
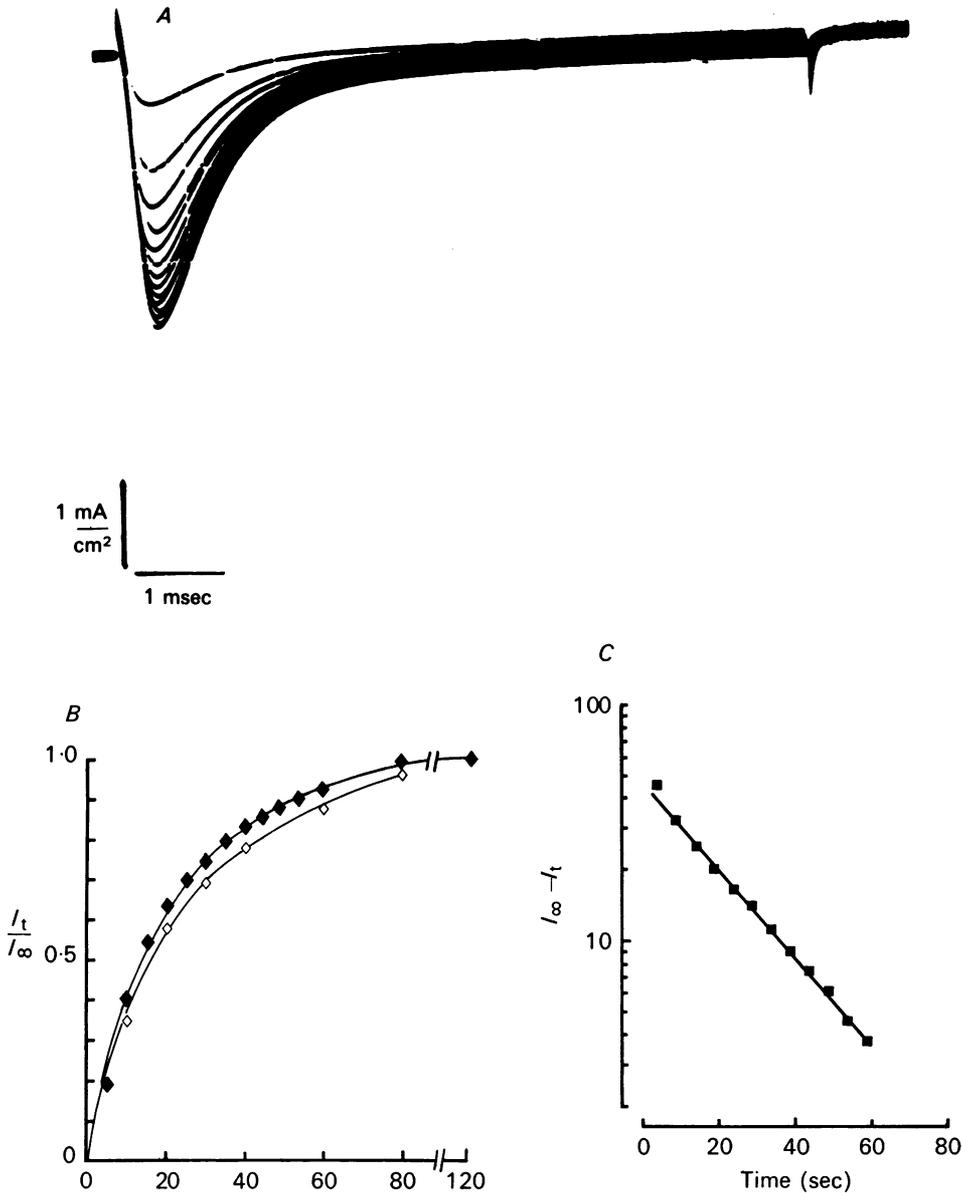
Fig. 1. *A*, current growth with subsequent pulses at intervals of 5 sec. The membrane was held at resting potential (-8 mV) for several minutes and then clamped to a V_{H} of -73 mV. 5 sec later, pulses which took the membrane potential to $+20$ mV were applied at 5 sec intervals. Temp. 5 °C. *B*, recovery from slow inactivation. Ordinates: ratio of peak current for the pulse n, t sec after the clamp had been started (I_t), over the final value I_∞. Abscissa: time in sec. Filled symbols obtained from the experiment in Fig. 1*A*. Open symbols obtained in the same axon as explained in the text. Currents corrected for leakage. *C*, semilogarithmic plot of the recovery from the filled symbols in Fig. 1*B*. Ordinates: steady peak current (I_∞) minus current for pulse n, t sec after starting the clamp (I_t). Abscissa: time in sec.

the current produced by a 100 mV depolarizing pulse was recorded. In these two experiments we observed the same current inhibition.

The phenomenon just described has, therefore, the characteristics of slow inactivation. It is induced by a long-lasting depolarization and recovers after a long-lasting hyperpolarization.

The following results suggest that the slow inactivation is a general property of squid giant axons, whether perfused or not. (*A*) It usually takes several minutes between the initiation of the perfusion with CsF and the drop in the resting potential. The phenomenon described in Fig. 1 was checked during this period. There is no appreciable inactivation until the resting potential depolarizes beyond -40 mV. (*B*) The phenomenon (Fig. 1) is also observed in intact axons with low values of resting potential. (*C*) The phenomenon was not observed in KF perfused axons with high resting potentials, or in mixtures of KF and Na or CsF that produce resting potentials below -60 mV.

The phenomenon can be observed under very different conditions, provided only that the resting potential is low. This suggests that the process might be a normal property of the Na channel. Also in support is the possibility that slow inactivation could explain adaptation of firing rate with time as proposed later in the Discussion.

3. *Recovery from slow inactivation*

Preliminary experiments indicated that slow inactivation developed completely after a 2 min depolarization. In the following experiments, 3 min depolarizations were used. Thus we were certain that we were measuring recovery from a condition of full steady-state inactivation.

We obtained the time course of recovery from experiments such as the one illustrated in Fig. 1*A*. The difference between the steady value of the peak current (I_∞) and the value at any time (I_t) after the clamp had started, plotted against time, shows that recovery follows an exponential time course. This is illustrated in Fig. 1*C*. The points do not seem to diverge significantly from a straight line. Assuming slow inactivation recovers with a single exponential time course we can estimate a time constant for recovery. For the experiment in Fig. 1, the time constant was 28 sec, measured at a V_H of -73 mV and a temperature of 5 °C. The recovery was tested in the same way for V_H between -100 and -60 mV. In one experiment, at 5 °C, the time constants obtained were: 11 sec at -100, 18 sec at -83 and 31 sec at -63 mV.

The first point in Fig. 1*C* is clearly off the straight line. Fox (1976) reported two time constants for slow inactivation in the frog node of Ranvier. It was therefore of interest to explore in more detail the early part of the time course of slow inactivation. Experiments in which points were obtained at 2 or 3/sec were used. The problem was explored in a total of five experiments. In three of these a single exponential was a good fit; in the rest, the early points were off the single exponential straight line for a semilogarithmic plot. The data could be fitted well by the function erf $\sqrt{t/\tau}$. A good fit is also provided by the sum of two exponentials. It is clear that very precise data, particularly at earlier times, are required to obtain the time course of slow inactivation with precision. This seems worthwhile. Since slow inactivation is so much slower than other channel transitions it is reasonable to assume that the time course of the process reflects in a more direct fashion the molecular transitions responsible for it, independent of, for example, the transitions from closed to open states. Neumcke, Fox, Drouin & Schwarz (1976) have developed an electrodiffusion model to explain slow inactivation. The model derived predicts an erf (error function) of $\sqrt{t/\tau}$ for the time course of slow inactivation. These authors also

demonstrated that the non-exponential time course of slow inactivation could be fitted by such a function.

