J. Physiol. (1979), **286**, *pp.* 157–171
With 8 text-figures
Printed in Great Britain

IMMOBILIZATION OF INTRAMEMBRANE CHARGE IN *MYXICOLA* GIANT AXONS

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(*Received 6 March 1978*)

SUMMARY

1. Immobilization of gating charge was examined in *Myxicola* giant axons dialysed with Cs^+.

2. With increasing pulse durations the ratio Q_{OFF}/Q_{ON} decreases to as little as 0·25 with a time constant of 3·2 msec determined from composite data. Na inactivation time constants measured in the same axons ranged from 0·8 to 1·5 msec. A slow component of Q_{OFF} tends to appear as Q_{OFF}/Q_{ON} decreases.

3. Both Q_{ON} and Q_{OFF} may be decreased by a prepulse with no change in their time course. In this case the time course of the decrease is similar to that observed for I_{Na}.

4. The normalized steady-state charge immobilization curve is shifted in the depolarized direction by 15–20 mV from the normalized Na inactivation curve.

INTRODUCTION

In a variety of systems including squid giant axons (Armstrong & Bezanilla, 1974; Keynes & Rojas, 1976), myelinated nerve (Neumcke, Nonner & Stampfli, 1976), and *Myxicola* giant axons (Schauf, Bullock & Pencek, 1977; Bullock & Schauf, 1978) the total displacement current during a depolarizing pulse from a relatively large negative holding potential is slightly larger than that during an equal hyperpolarizing pulse. Comparison of these current differences (asymmetry currents) with the kinetics of Na activation suggests that such asymmetry currents may be related in some way to sodium channel gating processes (Almers, 1978), although probably not as a direct reflexion of the redistribution of charged Hodgkin–Huxley 'm' particles. (Neumcke *et al.* 1976; Armstrong & Bezanilla, 1977; Almers, 1978; Bullock & Schauf, 1978).

The latter conclusion rests partly upon detailed comparisons between the time courses of asymmetry and sodium currents (Neumcke *et al.* 1976; Schauf *et al.* 1977; Bullock & Schauf, 1978). but it is also strengthened by the observation in squid axons that the intramembrane charge appears to become immobilized during a depolarizing pulse of sufficient duration in that the ratio of the integral of asymmetry current at the end of the pulse (Q_{OFF}) to that at the start of the pulse (Q_{ON}) is less than unity (Armstrong & Bezanilla, 1977; Meves & Vogel, 1977). In a similar way depolarizing prepulses have been shown to be capable of reducing the magnitude of the charge displaced during subsequent paired test pulses (Bezanilla & Armstrong, 1974; Armstrong & Bezanilla, 1977; Meves & Vogel, 1977). However, the relationship

between the time course of these effects and the process of Na inactivation is a matter of greater uncertainty and in an attempt to clarify these issues we have made measurements of charge immobilization in *Myxicola* giant axons.

METHODS

Methods for simultaneous voltage-clamp and internal dialysis of *Myxicola* axons have been previously described (Bullock & Schauf, 1978). The only change from those procedures was to replace the $K^+ + TEA^+$ dialysate used in previous studies by a K^+-free solution containing 600 mM-Cs^+ and 50 mM-F^- (plus 550 mM-glutamate and 1 mM-Hepes) to eliminate completely currents through the K^+ channel for long duration, large amplitude depolarizations. The external solution was K^+-free artificial sea-water (Schauf *et al.* 1977) in those cases where Na^+ currents were measured, while the external Na^+ was replaced by Tris (hydroxymethyl) aminomethane with 10^{-6} M-tetrodotoxin added for the measurement of asymmetry currents. Internal and external pHs were $7{\cdot}3 \pm 0{\cdot}1$ and $7{\cdot}8 \pm 0{\cdot}1$ respectively. Temperatures ranged between 5 and 7 °C.

The method of calculation of charge movements deserves some comment. As in previous studies (Bullock & Schauf, 1978) asymmetry currents were measured by adding to the displacement currents obtained in response to a specific sequence of depolarizing pulses the displacement currents obtained during an identical sequence of pulses of opposite polarity taken from the same holding potential. This protocol yielded the same results as the use of a divided pulse procedure (Schauf *et al.* 1977) for holding potentials in excess of -100 mV.

In most cases the resulting asymmetry currents have significant rising phases (see Fig. 1 for the ON response and Fig. 3 for the OFF response). As we previously noted (Schauf *et al.* 1977), this rising phase could not be eliminated by adoption of a divided pulse procedure (Armstrong & Bezanilla, 1974). In order to calculate total charge movement it must be assumed either that this rising phase is an artifact produced by the experimental procedure or that it represents a lag in the response of the gating charge to changes in the membrane field (see Discussion). In the latter case it would be particularly critical to accurately record asymmetry currents during a rising phase which varies between axons but is typically 50–100 μsec. Our present data acquisition system is limited to a resolution of 10 μsec and it is not unusual for the displacement current during the first 20–30 μsec to saturate the amplifier (we have shown this produces no errors in later points, however). Considering that the voltage clamp requires at least 10 μsec to control membrane potential, and that large errors in integral charge could be introduced by small errors in current measurement at these early times, we do not feel confident in attempting such an analysis in *Myxicola* with our present techniques. Therefore, we have chosen to calculate integral charge movement by performing a least-squares exponential fit to the asymmetry current data at times in excess of time to peak, extrapolating the resulting curve to zero time (giving a current I_o), and using the measured time constant, τ, calculating $Q = I_o\tau$.

