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THE TIME COURSE OF SODIUM INACTIVATION IN SQUID GIANT AXONS

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SUMMARY

1. The time course of Na inactivation was studied in intact and perfused squid giant axons under voltage-clamp conditions.

2. The pulse programme consisted of a conditioning pulse of varying duration, followed after an interval of 3–8 msec by a test pulse. The measurements were done in sea water with 1/3 or 1/5 of the normal Na concentration. In most experiments a 100 μsec conditioning pulse was sufficient to reduce $I_{\text{Na peak}}$ elicited by the test pulse. In some experiments even a 50 μsec conditioning pulse produced a clear reversible decrease of $I_{\text{Na peak}}$. We conclude that the upper limit for an initial delay in the development of inactivation is 50–100 μsec; this applies to temperatures between 0 and 13 °C and membrane potentials between -40 and 15 mV. The decrease of $I_{\text{Na peak}}$ with increasing duration of the conditioning pulse was consistent with an exponential decay starting at 50 or 100 μsec.

3. With large Na currents in full Na sea water the time course of inactivation became sigmoid. This is attributed to a long-lasting tail of inward current which follows the conditioning pulse and produces a voltage drop across the series resistance.

4. If the conditioning pulse and the test pulse were not separated by an interval, $I_{\text{Na peak}}$ showed a sigmoid dependence on the duration of the conditioning pulse. This phenomenon is predicted by the equations of Hodgkin & Huxley (1952) as first pointed out by Kniffki, Siemen & Vogel (1978). With sufficiently strong conditioning pulses $I_{\text{Na peak}}$ could even increase in size.

INTRODUCTION

This paper is concerned with the question whether the time course of inactivation is exponential or sigmoid. Hodgkin & Huxley (1952) showed that, upon depolarization, inactivation develops along an exponential time course without an obvious initial delay. This was confirmed by Chandler, Hodgkin & Meves (1965) on perfused squid axons. However, a sigmoid time course with a pronounced initial delay was found by Goldman & Schauf (1972) on *Myxicola* axons and by Bezanilla & Armstrong (1977) on perfused squid axons. The question whether the time course of inactivation is exponential or sigmoid is related to the question whether activation and inactivation are independent or coupled processes (see Meves, 1978, for review).

Recently, Kniffki, Siemen & Vogel (1978) pointed out that measurements with a prepulse followed *immediately* by a test pulse can lead to an *apparently* sigmoid

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time course of inactivation, a phenomenon predicted by the equations of Hodgkin & Huxley (1952). We began our experiments by confirming the work of Kniffki *et al.* (1978). Soon it became obvious that another source of error which may cause an *apparently* sigmoid time course of inactivation is the voltage drop across the resistance in series to the membrane (Hodgkin, Huxley & Katz, 1952; Taylor, Moore & Cole, 1960; Chandler & Meves, 1970*b*). If both errors are avoided, the time course of inactivation (measured at 0–13 °C) is exponential, at least for times greater than 100 μsec.

METHODS

The experiments were done on giant axons of *Loligo forbesi* with diameters between 600 and 1000 μm.

The axons were perfused by the method of Baker, Hodgkin & Shaw (1962) and, if excitable, mounted horizontally in a Perspex chamber. The chamber was 20 mm long, 3·5 mm wide and 4·5 mm deep and contained artificial sea water. Its temperature was kept constant at a value between 0 and 13 °C by an appropriate cooling device and was measured by a small thermistor.

The voltage clamp amplifier was similar in design to that described by Bezanilla, Rojas & Taylor (1970). Compensated feed-back was employed in order to reduce the error produced by the resistance in series to the membrane; we estimate that we were able to compensate at least 70% of the series resistance. The internal electrode consisted of a 100 μm glass capillary for measuring the potential and a 70 μm Pt wire for passing current, attached to the capillary. The 100 μm glass capillary was filled with 0·6 M-KCl and contained a 20 μm bright Pt wire. The external 70 μm Pt wire was insulated except for the distal 12 mm which were carefully cleaned and platinized. The external current electrode consisted of a pair of platinized Pt plates which were connected electrically and kept at virtual ground potential. The width of each plate was 2 mm. Two further pairs of platinized Pt plates served as guards.

The membrane was held usually at a potential equal to the resting potential, i.e. the sending of holding current was avoided. Voltage clamp currents were photographed occasionally on 35 mm film. In most experiments the currents were recorded on-line with a PDP 11 computer (Digital Equipment Corporation, Maynard, Mass.) and stored on cartridge disks (see Kimura & Meves, 1979).

In most experiments, sea water with 1/3 or 1/5 of the normal Na concentration was used in order to reduce the Na current and hence the voltage drop across the series resistance. It was obtained by mixing Na artificial sea-water (470 mM-NaCl, 11 mM-$CaCl_2$, 55 mM-$MgCl_2$, 5 mM-Tris HCl buffer, pH 7·5) and Tris artificial sea-water (524 mM-Tris base, 11 mM-$CaCl_2$, 55 mM-$MgCl_2$, HCl added to give a pH of 7·5 at room temperature). The internal solution was 218 mM-KF + 54 mM-tetraethylammonium chloride + sucrose; it was obtained by mixing isotonic solutions of KF (0·6 M), tetraethylammonium chloride (0·6 M) and sucrose (1 molal).

RESULTS

An apparent initial delay

Figs. 1 and 2 illustrate an experiment which was done in the simplest possible way, namely on an intact axon in full Na sea water without computer, using only oscilloscope and camera; to reduce the K currents drastically 15 mM-4-aminopyridine were added to the external solution (Yeh, Oxford, Wu & Narahashi, 1976; Meves & Pichon, 1977). The pulse programme (Fig. 2*A*) consisted of two pulses, a conditioning pulse and a test pulse, separated by a 5 msec gap. The duration of the conditioning pulse (Δt) was 0, 100 and 200 μsec in *A*, *B* and *C*, respectively. The peak Na current was slightly smaller in *B* and *C* than in *A* (see numerical values in Fig. 1), suggesting that a 100 or 200 μsec pulse to -30 mV is sufficient to produce some inactivation of

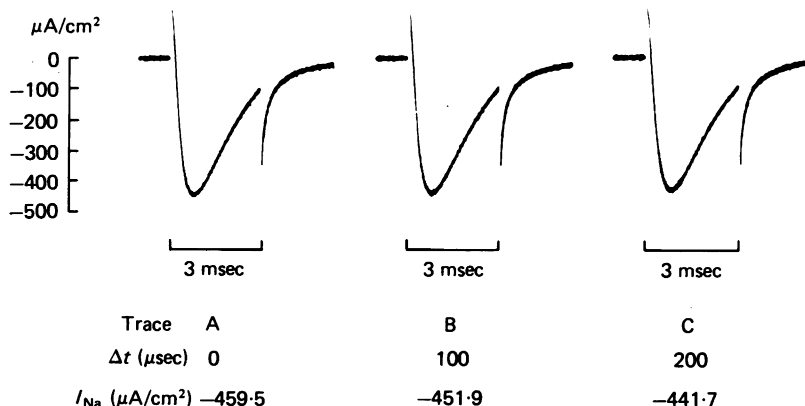

Fig. 1. Effect of a short-lasting conditioning pulse on the Na inward current associated with a test pulse to 0 mV. Pulse programme shown in Fig. 2*A*. Δt = duration of conditioning pulse = 0 μsec in *A*, 100 μsec in *B*, 200 μsec in *C*. Intact axon in full Na sea-water with 15 mM-4-aminopyridine. Currents recorded with oscilloscope and camera. Axon diameter 784 μm, temperature 5·4 °C.