A model for inactivation of the monazomycin-induced conductance in black lipid films has been proposed by Heyer, Mueller & Finkelstein (1976). Such a model applied to slow inactivation would be consistent with an electrodiffusion process, in which the channel (or parts of it) moved to the opposite side of the membrane from the one in which they are located at rest, if the axon is depolarized for a long time. The return of these inactivated portions of the channel could correspond to the 'reversed gating currents' observed by Keynes, Rojas & Rudy (1974). This model could also explain why Zn, when internally applied to the squid giant axon, blocks the Na and the Na-gating currents, but not the reversed gating currents (Meves, 1976).

4. Time course of onset of slow inactivation

The onset of slow inactivation was studied by letting the membrane depolarize at its resting potential for different lengths of time. After this period the axon was clamped at a potential between -70 and -80 mV. Five sec later, a depolarizing pulse was applied and the peak Na current recorded. The time course of onset of slow inactivation was obtained from plots of the peak Na current against the duration of the initial depolarization. This could be fitted to a single exponential; the time constant varied between 10 and 20 sec, depending on the resting potential, at 5–7 °C.

Fig. 3 collects various values for the time constants both for recovery and onset of slow inactivation.

5. Temperature dependence of the slow inactivation

The effect of temperature on the slow inactivation was tested in three axons with an experiment similar to that shown in Fig. 1A and following the treatment of the data shown in Fig. 1C. Two of the axons were perfused with CsF and one with NaF.

The main effect of temperature was to change the time constant of the recovery process, without any appreciable effect on either the time course or on the value of the ratio of peak current at 0 time to steady peak value. The temperature coefficient of the rate constant is positive. The Q_{10} was estimated over a temperature range of 4–13 °C. In three experiments in which it was determined, the Q_{10} was: 3·3, 3·5 and 2·9.

6. Steady and time-dependent characteristics of the slow inactivation. Comparison with the slow inactivation observed by Chandler & Meves (1970b) on NaF perfused axons

The currents recorded in a Na perfused axon show similar features to those recorded in a Cs perfused axon (Chandler & Meves, 1965, 1970a). Late outward current through the K conductance channel is absent, and there is a maintained component in the early current; thus the fast inactivation is incomplete. The maintained component of the early current is larger in a NaF perfused axon than in a CsF perfused axon. Most of the quantitative studies on slow inactivation reported by Chandler & Meves (1970b) were done on the maintained component. However, they showed that the inactivating component of the current had a similar slow inactivation.

Chandler & Meves induced slow inactivation by applying long depolarizing pulses and found that the fibres deteriorated rapidly. I had the same experience; therefore most of the quantitative studies were done by leaving the fibre depolarized at its own resting potential. This increased the survival of the axon, allowing several measurements to be done on a single fibre. Even under

these circumstances, the axon did not last as long as a perfused axon maintained under clamp. Therefore, the values reported here were collected from several fibres. This may be responsible for some of the scatter. Measurements in a few axons gave quantitative values similar to those reported by Chandler & Meves (1970*b*). Since these are difficult measurements, only a few points were taken at the beginning of experiments dedicated to exploring other problems.
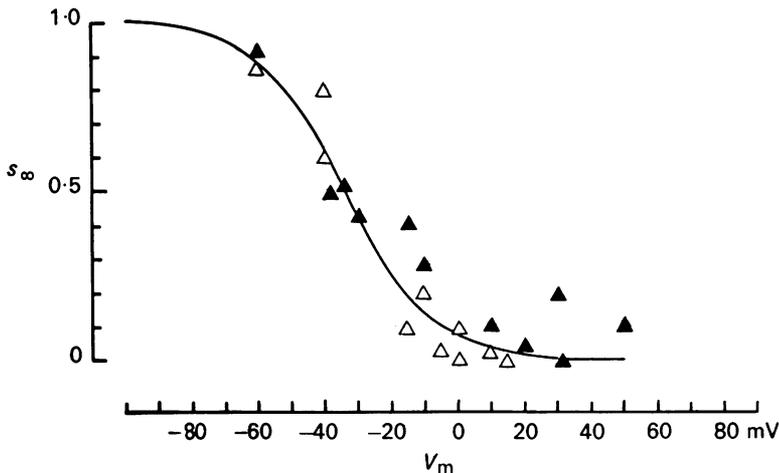

Fig. 2. Voltage dependence of the steady value of slow inactivation. Open symbols: values obtained from experiments like that illustrated in Fig. 1 clamping from various resting potentials. Several axons from 74–75 and 75–76 series perfused with either high or low CsF. Filled symbols: experimental values from Chandler & Meves (1970*b*). For the open symbols: ordinates: $I_0/I_\infty = s_\infty$. I_0 was obtained from the extrapolated value obtained in a semilogarithmic trace of the type illustrated in Fig. 1*C*. Abscissa: resting potential in mV. Curve fitted to the equation

$$s_\infty = \frac{1}{1 + \exp\left\{\dfrac{-(V_s^0 - V)}{k_s}\right\}}$$

$k_s = 13{\cdot}5 \text{ mV}$
$V_s^0 = -33{\cdot}2 \text{ mV (potential at which } s_\infty = 0{\cdot}5)$

One of the quantitative measurements of interest is the voltage dependence of the steady value of inactivation. For the fast inactivation Hodgkin & Huxley (1952*b*) introduced the variable *h*, with (1-*h*) being the fraction of Na conductance pathways that are inactivated. Similarly, Chandler & Meves (1970*b*) used s for the slow inactivation variable; in this paper I will use the same convention.

Fig. 2 contains the values of s_∞ (steady value of slow inactivation, I_0/I_∞) at different membrane potentials. Open symbols are my own experimental measurements, and filled symbols are Chandler & Meves' experimental values. My own values were obtained as follows. The membrane was left at its resting potential for 3 min, then clamped to -83 mV and pulsed at a rate of once every 3 sec, starting 3 sec after the membrane had been clamped, until full recovery was obtained. After correcting for leakage, the values of $I_\infty - I_t$ were then plotted against time on semilogarithmic paper and the straight line extrapolated to zero time. This value was considered as I_0. The points were collected from various experiments at different resting potentials. I_∞ is, as before, the peak Na current after full recovery.

Fig. 2 shows that the few points obtained correspond rather well with those of Chandler & Meves.

