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D_2O and the Sodium Pump in Squid Nerve Membrane

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Summary. In 10 K artificial seawater (ASW), D_2O replacement reduced the Na efflux of squid axons by about one third. In 0 K ASW, D_2O replacement had little effect. D_2O reduced the K^+ sensitivity of the efflux but increased the affinity for K^+. A 4° decrease in temperature mimicked the effects of D_2O. When axons were injected with arginine, to decrease the ATP/ADP ratio, they lost K^+ sensitivity in normal ASW, as expected. Their efflux into 0 K ASW became D_2O sensitive. The results are discussed in terms of conformational changes in the Na pump molecular complex.

Key Words heavy water · deuterium oxide

Introduction

Replacing normal solvent water with heavy water (D_2O) slows many aqueous reactions. In nerve membranes D_2O substitution prolongs the action potential and slows the conduction velocity. This is achieved by a reduction of the magnitude and slowing of the time course of the ionic permeability changes that underlie the nerve impulse (Conti & Palmieri, 1968). Significantly, D_2O replacement does not alter the polarization ('gating') currents that precede the sodium permeability change. This differential sensitivity of the ionic and the gating currents is one of several indications there are at least two steps in opening sodium channels and perhaps suggests that they occur in two different local environments (Meves, 1974; Schauf & Chuman, 1986).

D_2O slows the sodium pump. Keynes (1965) reported that D_2O replacement quickly and reversibly reduced the sodium efflux in frog muscles by about one third. Studies on the $(Na^+ + K^+)$-ATPase of rat brain revealed, in addition to reducing the ATPase activity, D_2O decreased the apparent affinity towards sodium and increased the apparent affinity towards potassium (Ahmed, Riggs & Ishida, 1971). The $(Na^+ + K^+)$-ATPase has a cyclic reaction scheme that features two conformational forms of the protein. Both conformations exist in phosphorylated and unphosphorylated forms. Figure 1 shows a simplified kinetic scheme indicating the forms of the enzyme (Jørgensen, 1982). Ahmed and Foster (1974) suggested that D_2O shifted the equilibrium for the enzyme towards the E_2 form.

To better understand the role of the conformational changes of the sodium pump I have measured some effects of D_2O substitution on the sodium efflux across the membrane of the squid giant axon.

Materials and Methods

The methods are similar to those of Caldwell et al. (1960). Segments of squid giant axons were mounted in a vertical chamber which could be collapsed into a 2-mm square channel. About 1 μC of ^{22}Na in 100 mM potassium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES), pH 7.2, was injected into the axoplasm along a 9–15 mm length, about 0.2 μl/cm. In some experiments 300 mM arginine was injected along 19 mm of axon centered on the 9 mm length containing the radioactive Na. There was an approximately 15-fold dilution as the injection equilibrated with the axoplasm. In most experiments after the injection the axon was tied just below the cannula, 2 cm above the injection site, and remounted in a water-jacketed tube (2 mm i.d.). This reduced the effects of layering on switching between solutions with different densities. Artificial seawater was pumped continuously at about 1 ml/min past the axon and collected over 1-min intervals in planchets, dried and counted in a low background beta counter. At the end of the experiment the radioactivity of the axon was measured and the fraction of radioactivity lost per minute was back calculated for each sample period. The normal 10 K ASW contained (in mM): 462 Na, 10 K, 10 Ca, 55 Mg, 600 Cl and 2 HEPES, pH 7.4. D_2O ASW was made by dissolving the chlorides in 99.8 mole-% D_2O (Bio-Rad, Sigma) and adding the buffer from a 0.5-M stock solution. The final solution was about 95% D_2O because of the water of hydration of the divalent salts and the water with the buffer. 0 K ASW and 0 K D_2O ASW were similar, with Na replacing the K. Intermediate ASWs were made by mixing. Unless noted, experiments were performed at room temperature (20–26°) and controlled ±1° within each experiment.

Results

Sodium pump function was studied by measuring changes of the fraction of tracer ^{22}Na lost from axons as the composition of the bath solution is varied (Fig. 2). Solutions were changed every 5 or 6 min and 2 min were allowed to completely exchange the fluid in the chamber. Data from the last 3 or 4 min were taken as characteristic for the solution. In most cases the data have been normalized by dividing the fraction of ^{22}Na lost per minute in the test solution by the average fraction lost per minute in the bracketing 10 K ASW solution. Thus, in this experiment, 0 K ASW reduced the sodium efflux to 0.40 of its value in 10 K ASW. In a series of freshly mounted axons at 23° ± 1, 0 K ASW reduced the efflux to 0.35 ± 0.04 [9] (mean ± SE[n]).

