SOLVENT SUBSTITUTION AS A PROBE OF CHANNEL GATING IN *MYXICOLA*

DIFFERENTIAL EFFECTS OF D_2O ON SOME COMPONENTS OF MEMBRANE CONDUCTANCE

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ABSTRACT Careful examination of the effects of solvent substitution on excitable membranes offers the theoretical possibility of identifying those aspects of the gating and translocation processes which are associated with significant changes in solvent order. Such information can then be used to develop or modify more detailed models. We have examined the effects of heavy water substitution in Cs^+- and K^+-dialyzed *Myxicola* giant axons. At temperatures of 4–6°C, the rates of Na^+, K^+, and Na^+ inactivation during a maintained depolarization were all slowed by ~ 50% in the presence of D_2O. In contrast, the effects of solvent substitution on the time-course of prepulse inactivation and reactivation were much larger, with slowing averaging 160%. Studies at higher temperatures yielded Q_{10}'s for Na^+ activation and K^+ activation which were essentially comparable (0.72) and slightly but significantly smaller than that for inactivation during a maintained depolarization (0.84). In contrast, the Q_{10} for the D_2O effect on prepulse inactivation was ~ 0.48. Heavy water substitution decreased $\bar{G}_K$ to a significantly greater extent than $\bar{G}_{Na}$, while the decrease in the conductance of the Na^+ channel caused by D_2O was independent of whether the current-carrying species was Na^+ or Li^+. Sodium channel selectivity to the alkali metal cations and NH_4^+ was not changed by D_2O substitution.

INTRODUCTION

We have previously described studies which indicate that deuterium oxide solvent substitution slows both the Na^+ and K^+ ionic conductances (G_{Na} and G_K) in *Myxicola* giant axons in a temperature-dependent fashion, while leaving the asymmetry current thought to be associated with Na^+ activation unaffected (Schauf and Bullock, 1979). At that time, the data was insufficient to resolve whether all of the various kinetic processes (Na^+ activation, Na^+ inactivation, K^+ activation) were similarly affected by D_2O or whether some differential effects might exist. The magnitude of the D_2O effect in *Myxicola,* combined with its unusually strong temperature dependence, suggests that formation of conducting channels involves appreciable changes in local solvent structure (Schauf and Bullock, 1979). Thus it is reasonable to expect that components of the ionic current kinetics might show different isotope sensitivities corresponding to differences in local solvent interactions.

We report here studies showing that D_2O substitution in *Myxicola* does in fact produce differential effects on Na^+ and K^+ kinetics. In addition, the maximum K^+ and Na^+ channel conductances also seem to be differentially affected, whereas the selectivity of a particular channel to the alkali cations and NH_4^+ remains unaltered. The latter result can be compared

with similar studies on gramicidin-doped lipid bilayer membranes (Tredgold and Jones, 1979). Overall, our findings indicate that systematic solvent substitutions may provide a useful means of probing molecular events occurring during channel gating.

METHODS

We have previously reported the methods of simultaneous internal dialysis and voltage-clamp of *Myxicola* giant axons (Bullock and Schauf, 1978) as well as the general protocol we follow in our experiments involving solvent substitution (Schauf and Bullock, 1979). This includes the necessity for recompensation of membrane series resistance to allow for the lower equivalent conductivity of electrolyte solutions in D_2O (Swain and Evans, 1966). When potassium currents were measured the internal solution was composed of 450 mM K glutamate, 50 mM KF, and 30 mM K_2HPO_4, and was buffered to pH 7.3 ± 0.1 with 1 mM Hepes. Otherwise, the dialyzate contained 600 mM Cs^+ and was K^+-free. The external solution was K^+-free artificial seawater containing 430 mM NaCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$, and 20 mM Tris, adjusted to pH 7.3 ± 0.1, except in those cases where sodium channel selectivity was measured.