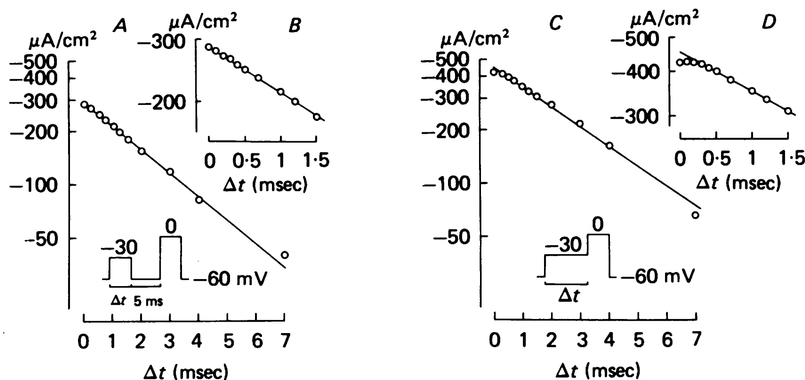

Fig. 2. *A*, quantitative analysis of the experiment in Fig. 1. Ordinate: $I_{Na\ peak} - I_{\infty}$. I_{∞} is the peak Na current measured with Δt = 25 msec. $I_{Na\ peak}$ and I_{∞} were corrected for the tetrodotoxin-insensitive current which was recorded after adding 1 μM-tetrodotoxin to the external solution. Abscissa: Δt = duration of conditioning pulse. Straight line fitted by eye with τ_h = 3·28 msec. Inset shows pulse programme. *B*, same plot (with additional points and expanded ordinate and abscissa scale) for early times. *C*, measurements on same fibre with a different pulse programme (no gap between conditioning pulse and test pulse, see inset). Straight line fitted by eye with τ_h = 3·90 msec. *D*, same plot for early times.

the Na permeability. In conclusion, any initial delay of the inactivation process must be shorter than 100 μsec (at −30 mV and 5·4 °C).

The measurements shown in Fig. 1 were followed by similar measurements in the presence of 1 μM-tetrodotoxin and the peak Na currents were corrected for the tetrodotoxin-insensitive current. The peak Na current obtained with Δt = 25 msec (I_{∞}) was subtracted and the difference ($I_{Na\ peak} - I_{\infty}$) was plotted on a logarithmic scale against Δt. As shown in Fig. 2*A*, the points follow an exponential time course

with a time constant $\tau_h = 3{\cdot}28$ msec. The points measured with the shortest Δt values (100, 200, 300 μsec) are also well fitted by this time constant (see plot on expanded scale in Fig. 2*B*).

The measurements were repeated without gap between conditioning pulse and

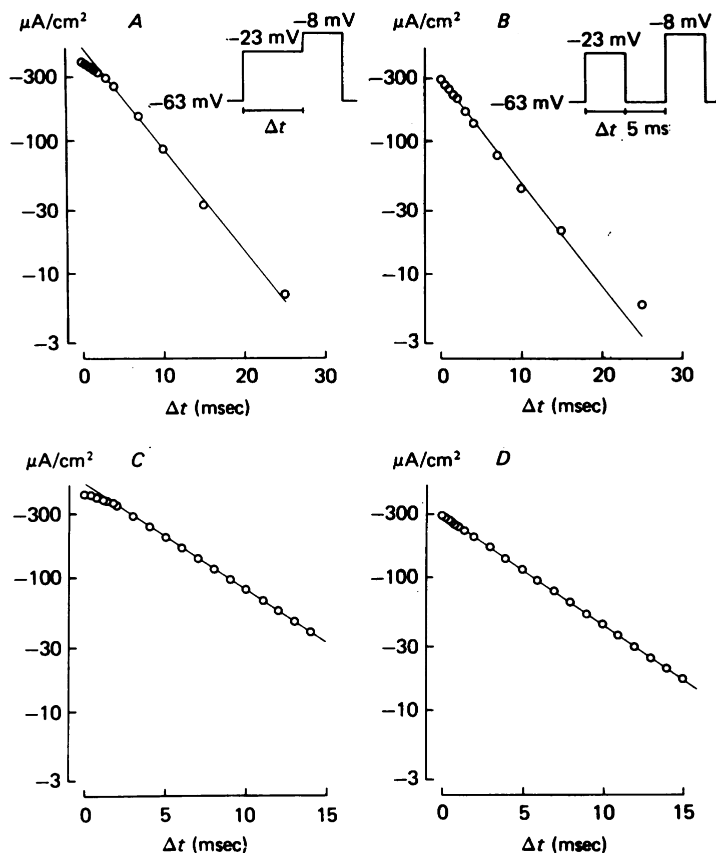

Fig. 3. Repeat of the experiment of Figs. 1 and 2 on a perfused axon and simulation of the results by the equations of Hodgkin & Huxley (1952). Ordinate: $I_{Na\ peak} - I_{\infty}$ (as in Fig. 2, but I_{∞} determined with Δt = 45 msec in *A*, *B* and with Δt = 42 msec in *C*, *D*). Abscissa: Δt = duration of conditioning pulse. *A*, *B*: measurements on a perfused axon (diameter 882 μm, temperature 3·8 °C, 1/3 Na sea-water) with the pulse programmes shown; straight lines drawn with τ_h = 5·74 msec in *A* and τ_h = 5·55 msec in *B*. *C*, *D*: calculations for the same two pulse programmes from the equations of Hodgkin & Huxley (1952) with $\bar{g}_{Na}$ = 50 mmho/cm², V_{Na} = 60 mV and 3·8 °C; straight lines drawn with τ_h = 5·30 msec in *C* and *D*.

test pulse (Fig. 2*C*, *D*). Under these conditions the values for $(I_{Na\,peak} - I_{\infty})$ at $\Delta t = 0$ and $\Delta t = 200$ μsec are identical (424·2 μA/cm²) and clearly below the straight line (drawn with $\tau_h = 3{\cdot}90$ msec) which fits the points for $\Delta t \geqslant 300$ μsec. Thus, there is an apparent delay of 200–300 μsec in the development of inactivation.

Similar results were obtained when the experiment was repeated on a perfused axon immersed in 1/3 Na sea-water (Fig. 3*A*, *B*). The currents were recorded by

