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Activation, inactivation and recovery in the sodium channels of the squid giant axon dialysed with different solutions†

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SUMMARY

Comparisons were made between families of ion currents recorded in voltage-clamped squid axons dialysed with 20 mM NaF and 330 mM CsF or TMAF, and bathed in a solution in which four fifths of the Na was replaced by Tris. The permeability coefficient $P_{Na,fast}$ for the fast-inactivating current in the initial open state was calculated as a function of test potential from the size of the initial peak of I_{Na}. The permeability coefficient $P_{Na,non}$ for the non-inactivating open state was calculated from the steady-state I_{Na} that persisted until the end of the test pulse. Dialysis with TMA had no direct effect on the Q_V curve for gating charge. The reversal potential for $I_{Na,non}$ was always lower than that for $I_{Na,fast}$, the mean difference being about -9 mV when dialysing with Cs, but only about -1 mV with TMA. Except close to threshold, $P_{Na,fast}$ was roughly halved by dialysis with TMA as compared with Cs, but $P_{Na,non}$ was substantially increased. The time constant τ_h for inactivation of the sodium system was slightly increased during dialysis with TMA in place of Cs, and there were small shifts in the steady-state inactivation curve, but the rate of recovery from inactivation was not measurably altered. The flattening off of the τ_h curve at increasingly positive test potentials corresponded to a steady reduction of the apparent inactivation charge until a value of about $0.2e$ was reached for pulses to 100 mV. The instantaneous I–V relationship in the steady state was also investigated. The results have a useful bearing on the effects of dialysis with TMA, on the differences between the initial and steady open states of the sodium channel, and on the relative voltage-dependences of the transitions in each direction between the resting and inactivated states.

1. INTRODUCTION

The experiments to be discussed in this paper formed part of parallel investigations carried out in 1986/8 into the kinetics of the macroscopic sodium current (I_{Na}) and of the sodium gating current (I_g) in the squid giant axon (Greeff *et al.* 1982; Keynes *et al.* 1982; Keynes 1983; Bekkers *et al.* 1987; Keynes *et al.* 1990), whose ultimate object was to throw light on the molecular mechanism of the voltage-gated sodium channel. A preliminary report by Bekkers *et al.* (1987) showed that when the axons were dialysed with TMA instead of the Cs used previously to block the potassium channels (Greeff *et al.* 1982), there were marked differences between the normal and non-inactivating (Chandler & Meves 1970) open states in the behaviour of the macroscopic sodium current, as had also been noted by Oxford & Yeh (1985). Further analysis of the results was held up for some while by the difficulty of accounting for the consistent location of the mid-point of the activation curve at about $+6$ mV instead of the expected value in the neighbourhood of -20 mV (Hodgkin & Huxley 1952) when dialysing with Cs, but it eventually transpired (Keynes *et al.* 1991) that the shift arose from a voltage-dependent blocking of the channels by external Tris. Better sense could then be made of the properties of I_{Na}, and an examination is presented here of the effects of internal Cs and TMA on various properties of the sodium system.

2. METHODS

(*a*) *Apparatus*

Giant axons from *Loligo forbesi* were dissected and mounted in an air-gap voltage-clamp chamber (Fishman 1970) with a central 5 mm recording region and 6 mm guard regions. Porous dialysis tubing was threaded inside the axon as described by Bullock & Schauf (1978), through which a solution controlling the internal ionic composition flowed under gravity feed. The axon was bathed in a solution maintained at

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a constant temperature which was circulated through the chamber by a peristaltic pump.

An eightfold increase in the signal:noise ratio of the voltage-clamp system was achieved (Bekkers *et al.* 1986; Forster & Greeff 1990) by redesign of the voltage-clamp amplifier, by the introduction of partitions in the recording chamber, and by using a dual voltage-sensing electrode with improved frequency response (Levis & Bezanilla, 1983) that comprised a conventional KCl-filled pipette connected in parallel with a Pt wire insulated in a 50 μm glass tube except at its tip, which was capacitatively coupled to the differential amplifier input stage.

Command pulses were provided by a PDP 11/73 computer (Digital Equipment Corporation) via an analogue interface (Cambridge Electronic Design Type 502). The membrane currents were passed through a 100 kHz (−3 dB) 4-pole Bessel filter, and sampled to 16 bit resolution by the same interface. Capacity transients were subtracted using an appropriately scaled back reference pulse (Bekkers *et al.* 1984). Compensation for the Schwann cell series resistance R_s was applied in the usual manner (Bezanilla *et al.* 1982), and adjusted for exactly critical damping of the capacity transient (Greeff *et al.* 1982). Checks showed that the junction potential between the recording electrodes never drifted by more than 1.5 mV in the course of an experiment. A final recalibration of the amplitudes of the test pulses as compared with the values assumed in our earlier paper (Keynes *et al.* 1990) necessitated a slight readjustment of the voltage axes of some of the curves, but did not materially affect the conclusions. DAOS software (Laboratory Software Associates, Melbourne, Australia) was used to write the programmes both for running the experiments and for data acquisition and analysis.

(*b*) *Solutions*

The solutions for internal dialysis when recording sodium currents normally contained 330 mM CsF or TMAF, 20 mM NaF, 400 mM sucrose and 10 mM HEPES buffer pH 7.3. The external bathing solution contained 103 mM NaCl, 11 mM $CaCl_2$, 55 mM $MgCl_2$ and 421 TrisCl pH 7.3, to which 1 μm tetrodotoxin (TTX) was added in order to record families for subtraction of the contribution of the tail of I_g to the initial peak of I_{Na}. The full [Na] ASW contained 514 mM NaCl, 10 mM TrisCl pH 7.3, 11 mM $CaCl_2$ and 55 mM $MgCl_2$.

When recording gating currents, the dialysis solution contained 350 mM CsF or TMAF, 400 mM sucrose and 10 mM HEPES buffer pH 7.3. The bathing solution contained 524 mM TrisCl pH 7.3, 11 mM $CaCl_2$, 55 mM $MgCl_2$ and 1 μm TTX.

3. RESULTS

(*a*) *The blocking of the potassium channels by internal cations*

The starting point of the investigation was a check on the efficiency with which the last traces of outward current flowing through the potassium channels were blocked by dialysis with CsF. Although caesium had advantages for studies of the rapidly relaxing components of I_g, it was important to eliminate any such currents when attempting to record the small component I_{g4} (Keynes *et al.* 1990) that relaxed with the inactivation time constant τ_h (Hodgkin & Huxley 1952). As may be seen in figure 1, it turned out that a complete replacement of CsF by TMAF was needed to achieve this aim, and that for V_p +74 mV the late outward current of Cs^+ was 20 μA cm^{-2} when dialysing with 350 mM CsF, and was still 10 μA cm^{-2} with 50 mM CsF and 300 mM TMAF. The change had no obvious effect on the gating current, and as may be seen in figure 2, the Q_v curve relating total gating charge to pulse potential between −140 and 80 mV was identical whether dialysing with 350 mM CsF or TMAF.

However, both Cs^+ (Schauf & Bullock, 1978) and TMA^+ (Schauf *et al.* 1976; Schauf 1983; Oxford & Yeh 1985) had been reported to interact with sodium gating, and experiments were therefore undertaken to explore the question in greater detail.

(*b*) *The effect of internal cations on* I_{Na}

The large size of the inward sodium current in an artificial sea water containing 524 mM NaCl would have strained the ability of the voltage-clamp system to maintain a perfect control. The measurements were therefore always made in a solution containing 20% by volume of full [Na] artificial sea water and 80% of Tris ASW. With this procedure, the contribution of the tail of I_g to the initial peak of I_{Na} could not be neglected, especially in the vicinity of the reversal potential V_{rev}. Families of $I_{Na}+I_g$ were recorded while dialysing first with 20 mM NaF + 330 mM CsF, and then with 20 mM NaF + 330 mM TMAF. Lastly I_g alone was recorded in a solution containing 1 μm TTX, and this final family was subtracted from the other two. Typical I_{Na} families thus obtained for an axon dialysed first with Cs and then with TMA are illustrated in figure 3*a*,*b*.

