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D_2O-Induced Cell Excitation

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Summary. The effects of deuterium oxide (D_2O) on giant internodal cells of the fresh water alga *Chara gymnophylla,* were investigated. D_2O causes membrane excitation followed by potassium leakage. The primary effect consists of an almost instantaneous membrane depolarization resembling an action potential with incomplete repolarization. A hypothesis was proposed which deals with an "osmotic stress" effect of D_2O on membrane ion channels followed by the supression of the electrogenic pump activity. The initial changes (potential spike and rapid K^+ efflux) may represent the previously undetected link between the D_2O-induced temporary arrest of protoplasmic streaming and the early events triggered at the plasma membrane level as the primary site of D_2O action.

Key Words deuterium oxide · *Chara* · membrane transport · action potential · protoplasmic streaming

Introduction

Deuterium oxide (D_2O) has been widely utilized as an isotopic analog of water. It has been frequently used in membrane transport studies and as an almost exclusive solvent in NMR studies. However, it is well documented that deuterium oxide causes perturbations in biological systems (Katz & Crespi, 1970). These effects are mainly discussed in terms of hydrogen bond replacement by the deuterium bond. However, a substantial fraction of exchangable H atoms in native macromolecules exchanges slowly (up to several hours) with D atoms. This can only account for long term D_2O effects. Yet, it has been reported that D_2O induces significant alterations in living systems, observable minutes or even seconds after exposure (Alexandrov et al., 1964; Hübner, 1965). This rapid action should imply solvent isotope effects. Its mechanism, however, remains largely unknown. D_2O can cause an abrupt interruption of protoplasmic streaming (cyclosis) in algal cells (Hübner, 1965). On the other hand, it is known that cyclosis may be affected by factors, such as intracellular calcium (Williamson & Ashley, 1982), which induce changes of ATPase activity (Williamson, 1975; Tazawa, Kikuyama & Shimmen, 1976), or by mechanical and electrical stimulations which lead to the generation of action potentials (Kishimoto & Akabori, 1959; Williamson & Ashley, 1982). In animal cell membranes D_2O can slow the sodium pump as revealed by the reduced sodium efflux (Keynes, 1965), and increase the $(Na^+ + K^+)$-ATPase affinity towards K^+ while decreasing the apparent affinity towards Na^+ (Ahmed, Riggs & Ischida, 1971; Ahmed & Foster, 1974). However, there are no reports regarding the influence of D_2O on the transport mechanisms in plant membranes.

Materials and Methods

Biological Material

Laboratory-grown *Chara gymnophylla* algae were attached to an agarose base of the same composition as the surrounding growth medium (1 mM $NaHCO_3$, 0.4 mM $Ca(NO_3)_2$, 0.2 mM $MgCl_2$ and 0.1 mM KH_2PO_4). Prior to measurements, cells were kept for 24 hr at 25°C in one of the experimental solutions: APW ("artificial pond water": 1.0 mM NaCl, 0.1 mM KCl and 0.1 mM $CaCl_2$) or ^{2}H-APW (99.8% D_2O solvent, Stohler Isotope Chemicals, with the same salt composition).

Measurement of Membrane Potential Difference (PD)

Individual internodal cells were fixed in the flow-through Perspex chamber within a light-proof Faraday cage. Intravacuolar $Ag/AgCl_2$ microelectrode (tip diameter 3–5 μm) was filled with 3 M KCl. The external glass reference electrode was filled with agarose in 3 M KCl, and placed near the flow outlet. In this experimental arrangement the measured PD corresponds to the multiple-boundary system between vacuole and external medium. PD at various temperatures was continuously registered on a Keithley 616 electrometer connected to a dual-chart recorder (Esterline Angus Instrument). While examining the influ-