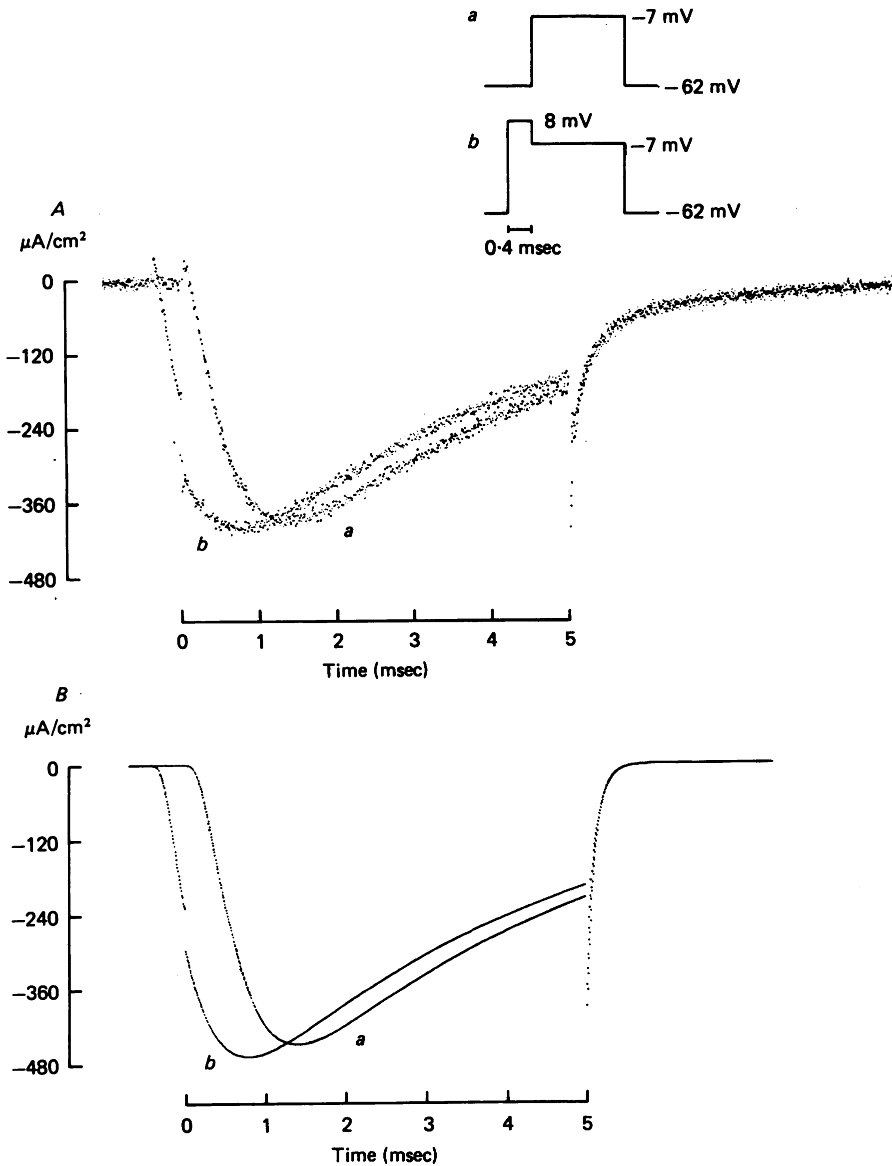

Fig. 4. Increase of $I_{Na\ peak}$ by a strong conditioning pulse. Pulse programme shown on top of Figure: *a*, control without conditioning pulse; *b*, strong conditioning pulse (Δt = 0·4 msec) to 8 mV followed by test pulse to −7 mV. *A*: experimental records (axon diameter 735 μm, temperature 4·1 °C, 1/3 Na sea water, tetrodotoxin-insensitive current subtracted). $I_{Na\ peak}$ 387·0 μA/cm² in *a*, 398·1 μA/cm² in *b*. Time to peak (measured from beginning of test pulse) 1·36 msec in *a*, 0·76 msec in *b*. *B*: calculations for the same pulse programmes from the equations of Hodgkin & Huxley (1952) with $\bar{g}_{Na}$ = 50 mmho/cm², V_{Na} = 60 mV and 3·8 °C. $I_{Na\ peak}$ 448 μA/cm² in *a*, 467 μA/cm² in *b*. Time to peak (measured from beginning of test pulse) 1·33 msec in *a*, 0·71 msec in *b*.

computer. Without a gap the early points in the semilogarithmic plot deviate from the straight line (Fig. 3A); with a 5 msec gap all points (and additional points at Δt = 100, 200, 300, 400 μsec) are well fitted by the straight line (Fig. 3B). This effect

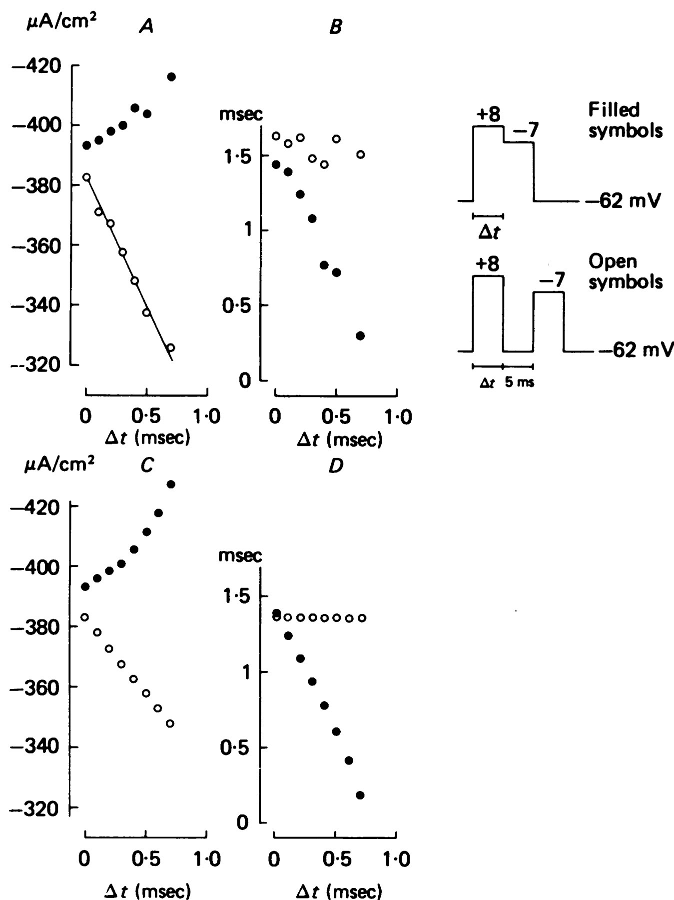

Fig. 5. Continuation of the experiment of Fig. 4. Pulse programmes shown in upper right corner: conditioning pulse to 8 mV immediately followed by test pulse to −7 mV (as in Fig. 4) or conditioning pulse and test pulse separated by 5 msec gap; measurements with the two pulse programmes indicated by filled and open symbols, respectively. A, B: experimental results showing $I_{Na\,peak}$ (A) and time to peak (B) as a function of Δt (= duration of conditioning pulse); curve in A drawn according to the equation

$$I_{Na\,peak} = (I_0 - I_\infty)\exp(-t/\tau_h) + I_\infty$$

with I_0 = 383 μA/cm², I_∞ = 128 μA/cm² and τ_h = 2·8 msec. C, D: $I_{Na\,peak}$ (C) and time to peak (D) calculated from the equations of Hodgkin & Huxley (1952) as in Fig. 4B.

could be simulated by a calculation from the Hodgkin & Huxley (1952) equations (Fig. 3C, D). We used the α_h and β_h values from Hodgkin & Huxley (1952) divided by 3 to account for the effect of internal fluoride and then scaled from 6·3 to 3·8 °C with a Q_{10} of 3.

Without a gap between conditioning pulse and test pulse $I_{Na\,peak}$ (elicited by

the test pulse) could not only exhibit a delayed *decrease*, but with a sufficiently strong conditioning pulse did actually *increase* in size (Figs. 4 and 5). This is shown by records *a* and *b* in Fig. 4*A* which were obtained with the test pulse alone (*a*) and with the test pulse preceded by a 0·4 msec conditioning pulse to 8 mV (*b*). In *b* the peak occurs earlier and is slightly larger than in *a* (see numerical values in legend of Fig. 4). This phenomenon could again be simulated by a calculation from the Hodgkin & Huxley equations (Fig. 4*B*) modified as described in the preceding paragraph.

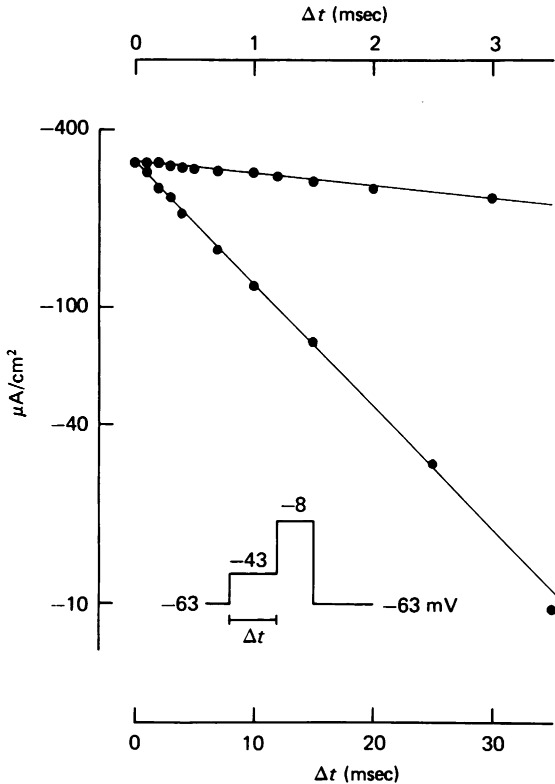

Fig. 6. Continuation of the experiment of Fig. 3. As in Fig. 3*A* the conditioning pulse is immediately followed by the test pulse; but the conditioning pulse is smaller than in Fig. 3. Ordinate and abscissa as in Fig. 3; I_{∞} determined with Δt = 45 msec. Points plotted against Δt (lower scale) and fitted by a straight line with τ_h = 10·55 msec. Points for Δt = 0–3 msec replotted on expanded scale (upper scale) and again fitted by a straight line with τ_h = 10·55 msec.