Fig. 3 is a comparison of the time constants of development and recovery of slow inactivation; my own values were obtained as described earlier. Chandler & Meves' values are the inverse of their rate constants. Since in their experiments these were
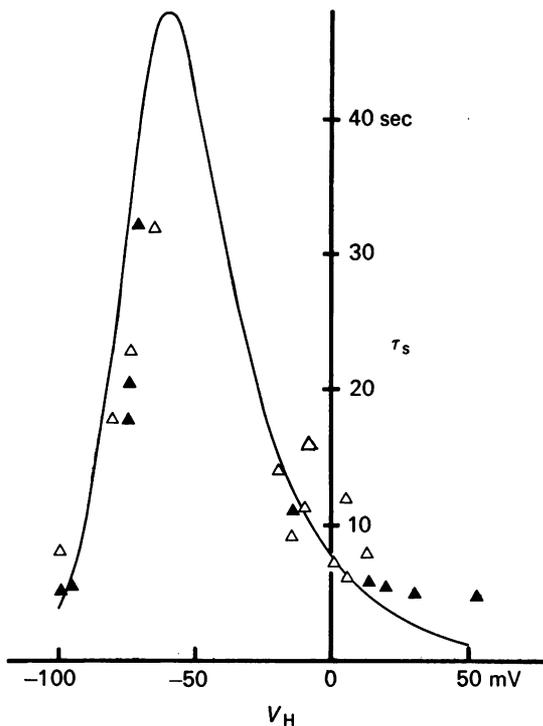

Fig. 3. Voltage dependence of the time constants of slow inactivation. Filled symbols: values from Chandler & Meves (1970 b). Open symbols: points to the left of − 50 mV represent time constant of recovery obtained from experiments of the type illustrated in Fig. 1 A as obtained in Fig. 1 C; different holding potentials (V_H). Points to the right, time constants for the onset of slow inactivation, at various resting potentials (V_R). Line fitted to the equation

$$\tau = \frac{1}{\alpha_s + \beta_s}$$

where

$$\beta_s = 0\cdot125 \exp (0\cdot035V),$$
$$\alpha_s = 0\cdot000016 \exp (-0\cdot097V).$$

The equation for α_s and β_s were obtained by fitting values obtained from:

$$\alpha_s = s_\infty/\tau_\infty,$$
$$\beta_s = (1 - s_\infty)/\tau_\infty.$$

obtained at 16–16·5 °C, the values were calculated for a temperature of 5 °C assuming a Q_{10} of 3. Again the correspondence with values obtained in different experimental conditions is striking.

7. *Pronase resistance of the slow inactivation*

Rojas & Armstrong (1971) reported that perfusion of squid giant axons with the proteolytic enzyme mixture pronase blocked the fast inactivation of the Na conductance (see also Armstrong, Bezanilla & Rojas, 1973). The Na currents are maintained during a normal depolarizing pulse. Later Rojas and Rudy (1976) demonstrated that one of the fractions of the mixture, alkaline proteinase b, was capable of producing
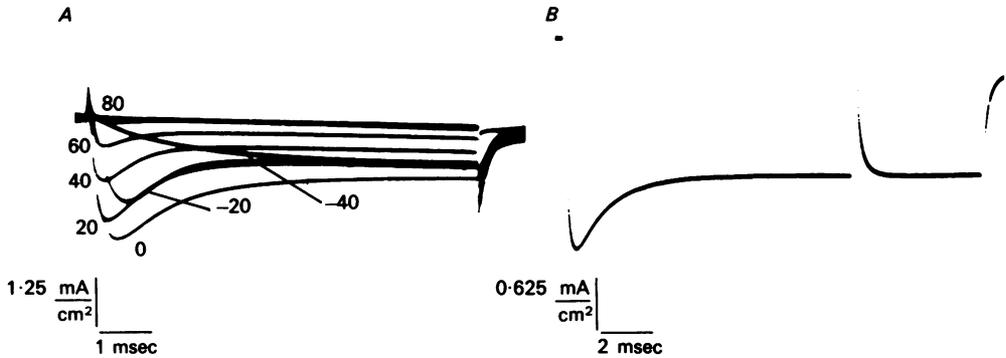

Fig. 4. Na currents after partial treatment with alkaline proteinase b. Expt. 13-D-74 II (74–75 series). *A*, superimposed membrane currents from a high Cs perfused axon. $V_H = -70$ mV. Membrane potential during the pulse shown in the Figure. Currents obtained after 10 min perfusion with alkaline proteinase b, followed by 5 min perfusion with high Cs solution. *B*, double pulse experiment. Two pulses were applied to the membrane. Same axon as *A*. Both pulses, separated by 500 μsec, depolarized the membrane from a V_H of -70 to $+20$ mV.

the effect by itself. Alkaline proteinase b was found to be much less damaging to the axons than pronase. Detailed quantitative studies were made on the behaviour of the Na conductance under such conditions showing that, except for the complete absence of fast inactivation and a variable decrease in the value of the maximum Na conductance, the Na conductance pathway behaves identically before and after the treatment.

Fig. 4*A* illustrates the effect of partial treatment with alkaline proteinase b on the Na currents recorded in a CsF perfused axon. At all the membrane potentials tested, the current rises to a peak value and decays to a level which is maintained during the depolarization. Fig. 4*B* shows that the partial block of inactivation can also be demonstrated with a double pulse experiment. A prepulse does not affect the maintained Na currents in a subsequent test pulse.

In the experiment illustrated in Fig. 5, slow inactivation was tested as in Fig. 1*A*, before and after the axon had been perfused with alkaline proteinase b. It is apparent that maintained Na currents, in which the fast inactivation is blocked, do exhibit slow inactivation.

The recovery from slow inactivation appears to follow the same time course both before and after the application of pronase. This is illustrated in Figs. 5 and 6. In the case of the experiment illustrated in Fig. 6, when the perfusion with dialysed pronase was stopped before full inactivation blockage had been reached, the current consists of an inactivating and a maintained component. The same experiment as that of

Figs. 1 and 5 was repeated under these conditions. The figure demonstrates that both the peak and the steady component recover together. The plot that accompanies the experimental records shows that the s_∞ and the recovery are similar for both components.

In other experiments, the same result was obtained if the recovery was compared, before and after full proteolytic treatment, at a V_H of -100, -80, and -70 mV (see Table 2).

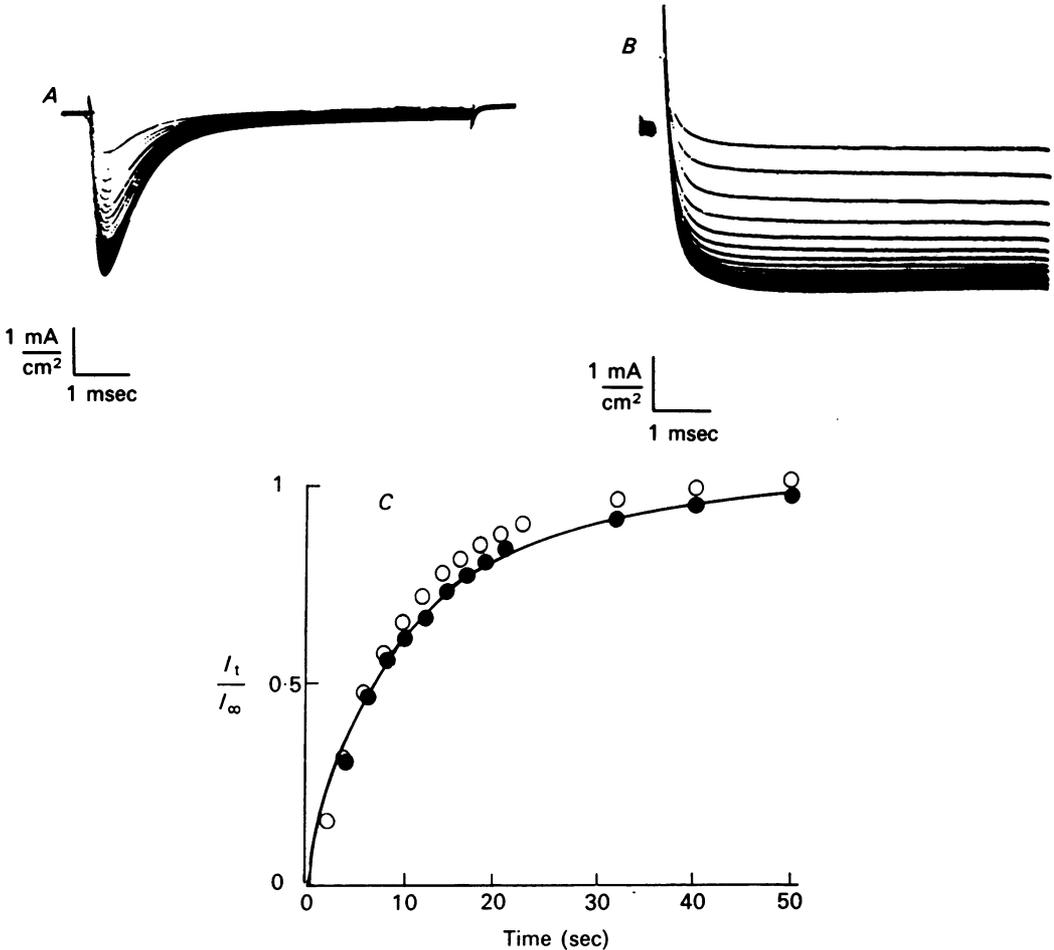The onset of slow inactivation after pronase was studied as described before for the

