Deuterium Oxide and Temperature Effects on the Properties of Endplate Channels at the Frog Neuromuscular Junction

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ABSTRACT The effects of deuterium oxide (D_2O) and temperature on the properties of endplate channels were studied in voltage-clamped muscle fibers from the frog *Rana pipiens*. Studies were performed at temperatures of 8, 12, 16, and 20°C. The single channel conductance (γ) and mean channel lifetime (τ) were calculated from fluctuation analysis of the acetylcholine-induced endplate currents. The reversal potential was determined by interpolation of the acetylcholine-induced current-voltage relation. The mean reversal potential was slightly more negative in D_2O Ringer's (-7.9 ± 0.1 mV [± SEM]) compared with H_2O Ringer's (-5.2 ± 0.6 mV, $P < 0.01$). The single channel conductance was decreased in D_2O. This decrease was greater than could be accounted for by the increased viscosity of D_2O solutions, and the amount of the decrease was greater at higher temperatures. For example, γ was 38.4 ± 1.3 pS (± SEM) in H_2O Ringer's and 25.7 ± 1.0 pS in D_2O Ringer's for a holding potential of −70 mV at 12°C. The mean channel lifetime was significantly shorter in D_2O, and the effect was greater at lower temperatures. There was not a strong effect of solvent on the temperature dependence of γ. On the other hand, the temperature dependence of the reciprocal mean channel lifetime, α (where $\alpha = 1/\tau$), was strongly dependent upon the solvent. The single channel conductances showed no demonstrable voltage dependence over the range of −90 to −50 mV in both solvents. The reciprocal mean channel lifetime showed a voltage dependence, which could be described by the relation $\alpha = B \exp(AV)$. The slope A was not strongly affected by either temperature or the solvent. On the other hand, the intercept B was a strong function of temperature and was weakly dependent upon the solvent, with most values greater in D_2O. The D_2O effects on α were what would be expected if they were due to the properties of D_2O as a solvent (solvent isotope effects), while the D_2O effects on γ must also include the exchange of D for H in the vicinity of the selectivity filter (primary and/or secondary kinetic isotope effects).

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INTRODUCTION

The current view of the acetylcholine (ACh)-activated channel at the neuromuscular junction is that it is a large aqueous pore (Lewis and Stevens, 1983). From considerations of what particular ions are permeant through the channel, its size is estimated at 6.4 Å in diameter (D. J. Adams et al., 1980; Watanabe and Narahashi, 1979). There are suggestions that hydrogen bonding is important to ion transport through the channel. Huang et al. (1978) found that the permeability of various organic compounds was correlated with their hydrogen-bonding ability. Ammonium ions are slightly larger than K ions, but appear to be more permeable than K with a permeability ratio relative to Na of 1.79:1.1 (D. J. Adams et al., 1980; Takeda et al., 1980). The ammonium ion has the ability to form hydrogen bonds and behaves in biological situations as though its effective radius were much smaller than its actual geometric radius.

Solvent substitution is one way to study the relative importance of hydrogen bonding for ion transport through the ACh-activated channel. Electrostatically there is no difference between deuterium oxide (D_2O) and H_2O because the electronic configurations and nuclei positions are the same. The result of substituting deuterium for hydrogen in water is that the increase in nuclear mass causes the lowest quantum mechanical energy level (zero-point energy level) to be lower. All of the observed isotope effects are a direct consequence of this.

There are two types of effects caused by deuterium substitution. Solvent isotope effects are one class of effects that are due to the behavior of liquid H_2O and D_2O as solvents. These two solvents have many similar physical properties, such as dipole moment, dielectric constant, hydrogen-bond length, and molecular dimensions (Nemethy and Scheraga, 1964). Some physical properties are different; D_2O has greater viscosity, a higher melting point, and greater heat capacity. These are presumably due to the fact that networks of D_2O molecules have a higher degree of structural order than do H_2O molecules because of more extensive intermolecular hydrogen bonding.

The second class of effects, equilibrium or kinetic isotope effects, result when H atoms on membrane proteins or other compounds exchange with solvent D. A primary effect occurs when the H or D is involved in a bond that is broken in a rate-limiting step. A secondary effect results if the H or D is attached to a chemical group that participates in the reaction. This substitution of D for H may change rates and equilibrium constants.

This paper reports on the effects of substituting D_2O for normal water on the following properties of the endplate channel: (*a*) the reversal potential, V_o, (*b*) the single channel conductance, γ, (*c*) the mean channel lifetime, τ, and (*d*) the voltage sensitivity of γ and τ. The temperature dependence of these properties was also investigated. The observations are that D_2O substitution causes a decrease in both the single channel conductance and in the mean channel lifetime. The conclusion reached is that the reduction in γ in D_2O Ringer's is probably due to a combination of both the increased viscosity of the solution and to primary and/or secondary isotope effects, with a D-H exchange occurring in the region of the selectivity filter. The shortening of τ in D_2O Ringer's, on the other

hand, is probably due to solvent isotope effects, with D_2O affecting the conformational structure of the channel such that the relative stability of the open or closed configuration is now changed. These results confirm the importance of hydrogen bonding to ion permeation through endplate channels.

Some of these results have appeared elsewhere in preliminary form (Lewis, 1984).

METHODS

The methods have been previously described (Lewis, 1979). The biological preparation is the cutaneous pectoris muscle from northern *Rana pipiens*, dissected down to a monolayer. A two-microelectrode (filled with 3 M KCl) voltage clamp was used, with a third microelectrode filled with ~3 M AChCl being used to apply ACh iontophoretically to the endplate region.

The solutions contained 115 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, and 4 mM HEPES (Sigma Chemical Co., St. Louis, MO) as the buffer. The solutions also contained 100 nM tetrodotoxin (Sigma Chemical Co.) to block Na channels. The D_2O (Merck Chemical Co., Rahway, NJ) was 99.8% pure. Ionic equilibria differ in D_2O because the self-ionization of D_2O is an order of magnitude smaller than for H_2O. As a consequence of this, pD does not equal pH. A correction factor for converting a pH reading with glass electrodes to pD is the following: $pD \cong pH_{(reading)} + 0.41$ (Katz and Crespi, 1970). The pD of the solutions was adjusted to 7.4.