The experiment of Fig. 4 was repeated with Δt, the duration of the conditioning pulse, varying between 0 and 1 msec. The results are shown by the filled symbols in Fig. 5*A*, *B*: the longer Δt, the more pronounced the increase of $I_{\text{Na peak}}$ (*A*) and the shortening of the time to peak (*B*). Calculations from the Hodgkin & Huxley equations (filled symbols in Fig. 5*C*, *D*) give the same dependence of $I_{\text{Na peak}}$ (*C*) and time to peak (*D*) on Δt.

Finally, the conditioning pulse and the test pulse were separated by a gap of 5 msec. Again, Δt was varied between 0 and 1 msec. The results (represented by the open

symbols in Fig. 5) show that $I_{\text{Na peak}}$ (A) decreases steeply with increasing Δt whereas the time to peak (B) stays constant. Similar results are obtained from the Hodgkin & Huxley equations (open symbols in Fig. 5C, D).

The experiments so far described demonstrate that a pulse programme consisting of a conditioning pulse immediately followed by a test pulse causes a delay in the decrease of $I_{\text{Na peak}}$ or even an increase of $I_{\text{Na peak}}$. Fig. 6 shows that no such effect occurs when the conditioning pulse is small, e.g. from -63 to -43 mV. Points plotted against Δt (lower scale) are well fitted by a straight line with $\tau_h = 10{\cdot}55$ msec. Points for $\Delta t = 0 - 3$ msec replotted on an expanded scale (upper scale) could again be fitted by a straight line of the same slope.

Another way to obtain an apparently sigmoid time course

As illustrated by Figs. 1–6 the time course of inactivation is exponential, provided there is an interval between conditioning pulse and test pulse which allows the activation variable m to return to its resting value. However, this statement must be qualified: when the Na currents are large a clear deviation from the exponential time course is seen even with intervals of 5 msec or greater. Fig. 7 shows an experiment with full external Na (A) and 1/3 Na (B) on the same fibre. The points in B are well fitted by an exponential equation ($\tau_h = 5{\cdot}75$ msec) whereas the points in A are not. The points in A *appear* to follow a time course which consists of an initial shoulder and a subsequent faster decay. It can be seen, however, that the points at $\Delta t = 0 - 0{\cdot}5$ msec and at $\Delta t = 15 - 45$ msec lie on the curve drawn with $\tau_h = 5{\cdot}75$ msec (as found for the points in B). The deviation from this curve begins at $\Delta t >$ 0·5 msec. At the same time a slow inward tail, following the conditioning pulse and not completely decaying to zero during the 5 msec interval, became conspicuous (see Fig. 9 which will be described later). We think that the voltage drop of this inward current across the series resistance shifts the steady-state inactivation curve to more negative internal potentials (cf. shift of activation curve in Fig. 13 of Chandler & Meves, 1970*b*) so that $I_{\text{Na peak}}$ (elicited by the test pulse) becomes smaller. This explanation is supported by the finding that reduction of the inward current by reduction of the external Na (or by a more positive holding potential) leads to an almost perfect exponential time course (Fig. 7B). However, closer inspection of Fig. 7B shows that there is still a small deviation for the two points at $\Delta t = 3$ and 4 msec.

Results similar to those in Fig. 7 were obtained from the Hodgkin & Huxley equations and are shown in Fig. 8. The points in B were calculated for a small Na current and a small series resistance and are well fitted by an exponential equation with a time constant $\tau_h = 2{\cdot}38$ msec. The points in A were calculated for a 5 times larger Na current and a 5 times larger series resistance and follow a sigmoid time course similar to that in Fig. 7A, consisting of an initial shoulder and a subsequent faster decay. With a 5 times larger Na current and a 3 times larger series resistance the deviation from the exponential time course was also present but much less pronounced. The pulse programme used for the calculations in Fig. 8 is similar to that used for the experiment in Fig. 7. The only major difference is in the gap length: 5 msec in Fig. 7, 2 msec in Fig. 8. For the calculations the gap length had to be drastically shortened because the Hodgkin & Huxley equations do not predict the slow tail of inward current which is seen experimentally.

The long-lasting tail of inward current is illustrated in Fig. 9 for a fibre in 1/3 Na sea-water at 10·6 °C. The record shows the current during a 1 msec depolarizing pulse to +7 mV and during repolarization to −93 mV; similar records were obtained for repolarizations to potentials between −70 and −100 mV. At 2·0 msec after the end of the depolarizing pulse the slow component of the tail has decayed to half its original value (−70 μA/cm^2), suggesting a time constant of 2·9 msec at 10·6 °C. (A slow tail following a 5 msec depolarizing pulse in 1/3 Na

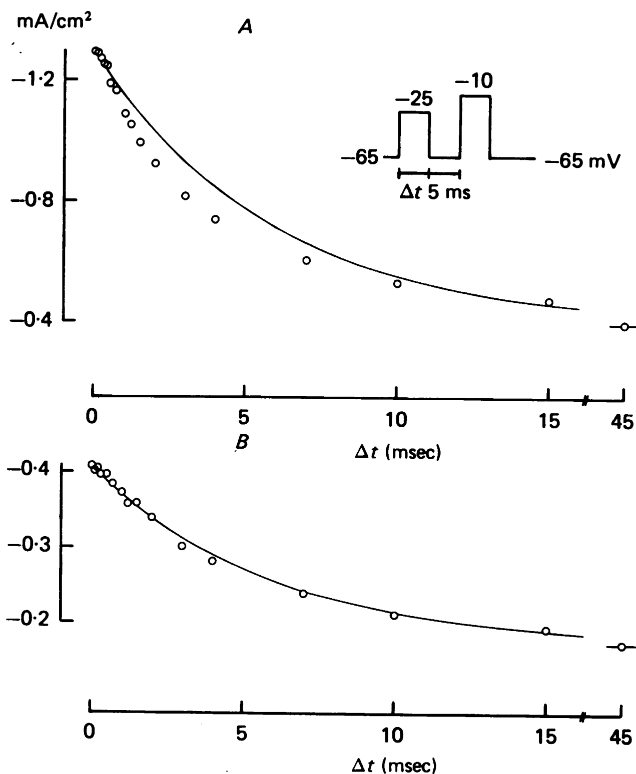

Fig. 7. Measurement of the time course of inactivation in full external Na (*A*) and in 1/3 Na (*B*) on the same fibre. Pulse programme see inset in *A*; interval between conditioning pulse and test pulse 5 msec. Ordinate: $I_{\mathrm{Na\,peak}}$ (elicited by test pulse) on a linear scale. Abscissa: Δt = duration of conditioning pulse. Currents in *B* (but not in *A*) were corrected for the tetrodotoxin-insensitive current which was recorded after adding 1 μM-tetrodotoxin to 1/3 Na sea-water. The points in *B* were fitted by the equation

$$I = (0{\cdot}409-0{\cdot}173)\exp(-\Delta t/5{\cdot}75)+0{\cdot}173 \qquad (\mathrm{mA/cm^2})$$

In *A*, the equation

$$I = (1{\cdot}294-0{\cdot}397)\exp(-\Delta t/5{\cdot}75)+0{\cdot}397 \qquad (\mathrm{mA/cm^2})$$

fits only the points at Δt = 0–0·5 msec and at Δt = 15–45 msec. Axon diameter 931 μm, temperature 3·8 °C.

sea-water at 4·1 °C is visible in Fig. 4*A* but not in the calculated Fig. 4*B*). The series resistance in 1/3 Na sea-water was 20 Ω cm^2 (determined in current clamp) and we think that at least 70 % of that was compensated. In 1/3 Na sea-water the voltage drop of the tail current across the uncompensated part of the series resistance will be small and will cause only a small shift of the curves relating permeability parameters to membrane potential, but the 3 times larger shift in full Na sea-water could have a clear effect, especially in the steep region of the inactivation or activation curve.