RESULTS

Absence of a slow component in the ON-response

The asymmetry current ON response in squid axons has recently been reported to contain two distinct temporal components (Armstrong & Bezanilla, 1977). We have carefully looked for a slow component in Cs^+ dialysed *Myxicola* axons but have failed to detect one as illustrated in Fig. 1. Here we have provided records of the asymmetry currents from several axons at different membrane potentials together with the assumed time-independent asymmetric component of the leakage conductance (baseline) and the resulting semilogarithmic plots of asymmetry current as a function of time. The best fit to the experimental data points determined by a least squares analysis clearly represents an adequate description to currents as small as 2–3 % of the peak asymmetry current and times of the order of 1 msec. In order to be unresolved the magnitude of an assumed slow component would have to be

extremely small (less than 5 %) and/or have a time constant greater than 3–4 msec, both values being well outside the range reported by Armstrong & Bezanilla (1977).

Complete inactivation of I_{Na} at positive potentials

In squid giant axons in the absence of internal Na^+ early channel inactivation is incomplete at large positive potentials (Chandler & Meves, 1970*a*, *b*; Bezanilla & Armstrong, 1977). Immobilization of the gating charge (Q) is also incomplete at such potentials (Armstrong & Bezanilla, 1977; Meves & Vogel, 1977). In order to compare these processes in *Myxicola* we first attempted to determine whether a comparable incomplete inactivation of I_{Na} occurs in this preparation under similar conditions.

Fig. 2 illustrates the results of such an experiment. Na currents were recorded in

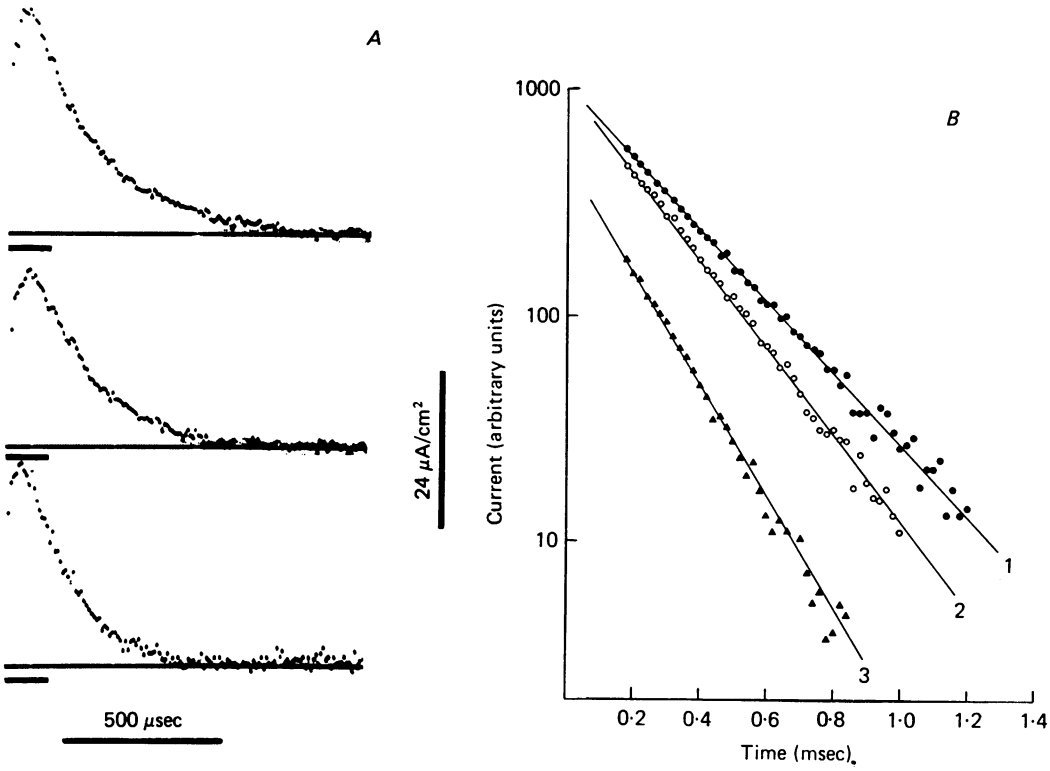

Fig. 1. *A*, asymmetry current ON responses in *Myxicola*. Top trace: sum of the displacement currents during 32 depolarizing and 32 exactly matched hyperpolarizing pulses of ± 100 mV from a holding potential of − 120 mV (axon 77M62). Middle trace: same but pulse amplitudes of ± 80 mV, holding potential − 100 mV. Bottom trace: same but 16 pulses of ± 100 mV, holding potential − 100 mV (axon 77M51). The short horizontal line indicates zero current while the longer line above it represents the baseline used to generate the plots in part *B*. Time scale is 500 μsec; current calibration is 24 μA/cm². Temperature 6 °C. Outward current is upward. *B*, semilogarithmic plots of the data in *A*. Continuous lines represent the computed least squares fit to the experimental points. Time constants are 272, 220, and 180 μsec for curves 1, 2, and 3 which correspond to the traces in part *A* taken from top to bottom. Data plotted in arbitrary units to avoid overlap. For curves 1 and 2, 100 units = 4·8 μA/cm²; for curve 3, 100 units = 19 μA/cm².

Cs^+ dialysed fibres for depolarizing voltage steps of 60–180 mV from a holding potential of −80 mV, while leakage currents were recorded for an equal series of hyperpolarizing pulses. Addition of these records gave the results shown in part *B* of the Figure. In an alternate method membrane currents were recorded on the same axons following addition of 10^{-6} M-tetrodotoxin and the corresponding records subtracted (part *A*). In neither method was there any evidence of a maintained Na conductance, the maximum possible noninactivating current consistent with the resolution of the experiment being approximately 2–3 %.