Two aspects of the present study deserve special comment. Since our interest was in resolving as precisely as possible small differential effects of solvent substitution on both kinetic and steady-state channel properties, all comparisons were done between parameters measured on the same set of axons. To compare solvent effects on Na^+ activation to those on Na^+ inactivation, experiments were done on axons in which the K^+ conductance was blocked by Cs^+ dialysis (Bullock and Schauf, 1978). In each axon Na^+ currents were first recorded during a series of maintained depolarizations, to measure the rate of Na^+ activation, and allow the calculation of the time constant of Na^+ inactivation for those channels known to have become conducting (τ_h^s, Schauf and Davis, 1975). Next currents were measured during a fixed test depolarization which had been preceeded by conditioning prepulses of variable amplitude and duration to define the prepulse inactivation time constant (τ_h^p, Schauf and Davis, 1975). Currents were leak corrected by adding the current records obtained during equivalent hyperpolarizing command pulses. All experiments in D_2O were bracketed in the sense that measurements were initially made in H_2O, generally at more than one temperature, then repeated 15–20 min after D_2O substitution, and finally repeated again following a return to H_2O as the solvent. Measurements in heavy water could then be corrected for any small change which might have occurred between the two bracketing H_2O runs.

Comparisons between Na^+ activation and K^+ activation, as well as between the maximum Na^+ and K^+ conductances were made in axons dialyzed with K^+ glutamate rather than Cs^+. Both internal application of tetraethylammonium (TEA^+)[1] and external application of tetrodotoxin (TTX) were initially used to separate the Na^+ and K^+ currents. In later experiments this was found to be unnecessary since comparable results could be obtained by simply comparing the effect of D_2O substitution on peak Na^+ current at a fixed potential, and on the time at which the rate of rise of G_{Na} achieved its maximum value (called t_{max}^{Na}, with the effect of D_2O on K^+ kinetic and steady-state properties at potentials near the sodium equilibrium potential (E_{Na}, see Results). Again measurements in D_2O were bracketed by measurements performed in H_2O.

Measurements of solvent effects on Na^+ channel selectivity also deserve some comment. In experiments using Li^+ and NH_4^+ the axons were dialyzed with a solution containing 25 mM Na^+ in addition to the usual 600 mM Cs^+. This was done to guard against any slow change in reversal potential arising from the gradual washout of residual internal Na^+. Channel selectivity to Li^+ and NH_4^+ was then measured by replacing all of the external Na^+ (430 mM) with the substitute cation (M^+), determining the change in reversal potential that occurred, and calculating the permeability ratio (P_{M^+}/P_{Na^+}) from the equation (Hille, 1971, 1972):

$$\Delta V_R = \frac{RT}{F} \ln \frac{P_{M^+}}{P_{Na^+}} \frac{[M^+]}{[Na^+]} \frac{\gamma_{M^+}}{\gamma_{Na^+}},$$

[1] *Abbreviations used in this paper*: TEA^+, tetraethylammonium; TTX, tetrodotoxin.

where γ_{Na}^+ and γ_M^+ are the activity coefficients of the two ions, $[Na^+]$ and $[M^+]$ and their concentrations, ΔV_R is the change in reversal potential, and R, T, and F have their usual values. Measurements of ΔV_R were made first in H_2O, then in D_2O, and again in H_2O to enable a correction to be made for any progressive change in selectivity between the two H_2O determinations. Activity coefficients for Na^+, Li^+, and NH_4^+ were taken from tabulated values in H_2O (Robinson and Stokes, 1959). In general, activity coefficients are only very slightly altered in D_2O (Marshall and Katz, 1974), and we thus used the same values for the activity coefficient ratio as in H_2O.

The disadvantage of the foregoing evaluation of channel selectivity is that measurements are not made at a constant membrane potential, and to some extent selectivity may be voltage-dependent (Hille, 1975). However, the alternative approach of determining selectivity also has disadvantages. For measurements to be made at constant membrane potential internal concentrations must vary and this is known to alter selectivity (Cahalan and Beginisich, 1976; Ebert and Goldman, 1976). In addition, changes in internal composition produced using dialysis are relatively slow (Bullock and Schauf, 1978) and we felt it was critical to perform these measurements over as short an interval as possible.