Fig. 5. Slow inactivation in the presence and absence of fast inactivation. Expt. 29-J-75 II (74–75 series). A, before enzymic treatment. The membrane was held at its resting potential (-8 mV) for three minutes and then clamped to -73 mV. Records obtained with pulses to $+20$ mV repeated at a frequency of 2 sec^{-1}. First pulse obtained 4 sec after the clamp was started. Temp. 8 °C. B, same experiment as in A, obtained after enzymatic treatment, and at 2 sec^{-1}. First pulse 2 sec after hyperpolarizing. Temp. 8 °C. C, recovery from slow inactivation before and after pronase. The ratio of the peak sodium current for the pulse n, t sec after the clamp had been started (I_t), over the final value I_∞ is plotted against time in sec. Currents corrected for leak. Filled symbols before pronase, open symbols after pronase. Time constants of recovery were calculated from semilogarithmic plots as 14·4 sec before pronase and 12·7 sec after pronase.

normal case. The results indicated that slow inactivation appeared to develop about twice as fast in the pronase treated axon than in the same axon before the treatment. This is best illustrated by the experiment shown in Fig. 7. This corresponds to the same experiment described earlier in reference to Fig. 6. Since both components are
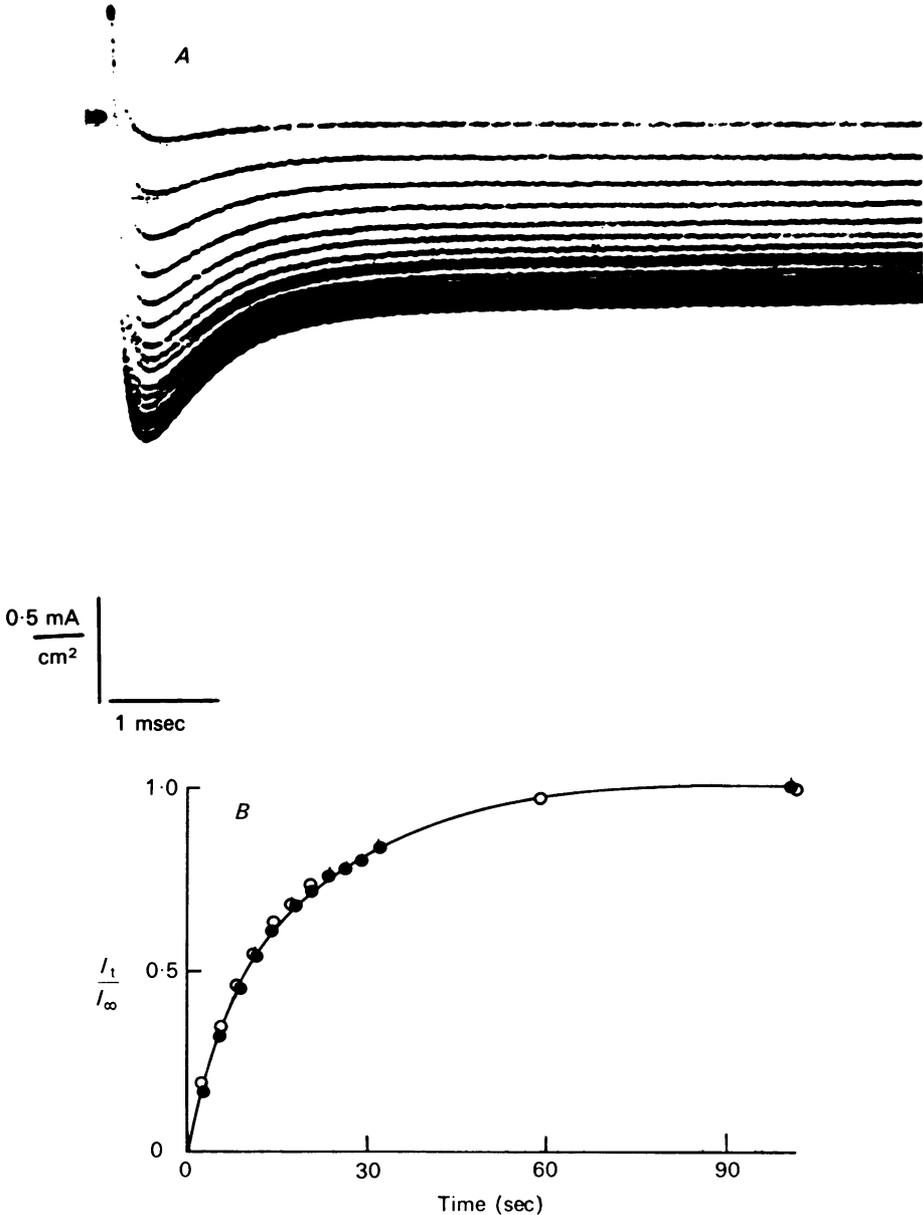

Fig. 6. Recovery from slow inactivation after pronase in a partially treated axon. Expt. 10-0-75 III (75–76 series). Low Cs perfused axon. *A*, the membrane was depolarized at resting potential (-5 mV) for 2 min and then clamped to -70 mV. Pulses to $+30$ mV applied at 3 sec intervals. Temp. 5·6 °C. *B*, I_t/I_∞ for the value of current at the peak (filled circles) and at the end of the pulse (open circles). Currents corrected for leakage.

present (maintained and rapidly inactivating) they can be compared directly. It is shown in the figure that indeed the slow inactivation develops faster for the maintained component, the rate constant being about 2·5 times larger for this component than for the rapidly inactivating component. Similar differences are observed if the comparison is made before and after full proteolytic treatment (Table 2).