Although the main part of the interference from I_g was satisfactorily eliminated in this way, there was often a slight slowing down of the capacity transient as the experiment proceeded, so that the initial part of the gating current transient was not always perfectly cancelled out for the families that had been recorded earliest, and the current at the start of the test pulse was not exactly zero. A difficulty also arose because although the effects of TMA on the Na channels (see Keynes *et al.* 1992) were readily reversable, the blocking of the late outward ionic current that flowed through the K channels was not quickly reversed. Errors from this source in measuring the size of the steady-state current $I_{Na,non}$ could be eliminated by the final subtraction of a family recorded with 1 μm TTX for axons dialysed only with CsF, but not for those that had also been dialysed with TMAF beforehand.

Three effects of dialysis with TMA were immediately apparent. First, except for the smallest test

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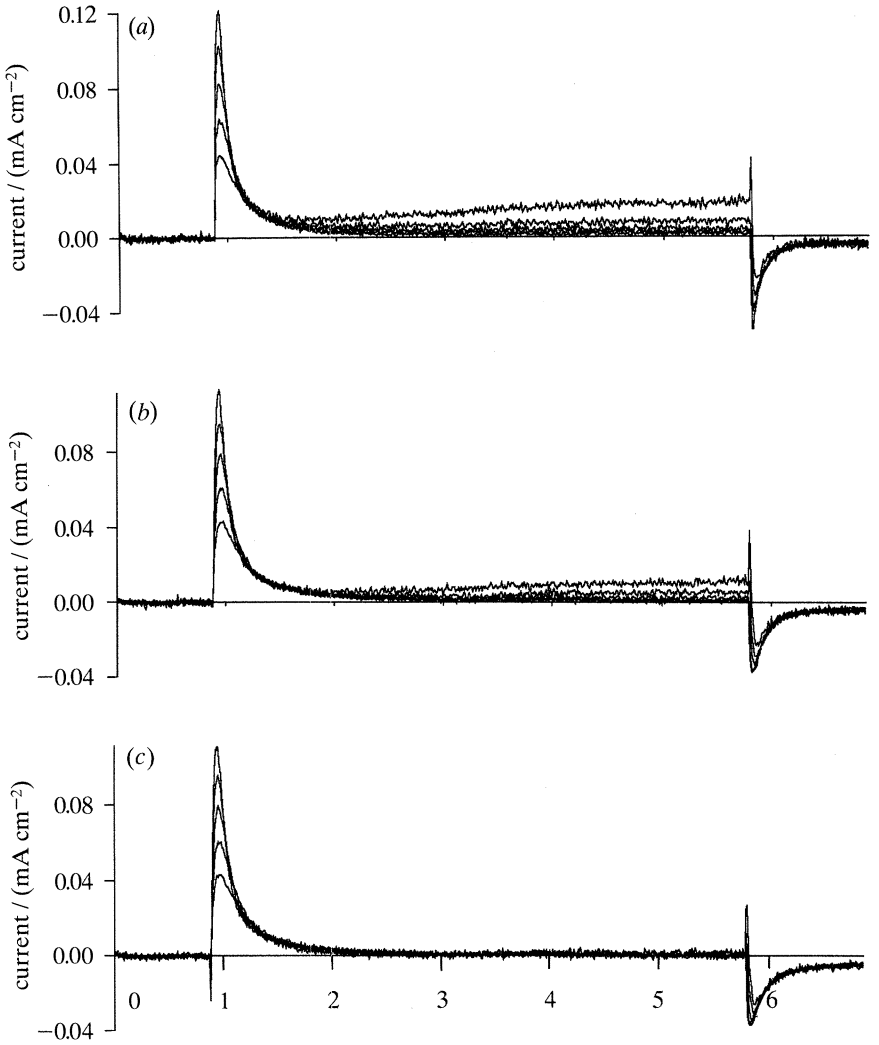

Figure 1. Gating current families for an axon dialysed with (*a*) 350 mM CsF, (*b*) 300 mM CsF + 50 mM TMAF, and (*c*) 350 mM TMAF, in each case plus 10 mM HEPES buffer pH 7.3 and 400 mM sucrose. Bathing solution contained 514 mM TrisCl pH 7.3, 11 mM $CaCl_2$ and 55 mM $MgCl_2$. Test pulses to −2.8, 16.4, 35.6, 54.8 and 74.0 mV. Sampling period 10 μs. Pulse length 5 ms. Axon diameter 840 μm. Temperature 5°C. Four sweeps were averaged. Data files I25oct.009/11.

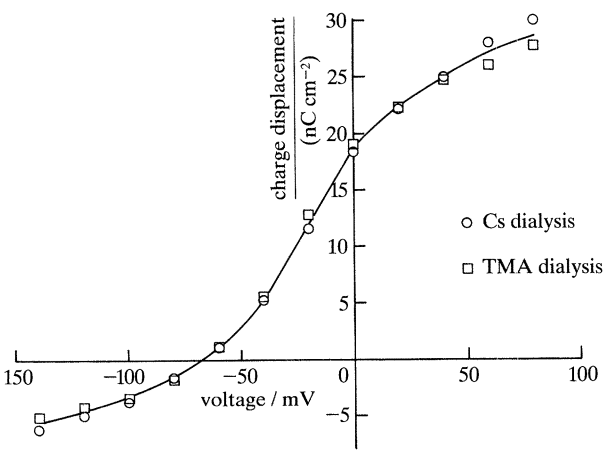

Figure 2. Total gating charge plotted against test potential for an axon dialysed first with 350 mM CsF (circles) and then with 350 mM TMAF (squares), and bathed in Tris SW. Temperature 5°C. Back-reference pulses −150 to −180 mV; for other details see figure 4 of Keynes *et al.* (1990). Data files I27nov.002/6.

pulses, there was a marked reduction in the size of the initial peak of the fast-inactivating current $I_{Na,fast}$. Second, there was an increase in the size of the final plateau of current $I_{Na,non}$ that flowed during the non-inactivating open state first described by Chandler & Meves (1970). Lastly, although with TMA $I_{Na,fast}$ and $I_{Na,non}$ were reversed at much the same pulse potential, when dialysing with Cs, $I_{Na,fast}$ was reversed at a lower potential than $I_{Na,non}$.

(*c*) *The reversal potentials for* $I_{Na,fast}$ *and* $I_{Na,non}$

Before examining the behaviour of the sodium permeability coefficient when dialysing with Cs and TMA, it is first necessary to consider the determinations of V_{rev} that are summarized in Table 1. Averages for a number of axons showed that with Cs dialysis the reversal potential for $I_{Na,non}$ was consistently 9 to 10 mV on the negative side of that for $I_{Na,fast}$. When dialysing with TMA the shift was appreciably less, averaging 1.6 mV. The values of V_{rev} increased by

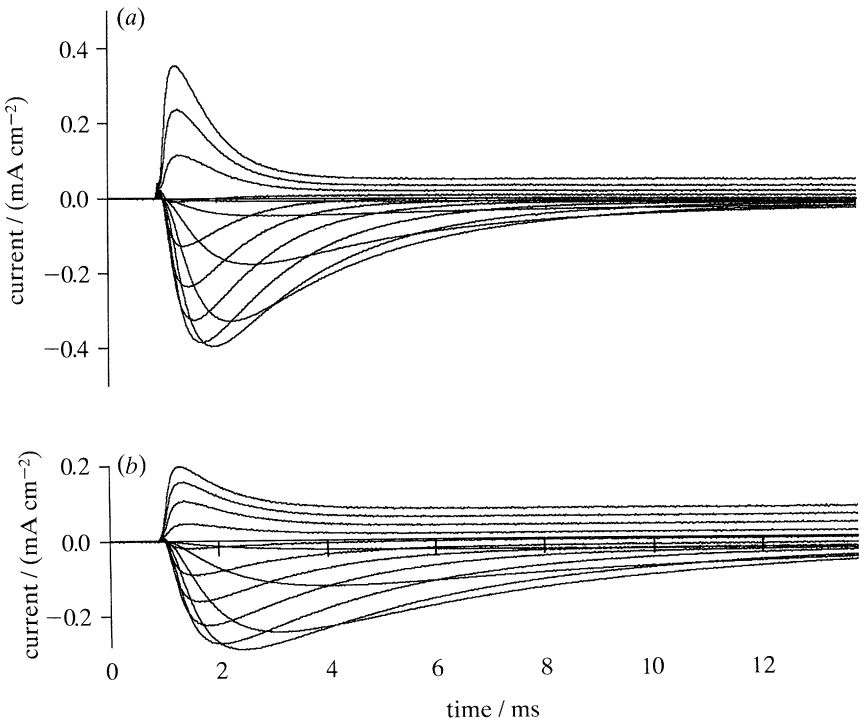

Figure 3. Sodium current families for an axon dialysed with (*a*) 330 mM CsF, and (*b*) 330 mM TMAF, plus 20 mM NaF, 10 mM HEPES buffer pH 7.3, and 400 mM sucrose. Bathing solution contained 103 mM NaCl, 411 mM TrisCl pH 7.3, 11 mM $CaCl_2$ and 55 mM $MgCl_2$. Test pulses −41 to 74 mV in steps of 9.6 mV. Sampling period 20 μs. Temperature 5°C. Axon diameter 620 μm. Corresponding I_g families have been subtracted. No averaging. V_{rev} at the peak of $I_{Na,fast}$ was 45.9 mV for (*a*) and 38.6 mV for (*b*); for the final plateau of $I_{Na,non}$ it was 35.0 mV for (*a*) and 37.6 mV for (*b*). Data files I17nov.s01/08.