The D_2O substitution studies of Na^+ and K^+ kinetics involved dialysis with either Cs^+ or K^+ in the absence of any internal Na^+. In the case that only one permeable cation (Na^+) is present externally and a different permeable cation (Cs^+ or K^+) is present internally (biionic conditions), determination of the reversal potential allows the direct calculation of the permeability ratios P_{Cs}^+/P_{Na}^+ and P_K^+/P_{Na}^+ (Ebert and Goldman, 1976; Bullock and Schauf, 1978) before and during D_2O substitution. The major concern here is that small amounts of residual internal Na^+ may make the calculated permeability ratios erroneously high (Results), although this should not affect the assessment of a D_2O effect.

RESULTS

D_2O on Maximum Channel Conductances

Heavy water substitution decreases the maximum sodium and potassium conductances ($\bar{G}_{Na}$ and $\bar{G}_K$) in both squid (Meves, 1976; Conti and Palmieri, 1968) and *Myxicola* (Schauf and Bullock, 1979) axons although previous data was insufficient to resolve whether or not there are comparable effects on both systems.

The results of our comparative studies in 17 axons can be summarized as follows. At 2–4°C, the average decrease in $\bar{G}_{Na}$ was 26 ± 2% (all results discussed here are given as means ± standard error) whereas the average decrease in $\bar{G}_K$ was 32 ± 2%, a difference which is statistically significant with $P < 0.002$ (unpaired t test). The difference is even more obvious if one only analyzes data from the 12 axons in which both $\bar{G}_{Na}$ and $\bar{G}_K$ were simultaneously measured using a paired t test. The average decreases in $\bar{G}_{Na}$ and $\bar{G}_K$ are unchanged, but the significance of the result is increased ($P < 0.0003$), and there are no instances in which D_2O decreased $\bar{G}_{Na}$ by more than $\bar{G}_K$ in any particular axon. We should note that initially $\bar{G}_{Na}$ and $\bar{G}_K$ were separately computed using TTX or TEA^+ for the current separation. However after making an extensive comparison between the effects of D_2O on peak inward current at a fixed membrane potential and on $\bar{G}_{Na}$ calculated (after blocking K^+ currents) by extrapolation of the falling phase of membrane current to zero time, we decided to simply measure the D_2O effect in axons with intact K^+ currents, as the results were identical in both procedures. Although relatively sparse, the data at 11–14°C shows no evidence for any temperature dependence of the isotope effects on $\bar{G}_{Na}$ and $\bar{G}_K$. At high temperatures the decrease in $\bar{G}_{Na}$ was 27 ± 5% and the decrease in $\bar{G}_K$ was 34 ± 3%, comparable to the effects seen at 2–4°C. It should be noted, however, that the Q_{10} for ionic conductances in bulk solution expected on the basis of the viscosities of the two solvents is only 0.97 (Hardy and Cottington, 1949), well within the scatter of this data.

In six axons (79M16–79M21) the isotope effect on sodium channel conductance was measured in both 430 mM Na^+ASW and in 430 mM Li^+ASW. The internal solution contained only Cs^+ in both cases. The decrease in Na^+ channel conductance by D_2O in these six axons was 24 ± 4% with Li^+ as the current carrier and 25 ± 4% with Na^+ as the current carrier (the latter is essentially identical to the 26% decrease derived from all axons examined). The ratio of Na^+ channel conductance in Li^+ ASW to that in Na^+ ASW was 0.84 ± 0.05 (n = 11) when measured in H_2O and 0.82 ± 0.04 (n = 5) when measured in D_2O. As might be expected, isotope effects on transient current activation and inactivation kinetics were completely independent of whether the current carrier was Na^+ or Li^+. In Na^+ ASW, time to peak sodium current was slowed in D_2O by 46 ± 5% whereas inactivation during a maintained depolarization was slowed by 48 ± 6%. In Li^+ ASW the D_2O induced changes were 47 ± 6 and 49 ± 5%, respectively.