TABLE 2. Rates of slow inactivation before and after proteolytic treatment*

I Time constants of onset

Experiment	Membrane potential (mV)	(Sec) Before	(Sec) After	Temperature (°C)
9-0-75 I	−10	22·09	10·82	5
6-0-75 I	−15	20·12	8·0	5
—	0	14·1	7·0	5

II Time constants of recovery

29-J-75 III	−80	16·74	13·5	8·5
10-0-75 III	−70	23·81	18·5	8
9-0-75 III	−70	20·5	21·6	9
—	−100	12·0	10·6	8
29-J-75 II	−73	21·65	19·5	8
—	−83	15·0	13·2	8

* In the experiments reported in the Table the measurements *after* proteolytic treatment refer to axons in which the inactivation had been fully removed. So in these cases, maintained sodium current was considered in obtaining the time constants. Peak Na currents were used for measurements before proteolytic treatment.

Current dependent processes (electrode polarization or ion accumulation) could affect this measurement. However, similar differences in the development of slow inactivation were found in several axons with rather different current densities. In one of the axons in which this difference had been recorded, the rate of onset of slow inactivation after pronase was checked for two different current densities by adding a small amount of TTX to the axon. The rate was the same as before the addition of TTX.

8. *Slow inactivation is not the result of a change in surface charge*

The long term changes in transmembrane potential that produce slow inactivation could result in changes in the membrane surface charge by, for example, a phospholipid flip-flop mechanism (McNammee & McConnell, 1973; McLaughlin & Harari, 1974). A long term depolarization could produce an increase in the negative charge density of the internal surface of the membrane, and, since the parameters which control the Na conductance are voltage dependent (Hodgkin & Huxley, 1952b) and they are affected by changes in surface potential (Chandler, Hodgkin & Meves, 1965), such a change in surface charge could be the cause of slow inactivation.

If such a change in charge density had ocurred during the time that the axon is depolarized to produce slow inactivation, the voltage-dependent parameters controlling the sodium permeability would shift to more positive potentials. However, no indication of a shift was observed.

There are some qualitative predictions of the previous hypothesis which are not met by experimental results.

The hypothesis could be specified as follows: during a long depolarization, the surface charge changes to a final value which depends on the value of the transmembrane potential; when the membrane is repolarized the surface charge relaxes to its original value. The time constants of those processes should somehow be proportional to the rates of development and recovery of slow inactivation. During the
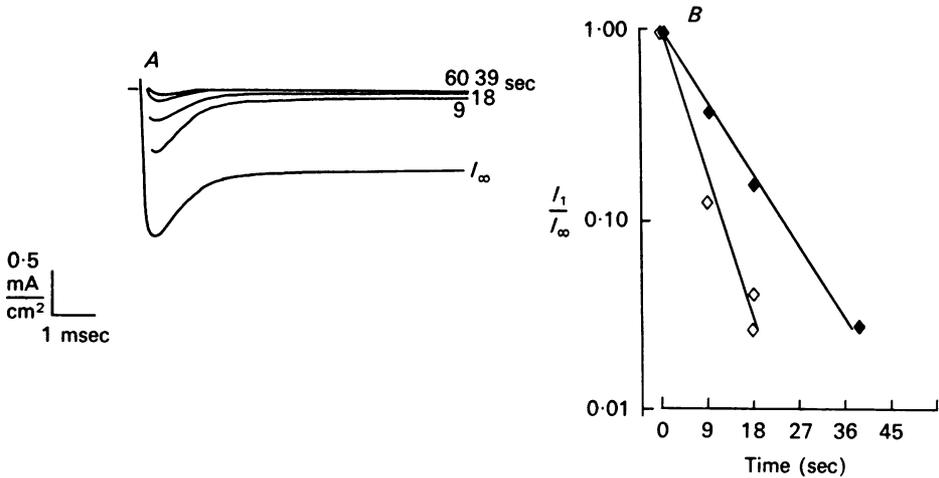

Fig. 7. Development of slow inactivation before and after pronase. A, the top four traces represent the Na current recorded for a pulse to $+20$ mV, applied 3 sec after clamping to -70 mV from a resting potential of -5 mV. The values on the right indicate the time in sec that the membrane was held at rest before being clamped. I_∞ is the current recorded for the same pulse after being held 3 min at -70 mV. Expt. 10-0-75 III (75–76 series). Low Cs perfused axon. B, semilogarithmic plot of I_1/I_∞ against time under depolarization at rest for the records shown in A. I_1 peak current (filled symbols) or maintained current (open symbols) for the pulse at t sec. I_∞ peak or maintained current for the I_∞ trace in A.

recovery period the surface charge is changing continuously, and since not only the magnitude but also the time course of the Na currents depends on potential, the time course of the recorded Na currents will be changing during the recovery period. This is not observed in any of the records shown earlier. Fig. 8 I shows recovering Na currents obtained at different potentials for the test pulse and therefore different time courses. In no case is such a change observed. The expected change can be estimated from the conductance voltage relationship for that particular axon, which is shown in Fig. 8 II.

In the experiment shown in Fig. 8 the long lasting depolarization was to -4 mV. At this potential the Na conductance is almost 90 % in slow inactivated state. The shift in surface charge would have to be such that when a depolarizing test pulse is applied, the actual membrane potential is below -40 mV, so the Na conductance will be activated less than 10 %. Since the magnitude of the shift depends on the value of the potential during the long lasting depolarization, it ought to be the same for all the cases shown in Fig. 8 (-4 mV). The minimum shift is defined by the experiment with the highest test pulse, that is to $+30$ mV (Fig. 8 IE); hence it must
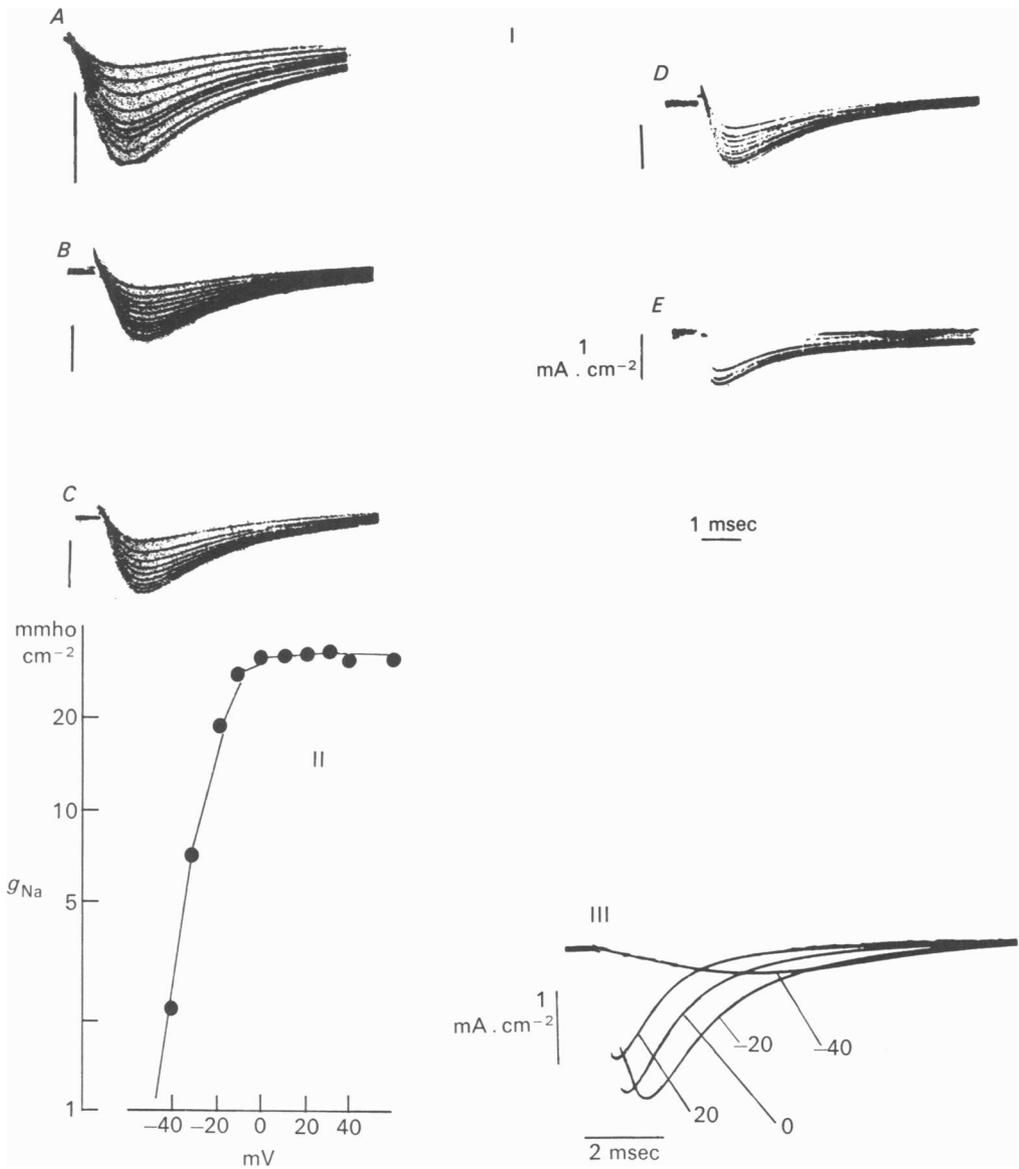
Fig. 8. I, recovery from slow inactivation for various test potentials. Expt. 2-0-75 I (75–76 series). $V_H = -70$ mV. Temp. 4 °C. Axon perfused with low Cs and bathed in K-free ASW. The axon was depolarized each time for 2 min at resting potential (-4 mV), then clamped to V_H and test pulses applied at a frequency of 3 sec^{-1}. Potential during pulse: A, -20; B, -10; C, 0; D, $+10$; E, $+30$ mV. Scales: vertical: 1·0 mA/cm^6; horizontal: 1 msec. Records after correction for leakage.