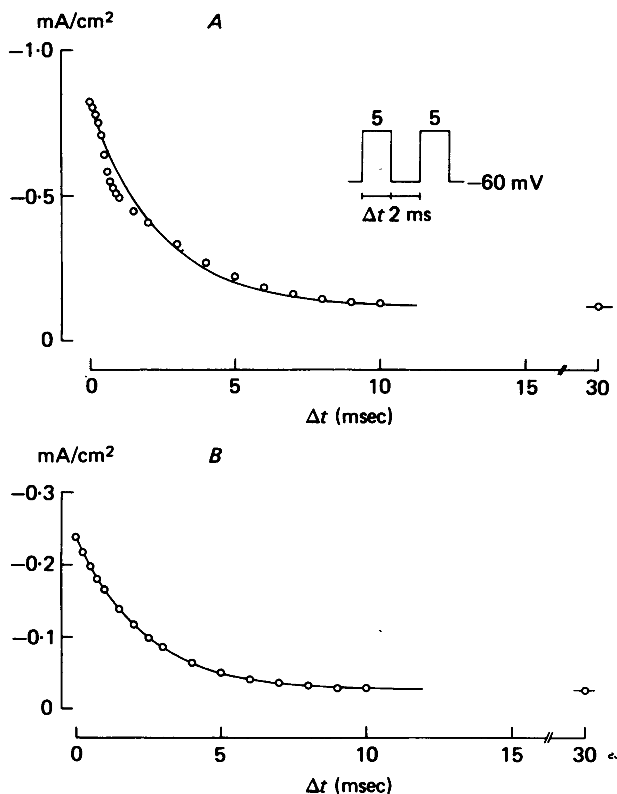

Fig. 8. Calculation of the time course of inactivation for a large Na current and a large series resistance (*A*) and for a small Na current and a small series resistance (*B*). Pulse programme see inset in *A*. Ordinate and abscissa as in Fig. 7. Na currents calculated from the equations of Hodgkin & Huxley (1952) by a modification of the Runge-Kutta method (Gill, 1951), using $V_{Na} = 55$ mV, temperature 0 °C, $Q_{10} = 2{\cdot}34$ for τ_m and $Q_{10} = 3{\cdot}79$ for τ_h (see Kimura & Meves, 1979). *A*: $\bar{g}_{Na} = 150$ mmho/cm², series resistance $R_S = 25\ \Omega\ \mathrm{cm}^2$. *B*: $\bar{g}_{Na} = 30$ mmho/cm², $R_S = 5\ \Omega\ \mathrm{cm}^2$. Curves drawn from the equations

$$I = (0{\cdot}825+0{\cdot}117)\exp(-\Delta t/2{\cdot}38)+0{\cdot}117 \qquad [\mathrm{mA/cm^2}]$$

and

$$I = (0{\cdot}238+0{\cdot}025)\exp(-\Delta t/2{\cdot}38)+0{\cdot}025 \qquad [\mathrm{mA/cm^2}]$$

in *A* and *B*, respectively. 2·38 msec is the value of the time constant τ_h calculated from Hodgkin & Huxley for a potential of 5 mV and a temperature of 0 °C with a Q_{10} of 3·79.

The presence of a long-lasting tail of inward current which has also been observed in *Myxicola* axons (Goldman & Hahin, 1978) might be the result of an uncompensated part of the series resistance. The configuration of the tail current is very sensitive to series resistance (see Fig. 3 of Ramón, Anderson, Joyner & Moore, 1975); observations reported on page 302 indicate that even in 1/5 Na sea-water the effect of series resistance is not negligible. However, when the Na current was reduced even further by using 1/10 Na sea-water, a small but distinguishable slow component could still be observed. It seems unlikely that in this case the slow component is due to an uncompensated part of the series resistance.

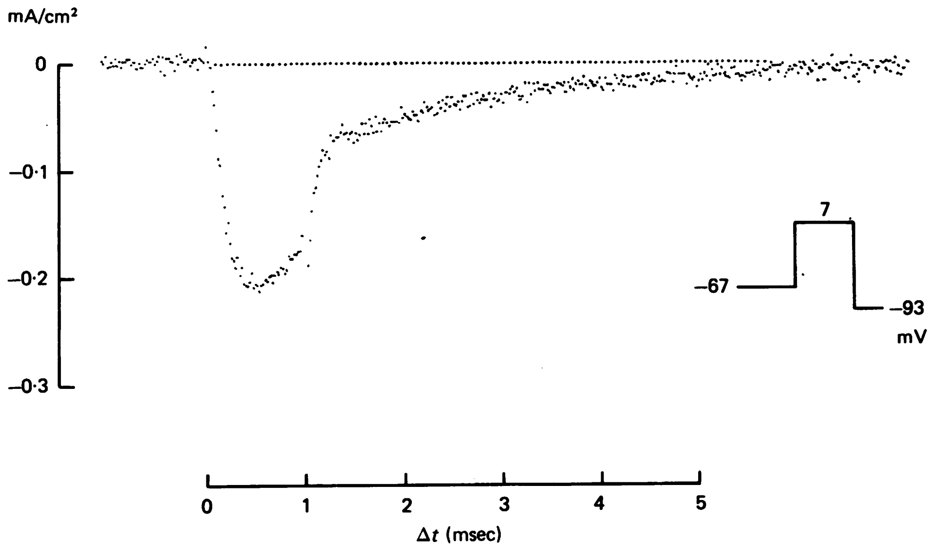

Fig. 9. Long-lasting tail of inward current in 1/3 Na sea-water. Pulse programme: holding potential − 67 mV, 1 msec depolarizing pulse to + 7 mV, repolarization to − 93 mV. Tetrodotoxin-insensitive current subtracted. Axon diameter 711 μm, temperature 10·6 °C.

Search for a real initial delay

The experiments so far described show (*a*) that $I_{\text{Na peak}}$ at $\Delta t = 100\,\mu$sec is smaller than $I_{\text{Na peak}}$ at $\Delta t = 0$ (Figs. 1, 2, 5*A*, 7*B*) and (*b*) that the decrease is approximately equal to the decrease expected for an exponential time course of inactivation (Figs. 2*B* and 5*A*). The experiments also show that the requirements for studying the time course of inactivation are (1) a pulse programme which consists of a conditioning pulse and a test pulse separated by a sufficiently long gap, (2) an external Na concentration which is small enough to reduce the error produced by the series resistance to a minimum.

In further experiments Δt was shortened to 50 μsec and the external Na concentration was reduced to 1/5 of its normal value. To improve the signal to noise ratio the currents were averaged (usually 5 sweeps). In order to distinguish between decrease due to inactivation and decrease due to run down we took control records with $\Delta t = 0$ before and after each record ('bracketing' procedure). A typical experiment consisted of 20–30 traces (each averaged from 5 sweeps) with $\Delta t = 0, 50\,\mu$sec, 0, 100 μsec, 0, 200 μsec, 0...0,30 msec, 0.

Original records from two such experiments are shown in Figs. 10*A*, *B* and 11. In Fig. 10*A* only the first and the last control records ($\Delta t = 0$) are shown, those in between are omitted. Fig. 11 shows the first seven traces of an experiment, including the interposed control records.

