THE GAP JUNCTION CHANNEL

Its Aqueous Nature As Indicated By Deuterium Oxide Effects

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ABSTRACT The effects of temperature and solvent substitution with deuterium oxide (D_2O) on axoplasmic (g_a) and gap junctional (g_j) conductances were examined in the earthworm septate median giant axon (MGA). The temperature coefficients (Q_{10}) for g_a and g_j were 1.4 and 1.5, respectively, between 5 and 15°C. Substitution with D_2O rapidly reduced both g_a and g_j by 20% and increased the Q_{10}'s to 1.5 and 1.8, respectively. The reduction in g_a upon substitution with D_2O and with cooling in either solvent reflects the changes that occur in solvent viscosity, which indicates that ion mobility in axoplasm, as in free solution, is primarily governed by viscous properties of the solvent. The similar initial reduction observed for g_j suggests that solvent occupies the gap junction channel volume and influences transjunctional ion mobility. With time there was a further reduction in g_j at 20°C and a larger Q_{10} in D_2O. The enhanced effects of D_2O on g_j cannot be accounted for by solvent viscosity alone and may be due to an increased hydration of the channels and/or the transport ions and by isotope effects of hydrogen-deuterium exchange on the channel protein that reduce g_j.

INTRODUCTION

Gap junction channels directly connect the cytoplasms of adjacent cells and presumably serve to coordinate both electrical and biochemical events in normal tissue function (for review see Bennett, 1977; Loewenstein, 1981; Bennett and Spray, 1985). In many systems, diffusion of low molecular weight probes between cells is rapid, and junctional membrane conductivity is high and linear over a wide voltage range (Johnston and Ramon, 1982; Verselis and Brink, 1984; White et al., 1985). An attractive hypothesis for such a highly communicative and ohmic pathway is that gap junctions are composed of large channels that are filled with an electrolyte solution, thus constituting a direct, aqueous pathway for cell-to-cell communication.

Passive diffusion processes, such as ionic conductance in bulk solution, are largely determined by solvent viscosity and are typified by Q_{10} values in the range of 1.1 to 1.5 (Robinson and Stokes, 1968; Prosser, 1973). Substitution of the natural biological solvent (H_2O) with deuterium oxide (D_2O) reduces ionic conductivity by an amount equal to the increase in viscosity of D_2O relative to H_2O. Such effects are termed solvent isotope effects because they reflect changes induced by properties of the solvent itself

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(Arnett and McKelvey, 1969). In addition to reduced conductance, the Q_{10} in D_2O is augmented because the viscosity of D_2O increases faster with cooling than does H_2O (Kazavchinskii et al., 1971). Therefore, if ions in the gap junction channel experience a solvent-like environment, reductions in junctional conductance should parallel the increases in solvent viscosity induced by cooling and solvent exchange. We tested for the presence of solvent isotope effects on axoplasmic (g_a) and gap junctional (g_j) conductances in the earthworm median giant axon (MGA). The MGA is a septate axon composed of large, cylindrical cells joined end-to-end at regions called septa, which are appositional membranes that contain extensive areas of gap junction (Brink and Dewey, 1978).

METHODS

Earthworms, *Lumbricus terristris*, were maintained at 5°C in a mixture of soil and mulch. Nerve cords were dissected as previously described (Brink and Barr, 1977) and bathed in either H_2O or D_2O saline containing the following salts; 120 mM NaCl, 1 mM $MgCl_2$, 5 mM choline chloride, and 50 μM carbachol, pH 7.5. The deuterium ion concentration of the D_2O saline was adjusted to 7.5 with the pH meter offset by +0.4 U (Wang and Copeland, 1973).