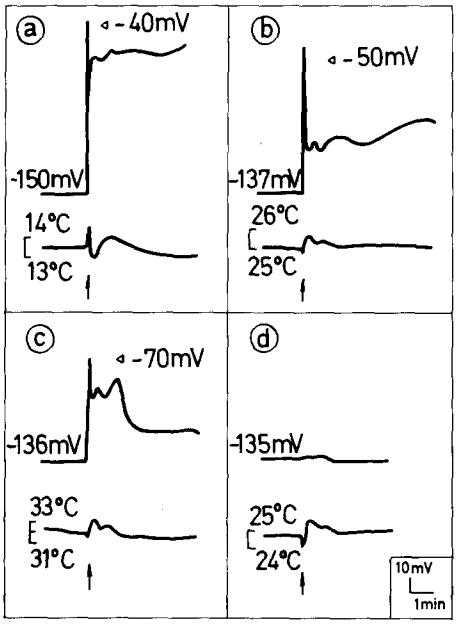

Fig. 1. D_2O-elicited potential spikes. (*a*), (*b*) and (*c*) Typical recordings of potential with temperature from cells exposed to the flow of ^{2}H-APW (at bottom arrow). (*d*) Example of control records (flow of APW increased at arrow without substituting the perfusion fluid). The initial resting potential value is given at the beginning of the upper tracing in each panel. Additional PD levels are indicated in upper tracings of *a*, *b* and *c*. A temperature scale is given for the lower tracing in each panel

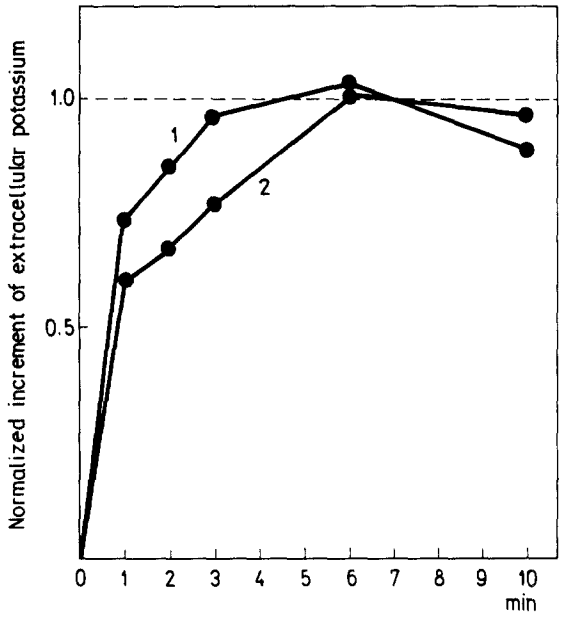

Fig. 2. Potassium leakage from internodal cells during incubation in deuterated media (^{2}H-APW); examples from two identical experiments (curves *1* and *2*). Data, corrected for volume changes, are expressed as increments of extracellular potassium concentration, normalized in respect to the 18 hr value (dashed horizontal line)

ence of D_2O, in order to speed up the exchange of perfusion fluids (^{2}H-APW for APW), the perfusion rate (0.5 ml/min) was increased to 24.0 ml/min during the first minute following the substitution of fluids.

Measurement of Extracellular Ions in ^{2}H-APW

After careful blotting on filter paper, 10 cells of measured length (approximately 10 mm each) were transferred to a vial containing 5 ml of ^{2}H-APW maintained at 25°C. Five 0.5-ml aliquots of the medium were withdrawn at various intervals during the first 10 min of incubation (with gentle mixing of the cell suspension before each withdrawal); the last aliquot was taken after 18 hr. After 10-fold dilution with distilled water, potassium content of aliquots was determined using a Varian 775 atomic absorption spectrophotometer. Extracellular chloride was determined by potentiometric titration of the ^{2}H-APWS solution (^{2}H-APW with 1 mM Na_2SO_4 substituted for 1 mM NaCl) in which the cells have been kept for 10 min. Titration was carried out with 1 mM $AgNO_3$ and the potential change was registered with a silver electrode. The ion concentration was calculated against total cell volume which was obtained by using the measured total cell length and the average cell radius of 0.2 mm.

Measurement of Cyclosis Temperature Dependence

A sample of cells was transferred to a thermostatically controlled Perspex chamber, filled with the experimental solution and prewarmed to 25°C. The temperature (thermistor probe, Yellow Springs Instruments, at the bottom of the chamber) was then slowly brought to the desired starting level. Prior to velocity measurements, the cells were allowed to adapt to the starting temperature for 30 min. A stopwatch and a stereomicroscope (Bausch & Lomb) with an ocular micrometer were used to follow the passage of cytoplasmic particles. In order to evaluate the temperature dependence of cyclosis, stepwise rewarming from the starting temperature was initiated at a rate of about 0.5°C/min between the steps. At each step in the heating series the temperature was allowed to stabilize (±0.5°C) for 15 min prior to v_c measurements.