II, voltage dependence of the peak Na conductance for the same axon. The Na conductance was calculated from the equation

$$g_{Na} = I_{Na}/(V - V_{Na}).$$

III, superimposed Na currents for depolarizations to the values indicated in the Figure. Same axon as in I. $V_H = -70$ mV. Temp. 4 °C.

TABLE 3. Recovery of slow inactivation at various test potentials

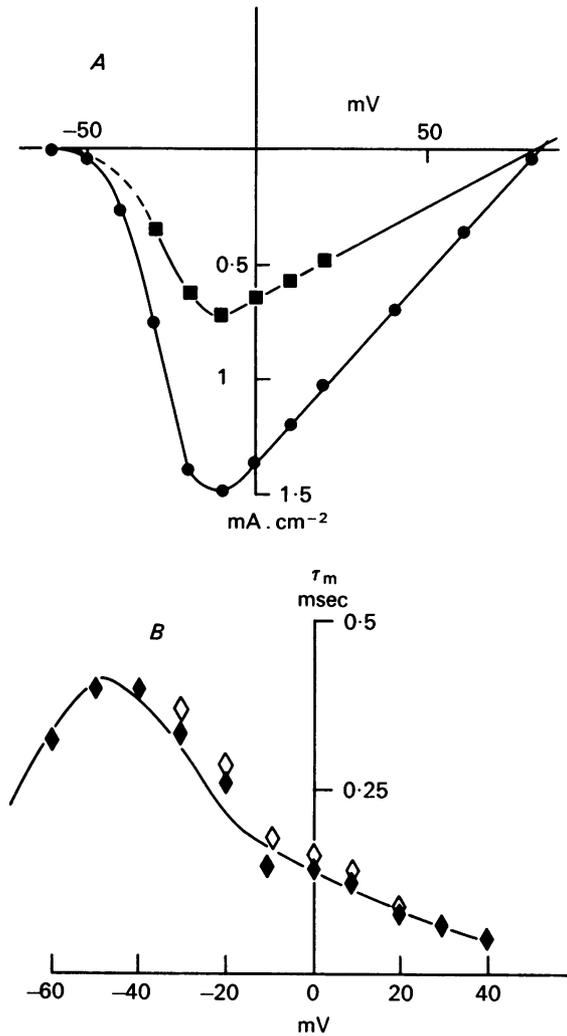| Records (Fig.8)	V_m	I_1/I_∞	τ_{rec} (sec)
A	−20	0·25	20·79
B	−10	0·25	25·98
C	0	0·27	20·79
D	10	0·32	22·50
E	30	0·27	—

Fig. 9. *A*, peak Na current–voltage curve during and at the end of recovery from slow inactivation. Same experiment as that illustrated in Fig. 8. Squares are the peak Na current at 15 sec after the start of recovery. Circles are the points obtained when full recovery was reached. *B*, time constant of activation of the Na conductance (τ_m) during and at the end of recovery from slow inactivation. τ_m was calculated by the method used by Hodgkin & Huxley (1952*b*). Filled symbols are the time constant for the Na currents at full recovery and open symbols those obtained at 15 sec after the start of the recovery period.

be of at least 70 mV. The series of sodium currents shown in Fig. 8 III illustrates just how much the time course of the Na currents ought to change if the membrane potential changes from any value below − 40 mV toward any of the values of the test potentials used in the experiments in Fig. 8. The change is considerable.

A more quantitative analysis is shown in Fig. 9. This demonstrates that there is no change in surface charge sensed by the Na conductance during the periods of time relevant for slow inactivation. The Figure illustrates the peak Na currents *vs.* voltage curve and the relaxation time *vs.* voltage curve obtained at the time of full recovery and one obtained after only 15 sec of recovery. There is no evidence for a shift.

Ehrenstein, Gilbert & Lipicky (1975) were unable to see a shift in membrane potential sensed by the K conductance even with 30 min depolarizations.

It is possible, however, that the change in surface charge is not homogeneous all over the membrane surface, but localized near the Na channels. The behaviour is 'all or none'. Na channels with such transition do not open at all due to the localized shift in surface charge, but the ones that open, and hence are responsible for the measured current, behave normally. If this were the case, one would predict that as the clamp imposed membrane potential is increased, one would overcome the shift in surface charge. Hence the I_1/I_∞ values would increase as the test depolarization is increased; where I_1 is the magnitude of the peak currents in the first pulse of each series, and I_∞, as before, the steady value for each series. Table 3 contains the values of I_1/I_∞ determined from the experiment illustrated in Fig. 8. There is not a consistent increase in I_1/I_∞ as the test potential is increased. Of course, it is still possible that the localized shift is larger than the potentials imposed in the last experiment.

DISCUSSION
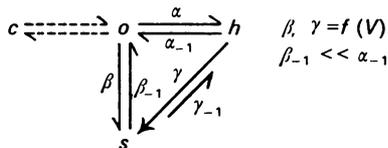
Kinetic schemes for slow inactivation

To my knowledge, every excitable tissue whose slow behaviour has been studied exhibits slow inactivation. It thus seems that slow inactivation is a general property of excitable channels. It could be proposed that it is an inherent property of these molecules. This contrasts with the apparent specificity of the sodium fast inactivation (*h*), which, although probably not exclusive of the Na channel, seems to be present in fewer systems. The results presented here suggest that, in the case of the Na channel, at least two distinct inactivation states exist.