The experiments were performed from December 1982 through February 1983. The frogs were kept in the laboratory, half of them at room temperature and the other half in the refrigerator at ~8°C. No differences were seen in the results with the two populations of frogs. The experiments were performed at temperatures ranging from 8 to 20°C using a Peltier device in the microscope stage to maintain the temperature.

Data acquisition and analysis were performed as described in Lewis (1979) with the following differences. 8-s samples of the low-gain (× 100) and high-gain (× 1,000) current were stored on magnetic tape using a 3960 Instrumentation tape recorder (Hewlett-Packard Co., Palo Alto, CA). The data were subsequently sampled at 1 kHz, bandpass-filtered at 1–400 Hz, and analyzed using a Nic Med-80 data processor (Nicolet Instrument Corp., Madison, WI). The reversal potential was estimated from interpolation of the ACh-induced endplate current vs. voltage relationship. Single time constant Lorentzians were fit to the difference power spectra by eye. The single channel conductance was estimated from the zero-frequency asymptote of the spectral density from the following equation:

$$\gamma = S(0)f_c\pi/[u_I(V - V_o)],$$

where u_I is the mean endplate current, f_c is the cutoff frequency or half-power frequency, and $S(0)$ is the zero-frequency asymptote of the spectral density (Stevens, 1972; Anderson and Stevens, 1973). The mean channel lifetime was estimated from the cutoff frequency according to the following equation:

$$\tau = 1/(2\pi f_c).$$

The stated error limits on the mean channel lifetime are calculated from the reciprocals of (f_c + SEM) and (f_c − SEM).

In statistically comparing two values for significance, the Student's t test was used. To quantify the temperature dependence of various parameters, values for Q_{10} were calculated for $T = 0$°C and $T = 10$°C.

RESULTS

More Negative Reversal Potential in D_2O Ringer's

The mean reversal potential values measured in the two solutions at temperatures ranging from 8 to 20°C are shown in Table I. Temperature over the range of 12–20°C appeared to have little effect on the reversal potential, so these values in H_2O or D_2O Ringer's were pooled together. The net result is that the reversal potential is slightly more negative in D_2O Ringer's compared with H_2O Ringer's (-7.9 ± 0.1 mV [± SEM] vs. -5.2 ± 0.6 mV) ($t = 4.44$, degrees of freedom = 6, $P < 0.01$). Permeability ratios can be calculated from the reversal potential values using the Goldman-Hodgkin-Katz potential equation (cf. Lewis, 1979) with the result that P_K/P_{Na} is 1.48 in D_2O Ringer's compared with 1.31 in H_2O.

TABLE I

Reversal Potential for the Solvents H_2O and D_2O at Different Temperatures

Temperature	Solvent	
	H_2O	D_2O
°C	mV	
8	−4.25±0.9* (n = 8)‡	−3.0±0.8 (n = 5)
12	−6.2±0.5 (n = 12)	−7.7±0.6 (n = 9)
16	−5.1±0.7 (n = 7)	−8.0±0.9 (n = 8)
20	−4.3±1.3 (n = 5)	−8.0±0.9 (n = 3)

* ± SEM.

‡ Number of determinations.

Decreased Single Channel Conductance in D_2O Ringer's

Fig. 1 *A* shows representative mean current (low-gain) records resulting from the iontophoretic application of ACh, while Fig. 1, *B* and *C*, shows the corresponding high-gain current records for normal Na Ringer's and D_2O Ringer's, respectively. Representative power spectral density plots are shown in Fig. 2. Fig. 2, *A* and *B*, shows power spectral density plots in normal H_2O Ringer's at 8 and 16°C for holding potentials of −90 and −50 mV, respectively, while Fig. 2, *C* and *D*, shows plots in D_2O Ringer's at the same temperatures and for the same holding potentials. There is more high-frequency scatter in the D_2O plots, but all the plots can be fit reasonably well with a single Lorentzian. Values for the single channel conductance and the mean channel lifetime can be calculated from the fitted Lorentzians as described in the Methods.

The single channel conductance is influenced by the solvent. For example, the γ values at 12°C and a holding potential of −70 mV are 38.4 ± 1.3 pS (± SEM; $n = 6$) and 25.7 ± 1.0 pS ($n = 7$) for H_2O and D_2O Ringer's, respectively ($t = 7.74$, degrees of freedom = 11, $P < 0.001$). The log γ values in the two solvents are shown in Fig. 3. The single channel conductance decreased in D_2O Ringer's, with this decrease being a function of temperature. There is a lot of scatter in the data, but there is a tendency for the decrease to be larger at higher temperatures.

Shortened Mean Channel Lifetime in D_2O Ringer's

The mean channel lifetime, τ, is also influenced by the solvent. The lifetime is shortened in D_2O compared with H_2O, and this is true for every holding potential (−90 to −50 mV) and for every temperature (8–20°C). For example, for a temperature of 12°C and a holding potential of −70 mV, the mean channel lifetime is 3.55 ± 0.11 ms (n = 6) for H_2O Ringer's and 3.01 ± 0.08 ms (n = 7)

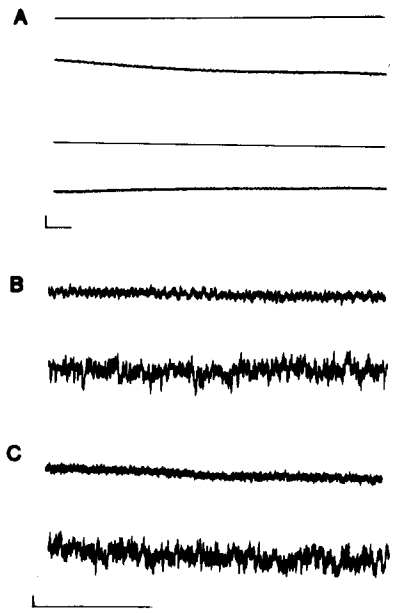

FIGURE 1. (*A*) Representative samples of the low-gain (× 100) current records obtained in normal Na Ringer's (above) and in D_2O Ringer's (below) in the presence of ACh. The horizontal scale corresponds to 1 s and the vertical scale to 4 nA. (*B* and *C*) Representative samples of the high-gain (× 1,000) current records obtained in normal Na Ringer's (*B*) and in D_2O Ringer's (*C*). In both *B* and *C*, the top trace was recorded in the absence of Ach and the bottom trace was recorded in the presence of ACh. The horizontal scale corresponds to 1 s and the vertical scale to 0.2 nA.

for D_2O Ringer's (t = 3.97, degrees of freedom = 11, $P < 0.01$). The log $(1/\tau)$ values in the two solvents are shown in Fig. 4. Once again, there is scatter in the data, but a tendency for the decrease to be smaller at higher temperatures can still be observed.