Isotope Effects on Na^+ Channel Selectivity

Data concerning the effect of heavy water substitution on Na^+ channel selectivity in 16 axons are given in Table I. Results for Li^+ and NH_4^+ were obtained from the changes in reversal potential (ΔV_R) measured after equimolar substitution for external Na^+ (with 25 mM Na^+ present internally), while data for K^+ and Cs^+ were calculated from the absolute reversal potentials (V_R) measured under biionic conditions with 430 mM Na^+ present externally. There is clearly no significant difference in either V_R or ΔV_R when measured in D_2O compared to H_2O.

The magnitudes of the selectivities themselves were calculated from the average of all measurements in H_2O and D_2O after correction for activity coefficients (Robinson and Stokes, 1959), and are given in the last columns. The ratio of P_{Li}/P_{Na} determined from reversal potential measurements was 1.07 compared to a conductance ratio G_{Li}/G_{Na} of 0.84 (see above). The former does not agree exactly with the value of 0.94 determined previously from reversal potential measurements in *Myxicola* (Ebert and Goldman, 1976). Although this difference may not be significant, we feel that the measured disparity between conductance and permeability ratios is real. We were unable to make an accurate comparison of conductance and permeability ratios for the other cations. The relatively large values of P_{Cs}/P_{Na} and P_K/P_{Na} compared to previous values (Ebert and Goldman, 1976) may be due to

TABLE I
REVERSAL POTENTIAL AND IONIC SELECTIVITY IN *MYXICOLA* AXONS*

Ion	$V_R^{H_2O}$	$V_R^{D_2O}$	$\Delta V_R^{H_2O}$	$\Delta V_R^{D_2O}$	$\left(\frac{P_x}{P_{Na}}\right)^{H_2O}$	$\left(\frac{P_x}{P_{Na}}\right)^{D_2O}$
	mV	*mV*	*mV*	*mV*		
Li^+	—	—	+4.6 ± 2.3	+4.7 ± 2.2	1.05 ± 0.10	1.07 ± 0.09
K^+	+61.1 ± 2.5	+60.7 ± 3.0	—	—	0.076 ± 0.008	0.077 ± 0.009
Cs^+	+79.4 ± 2.0	+79.2 ± 1.5	—	—	0.037 ± 0.003	0.037 ± 0.002
NH_4^+	—	—	−23.0 ± 1.2	−22.6 ± 3.4	0.41 ± 0.02	0.42 ± 0.06

*Data given as mean ± standard error. V_R is the reversal potential, ΔV_R is the change in reversal potential observed on equimolar substitution of the indicated ion for external Na^+, and P_x/P_{Na} is the permeability ratio calculated according to the methods described in the text.

the presence of some residual internal Na^+, but the important feature of the data is the lack of any change in selectivity caused by D_2O substitution.

Isotope Effects on Na^+ and K^+ Activation Kinetics

The effects of D_2O substitution on Na^+ and K^+ activation were directly compared in 10 axons dialyzed with K^+ glutamate. As an indication of the rate of Na^+ activation we measured both time to peak inward sodium current (t_{Pk}^{Na}) and the time of maximum rate of rise of I_{Na} (t_{max}^{Na}), while K^+ activation was described by the time to reach one-half maximum steady-state current at potentials near E_{Na} ($t_{1/2}^{K}$). This was done in axons in which both Na^+ and K^+ currents were intact in order to make as precise a comparison as possible. It should be noted that we demonstrated in independent experiments that measuring Na^+ activation in this way gave precisely the same magnitude for the D_2O effect as when activation kinetics were derived by removing an assumed exponential inactivation in axons dialyzed with Cs^+ to block the K^+ conductance. Also in other control experiments we showed that the D_2O effect on K^+ kinetics was the same whether or not axons were poisoned with TTX.