about 20% on raising the temperature from 5 to 18°C. In a small number of experiments performed later (see Keynes *et al.* 1992), the shifts of V_{rev} were very similar when choline was used instead of Tris as a substitute for external sodium. A possible explanation for these findings in terms of differences in the ionic selectivity of the Na channel between its two open states is deferred to the Discussion.

(*d*) *The sodium permeability coefficients* $P_{Na,fast}$ *and* $P_{Na,non}$

In order to eliminate the electrical driving force $V_p - V_{rev}$ as a variable, at the same time taking account of any non-linearity of the *I–V* curves, it was necessary to calculate values of the sodium permeability coefficients rather than those of the sodium conductance g_{Na}. For this purpose, the constant field relationship (Goldman 1943; Hodgkin & Katz 1949) for the case when there is no potential step at the edge of the membrane, that is $E' = 0$ in equations (4) and (5) of Frankenhaeuser (1960), leads to the equation

$$P_{Na} = I_{Na} \frac{RT}{F^2 V_p [Na]_o} \frac{\exp((V_p)F/RT) - 1}{\exp((V_p - V_{rev})F/RT) - 1}. \quad (1)$$

Figure 4 shows that during CsF dialysis $P_{Na,fast}$ had nearly reached a plateau at $V_p = 74$ mV, and that the mid-point $V_{mid,act}$ of the normalized activation curve lay at about +6 mV. The failure of $P_{Na,fast}$ to arrive at its plateau at a lower potential, and the shift of $V_{mid,act}$ by about +25 mV as compared with the behaviour of intact axons (Hodgkin & Huxley 1952), or with that seen in figure 5 for an axon bathed in a solution containing 514 mM NaCl and only 10 mM TrisCl, and dialysed with 350 mM NaF, were invariable. In seven axons dialysed with CsF at 5°C the average value of $V_{mid,act}$ was again +6 mV, and that of the plateau level of $P_{Na,fast}$ was estimated as 1.1×10^{-4} cm s^{-1}. For the axon of figure 5, $V_{mid,act}$ was −19 mV, and $P_{Na,fast}$ reached a value of 1.55×10^{-4} cm s^{-1} at 10°C.

It was discovered subsequently (Keynes *et al.* 1991) that the assumption that Tris could be used as an inert substitute for sodium in the bathing solution was incorrect, and that it exerted a pronounced blocking effect that decreased with the rise of V_p. This at once explained the positive shifts of $V_{mid,act}$ and of the potential for arrival at the plateau. For axons bathed in 20% Na ASW with choline as a substitute for the remainder of the sodium, $V_{mid,act}$ was moved back to around −20 mV, and $P_{Na,fast}$ reached a plateau of 1.4×10^{-4} cm s^{-1} at about 50 mV.

When the axon of figure 4 was dialysed with TMAF, $P_{Na,fast}$ was roughly halved in size, and after reaching a peak that averaged 0.5×10^{-4} cm s^{-1} at about 45 mV the curve turned downwards. This behaviour could be attributed to the voltage-dependant blocking effect described for single-channel currents in rat myotubes by Horn *et al.* (1981), which was evidently similar to the reduction of the single-channel conductance by tetraethylammonium ions (TEA^+) observed in the fluctuation analyses of Bekkers *et al.* (1986) on cut-open squid axons.

Table 1. Measurements of the reversal potential V_{rev} for the fast-inactivating and non-inactivating open states when dialysing with 20 mM NaF + 330 mM CsF or TMAF in an external medium containing 103 mM NaCl, 412 mM TrisCl pH 7.3, 11 mM $CaCl_2$ and 55 mM $MgCl_2$

		CsF dialysis		TMA dialysis	
data file numbers	temperature/°C	$V_{rev,fast}$/mV	$V_{rev,non}$/mV	$V_{rev,fast}$/mV	$V_{rev,non}$/mV
I17nov.s01/08	5	45.9	35.0	38.6	37.6
I21nov.s05/16	5	42.7	32.7	38.4	36.8
K03oct.s00/03	5	41.4	30.7	41.9	41.1
K04oct.s04	5			40.5	40.0
K05oct.s00	5	41.5	36.4		
K07oct.s00	5	45.5	35.6		
K07oct.s09	5			41.7	41.2
K09oct.s03	5	41.9	34.2		
K11oct.s02	5			37.3	33.5
K14oct.s06	15			44.8	43.6
K14oct.s07	5			42.9	43.3
K18oct.s03/00	15	45.8	35.9	46.7	45.5
K18oct.s05	15			46.2	42.7
K18oct.s04/02	5	45.2	37.6	45.3	42.4
K18oct.s06	5			44.6	42.8
L02nov.s00	10			44.9	39.4
L02nov.s01	18			53.5	50.6
L02nov.s02	10			47.4	46.3
L02nov.s03	18			48.2	47.1
L07nov.s00	10			44.4	43.4
L07nov.s01	18			45.0	44.4
L07nov.s02	10			44.9	44.4
means	5	43.4	34.6	41.2	39.9
	10			45.4	43.4
	15	45.8	35.9	45.9	43.9
	18			48.9	47.4

The values of $P_{Na,non}$ plotted in figure 6 show that the steady-state current rose steadily with increasing V_p, as had been noted by Chandler & Meves (1970), but was affected by TMA in the opposite direction to $P_{Na,fast}$, since the rate of rise was now increased by about half. A plateau for $P_{Na,non}$ could not be reached without going to excessively large test potentials, but as confirmed by Keynes *et al.* (1992), an inflexion in the plots was usually detectable at about $V_p = 50$ mV,

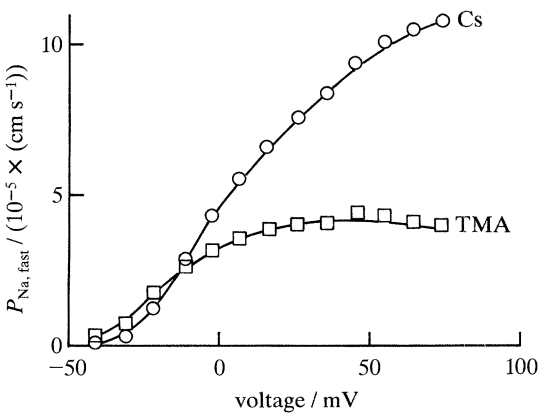

Figure 4. $P_{Na,fast}$ plotted against V_p for the records of figure 3. Circles, for Cs dialysis, $V_{rev} = 45.9$ mV. Squares, for TMA dialysis, $V_{rev} = 38.6$ mV.

though this was too close to V_{rev} for its position to be determined with precision. The inflexion point for $P_{Na,non}$ in figure 5 was also close to 50 mV.

A similar action of TMA had been reported for perfused squid axons by Oxford & Yeh (1985), although their results were not expressed in terms of conductances or permeability coefficients. The finding was first taken to indicate that the voltage-dependence of the block by TMA^+ of the single-channel conductance might be reversed in the non-inactivating open state. However, further consideration of the phenomenon (see Keynes *et al.* 1992; Keynes 1992) has suggested a better explanation in terms of a dual effect of TMA that increases the fraction of open channels to an extent which over-rides the simultaneous block of their conductance. Because the possibility could not be ignored of interactions both between Tris and TMA, and also of internal competition with Na^+ and other cations, additional evidence was needed on the effect of TMA in the absence of Tris. The results described by Keynes *et al.* (1992) fully confirmed those considered here.