To measure g_j, four low-resistance (5–10 MΩ) microelectrodes were inserted into the MGA, two each in adjacent axonal segments. The microelectrode pair in one axonal segment consisted of a ground and a voltage sensor, and in the other, consisted of a current source and a voltage sensor. The external bath was left floating from ground. The voltage difference recorded between axonal segments was clamped by feeding it and a command voltage step into the summing junction of a

control optional amplifier whose output was connected to the current-passing electrode. With electrode spacing below 800 μM, current flowed primarily down the axon core and through the septum (junction) to the ground electrode because the resistance of axoplasm and septum between electrodes was small compared with the parallel resistance out across the surface membrane, along the extracellular space, and back in across the membrane; in most cases the ratio was >20:1 so that >95% of applied current flowed internally (see Verselis and Brink, 1984). A direct measure of internal longitudinal conductance (g_i) was, therefore, obtained by dividing the applied current by the voltage step. The increase in internal resistance with cooling was not sufficient to alter the pattern of current flow and hence, measurement of g_j. Since g_i consists of axoplasmic (g_a) and junctional conductances (g_j) in series, recordings in each axon were also made within a single axonal segment (no intervening septum) to provide an independent measure of g_a. The value of g_j was computed by substracting the measured axoplasmic contribution from g_i.

To assess the effects of solvent substitution, we cut excised nerve cords in anterior and posterior halves; one half was placed in H_2O saline and the other was placed in D_2O. Soaking time was 1 h, which was sufficient to assure solvent equilibration. The following procedure was done on both halves of the nerve cord. The composite conductance ($g_i - g_a + g_j$) was measured and the Q_{10} was evaluated upon cooling. The preparation was warmed, two of the electrodes were moved so that all four electrodes were in the same segment, to measure g_a, and the Q_{10} was evaluated upon recooling. The order was reversed half of the time, i.e., g_a was measured first, then the composite g_i was measured. Preparations that did not return to control values after the first cooling or that displayed marked hysteresis were discarded. A dye injection was performed at the end of each experiment to verify the position of the septum (Brink, 1983). In all experiments, tetrodotoxin (1×10^{-7} M) and Tetraethylammonium (1×10^{-6} M) were added to the bath to abolish active currents. Preparations were cooled at a rate of 1°C/min with a bipolar temperature controller (Cambion, Cambridge, MA).

RESULTS

The temperature functions for g_i, g_a, and g_j in both H_2O (a) and D_2O (b) are illustrated in Fig. 1 by means of an Arrhenius plot. The solid lines with circles represent a best linear fit to the data for g_a (axoplasm) and the composite g_i (axoplasm + septum). The Q_{10} for g_j was computed by subtracting the expected conductance values (solid lines) for axoplasm from that of the composite. The curvature that arises on a log plot from subtraction of two log linear relations is negligible over the temperature range employed, and g_j is illustrated as a straight dashed line. All conductances represent measurements after soaking for 1 h in either solvent.

Nerve cords that were soaked in D_2O for 1 h had g_a values that were 20% lower at 20°C than those soaked in H_2O. Also, the Q_{10} increased from 1.4 to 1.5 (Table I). The effect of D_2O on g_a was rapid (not shown) and reached steady state in 2–3 min after which it remained unchanged. The time course of the D_2O effect probably represents exchange diffusion through the myelin. Both the 20% reduction in g_a at 20°C and the higher Q_{10} in D_2O closely reflect the differences in solvent viscosity between H_2O and D_2O, the expected result if solvent viscosity in the axoplasm governs ion mobility.

Compared with g_a the effects on g_j are different in several ways. The nerve cords soaked in D_2O for 1 h had g_j values reduced 30–35% at 20°C and the Q_{10} was steeper,
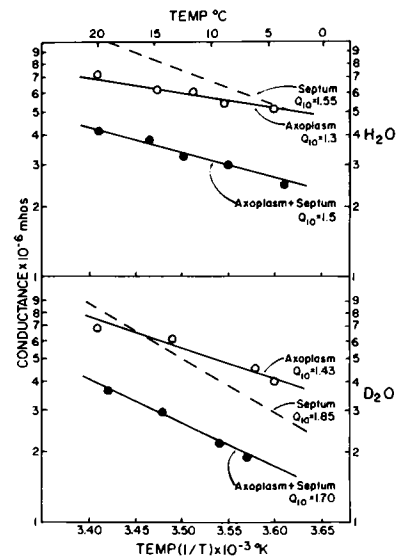