Table II summarizes the results. In these axons t_{Pk}^{Na} was increased by 43 ± 3% in D_2O at temperatures of 2–4°C, t_{Max}^{Na} was increased by 47 ± 3%, and $t_{1/2}^{k}$ by 40 ± 2%. These effects are identical within experimental error. At temperatures of 12–14°C the increases in t_{Pk}^{Na}, t_{Max}^{Na} and $t_{1/2}^{k}$ were 20 ± 2, 20 ± 3, and 17 ± 2%, respectively. The Q_{10}'s for the isotope effect on these parameters derived using the original data points were 0.84 ± 0.01, 0.83 ± 0.02, and 0.82 ± 0.02, respectively. Thus there appears to be no significant difference in either the magnitude or temperature dependence of the D_2O effect on Na^+ activation compared to K^+ activation, although in this particular group of axons the actual magnitude of the D_2O effect at 2–4°C, and thus the temperature dependence, was slightly less than observed in the majority of axons we examined (Table III).

Isotope Effects on Na^+ Activation and Inactivation

The effect of heavy water substitution on the rate of Na^+ activation was compared to that on the rate of Na^+ inactivation during a maintained depolarization (τ_h^s) in six axons, while in an additional nine axons the effect of D_2O on prepulse inactivation (τ_h^p) was compared to that on τ_h^s. These data are summarized in Table III.

At temperatures of 3–6°C D_2O substitution slowed τ_h^s by 52 ± 3%, a value no different than the 47 ± 2% slowing of Na^+ activation. At temperatures of 11–14°C, however, τ_h^s was still

TABLE II
COMPARISON OF ISOTOPE EFFECTS ON NA^+ AND K^+ ACTIVATION

Temperature	t_{Pk}^{Na}*	t_{max}^{Na}*	$t_{1/2}^{K}$*
2–4°C	1.43 ± 0.03 (39)	1.47 ± 0.03 (34)	1.40 ± 0.02 (26)
12–14°C	1.20 ± 0.02 (16)	1.20 ± 0.03 (16)	1.17 ± 0.02 (16)
Q_{10}	0.84 ± 0.01 (16)	0.83 ± 0.02 (16)	0.82 ± 0.02 (16)

*Ratios in D_2O compared to H_2O. The numbers in parentheses give the number of measurements performed. Data are given as means ± standard error.

TABLE III
COMPARISON OF ISOTOPE EFFECTS ON NA^+ ACTIVATION AND INACTIVATION

Temperature	t_{Na}^{Pk}*	t_{max}^{Na}*	τ_h^s*	τ_h^p*
3–6°C	1.47 ± 0.02 (98)	1.47 ± 0.02 (70)	1.52 ± 0.03 (50)	2.61 ± 0.21‡ (22)
11–14°C	1.18 ± 0.03 (47)	1.15 ± 0.02 (34)	1.37 ± 0.03 (29)	1.43 ± 0.05 (10)
Q_{10}	0.72 ± 0.02 (27)	0.72 ± 0.03 (21)	0.84 ± 0.03§ (31)	0.47 ± 0.07 (10)

*Ratios in D_2O compared to H_2O. Numbers in parenthesis give the number of measurements performed. Data are given as means ± standard error.
‡Significant with $P < 10^{-5}$.
§Significant with $P < 0.001$.

substantially slowed by D_2O (37 ± 3%), while the rate of Na^+ activation was much less affected (t_{Na}^{Pk} was slowed by 18 ± 3% and t_{Max}^{Na} by 15 ± 2%). Thus the Q_{10} for the D_2O effect on τ_h^s (0.84 ± 0.03) was significantly larger than the Q_{10} for the D_2O effect on Na^+ activation (0.72 ± 0.03). These differences were statistically significant with $P < 0.001$ (unpaired t test).