(*e*) *The kinetics of inactivation*

The time constant τ_h for the inactivation of the sodium system (Hodgkin & Huxley 1952) was deter-

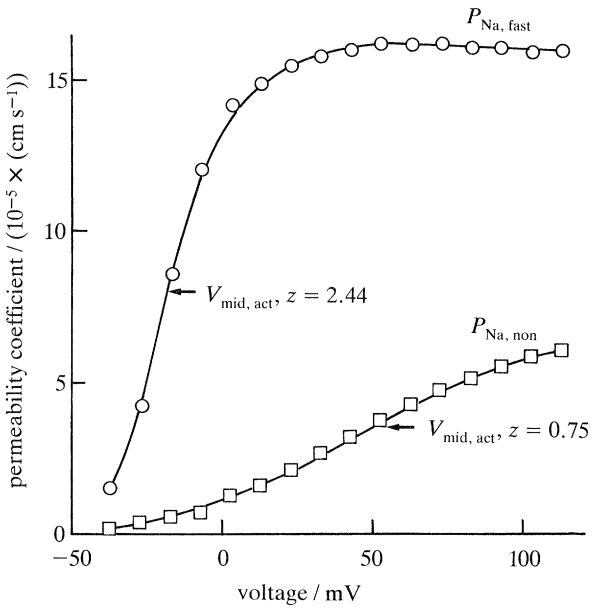

Figure 5. $P_{Na,fast}$ (circles) and $P_{Na,non}$ (squares) plotted against V_p for an axon dialysed with 350 mM NaF and bathed in full [Na] ASW + 16 nM TTX. I_{Na} family shown in figure 3*c* of Keynes (1991). V_{rev} was −1.1 mV for $I_{Na,fast}$ and −3.0 mV for $I_{Na,non}$. Arrows show $V_{mid,act}$ in each case. The values of P_{Na} shown in figure 4 of Keynes (1991) did not allow for the fraction of channels not blocked by TTX, which could be taken as 0.17 (Keynes *et al.* 1975). The normalized rate of rise of $P_{Na,fast}$ at V_p −19 mV was 1/40 mV^{-1}, corresponding to a charge of 2.44*e*. The normalized rate of rise of $P_{Na,non}$ at V_p 50 mV was 1/130 mV^{-1}, corresponding to a charge of 0.75*e*. Axon diameter 760 μm. Temperature 10°C. Data file L08nov.s04.

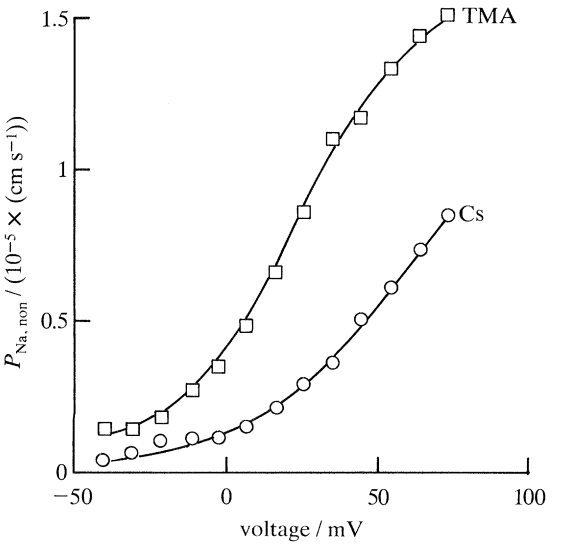

Figure 6. $P_{Na,non}$ plotted against V_p for the records of figure 3. Circles, for Cs dialysis, $V_{rev} = 35.0$ mV. Squares for TMA dialysis, $V_{rev} = 37.6$ mV.

mined by fitting a single exponential to each of the current records, starting routinely at the point where the slope was greatest. The results plotted in figure 7 on a semilogarithmic scale show the effects both of temperature and of dialysis with Cs and TMA. The inactivation displayed a tendency to slow down somewhat with fatigue of the axon, and measurements with Cs dialysis first at 15 and then at 5°C were therefore bracketed by similar runs with TMA. After thus taking fatigue into account, it turned out that above V_p 0 mV, τ_h was increased by an average of 22% at both 5 and 15°C when dialysing with TMA, but for negative V_ps the effect became smaller. An increase that was consistently nearly twice as great was observed in three other axons that were dialysed with Cs as well as TMA, but in each case the period with Cs came first, so that about half of the apparent increase of τ_h by TMA in these experiments may be attributed to fatigue. The increase by about one fifth that is not due to fatigue may perhaps be related to the smaller difference between the size of the initial peak of I_{Na} and of its steady-state value in the presence of TMA rather than to a direct effect on the rate of inactivation. The mean Q_{10} for the reciprocal of τ_h for V_p's between −22 and 102 mV averaged 3.27 for dialysis with Cs, and 3.44 for dialysis with TMA.

The values of τ_h plotted in figure 7 were obtained by fitting single exponentials to the current records, but although the fit always looked reasonably good on visual inspection, it could sometimes be improved slightly by fitting the sum of two exponentials, especially to records made at the higher temperatures. Figure 8*a* shows plots against V_p of averaged values of the faster time constant τ_{h1}, and of the slower time constant τ_{h2}, for the three runs at 15°C of figure 7, while figure 8*b* shows the corresponding sizes of the

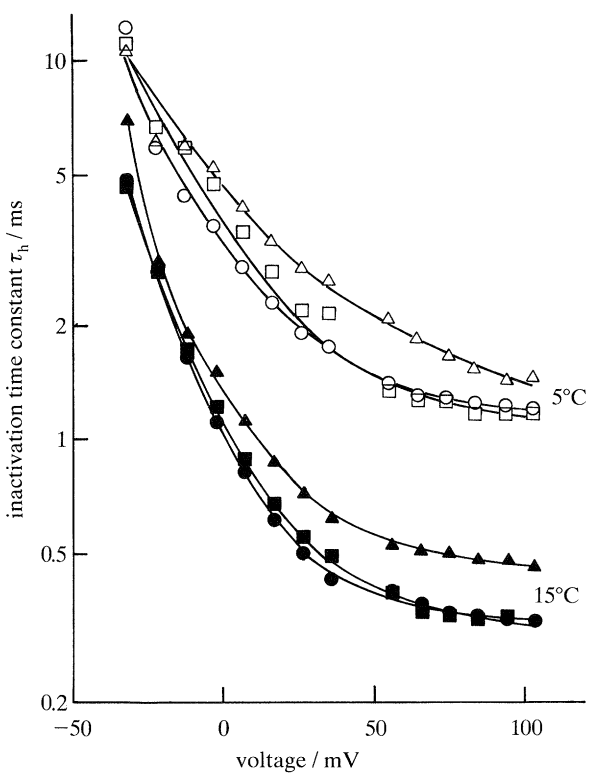

Figure 7. Semi-logarithmic plots of τ_h against V_p for I_{Na} families recorded at 15°C (solid symbols) and at 5°C (open symbols) for an axon dialysed first with TMA (squares), then with Cs (circles), and lastly with TMA again (triangles). Axon diameter 820 μm. Data files K18oct.s00/06.

