TETRAMETHYLAMMONIUM IONS ALTER SODIUM-CHANNEL GATING IN *MYXICOLA*

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ABSTRACT In *Myxicola* axons, substitution of tetramethylammonium (TMA^+) for Cs^+ alters intramembrane charge movements (gating currents). Although the total charge moved during and following a depolarizing step remains constant, with TMA^+ the ON response has additional slower component(s), and the OFF response is retarded. Concommitantly, TMA^+ produces the same voltage-dependent block of Na^+ inactivation in *Myxicola* as has been observed in other preparations. At large positive potentials as many as 70% of the Na^+ channels fail to inactivate in the steady state. In addition, TMA^+ slows Na^+ activation, retards the inactivation of those Na^+ channels that remain able to inactivate, and decreases the maximum Na^+ conductance. The steady-state Na^+ conductance induced by internal TMA^+ or Na^+ is consistent with a scheme in which these internal cations simply modify Na^+ channels in an all-or-none fashion so that a fraction become incapable of inactivating.

INTRODUCTION

Tetramethylammonium (TMA^+) is widely used as a K^+-channel impermeable ion in gating current experiments (Armstrong and Bezanilla, 1977), and as an inert substitute for Na^+ in various other protocols (Oxford, 1981). Thus, recent evidence demonstrating that internal TMA^+ can block Na^+ inactivation (Oxford and Yeh, 1979), and can occlude Na^+ channels (Oxford and Yeh, 1979; Horn et al., 1981) is cause for serious concern. If TMA^+ can generally alter Na^+ channel gating, then this must obviously be taken into account in interpreting not only ionic current records, but possibly also data on intramembrane charge movements. There exist many differences in behavior of Na^+-channel gating currents from one preparation to another (c.f. Schauf and Bullock, 1981), and it is possible that these arise in part from the varied composition of internal solutions used in different laboratories.

To further examine this issue we have recorded both Na^+ currents and intramembrane charge movements in *Myxicola* axons dialyzed with the two K^+ channel impermeable cations (Cs^+ and TMA^+) commonly used in gating current studies. In an attempt to define which cation produces behavior closest to that seen under normal physiological conditions, we also recorded gating currents in K^+-dialyzed axons in which the K^+ current was reduced by 4-aminopyridine. The results show that the choice of an internal solution can significantly affect gating currents, implying the need for extreme caution in interpretation of such experimental data.

METHODS

Myxicola giant axons were voltage-clamped, internally dialyzed, and their series resistance was compensated for by methods described in previous publications (Bullock and Schauf 1978; 1979). Leak and capacity currents were eliminated by an appropriate analog circuit. Internal solutions contained 550 mM M^+ glutamate plus 50 mM M^+F^- (where M^+ was either Cs^+, TMA^+, K^+, or Na^+) and were buffered with 20 mM HEPES. External solutions contained 86 mM NaCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, and 360 mM Tris (i.e., 20% normal [Na^+]) for measurement of inward Na^+ currents during Cs^+ or TMA^+ internal dialysis, but 430 mM NaCl for measurement of outward currents in Na^+ dialyzed axons (in general we reduced the transient current magnitude as much as possible to avoid possible series resistance errors). For asymmetry current measurements Tris was the sole external monovalent cation, and in addition 10^{-6} M tetrodotoxin was present. Asymmetry currents were generally measured using a $P/4$ protocol (Armstrong and Bezanilla, 1977). The initial holding potential from which 16 depolarizing steps ($+P$) were applied was -80 mV. Linear charge movement was subtracted by summing the currents during 64 steps of amplitude $-P/4$ from a reference potential of -160 mV. On a few occasions a second reference potential of $+120$ mV was also utilized to insure that large shifts in the charge-voltage curves were not taking place in the presence of TMA^+. Temperature was 5°C and external and internal pH was 7.30 ± 0.05 in all experiments.