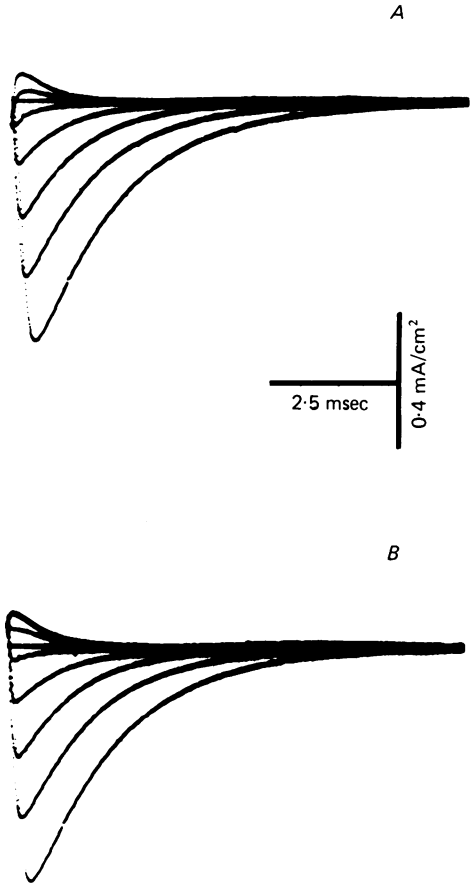

Fig. 2. Membrane Na currents in Cs^+-dialysed *Myxicola* axons. Total membrane current was measured in sea-water for a series of depolarizing pulses to between −20 and +100 mV (20 mV increments) and during equal but opposite hyperpolarizations. Addition of the corresponding records gave the result shown in *B*. Na currents were then blocked by Tris + tetrodotoxin and the total currents for depolarizing pulses remeasured. Subtraction of these records from the corresponding records obtained in sea water gave the result shown in *A*. Axon 77M51. Current and time calibrations are 0·4 mA/cm² and 2·5 msec respectively. Temperature 5·5 °C. Inward current is plotted downward.

Immobilization of intramembrane charge during sustained depolarizations

In *Myxicola* axons for pulse durations of 1 msec or less the magnitude of the charge movement measured following the termination of pulses of equal but opposite size

(Q_{OFF}) is equal to the magnitude of the charge movement (Q_{ON}) measured following the onset of the pulse (Bullock & Schauf, 1978). However, as the pulse duration is increased the magnitude of Q_{OFF} begins to decrease giving a ratio of Q_{OFF}/Q_{ON} less than unity. Figs. 3–5 summarize these data.

In Fig. 3*A* we have illustrated both ON and OFF membrane asymmetry currents at a potential of 0 mV for several pulse durations between 1 and 8 msec. In Fig. 3*B*

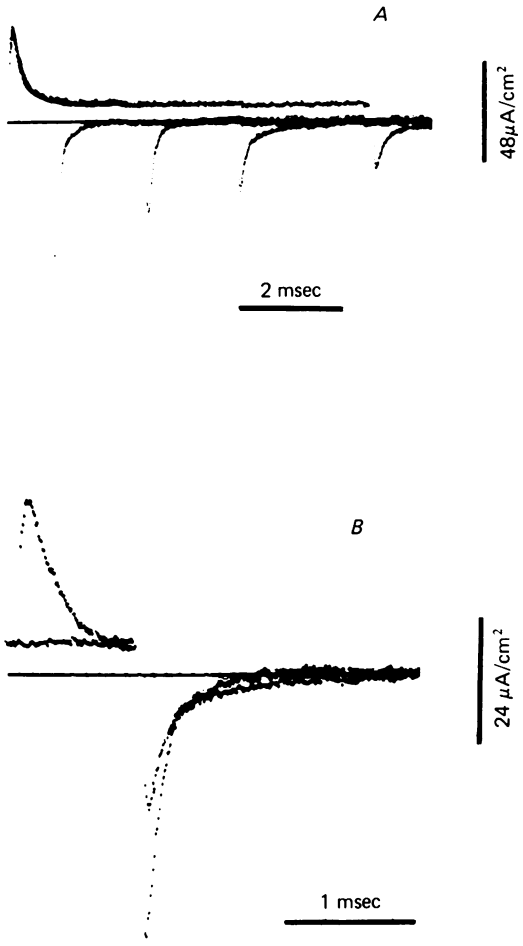

Fig. 3. In this experiment total membrane displacement currents were measured during 16 depolarizing and 16 exactly matched hyperpolarizing pulses 100 mV in amplitude from a holding potential of − 120 mV. Part *A* shows the result of adding such responses for four different pulse durations of 1, 3, 5, and 8 msec. In part *B* the OFF responses for the 1 and 5 msec duration pulses have been shifted horizontally so as to overlap and expanded both horizontally and vertically. Note that the OFF response for the 1 msec duration pulse is initially larger in amplitude and crosses over the OFF response for the 5 msec duration pulse at about 300 μsec so that for longer times the inward current following the termination of the 5 msec pulse exceeds that following the 1 msec pulse. The ON response for the 1 msec pulse is also visible as is the asymmetric component of the leakage current at the end of the 5 msec pulse. The continuous lines in both *A* and *B* represent zero current. Current and time calibrations are 48 μA/cm² and 2 msec in *A* and 24 μA/cm² and 1 msec in *B*. Axon 77M62. Temperature 6 °C.

the OFF responses for the 1 and 5 msec pulses have been shifted so that they coincide in time. As pulse duration increases, the initial amplitude of the OFF response clearly decreases. In addition, for longer durations, a slow component often appears in the OFF response. The degree to which this component can be resolved unambiguously varies from one axon to another and may change somewhat during the course of an experiment. It is most apparent when the axon is held at potentials more negative than -100 mV.