The first application of 10 K D_2O ASW reduced the efflux to 0.66 of the control level, very similar to Keynes (1965) observation in frog muscle. When different D_2O/H_2O mixtures were used, the inhibition was nearly linearly proportional to the D_2O mole fraction (Fig. 3). The response to D_2O substitution is as rapid and as reversible as the response to a change in K^+ concentration; either response probably occurs in less time than that required to change solutions. In four experiments when efflux samples were collected every thirty seconds the change in efflux was more than 90% complete in the second sample after the solution change. Two minutes is sufficient for the water to equilibrate on both sides of the nerve membrane (Nevis, 1958). The rapid onset and reversibility of the D_2O effect speak against deuterium exchange with the protein as the principal mechanism.

In D_2O the sodium efflux is less sensitive to K^+ removal (Fig. 2). The flux into 0 K D_2O ASW was 0.55 of the flux in 10 K D_2O ASW or 0.37 of the flux in 10 K ASW. The combined effects of reducing K^+ levels and D_2O replacement are shown in Fig. 4. The decrease in efflux associated with removing K from the external medium is smaller in D_2O ASW and occurs at lower K concentrations. The ratio of the efflux in 3 K ASW/the efflux in 10 K ASW is about 20% larger in D_2O than in H_2O. Ahmed et al. (1971) reported a corresponding increase in apparent affinity of the $(Na^+ + K^+)$-ATPase for K^+. In K-free ASW the efflux changes slightly with D_2O replacement.

Reducing the temperature also slows chemical reactions and the sodium pump. The viscosity of D_2O at room temperature is approximately equal to the viscosity of H_2O which is 4° cooler. Comparing Figs. 4 and 5 suggests that the effects of D_2O substitution on the Na efflux are very similar to 4° of cooling. Cooling has a larger effect on the efflux into 10 K ASW than in 0 K ASW. The relative change in K-free solution is smaller at lower temperatures, and the apparent affinity for K^+ is greater at the lower temperature. Ahmed and Judah (1965) reported an increase in the affinity for K^+ by the $(Na^+ + K^+)$-ATPase associated with cooling.

From the data presented thus far one might argue that D_2O acts only on the K^+-sensitive Na ef-

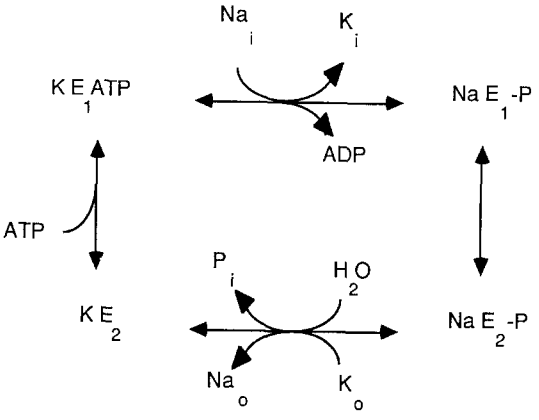

Fig. 1. A simplified generic kinetic scheme for the $(Na^+ + K^+)$-ATPase

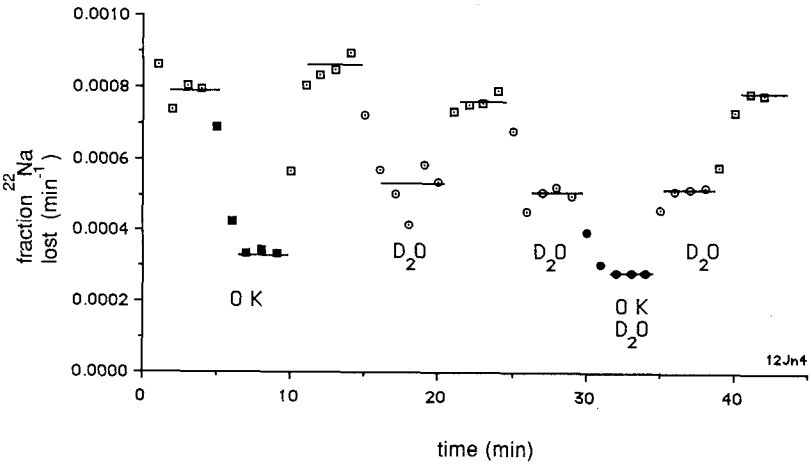

Fig. 2. Efflux of ^{22}Na from a squid giant axon. The effects of removing potassium from the surrounding seawater (filled symbols) and of D_2O substitution (round symbols). Temperature, 26°C