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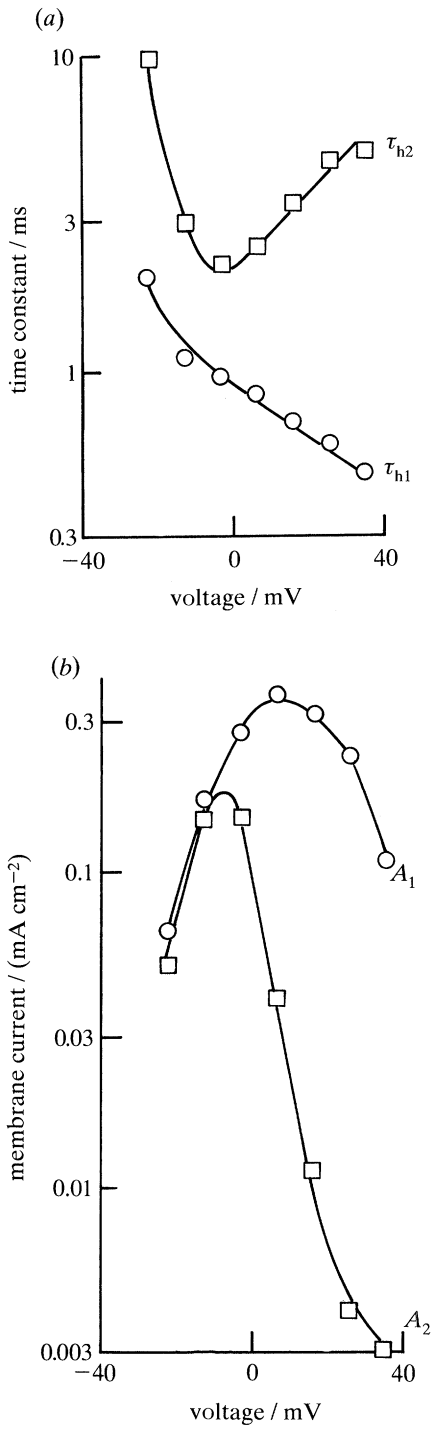

Figure 8. (*a*) Semi-logarithmic plots against V_p of the averaged values of τ_{h1} (circles) and τ_{h2} (squares) for the three runs at 15°C of figure 7. (*b*) Corresponding averaged sizes A_1 and A_2 of the current at the start of the fits.

current at the starting point of the fit. It will be seen that for the smallest test pulses the initial amplitudes of the two components were comparable, but above V_p 0 mV that of the slower one fell off rapidly while at the same time its time constant rose, until beyond V_p 50 mV it could no longer be detected and fitted. The reason for the disappearance of the slow component at 5°C appeared to be that with a threefold slowing down at the lower temperature, τ_{h2} simply became too large for the records to be fitted to two exponentials within the available time window.

In considering the mechanism of coupling between activation and inactivation, an important issue is the precise manner in which τ_h varies with pulse potential (Keynes 1991). It is obvious that the semilogarithmic plots in figures 7 and 8*a* do not behave as would be expected for a single voltage-dependent transition, because the curves for τ_h and τ_{h1} flatten off appreciably as V_p increases, as had been pointed out by Hodgkin & Huxley (1952). For a voltage-dependent system with a symmetrical energy barrier and an effective valency z that obeys Boltzmann kinetics, τ_h might be expected to be governed by the relationship

$$\frac{\tau_1 \text{ at } V_1}{\tau_2 \text{ at } V_2} = \frac{\exp(zF(V_2-V_{eq})/2RT)+\exp(-zF(V_2-V_{eq})/2RT)}{\exp(zF(V_1-V_{eq})/2RT)+\exp(-zF(V_1-V_{eq})/2RT)}, \quad (2)$$

where V_{eq} is the equilibrium potential for the transition, which from the mid-point of the steady-state inactivation curve (see p. 478) may be taken for the present purpose as −45 mV. This equation was used to calculate z for successive pairs of values of τ_h and V_p in each of the six curves shown in figure 7, and the averaged estimates of z thus obtained are plotted against $(V_{p1}+V_{p2})/2$ in figure 9.

Because for the smallest test pulses, e.g. $V_p = -40$ mV, there is no measurable inactivation, the τ_h curve goes off to infinity at the negative end. The correspondingly large values of z clearly reflect, as argued by Armstrong & Bezanilla (1977), the existence of a sequential coupling between activation and inactiva-

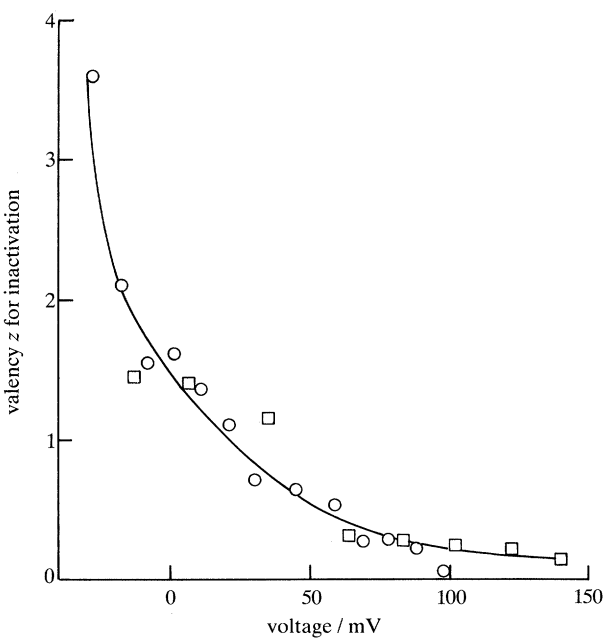

Figure 9. Circles: average values of z calculated from equation (2) and plotted against $(V_{p1}+V_{p2})/2$ for the six runs of figure 8. Squares: similar plots for two runs at 5°C for another axon dialysed with TMA, in which the potential range was extended to V_p 150 mV. Data files K18oct.s00/06 and K11oct.s00/02.

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tion, which means that equation (2) would not strictly be applicable to the initial part of the curve. The question then arises as to what extent the τ_h curve finally flattens off at the positive end. It may be seen that in figure 7 τ_h did reach a flat plateau at V_p 90 mV, but the behaviour of this axon was atypical in doing so, and normally τ_h was still falling slowly at the top end of the potential range. On one occasion the range was extended to V_p 150 mV, and as shown in figure 9, z did not fall much below 0.2 even at this potential. The average valency calculated from the rate of decline of τ_h at V_p 100 mV was close to 0.2.

A significant finding that emerged from figure 8 was that at 15°C, and for potentials between V_p −10 and 35 mV, the semilogarithmic plot of τ_{h1} lay on a nearly straight line, whose slope turned out to correspond to a nearly constant value of 1.36 for z. Five other axons examined at 15 or 18 °C behaved in exactly the same way, yielding an overall average of $z = 1.34$. At 5°C, where the data could only be fitted by a single exponential, the calculated values of z averaged about 1.5 at V_p −10 mV, but were only half as great at V_p 30 mV. The value of z thus derived from τ_{h1} at the higher temperatures between V_p −10 and +35 mV is close to the estimate of the quantal inactivation charge over the same potential range at which Greeff & Forster (1991) arrived from an isochronic analysis of the slow gating current and I_{Na} during the phase of macroscopic inactivation.

(*f*) *Steady-state inactivation*

The extent to which the sodium system was inactivated in the steady state was examined by applying a test pulse to 20 mV after holding the membrane potential for 20 ms at levels rising in appropriate steps from −100 to −10 mV, and recording the peak inward current. Errors arising from the tail of the gating current were again avoided by subtracting a family of traces for the same pulse protocol recorded after adding 1 μm TTX to the bathing solution. Figure 10 shows an example of a normalized steady-state inactivation curve, that is of $(1-h_\infty)$ as defined by Hodgkin & Huxley (1952), obtained for an axon dialysed first with Cs and then with TMA. The potential $V_{mid,inact}$ at which the channels were 50% inactivated during dialysis with Cs was −44.7 mV, and the slope of the curve at its mid-point was $-0.035\ mV^{-1}$. When dialysing with TMA, $V_{mid,inact}$ was shifted to −38.5 mV, and the slope was reduced to $-0.022\ mV^{-1}$. The differences between Cs and TMA were similar in other axons, the averages for four axons dialysed with Cs and four others dialysed with TMA being respectively −43.6 and −38.5 mV for $V_{mid,inact}$, and −0.032 and $-0.025\ mV^{-1}$ for the slope. One axon dialysed with 350 mM NaF, for which the current flowed outwards during the 20 mV test pulse, gave $V_{mid,inact} = -47.5$ mV and a slope of $-0.025\ mV^{-1}$.

It seemed probable that the apparent effect of TMA when using this experimental procedure was an indirect consequence of the simultaneous reduction in $I_{Na,fast}$ and increase in $I_{Na,non}$, and was not caused by a genuine alteration in the voltage-dependence of inactivation. When the size of the test response was measured relative to the steady current at the end of the prepulse, which became appreciable only beyond $V_{pre} = -40$ mV, the curves fell almost to zero at the positive end, but after normalization the shift of $V_{mid,inact}$ brought about by TMA was reduced to less than 2 mV.