The effects of D_2O substitution on prepulse inactivation were even more striking. At temperatures of 3–6°C prepulse inactivation was slowed by ~150% (τ_h^s increased to 2.61 ± 0.21 times normal) in the same axons in which τ_h^p was only slowed 50%. However, at 11–14°C the effect of D_2O on τ_h^p was similar to that on τ_h^s (an increase of 43 ± 5%), yielding a Q_{10} for the D_2O effect on τ_h^p of 0.47. An example of this is shown in Fig. 1 where the time-course of prepulse inactivation at −20 mV is plotted in both H_2O and D_2O. (This can be compared with

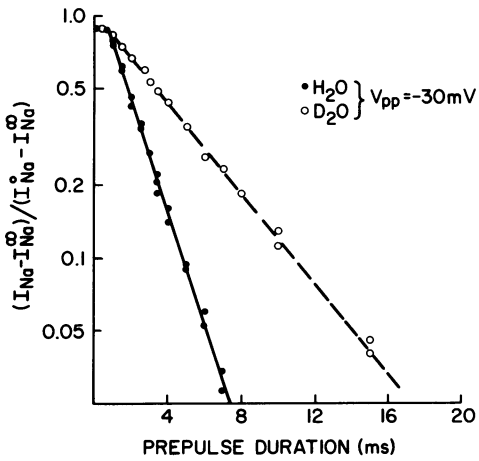

FIGURE 1 Time-course of prepulse inactivation in H_2O (●) and D_2O (○) at −30 mV. A prepulse to −30 mV of variable duration was followed by a test pulse to 0 mV, and the sodium current during the test pulse was measured, corrected for a linear leakage current, and normalized as indicated. Temperature was 5°C. The time constants of prepulse inactivation in this particular experiment were 1.8 ms in H_2O and 4.6 ms in D_2O.

the magnitude of the D_2O effect on step inactivation illustrated in Fig. 1 of Schauf and Bullock, 1979). Because the values for τ_h^s and τ_h^p at a particular voltage are comparable in Cs^+-dialyzed axons (Schauf and Bullock, 1979), the large differential effect of D_2O on τ_h^p at 3–6°C implies that the differences in τ_h^s and τ_h^p characteristic of intact (Goldman and Schauf, 1973) and K^+-dialyzed (Schauf et al., 1976; Schauf and Bullock, 1979) axons can be least partially restored in Cs^+-dialyzed axons by solvent substitution.

We also investigated the effect of D_2O substitution on recovery from inactivation (Schauf, 1976) in two axons. Using a protocol in which a 100-ms prepulse to −20 mV was followed by a return to a holding potential of −100 mV, and then by a brief test pulse to +20 mV, we found that in D_2O the time constant of recovery was increased to 2.44 ± 0.10 times its value in H_2O. In the same axons, however, time to peak I_{Na} was increased by 51 ± 4%, comparable to our other measurements. Thus recovery from inactivation shows the same large D_2O effect as prepulse inactivation itself.

Isotope Effects on Steady-State Inactivation

Fig. 2 shows steady-state Na^+ inactivation curves constructed by conventional protocols in Cs^+-dialyzed *Myxicola* axons in D_2O and H_2O. As was the case for the steady-state Na^+ and K^+ conductance-voltage curves (Schauf and Bullock, 1979), there is no effect of solvent substitution on the steady-state voltage dependence of Na^+ inactivation.