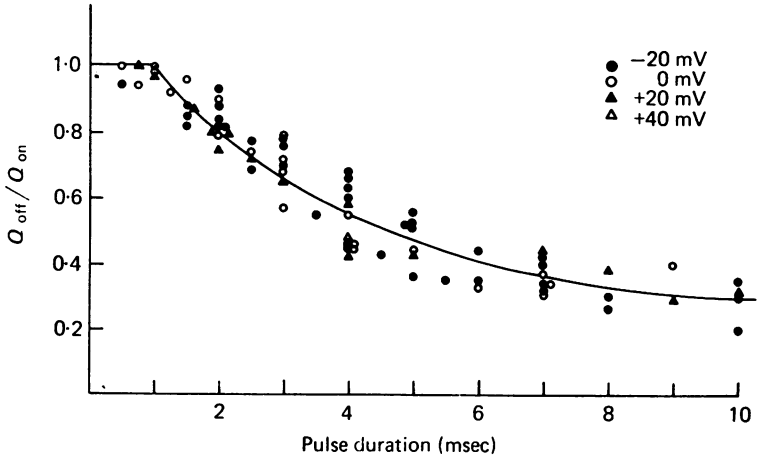

Fig. 4. Ratio of the integral of the fast component of the asymmetry current OFF response (Q_{OFF}) to the integral of the ON response (Q_{ON}) as a function of pulse duration. The integral charge movement, was calculated as $Q = I_0\tau$ where I_0 is the zero time intercept of the least-squares fit to the asymmetry current and τ is the time constant. Different symbols refer to the membrane potential during the depolarizing pulse of the various pulse pairs. Data from eight axons at several holding potentials between -120 and -80 mV. Temperature 5–7 °C. Continuous line is calculated from eqn. (1) of the text.

In Fig. 4 we have plotted the ratio Q_{OFF}/Q_{ON} as a function of pulse duration for potentials during the depolarizing step between -20 and $+40$ mV. Two kinds of analysis were performed to generate these data. For holding potentials of -80 mV a slow component is not readily apparent in the OFF response and integration was as previously described. For holding potentials of -100 and -120 mV many (but not all) records showed an obvious slow component whose contribution to charge movement was eliminated by calculating only the area under the fast component (Q^F_{OFF}) from the zero time intercept time constant in a manner similar to that used by Armstrong & Benzanilla (1977).

The time course of the decrease in Q^F_{OFF}/Q_{ON} does not seem to be significantly affected by the magnitude of the depolarizing pulse, although it should be noted that over this potential range the time constants for Na inactivation previously measured during a maintained depolarization (Goldman & Schauf, 1973) only vary by approximately a factor of 2. The data in Fig. 4, although showing a fair amount of scatter, can be described best by the expression

$$\frac{Q^F_{OFF}}{Q_{ON}} = 0{\cdot}25 + 0{\cdot}75 \exp\,(t - 1{\cdot}0)/3{\cdot}2 \qquad (1)$$

for $t > 1{\cdot}0$ msec. The time constant of 3·2 msec is substantially greater than the sodium inactivation time constants measured in these same axons which ranged from 1·5 msec (−20 mV) to 0·8 msec (+40 mV).

In squid axons, Armstrong & Bezanilla (1977) observed that with increasing pulse duration, charge movement in the slow component (measured at −140 or −150 mV) tended to increase as the magnitude of the charge movement in the fast component decreased, and that the ratio of the sum of these two components of charge movement to Q_{ON} remained near unity. Table 1 presents the result of an experiment in which our data were sufficiently good to quantitatively resolve the slow component of the OFF response at −120 mV.

TABLE 1. Components of the asymmetry current 'OFF' response in *Myxicola**

Pulse duration (msec)	$\frac{Q^F_{OFF}}{Q_{ON}}$	$\tau_F(\mu s)$	$\frac{Q^S_{OFF}}{Q_{ON}}$	$\tau_S(\mu s)$	$\frac{Q^T_{OFF}}{Q_{ON}}$
1·0	0·96	140	—	—	0·96
2·0	0·81	100	0·12	780	0·93
2·5	0·73	112	0·39	944	1·12
3·0	0·66	112	0·31	760	0·97
3·5	0·77	112	0·19	710	0·96
4·0	0·61	116	0·24	700	0·85
4·5	0·73	112	0·27	836	1·0
5·0	0·44	120	0·69	860	1·13
8·0	0·41	112	0·67	820	1·08
1·0	0·87	128	0·15	930	1·02

Average 0·99 ± 0·03

* Q^F_{OFF}, Q^S_{OFF} are respectively the integral charge movement during the fast and slow components of the OFF response calculated as the product $I_i\tau_i$ where I_F and I_S are the zero time intercepts of the asymmetry current and τ_F and τ_S are the time constants of the fast and slow component. Q^T_{OFF} is the sum of Q^F_{OFF} and Q^S_{OFF}.

In order to generate the entries in this table the asymmetry current OFF responses shown in Fig. 3*A* as well as other records from the same axon were plotted semilogarithmically and fitted by the sum of two exponentials as illustrated in Fig. 5 for the 1·0 and 5·0 msec duration pulses. The 1·0 msec OFF response (triangles) could easily be described as a single exponential with $I_0 = 89\ \mu A/cm^2$ and $\tau = 140\ \mu$sec giving a Q_{OFF} of 12·5 nC/cm². The OFF response for the 5·0 msec pulse (filled circles) clearly has at least two components. If one assumes the existence of only two processes the best fit to the data points (continuous line) between 0·8 and 1·4 msec yields $I^S_0 = 11{\cdot}1\ \mu A/cm^2$ and $\tau_S = 860\ \mu$sec, giving $Q^S_{OFF} = 9{\cdot}5$ nC/cm². Subtracting this relation from the raw data yielded the data points shown as open circles which could be described as an exponential process with $I^F_0 = 50{\cdot}6\ \mu A/cm^2$, $\tau_F = 120\ \mu$sec, and $Q^F_{OFF} = 6{\cdot}1$ nC/cm².

For the 5·0 msec pulse the sum of Q^S and Q^F is somewhat larger than Q_{OFF} for the 1·0 msec pulse but this difference may in part be due to the fact that total charge movement in the ON response was 13·8 nC/cm² compared to only 12·9 nC/cm² for the 1·0 msec pulse. Normalizing to Q_{ON} gives ratios of Q^S_{OFF}/Q_{ON} and Q^F_{OFF}/Q_{ON} of

0·69 and 0·44 respectively (Table 1; note that this represents the largest deviation of Q^{T}_{OFF}/Q_{ON} from unity).