FIGURE 1 *Top*, Arrhenius plot of MGA conductances (in mΩ) in H_2O. Data were taken from a single experiment. The solid lines represent the best linear fit to the data taken from two regions of the axon, one containing a septum between the recording electrodes (axoplasm + septum) and one with no septum present (axoplasm). The Q_{10} for junctional conductance (dashed line) was obtained by subtracting the series axoplasmic resistance from the composite resistance (axoplasm + septum). The Q_{10}s are as indicated. *Bottom*, same as in *top* panel except in D_2O. The other half of the nerve cord from the experiment in the *top* panel was used.

changing from 1.55 in H_2O to 1.8 in D_2O. The initial effect of D_2O, however, was very similar to that for axoplasm. Junctional conductance was reduced ~20% within 5 min, a time course that is consistent with exchange diffusion time for solvent to enter the axon and diffuse into the channel. Whereas g_a remained unchanged after the initial reduction, g_j decreased further and reached steady state in ~30 min at a value that was 30–35% lower than in H_2O (Fig. 2). We interpret the further reduction in g_j as H^+/D^+ exchange on the channel protein.

DISCUSSION

The high ionic conductivity and ability of gap junctions to admit hydrophilic dyes suggest that the channels are large and aqueous in nature and, in essence, directly join one cell interior to another. Ion mobility in aqueous solution is primarily determined by the hydrated ion size and by

TABLE I
TEMPERATURE COEFFICIENTS OF JUNCTIONAL (g_j) AND AXOPLASMIC CONDUCTANCES (g_a)

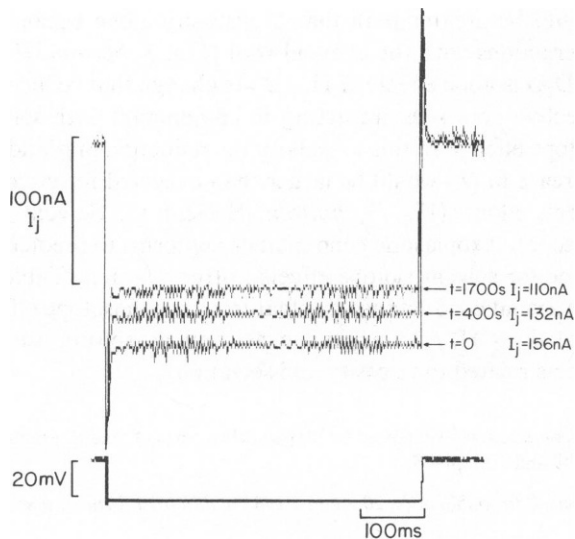| Solvent	Q_{10} (Axoplasm)	Q_{10} (Junction, septal membrane)
H_2O	1.4	1.55 (n = 6)
Range	1.37–1.45	1.4–1.6
D_2O	1.5	1.8 (n = 6)
Range	1.45–1.6	1.7–1.9
	Q_{10} H_2O/D_2O	Q_{10} H_2O/D_2O
	0.93	0.86

FIGURE 2 Voltage clamp of a septum before and after D_2O exchange. Junctional currents are shown at $t = 0$ upon substitution of D_2O and at two later times. For command steps of equal magnitude, the junctional current was decreased ~20% within minutes (shown at $t = 400$ s) and decreased further to a steady state reduction of 35% within 30 min (shown at 1,700 s).

viscosity of the solvent (Robinson and Stokes, 1968; Bockris and Reddy, 1970). With cooling, the solvent viscosity increases due to stronger intermolecular association between solvent molecules and is primarily responsible for determining the Q_{10} for conductivity. Because of the differences in its physical properties (e.g., greater intermolecular forces), D_2O is more viscous than H_2O at any given temperature and the fractional increase in viscosity with cooling is greater (Heppolette and Robertson, 1960). Consequently, the mobility of an ion is decreased in D_2O and a larger Q_{10} is observed.