Single Channel Conductance Voltage Dependence

The single channel conductance shows little dependence upon voltage for either solvent. Fig. 5 shows plots of γ vs. holding potential for the various temperatures. The lines in the figure are a linear least-squares fit to the data points. While the lines appear to be different in Fig. 5, the differences in the slopes are not

statistically significant. Consequently, because there is no significant effect of temperature, all the values were averaged together. For H_2O, the mean slope is 0.06 ± 0.06 pS/mV (± SD), while it is −0.07 ± 0.06 pS/mV in D_2O Ringer's. These values are not significantly different from zero, nor is there any significant difference in the values for the two solvents (t = 1.6, degrees of freedom = 6, P < 0.20).

The possibility exists that the single channel conductance may actually have a slight dependence upon voltage that is obscured by averaging data from many

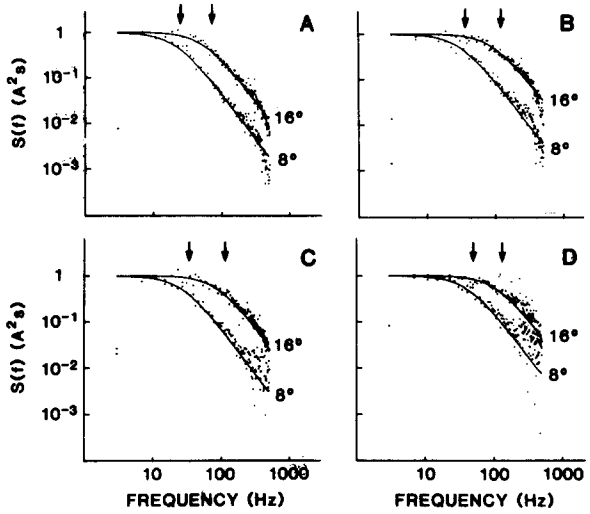

FIGURE 2. Power spectral density plots at temperatures of 8 and 16°C for various holding potentials. The arrows indicate the cutoff frequencies, f_c, and the straight line is a theoretical Lorentzian with the stated values for $S(0)$ and f_c. (*A*) H_2O Ringer's, −90 mV. 8°C: $S(0) = 1.767 \times 10^{-21}$ A² s, f_c = 21.6 Hz, γ = 31.2 pS. 16°C: $S(0) = 6.60 \times 10^{-22}$ A² s, f_c = 56.1 Hz, γ = 37.0 pS. (*B*) H_2O Ringer's, −50 mV. 8°C: $S(0) = 5.99 \times 10^{-22}$ A² s, f_c = 31.5 Hz, γ = 24.4 pS. 16°C: $S(0) = 2.21 \times 10^{-22}$ A² s, f_c = 95.5 Hz, γ = 42.6 pS. (*C*) D_2O Ringer's, −90 mV. 8°C: $S(0) = 8.65 \times 10^{-22}$ A² s, f_c = 27.7 Hz, γ = 22.9 pS. 16°C: $S(0) = 3.63 \times 10^{-22}$ A² s, f_c = 90.9 Hz, γ = 30.0 pS. (*D*) D_2O Ringer's, −50 mV. 8°C: $S(0) = 2.23 \times 10^{-22}$ A² s, f_c = 43.2 Hz, γ = 21.5 pS. 16°C: $S(0) = 9.44 \times 10^{-23}$ A² s, f_c = 114.4 Hz, γ = 28.3 pS.

cells. Consequently, I examined data from individual cells in which I made measurements at all three potentials. No consistent trends were observed for measurements in H_2O or in D_2O Ringer's.

Mean Channel Lifetime Voltage Dependence in Both Solvents

The mean channel lifetime, τ, is strongly voltage dependent in both solvents. The mean channel lifetime decreases as the holding potential is made more positive. For example, in D_2O Ringer's at 12°C, the mean channel lifetimes are 4.09 + 0.34 − 0.29 (plus and minus error limits), 3.01 ± 0.08, and 2.41 ± 0.08 ms for holding potentials of −90, −70, and −50 mV, respectively. Fig. 6 shows

plots of log $(1/\tau)$ vs. holding potential for temperatures of 8 and 16°C. The lines are a linear least-squares fit to the data points. The slopes and intercepts of the fitted lines are shown in Table II.

The slope A is not significantly affected by D_2O. The reciprocal of A gives an indication of the voltage change that is required to produce an e-fold change in the reciprocal mean channel lifetime. If the values for the different temperatures are averaged together, the result is that the mean $1/A$ value is 77 ± 11 mV (±

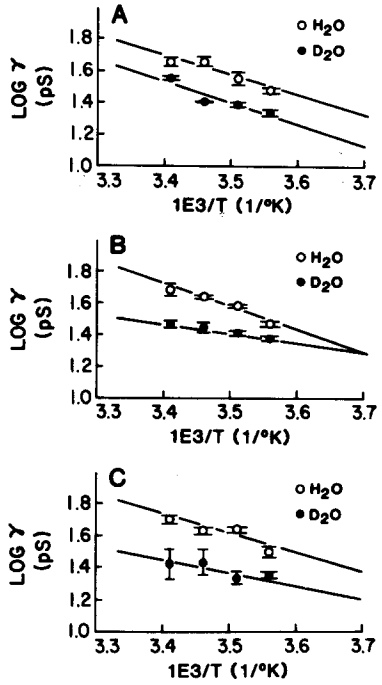

FIGURE 3. A plot of log γ vs. $1/T$. The error bars indicate ±1 SEM, and the straight line is a linear least-squares fit to the data points. The filled symbols indicate data with D_2O as the solvent and the open symbols are for H_2O. The graphs are for data at the following holding potentials: (A) −90, (B) −70, (C) −50 mV.