A similar analysis was performed using seven other pulse durations between 2·0 and 8·0 msec. The values of Q^{F}_{OFF}/Q_{ON} and Q^{F}_{OFF}/Q_{ON} for each pulse duration scatter considerably, perhaps because of the difficulties inherent in separating exponentials in the presence of fairly noisy data, but the sum of Q^{F}_{OFF} and Q^{S}_{OFF} is generally nearly equal to Q_{ON} (average $0{\cdot}99 \pm 0{\cdot}03$). Also there was no systematic tendency for the time constants of the fast or slow components to vary substantially with pulse duration.

Unfortunately the scatter of the data is too great to allow analysis of the time course of the shift of charge from the rapidly to the slowly returning state for comparison with the time course of I_{Na} inactivation at the test pulse potential. Note, however, that values of τ^{S} in both the experiment shown in Table 1 and in other experiments were consistently in the range of 0·7–1·1 msec which is significantly faster than the 2–4 msec time constant for *recovery* of I_{Na} from inactivation at potentials near -100 mV (Schauf, 1976).

Immobilization of intramembrane charge by depolarizing prepulses

Consider an axon in which Q_{ON} and Q_{OFF} are measured at the onset and termination of 0·5–1·0 msec test pulses (V_1) from a holding potential V_H. If a second measurement is made in which a depolarizing pulse to a potential $V_H + V_P$ preceeds the step of magnitude V_1 and a hyperpolarizing pulse of equal duration to a potential $V_H - V_P$ preceeds the step of magnitude $-V_1$ and the interval between the end of the prepulse and beginning of the test pulse is Δt, then the magnitudes of Q_{ON} and Q_{OFF} measured during the test pulses are found to be decreased as Δt is decreased. These results are illustrated by the records shown in Fig. 6 in which the asymmetry currents in the upper trace were obtained with no prepulse and the currents in the lower record were recorded 700 μsec following the end of a 10 msec prepulse of ± 80 from a holding potential of -100 mV.

The magnitude of both Q_{ON} and Q_{OFF} are decreased during the test pulses which had been preceded by the 10 msec prepulse. This was in general due primarily to a decrease in the zero time extrapolated current, rather than to a consistent change in the time constant of the ON (or OFF) responses. Fig. 7 illustrates the comparison between the time course of the recovery of I_{Na} from a depolarizing prepulse which causes complete inactivation and the time course of the recovery of gating charge for two different holding potentials. The data shown were normalized as follows. For I_{Na} the currents obtained during a test pulse to $+10$ mV following a recovery interval Δt were divided by the value of I_{Na} obtained in the absence of a prepulse ($\Delta t = \infty$) and plotted directly. For the asymmetry currents there was incomplete inactivation even for values of Δt as short as 200 μsec and consequently the data was additionally normalized by calculating and plotting the ratio $(Q - Q^{0})/Q^{\infty} - Q^{0})$ where Q^{0} is the average residual charge movement seen with $\Delta t = 200$ μsec and Q^{∞} is the charge movement in the absence of a prepulse. Values of Q^{0} averaged 30 % of Q^{∞} as expected on the basis of eqn. (1). When analysed in this way there is seen to be a close correspondence between the recovery from inactivation of I_{Na} and the comparable recovery of Q. Note that a plot logarithmic in time was chosen because of the

presence of both fast (Schauf, 1976) and slow (Schauf, Pencek & Davis, 1976) reactivation processes in *Myxicola* and the desire to compare both with recovery of charge in a single graph.

In these experiments both the prepulse and test pulse polarity were reversed to measure asymmetry currents. It is also in principle possible to measure the test pulse asymmetry current by leaving the depolarizing prepulse unchanged and