The peaks of the Na currents in Fig. 10*A* decay in an approximately exponential fashion with increasing Δt. The control record ($\Delta t = 0$) at the end shows a slightly

smaller Na current than the control record at the beginning (note also the end of the slow inward tail following the conditioning pulse, most conspicuous in record labelled $\Delta t = 2 \cdot 5$ msec). Records for small values of Δt in Fig. 10*B* demonstrate a small decrease for $\Delta t = 100\,\mu$sec but not for $\Delta t = 50\,\mu$sec; this was confirmed by comparison with the interposed control records (not shown). The experiment in Fig. 11, however, which was done at a somewhat higher temperature and with a shorter gap

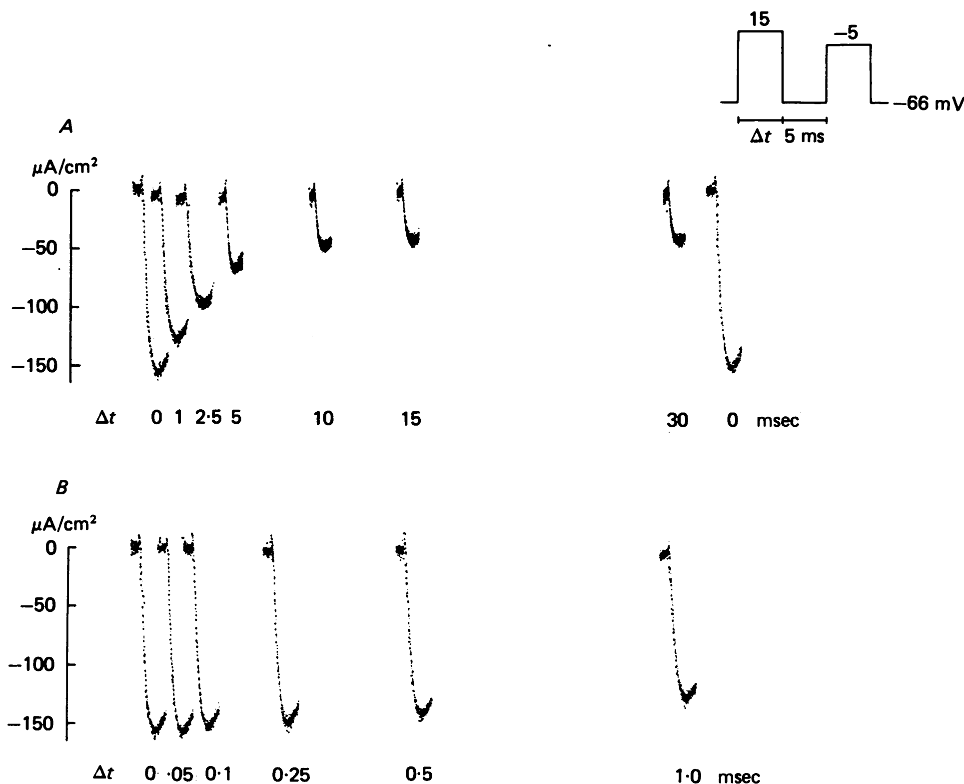

Fig. 10. Original records from an experiment in 1/5 Na sea-water with varying Δt. Pulse programme in upper right corner. Each trace is the average of 5 sweeps and shows the last msec of the pulse interval and the first 3·5–4·5 msec of the test pulse. Tetrodotoxin-insensitive current subtracted. *A*, first and last control record ($\Delta t = 0$) and records with $\Delta t = 1$, 2·5, 5, 10, 15, 30 msec. *B*, first control record ($\Delta t = 0$) and records with $\Delta t = 0 \cdot 05$, 0·1, 0·25, 0·5, 1 msec. Axon diameter 613 μm, temperature 1·2 °C.

shows a clear reversible decrease for $\Delta t = 50\,\mu$sec (and a more marked decrease for $\Delta t = 100$ and 250 μsec). Figs. 10 and 11 are typical for all our experiments: in most of them a clear reversible decrease of $I_{\text{Na peak}}$ for $\Delta t = 100\,\mu$sec was found (but see exception in Fig. 12*B*); a decrease for $\Delta t = 50\,\mu$sec was only seen in a few experiments, especially those with little run down and short gap. Thus, in our experiments the maximum value for the initial delay in the development of inactivation is 50–100 μsec.

The quantitative analysis of the experiment of Fig. 10 is shown in Fig. 12*A*. The

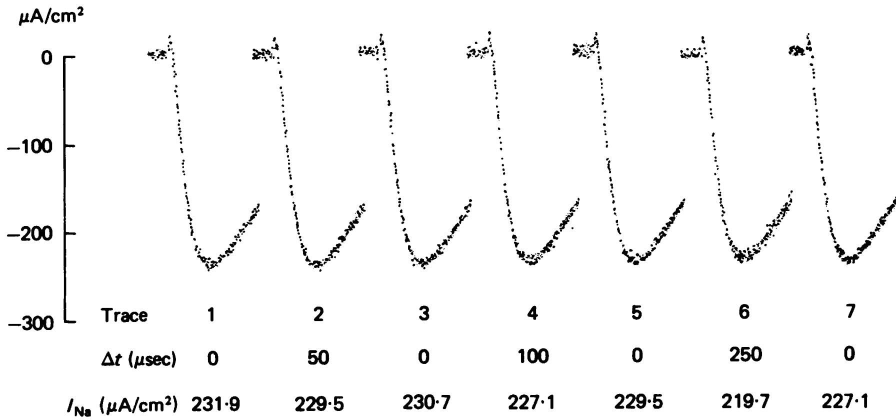

Fig. 11. Original records from another experiment in 1/5 Na sea-water with varying Δt. Pulse programme similar to that in Fig. 12*B*, but holding potential −74 mV, prepulse to −80 mV, conditioning pulse to 10 mV, 3 msec gap, test pulse to −10 mV. Each trace is the average of 5 sweeps and shows the last msec of the pulse interval and the first 5 msec of the test pulse. Tetrodotoxin-insensitive current subtracted. Axon diameter 735 μm, temperature 8·5 °C.

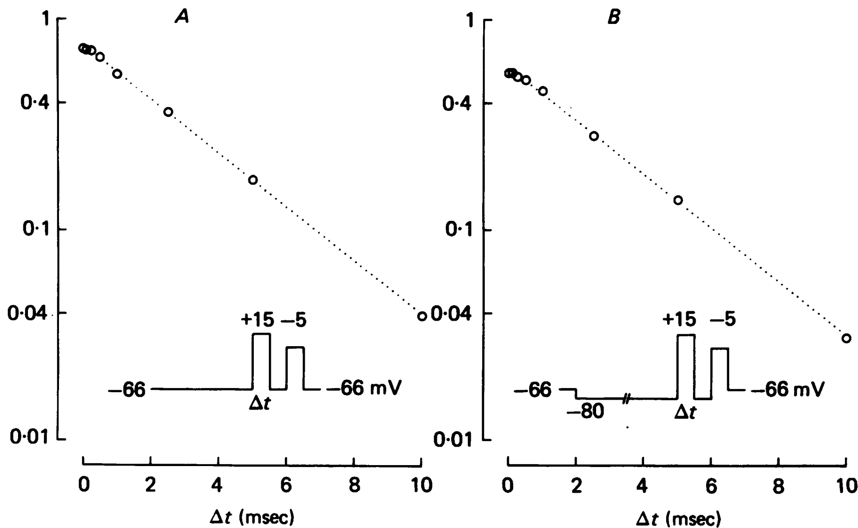

Fig. 12. Quantitative analysis of the experiment in Fig. 10 and of a similar experiment on the same fibre. Ordinate: $(I/I_0)-(I_\infty/I'_0)$ where I is $I_{Na\,peak}$ measured for a given Δt, I_0 is the average $I_{Na\,peak}$ for the two bracketing controls, I_∞ is $I_{Na\,peak}$ measured with Δt = 30 msec and I'_0 the average of its bracketing controls; all currents corrected for tetrodotoxin-insensitive current. Abscissa: Δt = duration of conditioning pulse. Pulse programmes as shown, gap 5 msec. Regression lines calculated. *A*: same pulse programme as in Fig. 10; regression line starts at 50 μsec, time constant τ_h = 3·389 msec. *B*: pulse programme modified by a 60–90 msec prepulse to −80 mV which lasts during the gap; regression line starts at 100 μsec, time constant τ_h = 3·405 msec.

peak current for a given Δt was divided by the average of the bracketing control records; the peak current I_∞ for $\Delta t = 30$ msec (also expressed as fraction of its bracketing control records) was subtracted; the difference was plotted on a logarithmic scale against Δt. The points were fitted by a regression line which started at 50 μsec (because the value for $\Delta t = 50$ μsec was not significantly smaller than the $\Delta t = 0$ value). The regression analysis gave 0·739 for the ordinate value at 50 μsec and 3·389 msec for the time constant τ_h. From these two values we calculated the values 0·728 for $\Delta t = 100$ μsec and 0·697 for $\Delta t = 250$ μsec. The calculated values are similar to the experimental values (0·713 and 0·705, respectively), indicating that the observed decrease of $I_{\text{Na peak}}$ at short times Δt is as large as expected for an exponential decay with a time constant of 3·389 msec, starting at 50 μsec.