SD) for H_2O and 88 ± 18 mV for D_2O Ringer's. These two means are not significantly different ($t = 1.04$, degrees of freedom = 6, $P < 0.30$).

The intercept B is only slightly dependent upon the solvent. Most of the values for B are higher in D_2O than in H_2O, but only the values at 8°C are significantly different (i.e., 365 ± 36 s^{-1} [± SD] and 579 ± 81 s^{-1} for H_2O and D_2O, respectively [$t = 4.16$, degrees of freedom = 4, $P < 0.02$]).

Neher and Stevens (1979) have presented a simple two-state model of a protein to show how the protein conformation might theoretically depend upon membrane potential. According to this simple model, the results just presented for the endplate channel indicate that the dipole moment change that occurs when the channel closes is not a function of the solvent or temperature. The observed

voltage dependence of α can be explained by this theory with a dipole moment change of 17.0 ± 2.9 D (± SD) in H_2O and 16.3 ± 2.2 D in D_2O Ringer's. These numbers are considerably lower than the value of 48.4 ± 2.6 D quoted in Magleby and Stevens (1972*b*). However, those authors apparently used an incorrect conversion factor, and a recalculation shows that their observed voltage dependence of α can be explained by a dipole moment change of only 10.0 ± 0.5 D.

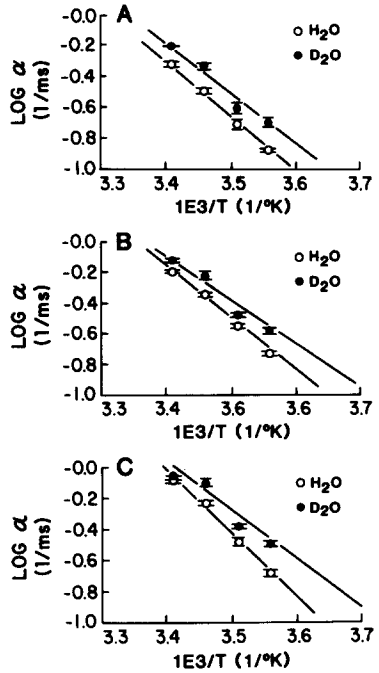

FIGURE 4. A plot of log α vs. $1/T$. The error bars indicate ±1 SEM, and the straight line is a linear least-squares fit to the data points. The filled symbols indicate data with D_2O as the solvent and the open symbols are for H_2O. The graphs are for data at the following holding potentials: (*A*) −90, (*B*) −70, (*C*) −50 mV.

Temperature Dependence of γ and τ

In the present series of experiments, γ shows some dependence upon temperature. Fig. 3 shows a plot of log γ vs. $1/T$ for the various holding potentials. The lines drawn through the data points are a linear least-squares fit. Table III lists the slopes of the lines that are used to calculate the Q_{10} values also listed in that table. (The slopes are calculated using natural log values instead of log values.) If the values at the three holding potentials are averaged together, then the result is that Q_{10} (γ) for H_2O Ringer's is 1.46 ± 0.02 (± SEM), while it is 1.29 ± 0.09 for D_2O Ringer's (t = 1.83, degrees of freedom = 4, $P < 0.2$), which indicates that the solvent does not have a strong effect on the temperature dependence of the single channel conductance.

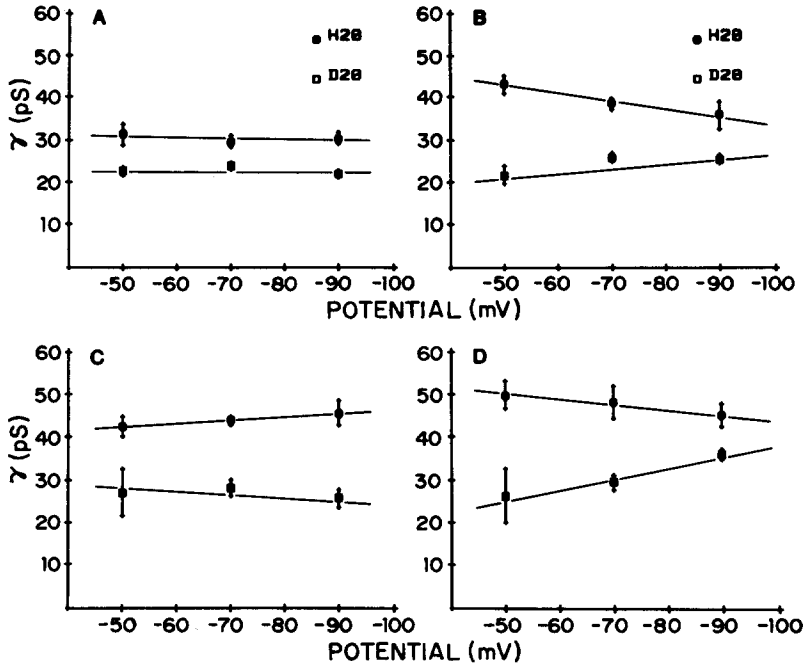

FIGURE 5. Plots of the mean single channel conductance values vs. holding potential for H_2O and D_2O Ringer's at various temperatures. The error bars indicate ±1 SEM, and the straight lines are a linear least-squares fit to the points. The filled symbols indicate data for H_2O Ringer's and the open symbols are for D_2O. In all four plots, the γ values in D_2O are less than the γ values in H_2O Ringer's. (*A*) 8, (*B*) 12, (*C*) 16, and (*D*) 20°C.