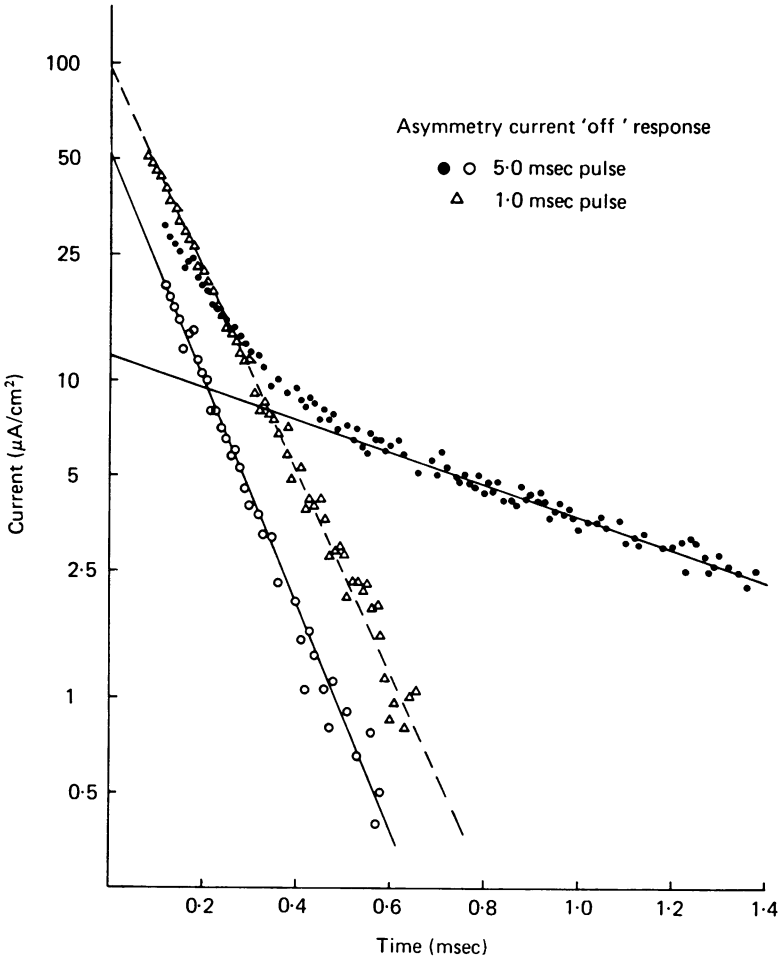

Fig. 5. Semilogarithmic plot of the OFF responses shown in Fig. 3*B*. The zero current base line was as indicated in the Figure. The trangles (△) represent the raw data points for the OFF response following the 1·0 msec pulse, while the filled circles (●) are the points following the 5·0 msec pulse. The open symbols were obtained by performing a least-squares fit to the data points (●) between 0·8 and 1·4 msec and subtracting the calculated regression relation $I(t) = I_0 \exp(-t/\tau)$. Values of I_0 and τ for each regression line were as follows: for the 1·0 msec OFF response (triangles) $I_0 = 89\ \mu A/cm^2$ and $\tau = 140\ \mu sec$; for the slow component of the 5·0 msec OFF response (filled circles) $I_0 = 11{\cdot}1\ \mu A/cm^2$ and $\tau = 860\ \mu sec$; for the fast component of the 5·0 msec OFF response (open circles) $I_0 = 50{\cdot}6\ \mu A/cm^2$ and $\tau = 120\ \mu sec$. Total charge movement for the ON response was 12·9 nC/cm² for the 1·0 msec pulse and 13·8 nC/cm² for the 5·0 msec pulse. Calculations using these figures yield the ratios listed in lines 1 and 8 of Table 1.

reversing only the polarity of the test pulse. This was attempted in several experiments, and for recovery intervals of 3 msec or greater the results were comparable. For shorter recovery intervals, however, analysis of the data was complicated by the fact that the total late displacement current in *Myxicola* is by no means a simple exponential process and time-dependent changes persist in the presence of tetrodotoxin and Cs^+ for at least 3 msec. Use of recovery intervals of 0·2 msec to 3 msec therefore results in records in which the ON and OFF responses are superimposed on a slowly decaying tail of late displacement current and are thus difficult to interpret (see Meves & Vogel, 1977, for similar observations). If one attempts to calculate Q_{ON} in these cases by subtracting a linear sloping baseline the results seem comparable, but quantitative analysis is questionable.

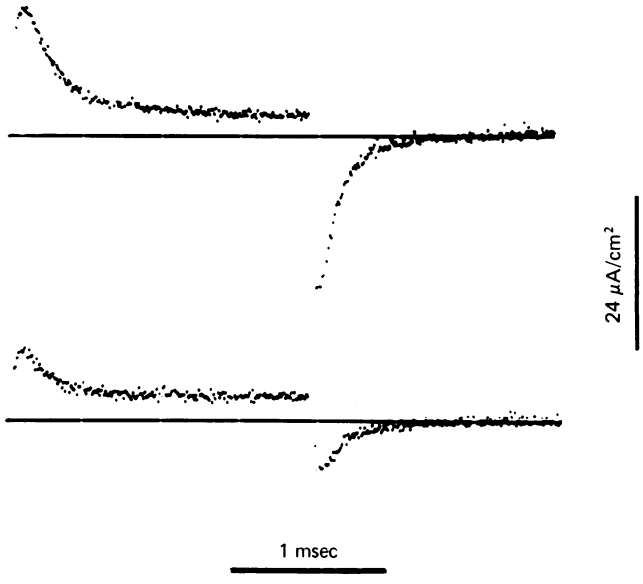

Fig. 6. Prepulse immobilization of charge movement in *Myxicola*. The asymmetry current resulting from the addition of the displacement currents during 32 depolarizing and 32 hyperpolarizing test pulses of ± 100 mV (holding potential − 100 mV) and 2 msec duration is shown in the upper trace. In the lower trace the depolarizing test pulse was preceeded by a 10 msec depolarization to − 20 mV (the end of which occurred 700 μsec prior to the beginning of the test pulse) while the hyperpolarizing test pulse was preceeded by a 10 msec hyperpolarization to − 180 mV also terminating 700 μsec before the beginning of the hyperpolarizing test pulse. Current and time scales and 24 μA/cm² and 1 msec respectively. Outward current shown upward. Axon 77M46. Temperature 5·5 °C.

Note that this protocol is analogous to the divided pulse procedure employed by Armstrong & Bezanilla (1977) in that the records taken to represent the linear component of late displacement current were obtained from a scaled version of the pulse protocol used for the total current. The scaling factor in our studies was − 1 while in those of Armstrong and Bezanilla this value was + 1/4. In addition, the linear currents were recorded at a more negative holding potential in the latter case. The assumption common to both procedures is that at sufficiently hyperpolarized

potentials, the amount of charge displaced by a step change in potential is linearly related to the magnitude of the step.

In several experiments we attempted to measure the equivalent of the steady-state I_{Na} inactivation relation as it pertains to the asymmetry currents. In these cases Δt was fixed at 700 μsec, prepulse duration was set to 50 msec, the prepulse magnitude was varied, and the effects on test pulse I_{Na} and Q determined. Both I_{Na} and Q were normalized by calculating $(I_{Na}-I'_{Na})/(I^{0}_{Na}-I'_{Na})$ and $(Q-Q')/(Q^{0}-Q')$ where I'_{Na} and Q' represent values obtained with prepulses to 0 mV and I^{0}_{Na} and Q^{0} were values obtained in the absence of a prepulse. The results are shown in Fig. 8 where the dashed line represents the curve expected from a simple 20 mV translation of the best fit to the I_{Na} inactivation curve in the depolarizing direction. It would appear that the charge inactivation data may be less steep, as well as shifted by 15–20 mV but quantitative analysis is difficult due to the considerable scatter.