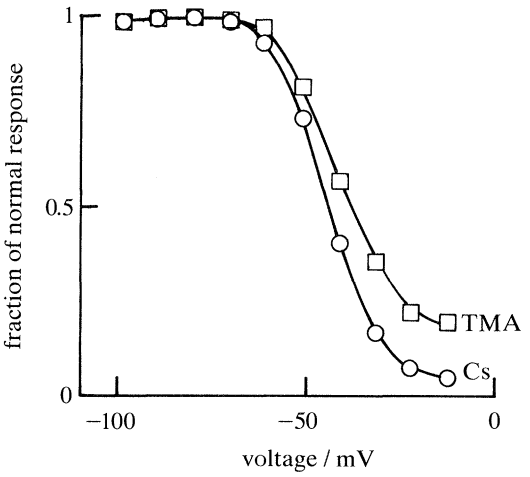

Figure 10. Normalized steady-state inactivation $(1-h_\infty)$ curves recorded at 5°C for an axon dialysed first with Cs (circles) and then with TMA (squares). Data files K03oct.s01/04.

It may be concluded that the centre of the normalized steady-state inactivation curve lies close to −45 mV at 5°C, and that its slope at this point is about $-0.030\ mV^{-1}$, which corresponds to a Boltzmann distribution for a particle carrying a charge of 3*e*. Similar figures have been reported by Vandenberg & Bezanilla (1991) from patch-clamp studies on cut-open axons.

(*g*) *The kinetics of recovery*

It has long been recognized that the rate of recovery of the sodium system from inactivation increases markedly when the membrane is returned to a more negative potential, and experiments were therefore undertaken to investigate the voltage-dependence of the recovery time constant τ_{rec}. After a 20 ms period of inactivation at 0 mV, the potential was returned to a negative holding level, and the response for a test pulse to 20 mV was determined as a function of the recovery interval. As before, gating currents were recorded using the same pulse protocols after adding 1 μm TTX to the bathing solution, and the families were subtracted in order to reduce errors from the contribution of the I_g tail to the peaks of the I_{Na} that flowed during the test pulse. As may be seen in figure 11, after a brief initial delay the recovery pursued an exponential timecourse.

Figure 12 shows typical plots of recovery time constant against return potential for an axon dialysed first with Cs and then with TMA. After reaching a peak at about −50 mV, τ_{rec} always fell back slightly until it could no longer be measured beyond −30 mV.

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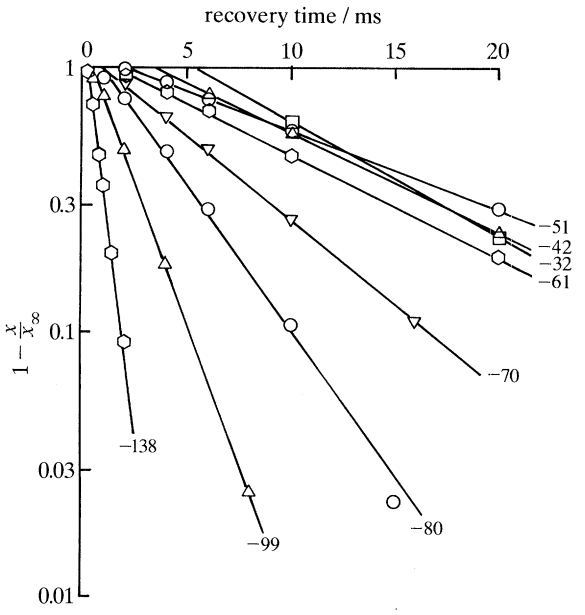

Figure 11. Semilogarithmic plots of the recovery of excitability after inactivation at 0 mV on returning to the potentials shown in mV on the right. For a given level of return potential V_h, the response x for a 20 mV test pulse was recorded after an appropriate series of recovery intervals t_r, and the points show the values of $1-x/x_\infty$ plotted against t_r where x_∞ was the response for complete recovery. The recovery time constant τ_{rec} for each V_h was given by the reciprocal of the slope of the straight line fitted to the points. Axon dialysed with TMA; diameter 620 μm. Temperature 5°C. Data files I21nov.s19/26.

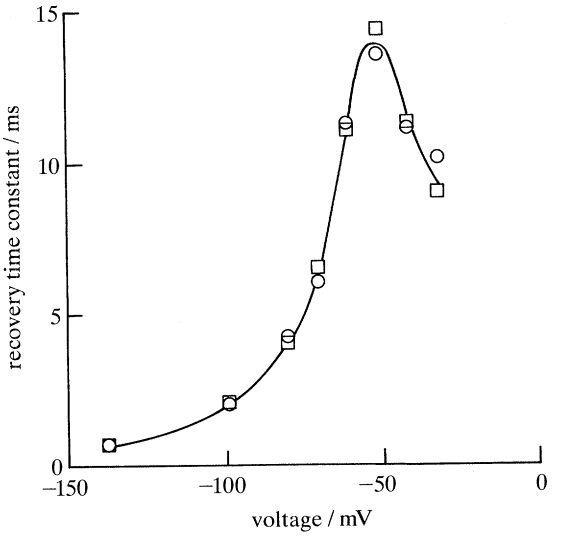

Figure 12. Squares: plots of the recovery time constant τ_{rec} against V_h for the data shown in figure 11. Circles: values for the same axon when dialysed with Cs.

Calculation with the aid of equation (2) of the effective charge carried by the recovery step gave a value of 1.31e between −100 and −140 mV (Keynes 1991).

Substitution of TMA for Cs in the dialysis solution had no significant effect on the rate of recovery.

(*h*) *The instantaneous I–V relationship in the steady state*

Starting with the membrane clamped at 74 mV in the non-inactivating open state, the potential was stepped to other levels in order to measure the instantaneous change in current and the time constants for arrival at a fresh open state. Typical families of records for an experiment of this kind with steps from both 74 and 150 mV during dialysis with TMA are shown in figure 13.

Backward extrapolation of the traces to the starting time of the step pulse enabled the immediate change in current at the new potential to be measured, and figure 14 shows instantaneous I–V curves determined in this fashion for the experiment of figure 13, and for another axon dialysed with Cs. It will be noted that the current when stepping from 150 mV was uniformly nearly 40% greater than when stepping from 74 mV, with a reversal potential close to 40 mV, as in the data listed in table 1. This behaviour is what would be expected if the steady-state probability of opening of the channels during dialysis with TMA was 1.4 times greater at 150 mV than it was at 74 mV, independently of the complications caused by the voltage-dependent blocking of the channels by TMA and Tris.

The timecourse of the transition from the steady state at 74 or 150 mV to that at the step potential was examined by fitting exponentials to the data of figure 13. It was found that as may be seen in figure 15, the traces were well fitted by the sum of two exponentials whose amplitudes were roughly equal, one relaxing about four times faster than the other. The time constants were roughly the same, as would be expected, for both of the starting potentials, and had maxima at around 50 mV, that is to say near the point of inflexion of the curve for the rise of $P_{Na,non}$.