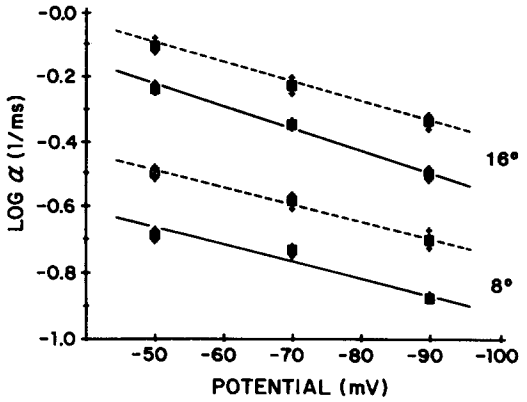

FIGURE 6. Plots of $\log_{10}(\alpha)$ vs. holding potential for H_2O and D_2O Ringer's at 8 and 16°C. The error bars indicate ±1 SEM, and the straight lines are a linear least-squares fit to the points. The filled symbols indicate data for H_2O Ringer's and the open symbols are for D_2O.

TABLE II

Voltage Dependence of the Reciprocal Mean Channel Lifetime, α ($\alpha = 1/\tau$), in Different Solvents

	A (1/V)		B (1/s)	
Temperature	H_2O	D_2O	H_2O	D_2O
°C				
8	10.8±1.2*	11.6±2	365±36	579±81
12	13.4±2.3	12.8±1.7	672±110	795±94
16	15.0±0.3	13.4±1.8	1,250±20	1,536±210
20	13.9±1.5	8.75±0.27	1,661±190	1,373±22

* ± SD.

As has been reported by others, τ shows a marked dependence upon temperature. Fig. 4 shows a plot of log α vs. $1/T$ with the lines calculated from a linear least-squares fit to the data points. The slopes and Q_{10} values were calculated as for the single channel conductance data and the values are listed in Table IV. The reciprocal mean channel lifetime has a higher Q_{10} in normal H_2O Ringer's than in D_2O Ringer's at every potential. If the values at the three holding potentials are averaged together, then the result is that the average Q_{10} (α) for H_2O Ringer's is 3.14 ± 0.14 (± SEM), while it is 2.69 ± 0.07 for D_2O Ringer's ($t = 2.87$, degrees of freedom = 4, $P < 0.05$). The temperature dependence of the mean channel lifetime, therefore, is strongly affected by the particular solvent.

If a single barrier is rate-limiting, various thermodynamic parameters describing the kinetic process can be calculated from the fitted lines in the Arrhenius plots in Figs. 3 and 4. Previous studies had indicated that an asymmetric Eyring rate theory model with a rate-limiting inner barrier was adequate for the endplate channel (Lewis and Stevens, 1979; Horn and Brodwick, 1980), although recent work has indicated that a more complicated model may be necessary (Dani and Eisenman, 1984; Dwyer and Farley, 1984). The Arrhenius activation energy (E_a)

TABLE III

Temperature Effects on the Single Channel Conductance with Either H_2O or D_2O as the Solvent

Holding potential	Slope	Q_{10} (γ)	$\Delta H°$
mV	*°K*		*kcal/mol*
H_2O			
−90	−2,844±465*	1.45±0.09*	5.1±0.9*
−70	−3,186±424	1.51±0.08	5.7±0.8
−50	−2,774±619	1.43±0.11	4.9±1.2
D_2O			
−90	−3,002±321	1.47±0.06	5.4±0.6
−70	−1,414±434	1.20±0.07	2.2±0.9
−50	−1,350±1,140	1.19±0.18	2.1±2.3

* ± SD.

can be calculated from the slope of the lines [i.e., $E_a = (R)$ (−slope), where R is the gas constant]. The activation enthalpy $\Delta H°$ is related to the Arrhenius activation energy by: $\Delta H° = E_a - RT$.

The results of analyzing the Arrhenius plots for γ and α are listed in Tables III and IV, respectively. The enthalpies of activation for γ are on the order of 5

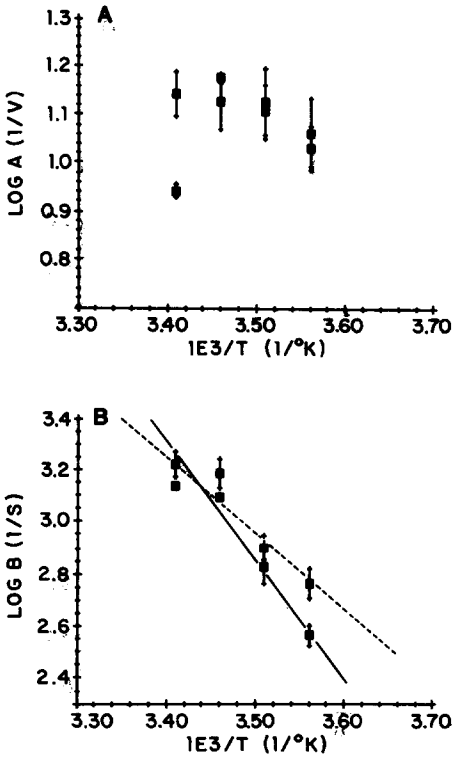

FIGURE 7. (*A*) A plot of $\log_{10}$ (*A*) vs. $1/T$ for both H_2O and D_2O Ringer's. The error bars indicate ±1 SEM. The filled symbols indicate data for H_2O Ringer's and the open symbols are for D_2O. (*B*) A plot of $\log_{10}$ (*B*) vs. $1/T$ for both H_2O and D_2O Ringer's. The error bars indicate ±1 SEM. The straight lines are a linear least-squares fit to the data points. The filled symbols and solid lines indicate data for H_2O and the open symbols and dotted lines are for D_2O.

kcal/mol for H_2O at all potentials and for D_2O Ringer's at −90 mV. The enthalpies are ~2 kcal/mol for D_2O Ringer's at −70 and −50 mV, but, because of the large uncertainty in the value for −50 mV, only the value at −70 mV appears to be different from the values in normal H_2O Ringer's ($t = 2.91$, degrees of freedom = 4, $P < 0.05$).