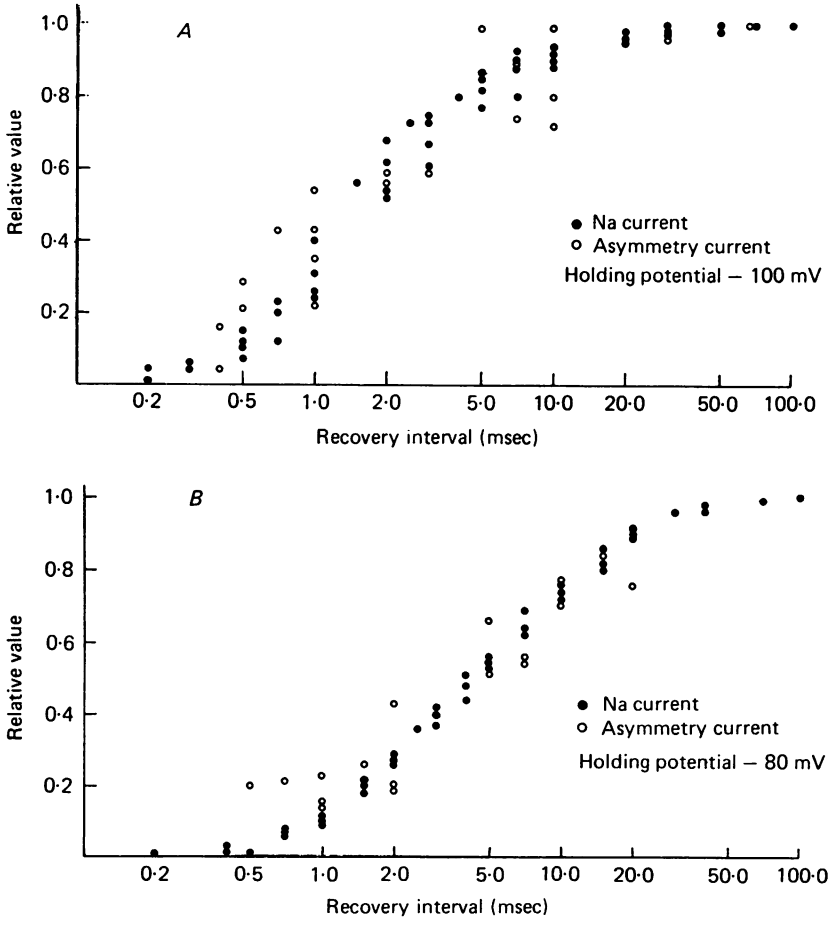

Fig. 7. Comparison between prepulse inactivation of I_{Na} and Q in several axons at two different holding potentials (*A* and *B*). Data from I_{Na} and Q are both calculated relative to the currents measured in the absence of a prepulse. Data from Q has in addition been corrected for the fact that average immobilization of Q was only 70% at recovery intervals where there was no residual I_{Na} (see text). Temperature 5·5–7 °C.

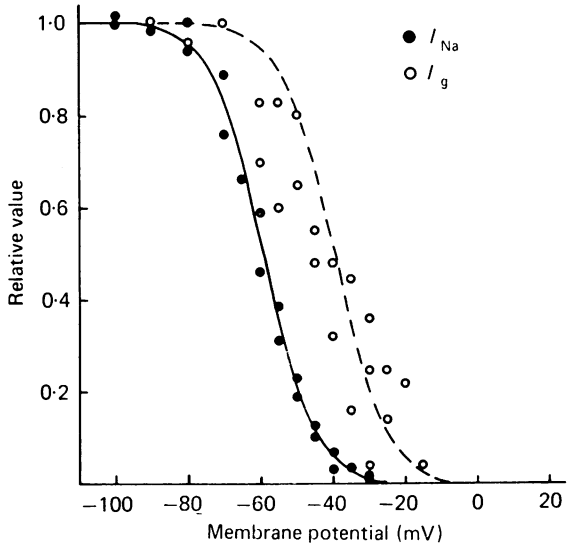

Fig. 8. Comparison between steady-state I_{Na} and Q inactivation. A test pulse to 0 mV was preceeded by a 50 msec prepulse of variable amplitude and separated from the test pulse by a 700 μsec return to the holding potential. Currents during the test pulse are plotted as a function of prepulse potential. In the case of Q the polarity of both the prepulse and test pulse was reversed to obtain asymmetry current. Inactivation of both I_{Na} and Q was incomplete due to the 700 μsec recovery interval at -100 mV and both sets of data were therefore normalized as described in the text. Temperature 5·5–7·0 °C.

DISCUSSION

We have carefully looked for, but have failed to find, any evidence for a slow component in the asymmetry current ON response in *Myxicola* axons. The data of Armstrong & Bezanilla (1977) demonstrate the presence of such a component in squid axons. The time constant of the slow component in their studies ranged from 340 μsec to 1·07 msec while its zero time intercept was 13–25 % of the initial value of the fast component (the fast component time constant ranging from 92 to 143 μsec, values comparable to those observed in *Myxicola*). A component of such magnitude should have been detectable by our procedures.

The absence of such a component in *Myxicola* may, however, not be so surprising. Armstrong & Bezanilla (1977) have interpreted the slow component of the ON response as being a result of the transition of the sodium channel between two different conducting states (x_1 and x_1y) and have supported their argument by observing that in squid the time constants of Na current tails and asymmetry current OFF responses vary with pulse duration (reflecting a transition between x_1 and x_1y). Since neither the Na current tails or asymmetry current OFF responses are appreciably sensitive to pulse duration in *Myxicola* (Schauf *et al.* 1977) it may well be that the x_1y state is either lacking or not substantially occupied at similar potentials in *Myxicola*, accounting for the lack of a slow component in the ON response.