RESULTS

Effects of TMA^+ on Asymmetry Currents

Intramembrane charge movements measured with 600 mM TMA^+ internally present were consistently very different from those obtained using 600 mM Cs^+. A typical pair of raw experimental records is shown in Fig. 1. The most striking difference was that, whereas the ON asymmetry current during Cs^+ dialysis invariably declined as a single exponential at all voltages (the data are plotted semilogarithmically as the solid squares (■) in Fig. 2; see also Bullock and Schauf, 1979), the asymmetry current obtained with 600 mM TMA^+ internally was lower in initial amplitude and had an additional slow component (solid circles, ●, in Fig. 2). These asymmetry currents were obtained only minutes apart in the same axon, and there-

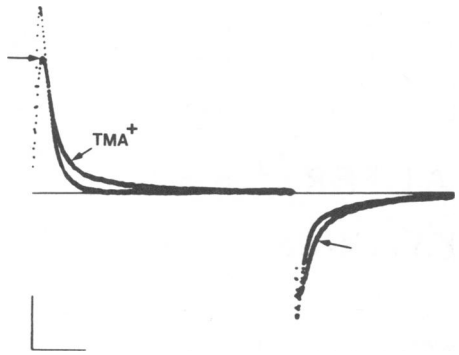

FIGURE 1 Effects of TMA^+ on intramembrane charge movements in *Myxicola*. Asymmetry current traces measured using a *P*/4 protocol (reference potential of −160 mV; holding potential −80 mV, test potential +20 mV) with Cs^+ (unmarked) and TMA^+ (→) dialysis shown superimposed. The base line represents zero current. Calibrations are 30 $\mu A/cm^2$ and 1.0 ms.

fore the differences observed in Cs^+ and TMA^+ seem likely to represent a real phenomenon. In this particular example the time constant of the TMA^+-induced slow component was 1.08 ms. The fast component of the decline in asymmetry current in TMA^+ was slightly slower than the decline measured in the presence of Cs^+, but the total charge moved, obtained by directly integrating the ON responses with Cs^+ or TMA^+ dialysis, was identical.

The results of a detailed analysis of such asymmetry current measurements in Cs^+ and TMA^+ as a function of membrane potential in eight axons are presented in Table I and illustrated by Fig. 3. Because the total charge moved for large depolarizations (Q_{max}) ranged from 14.1 to 21.4 nC/cm^2, the charge movements at other membrane potentials in each axon were normalized to the value of Q_{max} determined during Cs^+ dialysis. Four axons were first dialyzed with Cs^+ and then with TMA^+, while in the other four the sequence was reversed. The presence of a biphasic decline was seen only with TMA^+ dialysis in both instances, and thus was not due to a progressive change in the condition of the axons. The kinetic and steady-state data obtained at each voltage were averaged and the results expressed in Table I as the mean(s) ± standard error(s).

For voltages greater than −20 mV a substantial fraction of the total charge movement was contained in the TMA^+-induced slow component, and thus the asymmetry current could be unambiguously decomposed into the sum of two exponentials. We regard these data as quite accurate. For lower voltages, however, the slow component in TMA^+ was small (representing 15% or less of the total charge) and difficult to resolve. The procedure we followed in generating the data at −40 mV and −20 mV in Table I and Fig. 3 was to assume (*a*) that the base line of the ON response (leak pedestal) did not change in going from Cs^+ to TMA^+ or vice versa, and (*b*) that the time constant of the slow component in TMA^+ ought to be the same as (or slightly slower than) that measured at 0 mV. The fits thus obtained were hardly unique in the sense that a combination of a