The results are somewhat different for the thermodynamics of α. The enthalpies of activation are on the order of 16 kcal/mol for H_2O Ringer's and are lower at ~14 kcal/mol for D_2O Ringer's. The average enthalpy of activation in H_2O Ringer's at all three potentials is 16.9 ± 0.7 kcal/mol (± SEM), but it is

significantly lower in D_2O Ringer's, with an average value of 14.5 ± 0.4 kcal/mol ($t = 2.98$, degrees of freedom = 4, $P < 0.05$).

Voltage Dependence of the Reciprocal Mean Channel Lifetime

The dependence of log α on voltage [i.e., $\alpha = B \exp(AV)$] shows some dependence upon temperature, as shown in Fig. 7. Fig. 7*A* is a plot of the logarithm of the slope A vs. $1/T$ for the two solvents, and Fig. 7*B* is a plot of the logarithm of the intercept B vs. $1/T$. The Q_{10} (A) values are 1.25 ± 0.15 (± SD) for H_2O and 0.82 ± 0.09 for D_2O. The lines (not shown in Fig. 7*A*) are not very good fits, so the most that can be concluded is that the slope is not very temperature dependent with Q_{10} (A) values of ~1.

TABLE IV

Temperature Effects on the Reciprocal Mean Channel Lifetime with Either H_2O or D_2O as the Solvent

Holding potential	Slope	Q_{10} (α)	$\Delta H°$
mV	*°K*		*kcal/mol*
H_2O			
−90	−8,598±277*	3.04±0.11*	16.5±0.6*
−70	−8,362±262	2.95±0.10	16.0±0.5
−50	−9,494±403	3.42±0.18	18.3±0.8
D_2O			
−90	−8,008±383	2.82±0.14	15.3±0.8
−70	−7,490±346	2.64±0.12	14.3±0.7
−50	−7,368±219	2.60±0.07	14.0±0.8

* ± SD.

The intercept B exhibits a strong temperature dependence. The Q_{10} (B) values are 3.8 ± 0.4 (± SD) for H_2O and 2.3 ± 0.2 for D_2O. The corresponding enthalpies of activation are 20.0 ± 1.7 (± SD) and 12.3 ± 1.5 kcal/mol for H_2O and D_2O, respectively. The temperature dependence of the intercept is significantly decreased in D_2O ($t = 3.07$, degrees of freedom = 6, $P < 0.05$).

DISCUSSION

The substitution of D_2O for normal water in the extracellular solution has various effects on the properties of single ACh-activated channels. The reversal potential is more negative in D_2O Ringer's, which indicates that the permeability ratio P_K/P_{Na} is slightly larger in D_2O than in H_2O Ringer's. The single channel conductance and the mean channel lifetime are both substantially decreased in D_2O Ringer's.

Temperature also influences the properties of single ACh-activated channels. There is no significant effect on the reversal potential, but the single channel conductance increases and the mean channel lifetime decreases with increasing temperature. The Q_{10} values for the single channel conductances range from 1.2 to 1.5 in D_2O and H_2O Ringer's, with corresponding enthalpies of activation of 2–6 kcal/mol. The Q_{10} values for α range from 2.95 to 3.42 in H_2O and from 2.6 to 2.82 in D_2O Ringer's.

The single channel conductance shows no demonstrable voltage dependence over the range of −90 to −50 mV. The reciprocal mean channel lifetime is strongly voltage dependent, with an e-fold change occurring per 77 ± 11 mV (± SD) in H_2O and 88 ± 18 mV in D_2O Ringer's. D_2O has no significant effect on the voltage sensitivity of the single channel conductance and little effect on the voltage sensitivity of α. On the other hand, D_2O has a large effect on the temperature dependence of the intercept (B).

Comparison with Previous Results

Other investigators have examined the effects of temperature on the single channel conductance of ACh-activated channels with various results. Anderson and Stevens (1973) found no pronounced effect of temperature on γ. Several investigators observed a discontinuity in the relation of γ vs. temperature (Dreyer et al., 1976; Lass and Fischbach, 1976; Fischbach and Lass, 1978). Still other investigators have observed a monotonic temperature dependence, with the single channel conductance increasing with temperature. Q_{10} values for γ have been reported that range from 1.3 to 1.95 (Sachs and Lecar, 1977; Nelson and Sachs, 1979; Gage and Van Helden, 1979; Hoffmann and Dionne, 1983). These published results agree quite well with the results reported here of Q_{10} values ranging from 1.4 to 1.5 in H_2O solutions.

The reported effects of temperature on the mean channel lifetime or the miniature endplate potential current (MEPC) decay rate are much more consistent. Decreasing the temperature increases the MEPC decay rate or the mean channel lifetime and, conversely, decreases the reciprocal mean channel lifetime, α. Q_{10} values for mean channel lifetime have been reported that range from 2.1 to 5.3 (Anderson and Stevens, 1973; Dreyer et al., 1976; Sachs and Lecar, 1977; Fischbach and Lass, 1978; Nelson and Sachs, 1979; Gage and Van Helden, 1979). The present results of Q_{10} values for α of 2.95 ± 0.10 to 3.42 ± 0.18 in H_2O Ringer's agree quite well with these published results.

The present results indicate that temperature has little effect on the reversal potential of ACh-activated channels. This agrees with the observations of Sachs and Lecar (1977) using chick myoballs.

A few other investigators have examined the voltage sensitivity of the single channel conductance for endplate channels. Anderson and Stevens (1973) found no demonstrable voltage dependence. My previous studies (Lewis, 1979) indicated that there was a tendency for γ to increase with hyperpolarization. Several investigators have found that the single channel conductance increased with depolarization, but that a wide voltage range was required in order to detect the change (Gage and Van Helden, 1979; Van Helden et al., 1979; Takeda et al., 1980). My results do agree with those of Dani and Eisenman (1984), who found that the slope conductance was constant over the voltage range of −150 to +100 mV; however, they used symmetrical NaCl solutions, so their results may not be directly comparable.