In one respect the results shown in figure 14 departed significantly from our initial expectations. For a system that obeys the constant field relationship, the ratio of the limiting slopes of I–V for inward and outward currents would be expected to approximate to the ratio of the chemical driving forces $[Na]_o/[Na]_i$, which was about 5. When dialysing with TMA, the ratios of the limiting slopes were appreciably greater than this, being about 10 when stepping from 74 mV, and 12 when stepping from 150 mV. When dialysing with Cs, however, the slope of the I–V curves exhibited an opposite trend. In the experiment illustrated in figure 14 the limiting ratio was about 0.5, whereas in others it was closer to unity. Under these conditions, the constant field I–V curve would, of course, be multiplied by any voltage-dependent blocking of the channels that was taking place at the same time, i.e. a block decreasing with a more positive V_p for Tris (Keynes *et al.* 1991), but increasing with V_p for TMA (Horn *et al.* 1981; Keynes *et al.* 1992). In addition, a marked flattening off of the instantaneous I–V curve for large negative potentials has recently been reported by Vandenberg & Bezanilla (1991), and following Woodhull (1973) and others, has been shown to be due to a block of the channels by Ca^{2+} and Mg^{2+} in the external medium. The departures

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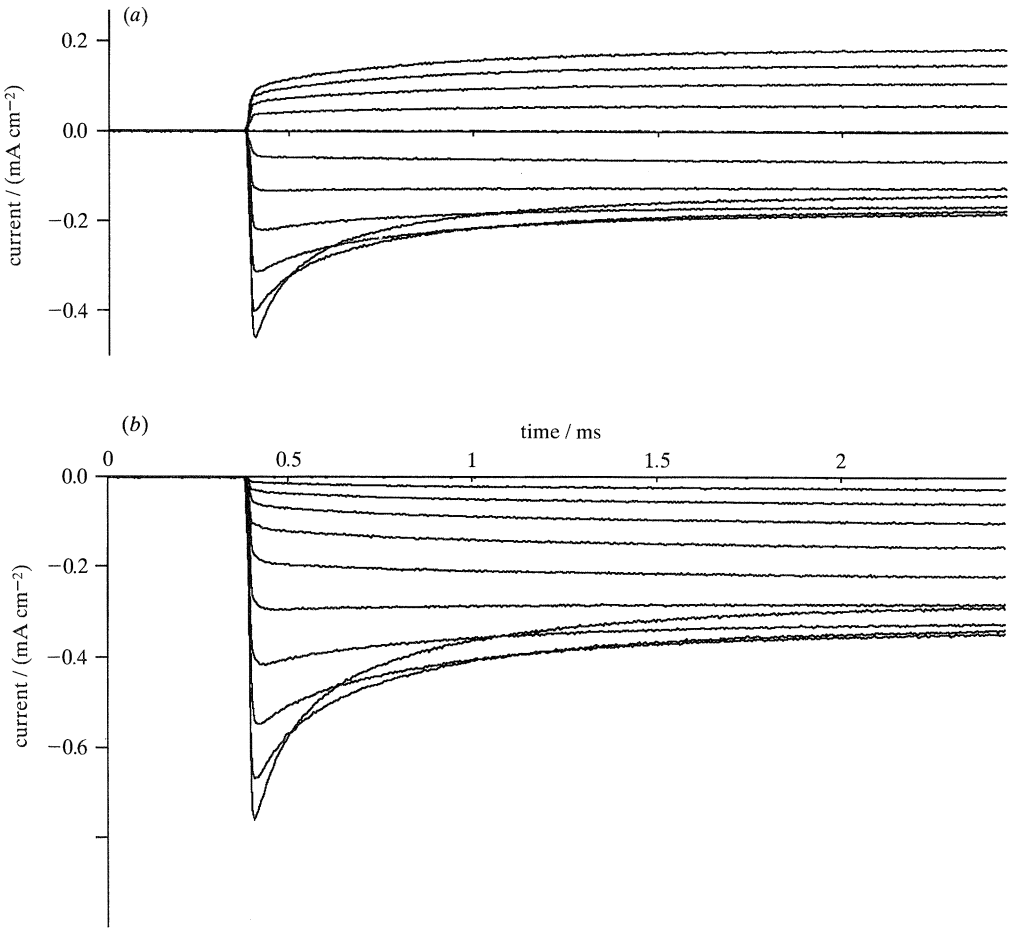

Figure 13. Step experiments for an axon dialysed with TMA in which the potential was stepped in turn to −42, −22, −3, 16, 35, 45, 74, 93, 112, 131 and 150 mV. (*a*) Starting from the steady state after 20 ms at V_p 74 mV, where the outward current was 0.118 mA cm^{-2}. (*b*) Starting from the steady state at V_p 150 mV, where the current was 0.286 mA cm^{-2}. Temperature 5°C. Data files K11oct.s01/03.

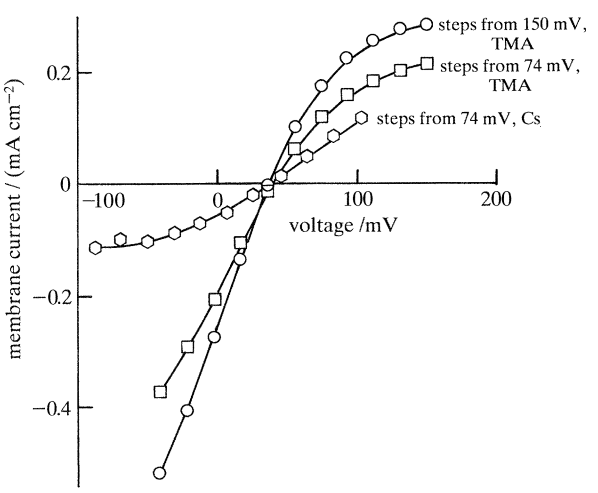

Figure 14. Instantaneous *I–V* curves for the steady-state current. Data from figure 13 for dialysis with TMA, stepping from 150 mV (circles) or 74 mV (squares). Octagons: stepping from 74 mV for an axon dialysed with Cs, Data file K09oct.s02.

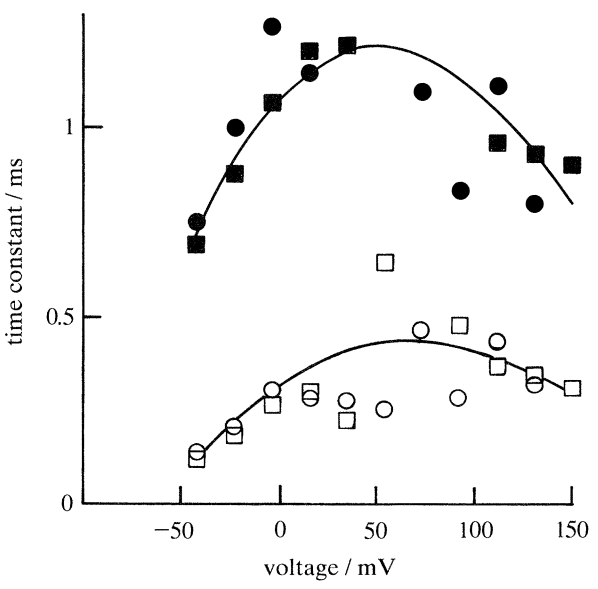

Figure 15. Slow time constants (solid symbols) and fast time constants (open symbols) for the transitions to the new steady states in the experiment of figure 14. Circles: steps from 150 mV. Squares: steps from 74 mV.

from the constant field I–V curve seen in figure 14 can evidently be attributed to the simultaneous occurrence of these several types of block, but more experiments are needed to determine the relative sizes of their contributions, and the extent to which there may be competition between Na* and the other cations.

By stepping from several different potentials in turn, it should be possible to make observations on the voltage-dependence of the mean open time of the channels in the steady state without the complications arising from alterations in single-channel conductance due to the blocking cations. Further studies of this kind should also be profitable to examine the kinetics of entry into the steady state, and the precise source of the two time constants seen in figure 15.

4. DISCUSSION

These findings place a fresh emphasis on the importance of the non-inactivating open state of the sodium channel (Chandler & Meves 1970), and reveal that its properties differ significantly from those of the normal open state in more than one respect. It has sometimes been thought that this second open state might be a consequence of perfusion or dialysis with solutions containing fluoride, but its existence has been demonstrated in intact squid axons by Shoukimas & French (1980), and in myelinated nerve fibres by Schmidtmayer (1989), so that it does not arise exclusively when F^- ions are present at the inner surface of the membrane. It has also been observed in patch-clamp recordings from cut-open squid axons (Bezanilla & Correa 1991) that 90% of the openings of a single channel take place with a very brief latency, while the remaining 10% that generate the steady-state current display a much longer latency. This shows that each channel has both normal and non-inactivating open states, and argues strongly against the possibility that two different types of channel might be present.