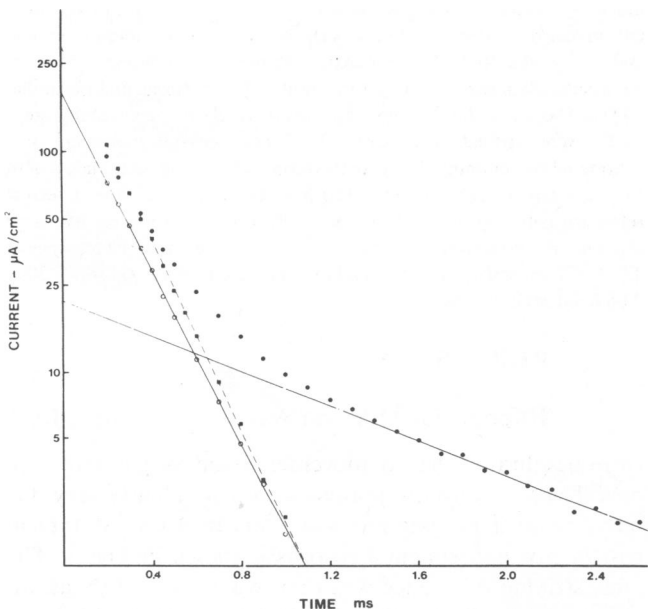

FIGURE 2 In this graph the ON responses for the asymmetry currents with Cs^+ (■) and TMA^+ (●) dialysis are plotted semilogarithmically. The dashed line (---) is the best least-squares fit by a single exponential to the ON response with Cs^+ dialysis, while the two solid lines are the best two time-constant fits to the data following TMA^+ dialysis (○, the calculated fast component in TMA^+). In Cs^+, τ_{ON} = 200 μs while for TMA^+ dialysis, the time constants τ^f_{ON} and τ^s_{ON}) were 220 and 1,080 μs, respectively. Temperature was 5°C.

TABLE I
EFFECTS OF TMA^+ ON INTRAMEMBRANE CHARGE MOVEMENT IN *MYXICOLA*

V_m	τ^f_{on}	τ^s_{on}	Q^s_{on}/Q^{Tot}_{on}	Q_{Tot}
(mv)	*(μs)*	*(μs)*		
Cs^+-dialyzed axons				
−40	272 ± 28			0.12 ± 0.04
−20	310 ± 36			0.31 ± 0.04
0	284 ± 33			0.62 ± 0.06
+20	212 ± 28	no slow component		0.84 ± 0.05
+40	147 ± 22			0.96 ± 0.04
+60	110 ± 23			0.99 ± 0.03
+80	98 ± 17			1.00
TMA^+-dialyzed axons				
−40	305 ± 37	1220 ± 440	0.09 ± 0.05	0.15 ± 0.04
−20	345 ± 32	1330 ± 390	0.16 ± 0.07	0.35 ± 0.09
0	320 ± 34	1090 ± 260	0.25 ± 0.06	0.66 ± 0.08
+20	230 ± 26	1110 ± 210	0.30 ± 0.07	0.86 ± 0.07
+40	177 ± 20	970 ± 140	0.32 ± 0.06	0.94 ± 0.05
+60	129 ± 24	780 ± 155	0.37 ± 0.05	0.98 ± 0.04
+80	117 ± 19	630 ± 145	0.40 ± 0.07	1.02 ± 0.04

Total charge movment (Q_{Tot}) was obtained by direct integration of the experimental records and normalized to the maximum charge movement obtained during Cs^+ dialysis. The ratio Q^s_{on}/Q^{Tot}_{on} was calculated in TMA^+ dialyzed axons from the value of τ^s_{on} and the extrapolated zero time intercept of the slow component. Data are given as mean ± standard error.