Effects of Other Solvent Substitutions

In addition to D_2O, we attempted early in the course of these experiments to examine the behavior of membrane currents in other ionizing solvents with very different physical properties. The first substance examined was formamide. Unfortunately at a concentration of 25% formamide makes the axons extremely sensitive to dielectric breakdown, so that even moderate depolarizations cause a progressive increase in leak conductance and a rapid loss of ionic currents. At higher concentrations the increase in leak occurred immediately upon exposure to formamide. This is consistent with the observation that lipid bilayer membranes

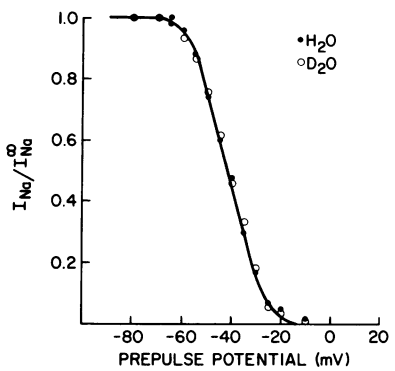

FIGURE 2 Steady-state inactivation curves in H_2O (●) and D_2O (○). In these experiments a prepulse 100 ms in duration was varied in amplitude and was followed by a fixed test pulse to 0 mV. The current during the test pulse was corrected for a linear leak and normalized to the maximum inward current obtained with a prepulse to −120 mV. Temperature, 5°C.

seem mechanically unstable when formed in the presence of formamide. (F. Cohen. Personal communication.)

DISCUSSION

To begin let us review and summarize the effects of solvent substitution in *Myxicola* axons. When H_2O is replaced by 99.8% D_2O both externally and internally certain biophysical phenomena are entirely unaffected. This includes the nonlinear charge movement thought to be associated with activation of the Na^+ channel (Schauf and Bullock, 1979), the steady-state voltage dependence of the Na^+ and K^+ channel conductances (Schauf and Bullock, 1979), steady-state Na^+ inactivation, and intracationic selectivity. In fact, the only nonkinetic parameters which are affected by D_2O appear to be the maximum conductances themselves. $\bar{G}_K$ is decreased to a significantly greater extent than $\bar{G}_{Na}$ in D_2O and the decrease in conductance of Na^+ channels in D_2O appears to be quite independent of the nature of the current-carrying ion.

All of the kinetic parameters are slowed by D_2O. At temperatures near the temperature of maximum density of liquid H_2O, Na^+ and K^+ activation are slowed by 43–47 and 40%, respectively. The time-course of Na^+ inactivation during a maintained depolarization (τ_h^s) is slowed by 52% at these temperatures. The differences in the magnitude of the preceeding effects may not be significant. However, at 2–4°C the time constants for prepulse inactivation (τ_h^p) and reactivation are increased by 2½ times in D_2O, a difference so large as to preclude interpretation as a minor effect. At higher temperatures (12–14°C), where the differences in the structural properties of H_2O and D_2O are smaller (Heppolette and Robertson, 1960), the kinetic effects of isotope substitution are decreased. Both Na^+ activation and K^+ activation are only slowed by 17–20%, while τ_h^s and τ_h^p are increased by 37 and 43%, respectively. Expressed as Q_{10}'s these data produce values of 0.72 for Na^+ activation, 0.84 for τ_h^s, and 0.47 for τ_h^p.

As we previously discussed (Schauf and Bullock, 1979), the magnitude of the isotope effects we observe, combined with the strong temperature dependence, suggests that we are dealing with processes in which changes in the structural order of the solvent are of primary importance (Laughton and Robertson, 1969). Viewed most directly, the measured Q_{10}'s would support the view that prepulse inactivation and reactivation involve the most extensive changes in solvent order, with inactivation during a maintained depolarization involving the least change. Gating current, on the other hand, seems to arise from processes which preceed by several steps the creation of a conducting channel and involve little or no solvent interaction. Thus they can be temporally dissociated by D_2O.