Critical evaluation of the method

Several types of experiment were concerned with the question whether the pulse programme employed (a conditioning pulse of varying duration followed after a gap of a few msec by a test pulse) gives a correct measurement of the time course of inactivation.

First, we demonstrated that the value obtained for τ_h does not depend on the membrane potential during the gap, i.e. on the rate of recovery. For this purpose the experiment of Figs. 10 and 12*A* was repeated with a hyperpolarizing prepulse to -80 mV which lasted during the gap (Fig. 12*B*). This made recovery faster and I_∞ larger, consequently the ordinate values became smaller than in Fig. 12*A*. In Fig. 12*B* the regression line starts at 100 μsec because the peak current for $\Delta t = 100$ μsec was not significantly smaller than the control value. The regression analysis gave 0·576 for the ordinate value at 100 μsec and 3·405 msec for the time constant τ_h, i.e. a value close to the τ_h value in Fig. 12*A*.

Secondly, we showed that the value obtained for τ_h is the same as the τ_h value determined from the decay of I_{Na}. Fig. 13 is from the same experiment as Figs. 10 and 12 and shows the Na inward current during an 18 msec long pulse to 15 mV, following a prepulse to -80 mV. The Na current was fitted by eqn. (3) of Kimura & Meves (1979) which is derived from Hodgkin & Huxley (1952) and contains the four adjustable parameters I'_{Na}, τ_m, h_∞ and τ_h. For τ_h the value 3·314 msec was found which is similar to the values 3·389 and 3·405 in Fig. 12*A*, *B*.

The experiment in Fig. 13 was repeated without the prepulse to -80 mV. The current associated with the 18 msec pulse to 15 mV was now smaller and decayed with a time constant of 3·854 msec, i.e. more slowly than with the prepulse. This was also seen in two other fibres (see also Fig. 11 of Meves, 1978) and could be caused by a shift of the τ_h curve due to voltage drop across the uncompensated part of the series resistance. In accord with this hypothesis (which was suggested to us by Drs Kniffki and Vogel), τ_h measured with the double pulse method was not reduced by the hyperpolarizing prepulse; it rather showed a small (probably not significant) increase (Fig. 12*A*, *B*).

Thirdly, we demonstrated that the value obtained for τ_h does not depend on the length of the gap. Measurements on six axons with gap lengths between 0 and 8 msec are collected in Table 1. Experiments *a*, *c* and *e* show no significant effect of gap length on τ_h. The effects seen in experiments *b*, *d* and *f* are in opposite directions and do not exhibit a systematic trend. The six experiments in Table 1 are arranged in

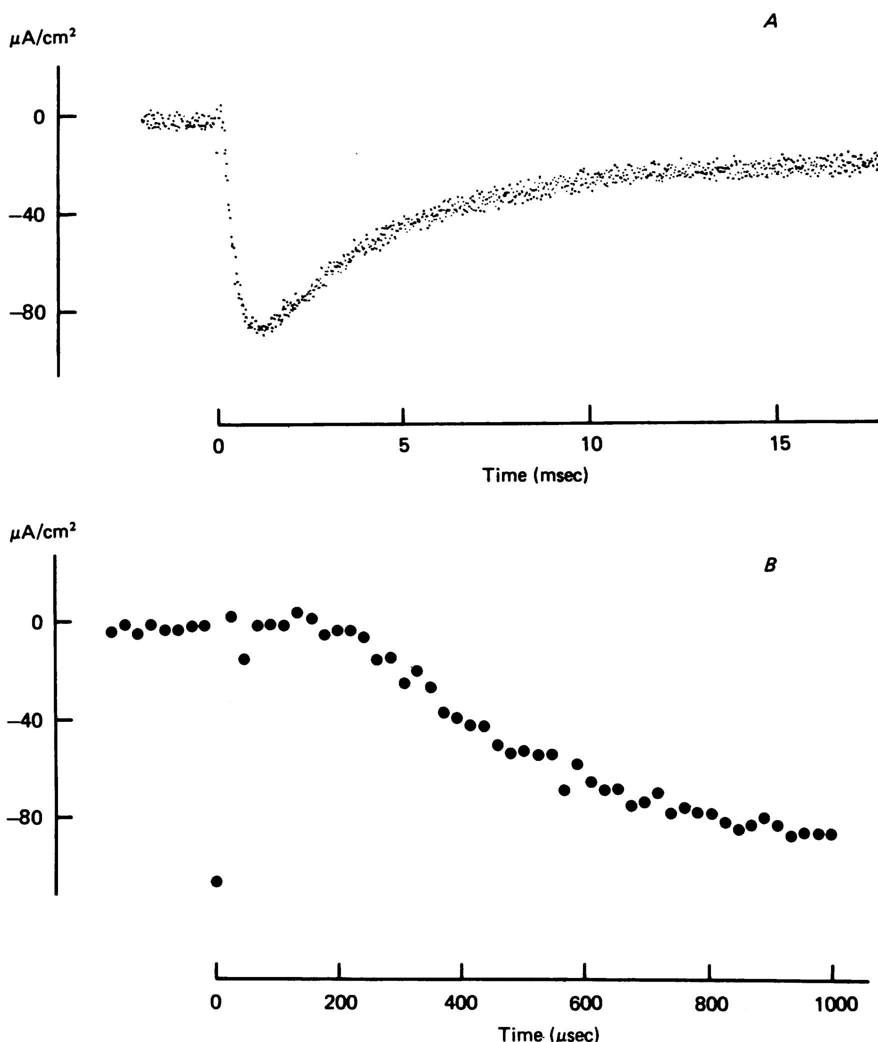

Fig. 13. Na current (minus tetrodotoxin-insensitive current) from the experiment of Figs. 10 and 12. Prepulse to −80 mV (as in Fig. 12*B*) followed by an 18 msec pulse to 15 mV. *A*: plot of current during 18 msec pulse. *B*: current during first msec replotted on expanded time scale, 20 µsec per point, points retouched.

two groups: three experiments at 11·0–12·7 °C and three experiments at 3·8–5·4 °C. In each group the expected decrease of τ_h at more positive potentials is noticeable. Experiment *d* was done on an intact axon; in accord with previous observations (see review of Meves, 1978) its τ_h values are much smaller than those of the perfused axon *e* which were measured at about the same temperature and potential.

Fourthly, we tried to find the shortest gap length which is suitable for measuring the time course of inactivation, i.e. does not produce an apparent initial delay as seen in Figs. 2*C*, *D* and 3*A*, *C*. It is clear that a very short gap will have the same effect as no gap. We studied recovery from inactivation by varying the length of the

TABLE 1. Time constant of inactivation (τ_h) determined by double pulse method with varying gap length. V_1 = potential during conditioning pulse. * denotes perfused axon in full Na sea-water, **intact axon in full Na sea-water; all others are perfused axons in 1/3 Na sea water.