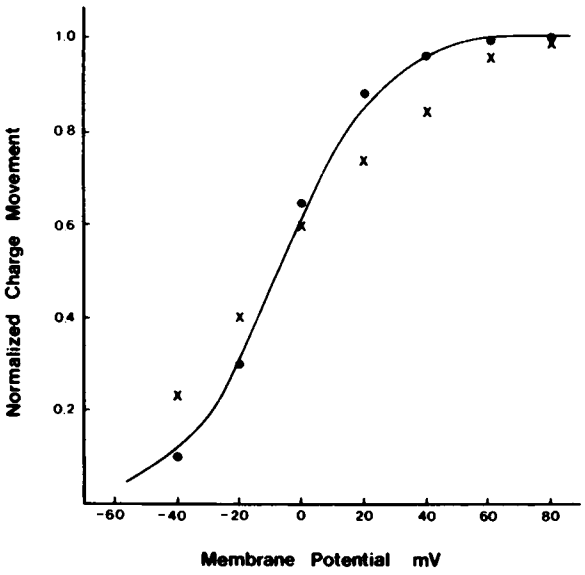

FIGURE 3 Charge voltage curves in Cs^+ and TMA^+. The solid line represents the relationship determined by direct integration of the asymmetry current records in Cs^+ and TMA^+ (column 5 of Table I). ● and X are the charge contained in the fast and slow components of the asymmetry current in TMA^+, respectively, obtained by fitting two exponentials to the falling phase of the asymmetry current, and then calculating $Q_f = I_f^0 \times \tau_{ON}^f$ and $Q_s = I_s^0 \times \tau_{ON}^s$ where the I^0's are the extrapolated zero time intercepts obtained from the semilogarithmic plots. Although the points are the means of several measurements, error bars have been omitted for clarity. Because of the scatter in the data and the uncertainty in fitting two exponentials to the data for $V < 0$ mV, the differences in these curves do not turn out to have statistical significance and thus must be regarded as only suggestive. The charge moving in the slow component amounted to 40% of the total charge at large positive potentials, but the $Q_f(V)$ and $Q_s(V)$ curves have been normalized so that the slopes can be compared.

slightly higher leak pedestal and a smaller value for τ_{on}^s would have yielded an equally good fit and, moreover, the standard deviations of the fitted parameters were quite large. However, they may at least serve to illustrate something of the qualitative characteristics of the TMA^+-induced changes at these voltages.

The total ON charge movement observed in TMA^+ was the same as that in Cs^+ (see the last line of column 5 in Table I). Moreover, the shape of the $Q_{total}(V)$ curves obtained by direct integration of the measured asymmetry currents were also the same (see column 5 of Table I and the solid line in Fig. 3) with a maximum slope of 20 mV/e-fold change and a midpoint at −12 mV. The two components of charge movement in TMA^+ (Fig. 3) appeared to have a slightly different voltage dependence, with the $Q(V)$ curve for the slow component being less steep (32 mV/e-fold) than that for the fast component (16 mV/e-fold). However, as these slopes are in large part computed on the basis of the measurements at −40 mV and −20 mV, they should be regarded as only qualitative indications of behavior.

The time constant of the slow component of charge movement in TMA^+ ranged from 0.6–1.4 ms, although there was considerable scatter from one axon to another. The time constant of inactivation of the Na^+ conductance during a step depolarization (τ_h^s) declined smoothly from 2.0 ± 0.2 ms at −40 mV to 0.80 ± 0.15 ms at +40 mV when measured using Cs^+ dialysis. During TMA^+ dialysis, these values were ~40% larger (see below) with τ_h^s averaging 2.7 ± 0.3 ms at −40 mV and 1.1 ± 0.2 ms at +40 mV. Thus, although for large steps the TMA^+-induced slow asymmetry current has a time course comparable to that of Na^+ inactivation, this correspondence is not seen at negative voltages. Also, with TMA^+ present internally, <10% of the total charge movement was contained in the slow component at −40 mV, 25% at 0 mV, and 40% at +80 mV. However, the fraction of Na^+ channels that were not able to inactivate in the presence of TMA^+ (see below) increased from 10% at 0 mV to 70% at +80 mV. Again, a quantitative correlation between modification of Na^+ inactivation and the appearance of a slow charge movement is not apparent.

The fast component of charge movement in TMA^+ was consistently slower than the charge movement in Cs^+, but the difference is relatively small. The OFF responses in TMA^+ at −80 mV were also generally slower than those in Cs^+. It should be stressed that the changes in the time course of intramembrane charge movement produced by TMA^+ were solely due to effects of TMA^+ on the total displacement current measured during and following the depolarizing pulses. The displacement currents obtained from the reference potential during the $P/4$ steps were unchanged following TMA^+ substitution.