In the earthworm MGA both axoplasmic (g_a) and gap junctional (g_j) conductances (Fig. 1) in either solvent have Q_{10} values that are within expectations of a free diffusion process. For axoplasm the consistent reduction in g_a of 20% at 20°C and the larger Q_{10} (the ratio is ~0.90 for H_2O/ D_2O) directly correspond to the changes that occur in solvent viscosity upon substitution with D_2O (see also Brink, 1985). Although the bulk viscosity inside a cell is high, the microviscosity, which determines ion mobility, is comparable to water (Gilbert, 1975; Rubinson and Baker, 1979). It has long been known that diffusion coefficients for ions in axoplasm are similar to those in bulk solution (Hodgkin and Keynes, 1953). Since cytoplasm represents a free diffusion realm for small ions, it follows that the solvent isotope effects on g_a in the MGA are in accordance with predictions made for an electrolyte solution.

The behavior of g_j differed in several ways. The range in Q_{10} values in H_2O (1.40–1.60) and in D_2O (1.70–1.90) are higher than the Q_{10} values for viscosity of either solvent (H_2O/D_2O ~ 0.86). Initially upon soaking in D_2O, the decrease in g_j was much like that in g_a, but was subse-

quently reduced further, reaching steady state in ~30–40 min. g_j was consistently reduced by 30–35% at 20°C compared to H_2O and the Q_{10} was enhanced more than predicted by viscosity alone.

Since viscosity and hence solvent isotope effects cannot explain the effects of D_2O on g_j we looked to isotope effects for an explanation. The explanation requires that the ions which carry the current have restricted mobilities in the gap junction channel, so solvation can influence ion mobility (Brink, 1983). Strongly ionized solutes are enveloped by a solvent sheath and together constitute a single diffusion entity. Compared with H_2O, mobility in D_2O is reduced, in part because stronger intermolecular association creates a larger solvent sheath at any temperature. Similarly, solvation of the channel wall would reduce its size which would further limit ion mobility. The effects of solvation, which are categorized as solvent isotope effects, are subtle and would not be expected to be evident in axoplasm or in a large aqueous channel. However, if isotope effects such as H^+/D^+ exchange on the channel protein, which are not solvent effects, alter the channel structure in a manner that reduces the effective channel radius, solvent isotope and isotope effects would sum and reduce g_j more than would result from the viscosity change alone. Also, the reduction in channel size may augment the effects of solvation because diffusion is further restricted and the decrease in channel size would manifest itself as a steeper Q_{10}. In this case the increase in Q_{10} is not caused directly by greater H^+/D^+ exchange with cooling, but is caused indirectly by a reduction in effective channel size allowing greater expression of solvation, a solvent isotope effect.

Alternatively, in a channel that is not aqueous, if ions jump between energy wells within the channel and this process is rate-limiting, H^+/D^+ exchange at binding sites could significantly reduce the transport rate thus reducing g_j. But because of the energy requirements for chemical processes (i.e., binding and release), the Q_{10} would not be expected to change in D_2O (for discussion see Schauf and Bullock, 1979). In this case, g_j could be reduced >20%, but the Q_{10} would remain unchanged ($Q_{10}H_2O/D_2O = 1$), which is not consistent with our observations (Table I).

In support of our results, the diffusion of fluorescent dye molecules, being larger than the current-carrying ions, were shown to be substantially reduced in D_2O with unexpectedly large Q_{10}s (Brink, 1983). Furthermore, diffusion block occurred at 4°C which was attributed to solvation which rendered the dye-solvent complex too large to diffuse through the channel. Higher block temperatures were observed for dyes with a larger number of ionized groups because they are more extensively solvated (Brink et al., 1984). Since charge groups with the most dense electric fields maintain larger hydration shells in water the predicted selectivity sequence for the gap junction channel for the alkali metals would be $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$, the reverse of the crystallographic radii. This sieving

model is not unreasonable for the gap junction channel because of its unusually large size. In other membrane channels alternate mechanisms of ion transport have been described to explain the variety of selectivity sequences encountered (Diamond and Wright, 1969; Hille, 1984).