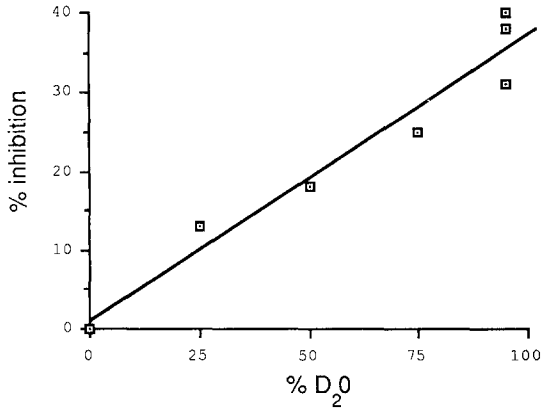

Fig. 3. Inhibition of sodium efflux as a function of D_2O concentration. Temperature, 26°C

flux and thus it is the hydrolysis of E_2-P which is being slowed by D_2O replacement. That this is not the case can be seen by manipulating the ATP/ADP ratio. Squid axoplasm has high arginine phosphokinase activity; when arginine is injected the ATP/ADP ratio decreases. Concomitantly, sodium efflux becomes less sensitive to external K^+ and the pump instead exhibits Na–Na exchange properties (Caldwell et al., 1960; Baker et al., 1969; DeWeer, 1970).

Injecting argining to a final concentration near 20 mM reduced the K^+ sensitivity of the Na efflux in H_2O ASW by about 50% (Fig. 6, Table). In 10 K ASW the sensitivity to D_2O is slightly increased. The flux remaining in 0 K ASW is now sensitive to D_2O replacement, and the relative flux remaining in

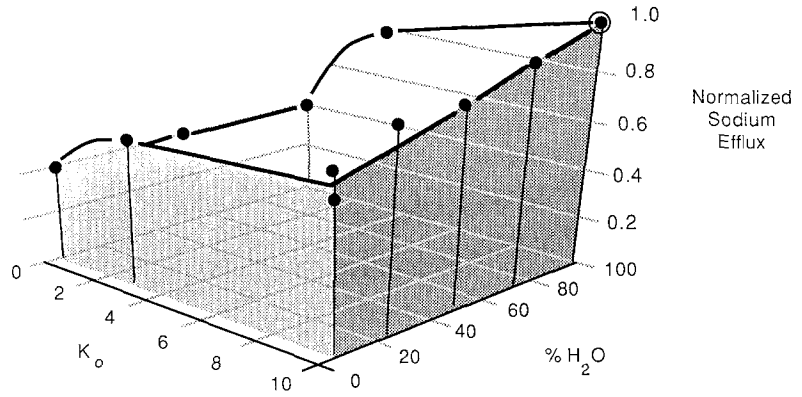

Fig. 4. Reduction of normalized ^{22}Na efflux by reducing external K concentration and/or increasing D_2O concentration. Temperature, 26°C

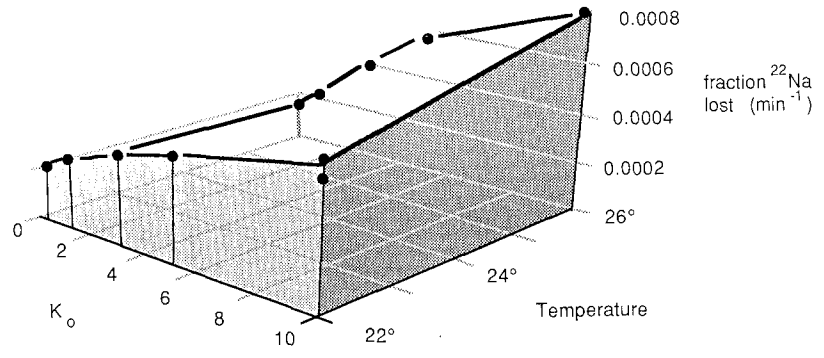

Fig. 5. Combined effects of external K concentration and temperature on ^{22}Na efflux