According to the constant field equation (Goldman 1943; Hodgkin & Katz 1949; Lakshminarayanaiah 1984), the potential V_{rev} at which the net ionic current is zero is given for an axon dialysed with Cs by

$$V_{rev} = \frac{RT}{F} \ln \frac{P_{Na}[Na]_o + P_{Tris}[Tris]_o + 4P_{Ca}[Ca]_o + 4P_{Mg}[Mg]_o + P_F[F]_i}{P_{Na}[Na]_i + P_{Cs}[Cs]_i + P_{Cl}[Cl]_o}, \quad (3)$$

where the quantities in square brackets are the internal and external ionic activities, and the Ps are the permeability coefficients for the principal ions present in the bathing and dialysis solutions. Because under the conditions of our experiments the external concentrations of $Tris^+$, Ca^{2+}, Mg^{2+} and Cl^-, and the internal concentration of F^-, were constant, then if the activity coefficients are assumed to be equal inside and outside, it follows that for dialysis with CsF at 5°C

$$V_{rev} = 24.0 \text{ mV} \ln \frac{[Na]_o + (P_X/P_{Na})[X]_o + (P_F/P_{Na})[F]_i}{[Na]_i + (P_{Cs}P_{Na})[Cs]_i + (P_{Cl}/P_{Na})[Cl]_o}, \quad (4)$$

where the X term represents the combined contribution of the external cations $Tris^+$, Ca^{2+} and Mg^{2+}. If $P_{Cl}/P_{Na} = 1/300$ (Chandler & Meves 1965), and P_F/P_{Na} is of the same order, while $[Cl]_o = 656$ mM and $[F]_i = 350$ mM, then with $[Na]_o = 103$ mM and $[Na]_i = 20$ mM,

$$V_{rev} = 24.0 \text{ mV} \ln \frac{104.2 + (P_X/P_{Na})[X]_o}{22.2 + (P_{Cs}/P_{Na})[Cs]_i}. \quad (5)$$

Now for $I_{Na,fast}$, it has been estimated by Chandler & Meves (1965) that $P_{Cs}/P_{Na} = 1/61$; and from table 1 the mean value of $V_{rev,fast}$ during dialysis with Cs at 5°C was 43.4 mV. From equation (5) it follows that $(P_X/P_{Na})[X]_o = 64.2$ mM. On dialysis with TMA, the average value of $V_{rev,fast}$ fell to 41.2 mV (table 1), so that if the X term had remained unaltered, then P_{TMA}/P_{Na} would be 1/41. In the initial open state this calculation would therefore suggest that the channels were slightly more permeable to TMA^+ ions than to Cs^+. The numbers change slightly if allowance is made for the greater blocking of the channels by external Tris with Cs dialysis than with TMA (see Keynes *et al.* 1991), but the qualitative conclusion still holds good that if the X term was always the same, the negative shift of V_{rev} in the presence of internal TMA could only mean that the relative permeability for TMA was appreciably greater than that for Cs. This directly contradicts the conclusions of Cahalan & Begenisich (1976) for squid axons, Hille (1971) for frog nerve, and Heggeness & Starkus (1986) for crayfish axons that P_{TMA}/P_{Na} is actually less than 1/500, and a more readily acceptable explanation for the shift in V_{rev} would be that during dialysis with TMA the permeability of the channel to Ca^{2+} and Mg^{2+} in the initial open state is reduced by a factor of about 3, so that the X term falls to 22.2 mM.

We then have to account for the shifts in V_{rev} between the initial and non-inactivating open states, which were −8.8 mV with Cs and −1.3 mV with TMA. Taking P_{Cs}/P_{Na} as 1/61 and P_{TMA}/P_{Na} as 1/500 in both states, the values of the X term in equation (5) when dialysing with Cs would fall from 64.2 to 12.6 mM, while when dialysing with TMA they would change only from 22.2 to 15.4 mM.

Although these calculations depend unavoidably on assumptions that are less than secure, the observed shifts in the reversal potential were entirely consistent, and the simplest explanation for the data would be that when dialysing with Cs, the relative permeability of the sodium channels to divalent cations is appreciably lower in the steady state than in the initial open state. When dialysing with TMA, the permeability to divalent ions is reduced in both open states, and the fall on passing to the final steady state is relatively small. It seems conceivable that this aspect of the behaviour of the sodium channel may reflect the possible difference in its degree of hydration in the two open states suggested by Keynes (1992), and be related to competition between the various cations.

The most obvious explanation for the differences in the overall permeability coefficients for the macroscopic sodium current flowing through the open channels would invoke the type of concentration and voltage-dependent blockage by competing mono-

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valent cations that has been described by Cahalan & Begenisich (1976), Begenisich & Cahalan (1980*a*,*b*) and Hille (1992), with contributions from protons (Begenisich & Danko, 1983), Tris (Keynes *et al.* 1991) and divalent cations (Vandenberg & Bezanilla 1991) that further complicate the situation. As far as the initial open state is concerned, this idea is fully supported by the observations of Horn *et al.* (1981) on the effect of internal TMA on sodium channels from rat myotubes, and by the fluctuation analyses of Bekkers, Greeff & Keynes (1986) which showed that in the squid giant axon the single-channel sodium conductance was halved by the presence of 50 mM TEA at the inner side of the membrane. However, it has to be questioned whether such an action on the conductance of open channels would be readily capable of accounting at one and the same time for both the decrease of $P_{Na,fast}$ and the increase of $P_{Na,non}$ that are seen to be brought about by internal TMA in squid. It may therefore be suggested that an entirely different kind of process takes place alongside the blocking of the channels once they have been opened, which involves a voltage and TMA-dependent alteration of the mean open time in the steady state. The evidence for this idea is discussed by Keynes *et al.* (1992), and its implications for models of the sodium channel are considered further by Keynes (1992).

As has been argued elsewhere (Keynes 1991; Greeff & Forster 1991), there is good reason to suppose that in the squid giant axon inactivation is controlled by a voltage-dependent mechanism. A strong argument in support of this proposition is the steep dependence on potential of the rate of recovery from inactivation that is seen in figure 12, and which corresponds to a recovery charge of 1.31*e*. Moreover, evidence has been presented by Forster & Greeff (1989) and Greeff & Forster (1991) for the existence of a quantal inactivation charge of about 1.2*e*. The model put forward by Keynes (1990, 1991) accordingly incorporated a step carrying this amount of charge between states A and B1 of voltage-sensor S4d, and computer simulations showed that the voltage-dependences of both τ_{rcc} and τ_h were predicted well but not perfectly for potentials between -140 and 20 mV. The identification in figure 8 of a fast component of τ_h that can be recorded at 15°C, and that between -10 and 35 mV falls off at a rate corresponding to the same inactivation charge, further strengthens the argument (Keynes 1992).

It has to be admitted, however, that the flattening off of the τ_h curve that is always observed for more positive potentials (see figures 7 and 8) until at V_p 100 mV the calculated charge has fallen to 0.2*e*, raises a major difficulty for the proposition that inactivation is a strongly voltage-dependent process. The explanations considered by Keynes (1991) involving cooperativity in the activation of the individual S4 units, and a weakening of the electric field at the upper end of the potential range, might go some way towards resolving the problem, but could not be claimed to provide a really convincing explanation for the flattening of the τ_h curve.

It has been pointed out to us by Professor Martin Rayner that if a relatively slow and voltage-independent step was interposed between the completion of the voltage-driven stages of activation and the actual opening of the channels, it would affect the rate of arrival at the initial open state, and in all probability that at the steady state as well. The case for including solvent-sensitive but voltage-insensitive transitions of this kind in modelling the kinetics of voltage-gated ion channels to account for the effects of heavy water and hyperosmolar media is a strong one, not only for squid axons (Conti & Palmieri 1968; Meves 1974; Conti *et al.* 1984), but also for other species and other types of channel (Schauf & Bullock 1979; Alicata *et al.* 1990; Chen & Hess 1990; Rayner *et al.* 1992). The existence of such a rate-limiting step in the activation pathway would account for the flattening of the curve relating τ_m to V_p that was already evident above 20 mV in figure 8 of Keynes & Kimura (1983); and in an axon dialysed with Cs we found that beyond 100 mV the time to peak had reached a flat plateau, although at this potential the rate of relaxation of the gating current was still falling. In a similar way, τ_h would be prevented from falling below a limiting value for large positive pulses.

The idea that the processes of activation and inactivation are subject to rate-limiting steps whose effect has hitherto been ignored in modelling the sodium system is consequently a most attractive one, which might readily account for the flattening off of both the τ_m and τ_h curves. Hydration steps have therefore been included in the series-parallel model put forward by Keynes (1992). However, it is only possible to speculate as to precisely how they might fit in, and there is no direct evidence on the details of their kinetics. They would also affect the rate of recovery of the system, and further investigations are needed into their effects on the ionic tail currents.

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