In a few experiments we attempted to determine whether Cs^+ or TMA^+ dialysis produced results that were

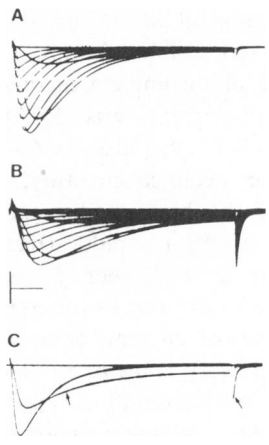

FIGURE 4 Effects of TMA^+ substitution for Cs^+ on the Na^+ current in *Myxicola*. In *A* are shown Na^+ currents during step depolarizations from −30 to +60 mV (10 mV increments) during 600 mM Cs^+ dialysis. Note the small size of the tail currents following repolarization. In *B*, Na^+ currents in the same axon for the identical voltage steps have been recorded following 20 min of dialysis with 600 mM TMA^+, and in *C* the currents for a step to +10 mV in Cs^+ (unmarked) and TMA^+ (→) are compared. Note the presence of both steady-state inward currents and large inward Na^+ tails with TMA^+. Calibrations are 0.2 mA/cm^2 and 2.0 ms.

more like physiological behavior by comparing charge movements with K^+- and Cs^+-dialysis. For these experiments 10 mM 4-aminopyridine (4AP) was used to block the K^+ current. Although 4AP does not completely eliminate the delayed rectifier, it does allow the measurement of asymmetry currents for pulses of 2 ms or less, provided the voltage steps are not too large (Schauf et al., 1977). Gating currents measured in axons exposed to 4AP were not altered when the internal K^+ was replaced by Cs^+, suggesting that Cs^+ is relatively inert, at least under the conditions of these experiments, and that TMA^+ is indeed the pharmacologically active species.

Effects of TMA^+ Substitution on Ionic Current

Tetramethylammonium alters the ionic currents in *Myxicola* in much the same way as has been reported for other preparations (Oxford and Yeh, 1979; Horn et al., 1981). With 600 mM Cs^+ dialysis (top records in Fig. 4), the inward Na^+ currents completely inactivated during an 18 ms depolarizing pulse as evidenced by the absence of any significant tail currents following repolarization to −80 mV. In contrast, when the internal solution was changed to 600 mM TMA^+, the Na^+ currents only partially inactivated so that a quasi–steady-state inward current remained at the end of the pulse (middle records in Fig. 4 — note also the comparison of the membrane currents for a depolarization to +10 mV shown at the bottom). If the pulse duration was made very long (not shown) this residual inward current declined with a time constant of 100–300 ms. That this was indeed a Na^+ current was shown by measurements of reversal potential (in axons in which $[Na^+]_i$ was increased) and by its TTX sensitivity. Following termination of an 18 ms depolarizing pulse, TMA^+ dialyzed axons exhibited a large inward Na^+ tail current, indicative of incomplete inactivation. The time course of these tail currents was slower than that seen following shorter (1–2 ms) pulses in Cs^+ dialyzed axons (see below), but they declined smoothly, without a hook.

The fraction of the Na^+ conductance that does not inactivate during an 18 ms pulse (termed h_{ss}) can be calculated either as $I^{\infty}_{Na}/I^{pk}_{Na}$, where I^{∞}_{Na} is the steady-state Na^+ current at the end of the 18 ms command pulse and I^{pk}_{Na} is the peak inward current, or as $I^{tail}_{Na}(V_h - E_{Na})/I^{pk}_{Na}(V - E_{Na})$ where I^{tail}_{Na} is the amplitude of the Na^+ tail current (extrapolated to the time of repolarization); V and V_h being the command and holding potentials, respectively. For moderate depolarizations both these measurements gave comparable values for h_{ss}. For larger depolarizations the Na^+ currents were small, and thus h_{ss} was best estimated from tail currents. With Cs^+ internally $I^{\infty}_{Na}/I^{pk}_{Na}$ was <0.05 between −30 and +80 mV. However, with 600 mM TMA^+ the ratio $I^{\infty}_{Na}/I^{pk}_{Na}$ increased from a negligible value at −30 mV to ~0.1 at 0 mV, 0.35 at +40 mV, and finally to between 0.6 and 0.7 at +100 mV. At all voltages the magnitude of this TMA^+-induced steady-state Na^+ conductance was two to three times larger than that previously seen with 600 mM Na^+ present internally (Schauf and Bullock, 1979). These data are qualitatively consistent with recent observations by Oxford and Yeh (1979) on internally perfused squid giant axons. Note that the values for h_{ss} obtained formerly using Na^+ dialysis (Schauf and Bullock, 1979) were based on outward current measurements, while those with TMA^+ reported here were derived under conditions where I_{Na} is inward. Because, if anything, an inward Na^+ flux should clear the channel of TMA^+, the results suggest that TMA^+ binds much more strongly than Na^+ to some site near the internal membrane surface that must remain free for normal inactivation to occur.