Another striking effect of D_2O substitution is the recovery of a protocol-dependent inactivation time constant difference previously eliminated by Cs^+ dialysis. Some years ago it was noted that in intact *Myxicola* axons the time-course of Na^+ inactivation during a maintained depolarization (τ_h^s) was substantially faster than the rate of prepulse inactivation (τ_h^p) at the same membrane potential (Goldman and Schauf, 1973). Although not apparent in squid axons (Benzanilla and Armstrong, 1977) this behavior appears not to be an artifact for several reasons. First, the values of τ_h^p and τ_h^s are exactly the same when measured in K^+- + TEA^+-dialyzed axons at different concentrations of external Na^+ producing absolute

membrane currents differing by a factor of 5 (Bullock and Schauf, 1978). Further, the same results are obtained whether Na^+ currents are recorded directly in the presence of TEA^+, or indirectly via TTX subtraction procedures (Schauf et al., 1976). Finally, the difference in τ_h^p and τ_h^s can be eliminated by substitution of Cs^+ for K^+ internally without changing the absolute magnitude of I_{Na} during step depolarizations (Schauf and Bullock, 1979).

These observations are completely inconsistent with the view that some current-dependent artifact is responsible for the difference between τ_h^p and τ_h^s. On the contrary, the effect of Cs^+ in selectively decreasing τ_h^p while leaving τ_h^s unchanged (Schauf and Bullock, 1979), and now of D_2O in increasing τ_h^p only at low temperatures by an amount five times as large as the effect on τ_h^s, suggests that, at least in *Myxicola,* inactivation produced by changes in initial conditions (prepulses), and presumably reactivation as well, represent different physical processes than those occurring when an open channel inactivates during a maintained depolarization. This is similar to the conclusion drawn from the differences in the temperature dependence of the isotope effects themselves.

Sodium channel selectivity in *Myxicola* is essentially comparable to that measured in other systems (reviewed in Hille, 1972; Ebert and Goldman, 1976). In particular we confirm the observation of Hille (1972) that the P_{Li}/P_{Na} ratio determined from reversal potential measurements is greater than the ratio G_{Li}/G_{Na}, a finding suggestive of saturable binding sites within the Na^+ channel (Hille, 1975). In any case, D_2O substitution has no effect on either permeability or conductance ratios for the alkali metal cations.

The single channel conductance of gramicidin A for Li^+ is only slightly (3%) decreased by D_2O substitution whereas significant decreases are seen for the other alkali cations. Further, the isotope effect increases with increasing ionic radius from 10% for Na^+ to 14% for Cs^+ (Tredgold and Jones, 1979). This behavior closely parallels the structure-breaking activity of these ions. The Li^+ ion has little or no effect on solvent structure, while Cs^+ is the strongest structure breaker (Friedman and Krishnan, 1973; Voice, 1974). The latter also has the highest absolute conductance through the gramicidin channel (Tredgold and Jones, 1979).

Because the thermodynamic property of structure breaking is associated at the molecular level with the disordering of solvent molecules outside of the electrostricted primary hydration shell, this type of effect can only operate when the ion can influence a fairly large number of water molecules. In bulk solution, ionic conductivity is enhanced by structure breaking activity; however, the absolute magnitude of the isotope effect on conductivity is greater for bulk solution (23% at 20°C) than for the gramicidin channel whereas the differences between the ions for both absolute conductivities and isotope effect ratios are smaller (Swain and Evans, 1966). In addition, Läuger (1976) has calculated that the approach of ions to the channel in bulk solution is not rate-limiting for channels with conductances as low as that of gramicidin. These findings suggested to Tredgold and Jones (1979) that there may be considerable solvent within the channel, and that Li^+ might pass through in a different way than the other alkali metal cations, perhaps by occupying a series of binding sites along the side of the channel. An additional possibility is that the observed effects are the result of the structure breaking influence of the ions on a region of solvent at the membrane-solution interface associated with the hydration of the membrane itself.

It is significant that the behavior of the sodium channel of nerve is distinctly different from that of gramicidin. The Na^+ channel does not discriminate well between Na^+ and Li^+, but

effectively excludes the larger cations. The isotope effect on conductance is the same for both permeant ions and is comparable to the corresponding reduction in free solution mobilities. These results might suggest that structure breaking may not be a rate influencing step in the sodium channel and that the observed isotope effects have some other basis.

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