Expt.	Temperature (°C)	V_1 (mV)	Gap length (msec)	τ_h (msec)
a	11·0	−44	0	8·95
			5	9·00
*b**	12·7	−20	0	2·88
			4	3·28
c	11·2	−6	4	3·20
			7	3·12
*d***	5·4	−30	0	3·90
			5	3·28
e	3·8	−23	0	5·74
			5	5·55
			8	5·80
f	4·1	8	5	2·80
			8	2·35

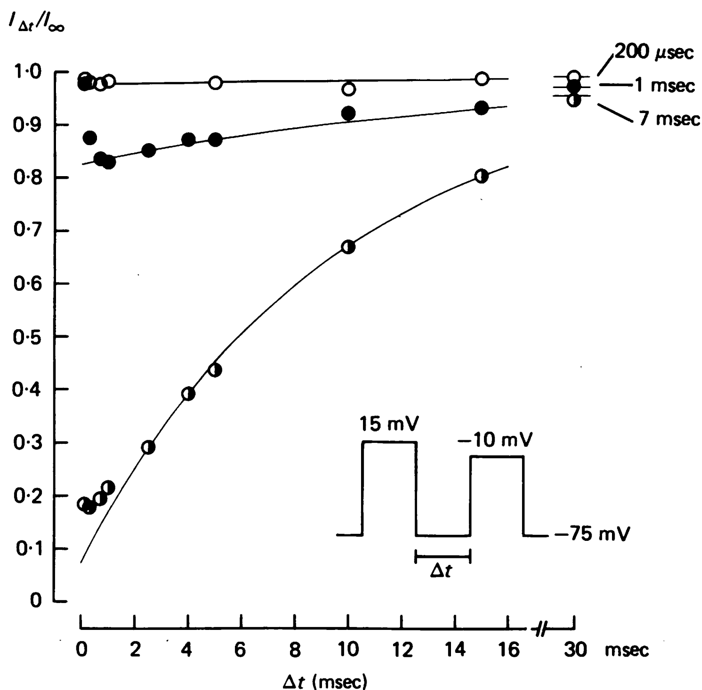

Fig. 14. Recovery from inactivation studied by varying the gap duration. Pulse programme as shown in inset; duration of conditioning pulse constant, duration of gap ($= \Delta t$) varied. Ordinate: $I_{\Delta t}/I_\infty$ = current elicited by test pulse, expressed as fraction of current measured with a very long gap; currents corrected for tetrodotoxin-insensitive current. Abscissa: Δt = duration of gap. Measurements are for three different durations of the conditioning pulse (200 μsec, 1 and 7 msec). Curves drawn from the equation

$$I_{\Delta t}/I_\infty = 1-(1-I_0/I_\infty)\exp(-\Delta t/\tau)$$

(where $I_{\Delta t}/I_\infty = I_0/I_\infty$ at $\Delta t = 0$) with I_0/I_∞ = 0·975, 0·825, 0·075 and τ = 15·5, 15·5, 9·6 msec for the 200 μsec, 1 and 7 msec conditioning pulse, respectively. Axon diameter 637 μm, 1/5 Na sea-water, temperature 2·6 °C.

gap between conditioning pulse and test pulse. Fig. 14 shows the results for three different durations of the conditioning pulse (200 μsec, 1 and 7 msec). Each point was obtained by dividing a measurement for a given gap length Δt ($I_{\Delta t}$) by two bracketing control measurements with a very long gap (I_{∞}). The curve for a 200 μsec conditioning pulse demonstrates again that any initial delay in the development of inactivation must be shorter than 200 μsec at 15 mV and 2·6 °C; even at a gap length Δt = 30 msec the ordinate value (0·994) is still slightly smaller than the control value. The recovery curves for a 1 msec and a 7 msec conditioning pulse exhibit the dip described by Chandler & Meves (1970 *a*). For a gap length Δt = 100 μsec the values for the 200 μsec and the 1 msec conditioning pulse are very similar (0·987 and 0·978, respectively) whereas the value for the 7 msec conditioning pulse is much smaller (0·184): plotting the three ordinate values as a function of conditioning pulse duration (as in Fig. 2 or 3) would give a pronounced initial delay. For a gap length Δt = 1 msec the values for the 200 μsec and the 1 msec conditioning pulse are significantly different (0·979 and 0·830, respectively). Thus, gap lengths of 1 msec or more are sufficient to avoid an apparent initial delay.

The time constants of the recovery curves in Fig. 14 (see legend of Fig. 14) indicate that recovery is faster for a 7 msec pulse than for a 200 μsec and a 1 msec conditioning pulse; however, two other experiments did not confirm this observation and it seems likely that rate of recovery is not significantly dependent on the duration of the conditioning pulse.

DISCUSSION

The experiments show that after the first 50–100 μsec of a depolarizing voltage step the time course of inactivation is exponential. The value 50–100 μsec is an upper limit for an initial delay in the development of inactivation: the true duration of an initial delay at 0–13 °C may be anywhere between 0 and 100 μsec. Thus, the assumption that upon depolarization inactivation develops without an initial delay along an exponential time course (Hodgkin & Huxley, 1952) is, at least, a good approximation.

By contrast, Bezanilla & Armstrong (1977) and Goldman & Schauf (1972) had concluded that inactivation follows a sigmoid time course with a pronounced initial delay. Bezanilla & Armstrong used a conditioning pulse immediately followed by a test pulse. As pointed out by Kniffki *et al.* (1978) a delay observed with this method is 'not an *a priori* sign for a delay in the onset of inactivation'. The delay is due to the increase of m, the Na activation variable, during the conditioning pulse. When the test pulse begins, m is already increased; it therefore reaches its final value (determined by the test pulse potential) earlier, i.e. the time to peak is shorter. Because the peak occurs earlier, less inactivation has developed; this results in an increase of $I_{\mathrm{Na\,peak}}$ which obscures any decrease of $I_{\mathrm{Na\,peak}}$ due to inactivation. If this explanation is correct, a sufficiently strong conditioning pulse should produce an *increase* of $I_{\mathrm{Na\,peak}}$, accompanied by a substantial shortening of the time to peak. This is actually observed (see Figs. 4 and 5).

Goldman & Schauf (1972) in their experiments on *Myxicola* axons used a gap of several msec duration between conditioning pulse and test pulse. The sigmoid time course of inactivation described by Goldman & Schauf could be reproduced on squid

axons in full external Na. However, reduction of the external Na to 1/3 converted the sigmoid time course into an exponential (Fig. 7). Likewise, calculations from the Hodgkin & Huxley equations gave a sigmoid time course for a large Na current and a large series resistance and an exponential time course for a small Na current and a small series resistance (Fig. 8). This supports the idea that the sigmoid time course is an artifact caused by the series resistance. We think that the long-lasting tail of inward current which follows the conditioning pulse (cf. Goldman & Hahin, 1978) produces a voltage drop across the series resistance that shifts the permeability parameters *vs.* voltage curves to more negative voltages. The tail current is still present in 1/3 Na (see Fig. 9), but is so much reduced in size that its effect can be almost completely suppressed by the series resistance compensation. The sensitivity of τ_h to series resistance artifact is also demonstrated by the observation that a hyperpolarizing prepulse (which increases the Na current) leads to an apparent decrease of τ_h (see p. 14).

In conclusion, to measure the time course of inactivation correctly one must (1) separate the conditioning pulse and the test pulse by a gap of at least 1 msec (see p. 17), (2) lower the Na concentration in order to reduce the error caused by the uncompensated part of the series resistance. If these two conditions are fulfilled, the time course of inactivation is exponential for times larger than 50–100 μsec and the time constant is the same as that determined from the decay of the Na current (see p. 14).

At a time of 50–100 μsec after the beginning of a depolarizing pulse no Na current is flowing (see record of I_{Na} on expanded time scale in Fig. 13*B*). Because I_{Na} is proportional to the cube of the Na activation variable m, a large increase in m is required for a noticeable increase in I_{Na}. At 0 °C and 15 mV the time constant of m (τ_m) is 0·45 msec (Kimura & Meves, 1979). If m increases exponentially from 0 to 1 with τ_m = 0·45 msec, it has reached the value 0·20 at 100 μsec, i.e. inactivation starts long before *full* activation has taken place or, in other words, *full* activation is not a prerequisite of inactivation. This sets a limit to sequential models of the excitation process which are based on the scheme *resting* → *active* → *inactive*. Our observations would, however, be compatible with a scheme in which the reaction *resting* → *active* consists of several steps and the first few of these steps are necessary for inactivation to start.

The gating current exhibits a rising phase of 20–50 μsec at 8 °C (Armstrong & Bezanilla, 1975). Fig. 1 of Kimura & Meves (1979) suggests a rising phase of about 70 μsec at −0·75 °C. If the initial delay in the development of inactivation is less than 50–100 μsec, this means that inactivation begins before all gating particles have left the resting position. However, the possibility that the rising phase is due to an overlapping inward current (Armstrong & Bezanilla, 1975; Meves, 1976) has not yet been excluded.

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