In addition to a voltage-dependent block of Na^+ inactivation, internal TMA^+ altered the ionic currents in other significant ways. The maximum Na^+ conductance determined from the peak inward currents (which is thus not corrected for inactivation) was decreased by 28 ± 8% (n = 6) in TMA^+. Sodium activation (measured as time to peak I_{Na}) was slowed by 24 ± 3% (n = 17) at all voltages, while the time constant of the Na^+ tail current at −80 mV (following short pulses) was increased by 23 ± 6% (n = 19). The time constant for the decline of that portion of the Na^+ conductance which remained capable of undergoing inactivation during a maintained depolarization $\tau_h{}^s$ was increased by 37 ± 4% (n = 15) in TMA^+. However, there was no effect of TMA^+ on the position of the $g_{Na}(V)$ curve along the voltage axis, or on the shape or position of the $h_{\infty}(V)$ curve as determined in two pulse experiments for $V_{pp} \leq -20$ mV. No outward current was observed at any voltage with TMA^+ internally, whereas with Cs^+ the early channel current reverses at about +80 mV, consistent with a Na^+/Cs^+ selectivity ratio of 25:1 (Schauf and Bullock, 1980). The kinetic effects of TMA^+ are specific and were not seen in axons dialyzed with high $[Na^+]$ rather than K^+, or when internal K^+ was replaced by Rb^+ or Cs^+. The effects of TMA^+ were not use dependent.

Nature of the Steady-State Na^+ Conductance

One interesting question was whether the steady-state Na^+ conductance observed in *Myxicola* axons dialyzed with TMA^+ (or Na^+) arose from a population of sleepy Na^+ channels similar to those studied by Matteson and Armstrong (1982) in squid giant axons. In contrast to the peak I_{Na}, the steady-state Na^+ current in squid axons appears to be temperature independent. Occasionally, at low temperatures (1°C) the I_{Na} in squid activated, partially inactivated, and then slowly increased again. In addition, a depolarizing prepulse often increased the peak Na^+ current during a subsequent pulse following after an interval of ~ 100 ms (Matteson and Armstrong, 1972).

None of this behavior was observed in Na^+- or TMA^+-

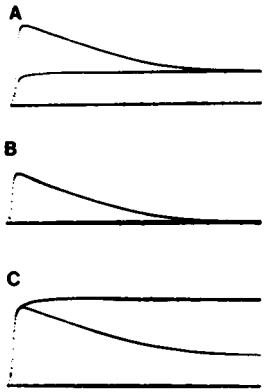

FIGURE 5 Example of prepulse separation of Na^+ current components in *Myxicola* axons dialyzed with 600 mM Na^+. In *A* are shown the Na^+ currents during an unconditioned depolarization to +20 mV (upper trace) and during an identical step preceded by a 50 ms prepulse to +20 mV (lower trace). The prepulse was separated from the test pulse by an 0.5 ms gap. The currents in *A* were subtracted and the result is shown in part *B*. Such processed records were used to compare the rate of inactivation of open channels under conditions in which inactivation was incomplete with that seen during Cs^+ dialysis. In *C* the current in *A* following a prepulse has been scaled to have the same maximum amplitude as that measured without a prepulse to compare the relative rates of activation.