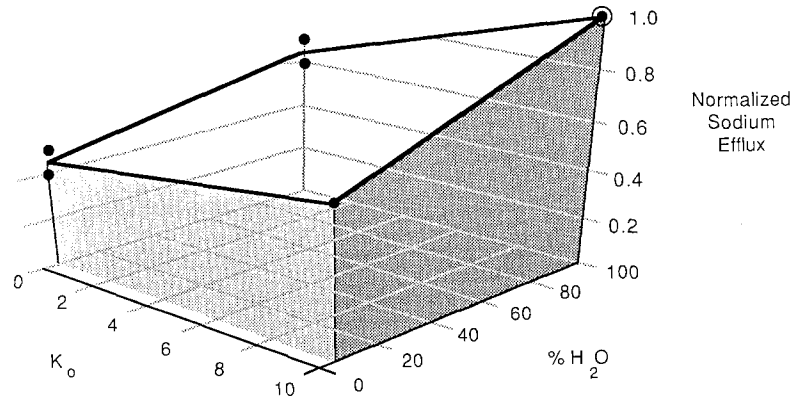

Fig. 6. The effects of external K concentration and D_2O on normalized ^{22}Na efflux after injection with 300 mM arginine. Temperature, 25°C

Table. Na efflux relative to 10 K (H_2O) ASW

ASW	Normal axons	Arginine-injected axons
0 K (H_2O)	0.35 ± 0.04 [9]	0.63 ± 0.04 [5]
10 K (D_2O)	0.66 ± 0.03 [15]	0.54 ± 0.04 [3]
0 K (D_2O)	0.34 ± 0.04 [5]	0.39 ± 0.03 [3]

0 K D_2O ASW is the same after arginine injection as in the normal condition. D_2O still reduces K^+ sensitivity, but the reduction is less after arginine treatment. This Na efflux in the absence of K^+ is now considered to represent Na–Na exchange involving the right-hand side of the reaction scheme shown in Fig. 1 and without E_2-P hydrolysis (Karlish, Yates & Glynn, 1978).

Discussion

Both in terms of its effects on the transport properties across intact membranes and on the ATPase activity of isolated molecules, D_2O appears to stabilize the E_2 conformation of the sodium pump. In the isolated complex the E_2 condition predominates in the absence of sodium and the presence of K^+; it is often referred to as the K-bound form or the K-form. In the absence of both Na^+ and K^+ the E_2 form is favored; it appears to be the lower energy form (Skou & Esmann, 1983). By shifting the equilibrium towards the E_2 form the Na efflux will be reduced and the apparent K^+ affinity increased because the complex spends more of its time in the K-form.

By measuring the ratio of (P_i released)/(E_1-P + E_2-P) Inoue, Fukushima and Tonomura (1975) also show that D_2O favors the E_2 form. In like manner cooling reduces the efflux and increases K^+ affinity by favoring the lower energy state. Fukushima and Tonomura (1975) measured the temperature dependence of the equilibrium constant and calculated an enthalpy change of 4–5 kcal/mole and an entropy change of 15–16 entropy units/mole. Ahmed et al. (1971) have shown that the effects of D_2O are not due to the change in the dissociation constant of water; the pump reactions have similar broad pH maxima in both light and heavy water.

In the context of protein folding or conformation, the most important difference between heavy and light water is hydrophilic bonding. The amount of hydrogen bonding and the strength of these bonds is greater in heavy water than in light (Nemethy & Scheraga, 1964). Water stabilizes the more hydrophilic configurations of the protein by hydrogen bonding, thus decreasing the entropy of the water-protein system. Heavy water is a better stabilizer.

In 0 K ASW with normal ATP and ADP levels, the pump spends more of its time in the E_1 or Na-form so D_2O or cooling has less effect on the remaining efflux. When the ADP/ATP level is elevated, the pump can exchange internal and external Na by converting E_2-P back to E_1-P. D_2O, by favoring the E_2 form, thus reduces the efflux.

From circular dichroism measurements on purified (Na^+ + K^+)-ATPase, Gresalfi and Wallace (1984) concluded that an extensive conformational change occurs with the net conversion of about 7% or 80 amino acids from α-helical in E_1 to β-sheet in E_2. The portion of the molecule involved in the transition could be larger but compensating in its effects on the CD spectra. Both forms have sufficient α-helix for the eight transmembrane helices suggested from amino acid sequence analysis (Kawakami et al., 1985; Shull, Schwartz & Lingrel, 1985). It is not difficult to imagine that a conformational change of this magnitude might lead to a change in the hydrogen bonding of 10–20 water molecules/pump molecule, which would be sufficient to account for the results described above.

Finally, the influence by D_2O must be in a region of the protein with access to the cytoplasmic and/or extracellular water space. In contrast, the major conformational change of the sodium channel molecule, seen as the 'gating' currents, is not slowed by D_2O substitution and thereby is more likely to be in the nonaqueous membrane portion of the protein. This difference in location of the changing portion of the molecules might be associated with the difference between the dramatic voltage dependence of the Na channel transition as opposed to the general lack or anomalousness of voltge dependence seen in the sodium pump (DeWeer & Rokowski, 1984).

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