Immobilization of charge associated with sodium channel gating processes has been studied in two laboratories. Armstrong & Bezanilla (1977) showed that Q_{OFF}

could be reduced to as little as 30 % of Q_{ON} for a 10 msec pulse and a holding potential of -70 mV where the slow component of Q_{OFF} cannot be distinguished from base line noise. The time course and voltage dependence of this reduction were found to be comparable to I_{Na} inactivation. At large negative potentials (-140 to -150 mV) Q_{OFF} could be observed to have fast and slow components (Q^F_{OFF}, Q^S_{OFF}) and the ratio $(Q^F_{OFF}+Q^S_{OFF})/Q_{ON}$ remained near unity. In separate experiments the recovery from prepulse inactivation of I_{Na} and Q were shown to have a similar time course and voltage dependence. In contrast to these experiments, Meves & Vogel (1977) found that although 50 % of Q could be immobilized, the time constant of the decrease in Q_{OFF} was 3–4 times slower than the time constant of Na^+ inactivation. Similarly, in the case of prepulse inactivation recovery of I_{Na} was substantially faster than the recovery of Q.

Our results are somewhat intermediate. During a depolarizing pulse Q_{OFF} can be decreased to 25–30 % of Q_{ON}, however the time constant of this decrease (3·2 msec) was 2–3 times slower than the time constant of the decline in I_{Na} measured at the same potentials in the same axons in qualitative agreement with the observations of Meves & Vogel (1977). We are able to confirm the Armstrong & Bezanilla (1977) observation that a slow component appears in the OFF response and increases in amplitude with increasing pulse duration in axons held at -120 mV, however, in contrast to their findings τ^S was found to be consistently smaller than the time constant for recovery of I_{Na} from inactivation. It should be noted that τ^S in our studies (0·7–1·1 msec) was consistently larger than those values (0·4–0·7 msec) observed by Armstrong & Bezanilla (1977) using more negative potentials. This causes the slow component of the OFF responses to be more difficult to resolve and may account for the increased scatter in our data.

In further agreement with Armstrong & Bezanilla (1977), the time course of the recovery of Q and I_{Na} from the effects of conditioning prepulses were found to be comparable, although the voltage dependence of these effects in *Myxicola* appeared to be significantly different for I_{Na} and Q.

In summary then the qualitative observations that both ON and OFF components of asymmetry current during a short test pulse can be reduced in magnitude by application of a depolarizing prepulse, and that as the duration of test pulses increases the magnitude of the fast component of Q_{OFF} decreases, appear to be confirmed by *Myxicola*. In addition, we have previously described Bullock & Schauf, 1978) a third method of immobilization closely correlated with slow sodium inactivation which is perhaps related to the immobilization produced by prolonged depolarizations in squid axons (Bezanilla & Armstrong, 1974; Meves & Vogel, 1977). Thus, all of the various procedures which result in the inactivation of I_{Na} appear to be associated with some type of immobilization of that charge redistribution producing the measured asymmetry currents. If these currents are in turn associated with the activation of Na channels, then inactivation may be viewed as holding the gating charge in the 'open' position, even though the channels are functionally 'closed' by means of the same process. Such a specific kind of interaction between activation and inactivation is by no means to be expected on the basis of any other evidence and so constitutes a major clue as to the molecular mechanism underlying the operation of the channel. The lack of detailed correspondence of the steady state

and kinetic characteristics of I_{Na} inactivation and immobilization is not surprising in view of the similar discrepancies which exist for activation, and serves to rule out only the simplest kind of relationships between them.

In comparing the studies presented here with those reported in other systems it should be noted that some quantitative differences may be related to the way in which such data are obtained. Asymmetry currents constitute only a small fraction of the total late displacement current and are isolated by subtracting from the total an appropriately scaled displacement current recorded in a potential range where it is assumed that there is no nonlinear charge movement. The region between -100 and -220 mV was utilized in our studies, whereas the corresponding region in studies using the divided pulse technique was generally between -130 and -170 mV.

Measurements of the quantity of intramembrane charge using these two techniques should differ at most by the small amount of nonlinear charge movement which may occur between -130 and -100 mV. The major advantage of the divided pulse procedure is that for some experimental protocols the 'equal and opposite' procedure would require hyperpolarizing the axon to potentials which it could not tolerate. Thus, it was not possible in our studies to investigate recovery from immobilization at potentials more negative than -120 mV.

Also related to the choice of experimental protocol is the question of the initial rising phase of the asymmetry current (apparent in Fig. 3). Because they found the rising phase to be less pronounced at more negative holding potentials using a divided pulse procedure, Armstrong & Bezanilla (1974) hypothesized that this feature of asymmetry currents had its origin in nonlinear charge movement during the hyperpolarizing pulse. Assuming the rising phase largely results from such a charge movement, the subsequent falling phase should still represent effects of the depolarizing pulse alone at times much longer than the time constant of the rising phase. Therefore, extrapolated exponential fits to the falling phase could be taken to represent charge movement during the depolarizing pulse corrected for any opposite going charge movement during hyperpolarization. This procedure has been followed by most investigators using the equal and opposite procedure (Keynes & Rojas, 1976; Meves & Vogel, 1977).

However, this hypothesis is not entirely adequate to explain the rising phase since it is generally observed that not only is there a rising phase in the OFF response but some rising phase persists even using the divided pulse procedure. Because of the possibility that the residual rising phase may be significant several laboratories (Armstrong & Bezanilla, 1977; Meves & Vogel, 1977) have also felt justified in integrating the asymmetry currents more or less directly in spite of some practical problems in doing so (see Methods). It should be noted that data at very early times have been generally ignored in such studies. The systematic deviation between values of charge obtained in this way and those obtained by exponential extrapolation would not be expected to be large; however, the question of which more nearly represents charge movement during depolarization remains open.

This work was supported by Career Development Award 1K04NS00004 to C.L.S., by the Morris Multiple Sclerosis Research Funds, and by training grants GM780 and GM2037 awarded to the Department of Biophysics and Theoretical Biology of the University of Chicago (J.B.).

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