dialyzed *Myxicola* axons at either high (13°–14°C) or low temperatures (1°–2°C). Both the peak and steady-state Na^+ currents increased on warming with a Q_{10} of 1.26 ± 0.08 (similar to the temperature dependence of peak I_{Na} previously measured in intact axons), and biphasic activation kinetics were never observed. Fig. 5 shows the result of an experiment in which a depolarizing prepulse was used to separate the inactivating component of g_{Na} from the steady-state component in a Na^+-dialyzed axon (Na^+ dialysis was used because large, easily measured, outward currents were present under these conditions. With TMA^+, g_{Na} has to be derived using tail currents). The only effect of a depolarizing prepulse sufficient to produce fast inactivation of the Na^+ channel was to first reduce, and then eliminate the peak Na^+ conductance during a subsequent test pulse with no effect on the steady-state conductance. If we used such prepulses experimentally to separate the inactivating and noninactivating conductances, we found that both components had the same kinetics of activation (Fig. 5 *C*) and that the rate of inactivation of the inactivatable component (Fig. 5 *B*) was the same as was measured during Cs^+ dialysis (when compared at the same voltages). If the interval between prepulse and test pulse was lengthened, the effect of the prepulse was diminished, and for gaps on the order of 50–100 ms the prepulse produced no change in peak I_{Na}.

Overall, our data seem consistent with a simple scheme in which internal Na^+ or TMA^+ simply modifies Na^+ channels in an all-or-none fashion so that a fraction become incapable of inactivating. This conclusion is supported by the fact that agents (for example D_2O, see Schauf and Bullock, 1980) which slow Na^+ activation, and reduce $\bar{g}_{Na}$ are equally effective on both the inactivatable and noninactivatable components. The behavior of *Myxicola* may thus be less complex than that observed by Chandler and Meves (1970 *a–d*) in squid axons perfused with NaF solutions.

DISCUSSION

In squid axons (Oxford and Yeh, 1979), TMA^+ substitution for Cs^+ greatly increases (from an initially nonzero value) the residual Na^+ current at the end of long depolarizing pulses. The only difference between these results and our own is that, in *Myxicola*, there is little or no steady-state Na^+ conductance in axons dialyzed with K^+ or Cs^+ (Schauf and Bullock, 1978; 1979). In the presence of TMA^+ the ratio $I_{Na}^{\infty}/I_{Na}^{pk}$ as determined both from tail currents and direct measurement of the residual I_{Na}, increases from negligible values at $V \leq -20$ mV to values of 0.6–0.8 at +100 mV. The fact that TMA^+ substitution does not alter the $h_\infty(V)$ curve for $V \leq -20$ mV suggests that those channels which are not affected by TMA^+ have normal steady-state inactivation properties. In addition to producing a voltage-dependent block of Na^+ inactivation in *Myxicola*, TMA^+ slows Na^+ activation, retards the inactivation of those Na^+ channels which remain able to inactivate, and decreases the maximum Na^+ conductance. The latter observation is comparable to that reported from macroscopic current measurements in squid axons (Oxford and Yeh, 1979) and from single-channel recordings in rat myotubes (Horn et al., 1981).

The most significant result of this study is the observation that TMA^+ alters the form of the measured asymmetry current from that recorded using Cs^+ or 4-aminopyridine. Although the total charge moved following a step depolarization remained constant when Cs^+ was replaced by TMA^+, the ON gating current developed a clearly defined slow component, and the OFF response was altered as well. The charge-voltage curve for the slow component appeared to be less steep than that describing the fast component. Clearly the concern expressed by Horn et al. (1981) that TMA^+ could, by blocking Na^+ channels in a voltage-dependent fashion, alter gating currents seems well warranted.

It is interesting to speculate concerning the origin of these changes. Because charge balance was observed with both Cs^+ and TMA^+ (that is, the total ON and OFF charge movement obtained by direct integration of the data records were equal to one another), and there was no change in the membrane currents recorded during the hyperpolarizing reference pulses, it is unlikely that the TMA^+ result was produced by some nonlinear ionic current. Also, total charge movement was quite unchanged. A variety of experimental evidence, including the fact that substitution of heavy water (D_2O) for H_2O slows the activation kinetics of the Na^+ channel without having any

detectible effect on gating currents (Schauf and Bullock 1979, 1980, 1981), suggests that most (if not all) gating charge movement occurs in transitions between distinct nonconducting states which are precursors to an open channel. Within this context the TMA^+-induced gating current changes seem consistent with a scheme in which TMA^+ retards the charge movement between two particular nonconducting states of the sodium channel, while leaving other transitions unaffected.

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