Since recovery from slow inactivation is similar, at various potentials, before or after the proteolytic treatment, it is fair to propose that the same state (*s*) is reached in both conditions. The faster development of *s* after proteolysis could be related to a direct effect of the enzymatic treatment or to the absence of fast inactivation (*h*). The fact that similar differences in the rates of development of *s* are found when maintained and peak currents are compared in NaF perfused axons (Chandler & Meves, 1970*b*) suggests that the faster development of *s* is related to the absence of *h*. The maintained component of the Na currents in those axons represents a fraction of the sodium which does not inactivate (fast inactivation).

It could be argued that the faster development of *s* is only apparent and that the inactivation measured is *h* slowed down by pronase; again the similarities between the enzymatic results and those of Chandler & Meves (1970*b*) support the notion that

the effect is real. It seems unlikely that fast inactivation will be equally slowed down by both treatments.

I propose the following kinetic scheme which accommodates both the results presented here and those of Chandler & Meves (1970*b*).

$$c \rightleftharpoons o \underset{\alpha_{-1}}{\overset{\alpha}{\rightleftharpoons}} h \qquad \beta, \ \gamma = f(V)$$
$$\beta \Big\Vert \beta_{-1} \ \gamma \Big/ \gamma_{-1} \qquad \beta_{-1} << \alpha_{-1}$$
$$s$$

The channel undergoes transitions over four main states: closed (*c*), open (*o*), *h* (fast inactivated), and *s* (slow inactivated). The forward rate constants dominate during a depolarization, while the backward rate constants dominate during hyperpolarization. The *s* state can be reached either from the *o* or from the *h* state. β is, in squid, about $2 \cdot 5 \gamma$, and $\beta/\alpha + \beta = 1 \times 10^{-2}$; thus under normal conditions in a short depolarization most of the channels enter the *h* rather than the *s* state from the open state. The extremely small number of channels which enter *s* from the open state only becomes apparent after long and repetitive stimulations, thus explaining the adaptation in the firing rate with time. However, if the $o \rightarrow h$ reaction is blocked either by proteolysis or by NaF, slow inactivation proceeds from the open state and its rate is $2 \cdot 5$ times faster than if it was reached from *h*.

Further support for the model proposed comes from studies of slow inactivation in *Myxicola* (Rudy, 1975, 1978, in preparation). In this preparation, during a short depolarization (less than 10 msec) 15 % of the Na channels enter an inactivated state from which recovery is slow. A state with similar recovery rates is reached by almost 100 % of the channels if the axon is depolarized for 1 or 2 min. The model proposed above can be applied to *Myxicola* except that $\beta/\alpha + \beta = \simeq 0 \cdot 15$ and β is much larger than γ. According to this model, the main difference between *Myxicola* and squid is in the rate constant β.

C. M. Armstrong & F. Bezanilla (personal communication) have demonstrated that most of the voltage dependence of the fast inactivation process can be explained if it is proposed that the open-to-fast inactivated rates are not voltage-dependent, but that the voltage dependence of the process is due to the variations with potential in the availability of various open states. Although in the model proposed here, slow inactivation is also coupled to the open states of the channel, it seems necessary to propose that the changes toward the slow inactivated state are voltage-dependent themselves. Differences in the order of seconds are observed in the time constants for the onset of slow inactivation at different potentials. This is much too slow compared to the rates of the previous processes.

Comparison with other reports on slow inactivation

As mentioned earlier, the phenomenon of slow inactivation seems to be a general property of voltage-dependent channels. Reports on slow inactivation of the sodium conductance in three different species are compared in Table 4.

There is good agreement, both in steady-state parameters as well as in the rates, between reports on squid giant axon, the node of *Ranvier* in frog nerve, and the

Myxicola giant axon. In the three species, the Na conductance enters a slow inactivated state with a rate on the order of tens of seconds.

An inactivated state with rates intermediate between the one considered here and the fast h inactivation has been described in the Na system in squid giant axons (Adelman & Palti, 1969a, b). A similar state has been described in the K system by Schwarz & Vogel (1971) in frog nerve. In both cases the slower inactivated state was observed. It will be interesting to test the effect of pronase on this intermediate state to establish its possible relation to the faster and to the slower inactivated states.

TABLE 4. Slow inactivation of the Na conductance in squid, *Myxicola* and frog

Preparation	Time constant[1]	$k_s^{(2)}$ (mV)	$V_s^{0'(2)}$ (mV)	Temp.[3] (°C)	Reference
Squid	20–100 sec	—	—	5–7	Adelman & Palti (1969b)
	10–30 sec	14	−35	5–7	Chandler & Meves (1970b)
	10–40 sec[4]	13·5	−33·2	5	This paper
Myxicola	10–20 sec	∼ 20	∼ −30	5–7	Rudy (1978, in preparation)
	∼ 10 sec	—	∼ −40	5	Schauf et al. (1976)
Frog node	∼ 200 sec[5]	11·7	∼ −50	5–7	Fox (1976)
of ranvier	∼ 80 sec			20	

(1) Approximate values at a membrane potential around − 80 mV.
(2) See legend to Fig. 2.
(3) A Q_{10} of 3 was used to normalize values.
(4) A second time constant, in the range of 2 sec was obtained in some experiments.
(5) A second time constant of about 3 sec at 20 °C is reported.

The papers of Adelman & Palti (1969a, b) require further discussion. Adelman & Palti used external K to depolarize the nerve fibre and proposed that the slow inactivation phenomena were produced by the interaction of K with certain sites in the membrane. In particular, they proposed that the recovery from inactivation induced in high K solutions, observed when long lasting hyperpolarizations are applied to the membrane, is due to the depletion of K from the Frankenhauser-Hodgkin space. This explanation was also applied to the phenomena of inactivation described by Narahashi (1964) in lobster axons bathed in K solutions. The experiments described here, as well as those reported by Chandler & Meves, were performed in K-free solution. It was originally thought, therefore, that the inactivation reported here was of a different nature, in that it was not produced by K but by depolarization of the membrane. However, Schauf et al. (1976) have demonstrated that in *Myxicola*, the slow inactivation phenomena produced by K are very probably due to the depolarization induced by this ion, rather than to any direct effect on the membrane.

Since similar tests as those performed by Schauf et al. (1976) have not been done on squid, it is difficult to make a final conclusion on this species. However, if an s_∞ curve is plotted from the data shown by Adelman & Palti (1969a, Fig. 3A) and compared to the curve shown in this paper and shifted 17 mV (the same shift observed on the h_∞ curves), both are very similar. A tentative conclusion is that squid is similar to *Myxicola* and that the effects of K are produced by depolarization. It is clear, however, that further tests must be performed to confirm this hypothesis.

REFERENCES

ADELMAN, JR., W. J. & PALTI, Y. (1969a). The influence of external potassium on the inactivation of sodium currents in the giant axon of the squid, *Loligo pealei. J. gen. Physiol.* **53**, 685–703.

ADELMAN, JR., W. J. & PALTI, Y. (1969b). The effects of external potassium and long duration voltage conditioning on the amplitude of sodium currents in the giant axon of the squid, *Loligo pealei. J. gen. Physiol.* **54**, 589–606.