Many investigators have noticed that the mean channel lifetime exhibits an exponential dependence on voltage, with τ larger at more negative potentials. Most investigators have found that an expression of the form $\alpha = B \exp(AV)$ was

adequate to describe their data. The values for the slope reported in the literature for frog endplate channels range from 3.2 to 17.9 V^{-1}, while the intercept values range from 170 to 1,670 s^{-1} (Anderson and Stevens, 1973; Magleby and Stevens, 1972*a*; Dionne and Stevens, 1975; Gage and McBurney, 1975; Miledi and Parker, 1980; Dwyer, 1981; Fiekers and Henderson, 1982; Auerbach et al., 1983). The intercept is very temperature sensitive (see Table II), and the range of values reported in the literature can be explained by the different temperatures that were used. My values for the intercept are similar to those reported in the literature at the same temperature. The wide range in the reported values for the slope is larger than can be accounted for by different experimental temperatures. One possibility is that there are species differences in the voltage sensitivity of the channel (e.g., from Fig. 3 in Neher and Stevens [1979], α is more voltage sensitive at hyperpolarized potentials in *Rana temporaria* than in *Rana pipiens*). Another possibility is that in some of the studies the endplate region was not adequately voltage-clamped. This would tend to decrease the apparent voltage sensitivity so that reported values that are on the high side are more likely to be correct. My reported value for the slope of 13.4 V^{-1} at 12°C is similar to the median value in the literature, which is ~10 V^{-1}. Kordas (1982) studied the voltage dependence of the decay rate of EPCs over a wide potential range of −150 to +50 mV and found that he needed to add a constant to describe the results adequately [i.e., decay rate = $B \exp(AV + C)$]. Over the voltage range that I studied, I found that the log α vs. V relationship was essentially linear. P. R. Adams and Sakmann (1978; Fig. 3) also observed a small effect of temperature on the slope A.

D_2O Effects on Ion Permeation and Rate Constants

SOLVENT ISOTOPE OR PRIMARY AND/OR SECONDARY KINETIC ISOTOPE EFFECTS The D_2O effects on single channel properties may be either solvent isotope effects or primary and/or secondary kinetic isotope effects. Detailed information on the origin of isotope effects can be obtained from texts on kinetics (e.g., Melander and Saunders, 1980). The first class of effects results because of differences in liquid D_2O compared with H_2O as a solvent. These differences are mainly due to the increased structure of networks of D_2O molecules because of stronger hydrogen bonding. The breakdown of structural order with increasing temperature occurs more rapidly in D_2O than in H_2O, so that at high temperatures the two solvents become more similar (Heppolette and Robertson, 1960). The Q_{10} of this effect may be quite large because the structural differences between D_2O and H_2O are much greater at low temperatures.

Because of the different properties of D_2O as a solvent, there are several effects that might be expected. The increase in viscosity would be expected to decrease the single channel conductance. For example, in a Nernst-Planck formulation for electrodiffusion, the flux of an ion is directly proportional to the mobility, and the mobility is inversely proportional to the viscosity of the solution (e.g., see Lecar, 1977). In an Eyring rate theory approach, the effect of increased viscosity can be modeled in one of two ways—either by increasing the barrier height experienced by an ion so that the flux through the channel is decreased, or by decreasing the jump frequency.

The increase in viscosity of D_2O might be expected to change the mean channel lifetime. According to a theory proposed by Kramers (1940), a chemical reaction can be modeled by Brownian motion in the presence of a potential energy barrier. This approach predicts that a rate constant should be inversely proportional to the viscosity of the solution. Kramers' equation has been extended to include the effect of dielectric constant differences (Gavish and Werber, 1979) and to include the possibility of local variations in viscosity (Gavish, 1980).

Another way in which D_2O could affect endplate channels as a solvent is through its effect on chemical equilibria. D_2O changes the pK_a of ionizable groups in the alkaline direction by an amount that depends upon the particular charge group. For example, simple carboxylic and ammonium acids have pK_a differences of 0.5 to 0.6 unit, while sulfhydryl acids have pK_a differences of 0.1 to 0.3 unit (Schowen, 1977). A shift in pH is not expected to have any effect on the reversal potential (Trautmann and Zilber-Gachelin, 1976; C. A. Lewis, unpublished observations) or on the single channel conductance (C. A. Lewis, unpublished observations). However, this pK_a change would be expected to affect the mean channel lifetime. Previous investigators have shown that the mean channel lifetime (or the EPC or MEPC decay rate) depend upon pH, with the lifetime (or decay rate) decreasing as the solution becomes more alkaline (Scuka, 1975; Trautmann and Zilber-Gachelin, 1976; Mallart and Molgo, 1978; Peper et al., 1982). The effective pH change expected in the present experiments is <0.6 pH unit in the alkaline direction. On the basis of my unpublished observations (i.e., τ was constant for pH 6.1, 6.6, and 7.4 and decreased by 20% at pH 8.4) and the data of Landau et al. (1981) (i.e., τ for *Rana pipiens* was constant over the pH range of 6.5–8.0), I would expect an effective pH change of that magnitude either to have no effect on τ or to decrease it only ~10%.

A third way in which D_2O could affect endplate channels through its properties as a solvent is by altering the structure of the channel. Deuteration of hydrogen-bonding sites in proteins can affect the conformation of proteins (Schowen, 1977). The amount of the change is usually so small that it cannot be detected by physical techniques such as X-ray crystallography (Schowen, 1977), but small structural changes in conformation might be expected to have large effects on the functioning of a protein.

The second class of effects, equilibrium or kinetic isotope effects, result when a D exchanges for an H on a chemical group involved in a chemical reaction. The kinetic isotope effect is caused by the difference in zero-point energy levels (ΔE_0) between D_2O and H_2O. The fairly strong C-D and C-H bonds have a ΔE_0 of ~1 kcal/mol, which would have a Q_{10} of 0.93 from 5 to 15°C (Schauf and Bullock, 1979). On the other hand, the difference in zero-point energies is smaller for hydrogen bonds (at ~0.24 kcal/mol [Némethy and Scheraga, 1964]), which would give a Q_{10} of ~1. In summary, kinetic isotope effects may be large (ratios up to 7) but will exhibit little temperature dependence (Q_{10} of ~1.0). Solvent isotope effects, however, may be smaller but may exhibit substantial temperature dependence.