In conclusion, Fig. 3 summarizes the predicted effects of solvent substitution on conductance. For a free diffusion process where solvent isotope effects are the only influence, the conductance should be reduced by ~20% and the ratio of the Q_{10}s should follow that for viscous flow (Fig. 3, top). This ratio, measured from bulk solution conductivity, is 0.92 between 5 and 15°C (Kazavchinskii et al., 1971). For a channel, where transport is more restricted, there are several possibilities. In a transport mechanism that occurs via ion binding, conductance can decrease (or increase) because of isotope effects (not shown), but the ratio of the Q_{10}s would approach unity. For an aqueous channel the Q_{10}
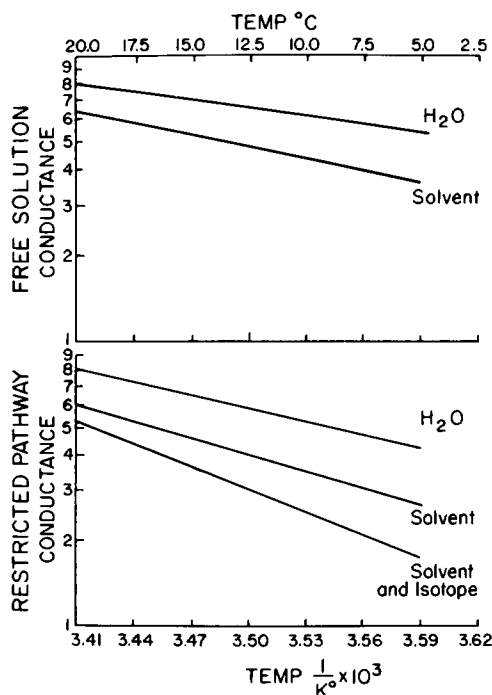
should be greater than that of viscosity alone because of interactions with the channel wall (Fig. 3, bottom, H_2O). In D_2O isotope effects of H^+/D^+ exchange that reduce the effective pore size are acting in conjunction with solvent isotope effects. In this case, both the reduction in g_j and the increase in Q_{10} would be larger than expected for viscosity effects alone (Fig. 3, bottom, Solvent vs. Solvent and Isotope). Axoplasmic conductance conforms to predictions made for solvent isotope effects in free solution (Table I). Junctional conductance has features of both isotope effects related to H^+/D^+ exchange as well as solvent isotope effects related to viscosity and solvation.

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FIGURE 3 Predicted behavior of conductances in free solution in both H_2O and D_2O based on known properties of ionic conductivity. The Q_{10}s are low (between 1.1 and 1.5) and only solvent isotope effects of viscosity are present. Conductance is reduced by 20% at 20°C and the Q_{10} in D_2O is slightly steeper; the Q_{10} ratio for viscosity of H_2O/D_2O is 0.92. Conductance is in arbitrary units. Bottom, predicted behavior of conductance through a somewhat restricted pathway. The Q_{10}s in H_2O are steeper than for viscosity alone because energy requirements become more demanding (line labeled H_2O). For a large aqueous channel solvent isotope effects alone, as in axoplasm, would reduce conductance by 20% at 20°C and exhibit a Q_{10} the same as that for viscosity (Solvent). Isotope effects that reduce the channel size would decrease conductance, but would not alter the Q_{10} (not shown). Isotope effects in conjunction with solvent isotope effects would reduce conductance below 20% at 20°C and further increase the Q_{10} by allowing greater expression of solvent isotope effects related to solvation (Solvent and Isotope). Conductance is in arbitrary units.

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