ADELMAN, JR., W. J. & SENFT, J. P. (1966). Voltage clamp studies on the effect of internal cesium on sodium and potassium currents in the squid giant axon. *J. gen. Physiol.* **50**, 279–293.

ARMSTRONG, C. M. & BEZANILLA, F. (1974). Charge movement associated with the opening and closing of the activation gates of the Na channels. *J. gen Physiol.* **63**, 533–552.

ARMSTRONG, C. M., BEZANILLA, F. & ROJAS, E. (1973). Destruction of sodium inactivation in squid axons perfused with pronase. *J. gen. Physiol.* **62**, 375–391.

BAKER, P. F., MEVES, H. & RIDGWAY, E. B. (1973). Calcium entry in response to maintained depolarization of squid axons. *J. Physiol.* **231**, 527–548.

BAKER, P. F. & RINK, T. J. (1975). Catecholamine release from bovine adrenal medulla in response to maintained depolarization. *J. Physiol.* **253**, 593–620.

BEELER, G. W. & REUTER, H. (1970). Membrane calcium current in ventricular myocardial fibres. *J. Physiol.* **207**, 191–209.

BEZANILLA, F. & ARMSTRONG, C. M. (1974). Gating currents of the sodium channels: three ways to block them. *Science, N.Y.* **183**, 753–754.

CHANDLER, W. K., HODGKIN, A. L. & MEVES, H. (1965). The effect of changing the internal solution on sodium inactivation and related phenomena in giant axons. *J. Physiol.* **180**, 821–836.

CHANDLER, W. K. & MEVES, H. (1965). Voltage clamp experiments on internally perfused giant axons. *J. Physiol.* **180**, 799–820.

CHANDLER, W. K. & MEVES, H. (1970a). Sodium and potassium currents in squid axons perfused with fluoride solutions. *J. Physiol.* **211**, 623–652.

CHANDLER, W. K. & MEVES, H. (1970b). Slow changes in membrane permeability and long lasting action potentials in axons perfused with fluoride solutions. *J. Physiol.* **211**, 707–728.

CHANDLER, W. K. & MEVES, H. (1970c). Evidence for two types of sodium conductance in axons perfused with sodium fluoride solution. *J. Physiol.* **211**, 653–678.

EHRENSTEIN, G. & GILBERT, D. L. (1966). Slow changes in potassium permeability in squid giant axon. *Biophys. J.* **6**, 533.

EHRENSTEIN, G., GILBERT, D. L. & LIPICKY, J. R. (1975). Does phospholipid flip-flop affect axon potassium channels? *Biophys. J.* **15**, 847–849.

FOX, J. M. (1976). Ultra-slow inactivation of the ionic currents through the membrane of myelinated nerve. *Biochim. biophys. Acta* **426**, 232–244.

HEYER, E. J., MULLER, R. U. & FINKELSTEIN, A. (1976). Inactivation of monazomycin-induced voltage-dependent conductance in thin lipid membranes. II. Inactivation produced by monazomycin transport through the membrane. *J. gen. Physiol.* **67**, 731–748.

HODGKIN, A. L. & HUXLEY, A. F. (1952a). The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo. J. Physiol.* **116**, 497–506.

HODGKIN, A. L. & HUXLEY, A. F. (1952b). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* **117**, 500–554.

KEYNES, R. D. & ROJAS, E. (1974). Kinetics and steady-state properties of the charged system controlling sodium conductance in the squid giant axon. *J. Physiol.* **239**, 393–434.

KEYNES, R. D., ROJAS, E. & RUDY, B. (1974). Demonstration of a first-order voltage-dependent transition of the sodium activation gates. *J. Physiol.* **239**, 100–101P.

LUTTGAU, H. C. (1960). Das Kalium-Transportsystem am Ranvier-Knoten isolierter markhaltiger Nervenfasern. *Pflügers Arch. ges. Physiol.* **271**, 613–633.

McLaughlin, S. & Harary, H. (1974). Phospholipid flip-flop and the distribution of surface charges in excitable membranes. *Biophys. J.* **14**, 200–208.

McNammee, M. G. & McConnell, H. M. (1973). Transmembrane potentials and phospholipid flip-flop in excitable membrane vesicles. *Biochemistry* **12**, 2951–2958.

Meves, H. (1976). The effect of zinc on the late displacement current in squid giant axons. *J. Physiol.* **254**, 787–801.

Moore, J. W. & Cole, K. S. (1963). Voltage clamp techniques. In *Physical Techniques in Biological Research*, ed. Nastuk, W. L. New York: Academic Press.

Nakajima, S., Iwasaki, S. & Obata, K. (1962). Delayed rectification and anomalous rectification in frog's skeletal muscle membrane. *J. gen. Physiol.* **46**, 97–115.

Narahashi, T. (1964). Restoration of action potential by anodal polarization in lobster giant axons. *J. cell. comp. Physiol.* **64**, 73–96.

Narahashi, Y. (1970). Pronase. *Meth. Enzym.* XIX, 651–654.

Neumcke, B., Fox, J. M., Drouin, H. & Schwarz, W. (1976). Kinetics of the slow variation of peak sodium current in the membrane of myelinated nerve following changes of holding potential or extracellular pH. *Biochim. biophys. Acta* **426**, 245–257.

Peganov, E. M., Khodorov, B. I. & Shishkova, L. D. (1973). Slow sodium inactivation in the Ranvier node membrane role of external potassium. *Bull. exp. Biol. Med. U.S.S.R.* **76**, 1014–1017.

Rojas, E. & Armstrong, C. (1971). Sodium conductance activation without inactivation in pronase-perfused axons. *Nature, New Biol.* **229**, 177–178.

Rojas, E. & Ehrenstein, G. (1965). Voltage clamp experiments with potassium as the only internal and external cation. *J. cell. comp. Physiol.* **66**, suppl. 2, 71–77.

Rojas, E. & Rudy, B. (1976). Destruction of the sodium conductance inactivation by a specific protease in perfused nerve fibres from *Loligo. J. Physiol.* **262**, 501–531.

Rojas, E., Taylor, R. E., Atwater, I. & Bezanilla, F. (1969). Analysis of the effects of calcium or magnesium on voltage-clamp currents in perfused squid axons bathed in solutions of high potassium. *J. gen. Physiol.* **54**, 532–552.

Rudy, B. (1975). Slow recovery of the inactivation of sodium conductance in *Myxicola* giant axons. *J. Physiol.* **249**, 22–24P.

Schauf, C. L., Pencek, T. L. & Davis, F. A. (1976). Slow sodium inactivation in *Myxicola* axons. *Biophys. J.* **16**, 771–778.

Schwarz, J. R. & Vogel, W. (1971). Potassium inactivation in single myelinated nerve fibres of *Xenopus laevis. Pflügers Arch.* **330**, 61–73.

Standen, N. B. (1974). Properties of a calcium channel in snail neurones. *Nature, Lond.* **250**, 340–342.

Tasaki, I., Watanabe, A. & Takenaka, T. (1962). Resting and action potential of intracellularly perfused squid giant axon. *Proc. natn. Acad. Sci. U.S.A.* **48**, 1177–1181.

Weight, F. F. & Vatava, J. (1970). Slow synaptic excitation in sympathetic ganglion cells: evidence for synaptic inactivation of potassium conductance. *Science, N.Y.* **170**, 755–758.