D_2O EFFECTS ON γ The decrease in γ in D_2O is expected because of the increase in viscosity, but the observed decrease is more than can be accounted

for by that explanation. The inverse viscosity ratios $[(1/\eta_D)/(1/\eta_H)]$ are 0.77, 0.78, 0.79, and 0.80 for 8, 12, 16, and 20°C (interpolated from Table II of Hardy and Cottington, 1949). The observed γ ratios vary from 0.80 to 0.50. There is a lot of scatter, but the data at −70 and −50 mV tend to show that the temperature dependence of the decrease is the opposite of what would be expected for a solvent isotope effect in that the D_2O effect is largest at 20°C. Q_{10} values for the D_2O effect on γ are ~0.80. I reached the conclusion that while solvent isotope effects probably occur because of the increased viscosity of D_2O, other effects must also be postulated to occur. It appears that the additional D_2O effects on γ are primary and/or secondary isotope effects caused by H-D exchange in a part of the channel influencing the selectivity filter. The slight change in the reversal potential seen in D_2O that is not temperature dependent is also consistent with a primary or secondary isotope effect on the selectivity filter such that barrier heights for Na and K are affected differently.

D_2O EFFECTS ON τ The τ values are decreased in D_2O, as would be expected for a solvent isotope effect. Furthermore, the change in the τ_{D_2O}/τ_{H_2O} ratio with temperature is also consistent with this hypothesis in that the change is greatest at 8°C. The Q_{10} values for the D_2O effect range from 1.09 to 1.33.

The observed effects of D_2O on mean channel lifetime are in the wrong direction to be explained by an effect of viscosity as described by Kramers' equation. $\tau \cong 1/\alpha$, where α is the closing rate constant if a two-state model of the channel is adequate and if the ACh concentration is kept low so that the opening rate is negligible in comparison with the closing rate. It was observed that the reciprocal mean channel lifetimes were larger in D_2O solutions, which have a higher viscosity. Furthermore, Dwyer (1981) has shown that the MEPC decay rate (and, by implication, the mean channel lifetime) is not affected by a high-viscosity solution. D_2O may be affecting τ both by changing the pK_a of an ionizable group in the alkaline direction and by altering the structure of the channel so that the stabilities of the open and closed configurations are changed relative to normal Ringer's.

Comparison with Previous Studies Using D_2O

The result presented here that D_2O decreases γ is similar to the results reported by other investigators for various channels. Tredgold and Jones (1979) studied the effects of D_2O on single channel conductivity for alkali cations in the gramicidin A channel. They observed a significant decrease in conductivity for all ions except for Li. This decrease was similar to the decrease in mobility of the different ions in D_2O as compared with H_2O. They concluded that the ions go through the channel in essentially hydrated form, except for Li, which may bind at sites along the wall of the channel. Schauf and Bullock (1979, 1980, 1982) and Schauf (1983) have performed an extensive study of the effects of D_2O on the Na and K channels in dialyzed *Myxicola* axons. They observed a decrease in peak Na and K current of ~30%, but saw no change in the steady state voltage dependence. Na and K channel kinetics were slowed, with a Q_{10} of ~0.70. Membrane asymmetry currents were not affected by D_2O, and the selectivity of the Na channel for the various alkali cations was not changed.

Schauf and Bullock concluded that the rate-limiting step that produces a conducting channel must involve significant local changes in solvent structure. Brink (1983) and Brink et al. (1984) have looked at the effect of D_2O on cell-to-cell transfer of the dye dichlorofluorescein in the septate giant axon of the earthworm. The permeability of K calculated from one-way fluxes decreased 42% at 25°C and 54% at 10°C. The conclusion is reached that the nexus channel is aqueous and that ions pass through in hydrated form.

The effects of substituting D_2O for ordinary H_2O have been studied extensively in several enzyme systems (see Katz and Crespi, 1970, for further information and references). For kinetic isotope effects, a wide variation in magnitude exists with k_H/k_D ratios of 1–45 being observed experimentally (Kresge, 1977). As a solvent, D_2O can have many different effects on enzyme reactions, such as: (*a*) catalytic rates may be depressed or unaffected, (*b*) protein structural equilibria may be altered or unaffected, and (*c*) rates of reaction may be increased or decreased (Schowen, 1977). As one example, yeast alcohol dehydrogenase has a ratio for K_H/K_D of 0.5 for an aldehyde substrate, while the ratio is 1.2 for an alcohol substrate (Klinman, 1977). The result presented here that the mean channel lifetime is shorter in D_2O is consistent with the above possibilities, with D_2O acting as a solvent to influence the mean channel lifetime.

Possible Sources of Error

A possible source of error that cannot be completely ruled out is unresolved flickering. If flickering occurs to any significant extent in D_2O Ringer's, then this could cause the calculated values of γ to be underestimated. Flickering might be expected to increase at higher temperatures, so the observed larger decrease in γ at high temperatures rather than at low temperatures, as expected for a solvent isotope effect, could be consistent with unresolved flickering. The fact that the power spectral density plots in Fig. 2 show more high-frequency noise in D_2O is also consistent with an occurrence of unresolved flickering; however, there is less high-frequency scatter at higher temperatures in D_2O, which is not consistent. One fact that argues against significant unresolved flickering is that this might be expected to cause a higher Q_{10} value for γ because the relevant rate constants probably are temperature dependent. The experimental results indicate the reverse in that γ may be slightly less temperature dependent in D_2O than in H_2O.

Conclusions

In spite of the fact that the occurrence of unresolved flickering cannot be ruled out, several conclusions can be drawn from the results presented here. One conclusion is a confirmation that hydrogen bonding is important to ion permeation through the channel and to determining the mean channel lifetime. A second conclusion is that Na and K ions must experience a slightly higher energy barrier in D_2O because of the larger energy required to dehydrate ions in D_2O. A view of the endplate channel that is consistent with the results reported here is that a site exists near the selectivity filter, which can exchange a D for an H and thereby affect the relative selectivities to Na and K ions. Furthermore, D

exchanging for H at some hydrogen-bonding sites on the channel protein is responsible for altering the relative stabilities of the open and closed conformations of the channel such that the mean channel lifetime is shorter in D_2O.

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