Results

Sudden exposure to D_2O by means of a rapid exchange of perfusion fluids (^{2}H-APW for APW) resulted always in a fast depolarization. This effect is similar in magnitude and in time course to the voltage characteristics of an electrically evoked plant cell action potential but only with a partial repolarization (Fig. 1*a–c*). Control tests using the same nondeuterated APW for sham substitution, remained without such an effect (Fig. 1*d*). Cells kept overnight in the deuterated medium, still showed the same substantially reduced PD values.

By following the time course kinetics of the extracellular concentration of potassium ions (Fig. 2), it was found that potassium leaks out of the cells during incubation in ^{2}H-APW (control experiments

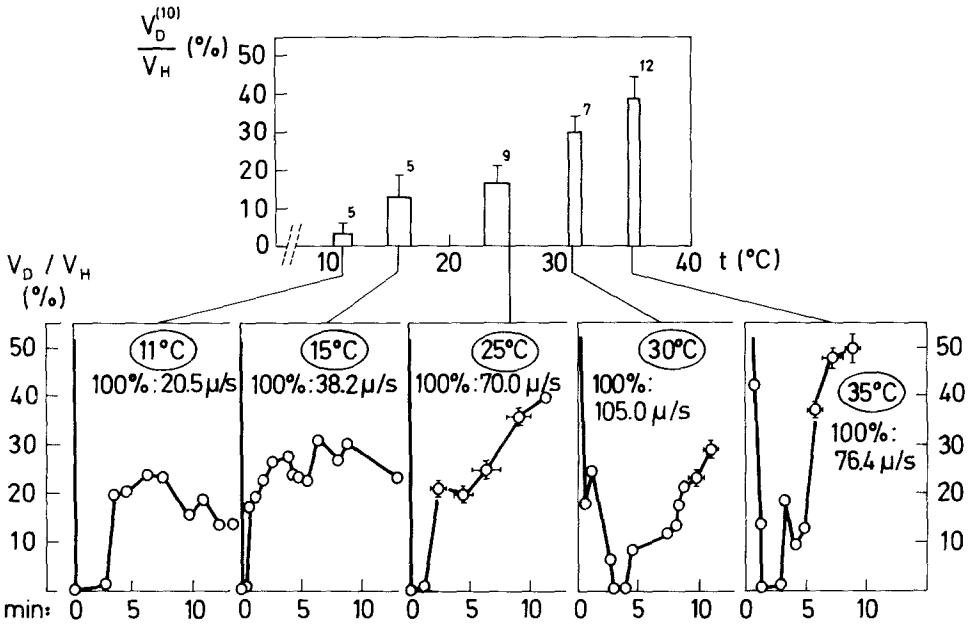

Fig. 3. Temperature dependence of the D_2O-induced suppression of cyclosis velocity (v_D, in ^{2}H-APW; v_H, in APW). Upper panel: average recovery levels after 10 min of incubation in the deuterated medium. Values expressed as percentage ratios between streaming velocities recorded 10 min after exposure to ^{2}H-APW, $v_D^{(10)}$, and the starting velocity in APW, v_H. Numerals along the SE extensions denote the number of cells explored within the temperature range indicated by bar width. Lower panels: rates of cyclosis (percentage fractions of control rates) after exposure to ^{2}H-APW at time zero (examples of experiments with single cells at four different temperatures). The "100%" values (in µm/sec) refer to control rates (v_H) recorded prior to exposure. Circles represent either single measurements of streaming velocity, or means ± SE from repeated measurements within time intervals indicated by the horizontal bars

with nondeuterated APW showed no such leak). The most rapid increase of extracellular potassium concentration occurred during the first minute following the exposure to ^{2}H-APW and there was little further change during the next 18 hr (Fig. 2, dashed horizontal line). The amount of potassium lost per cm^3 of cell volume was 17.5 ± 1.8 µmol. This value represents about 30% of the starting intracellular potassium which was estimated to be 53.8 ± 4.7 µmol/cm^3 (AAS of thermally ashed cells). Since the experimental solutions were not buffered, there was a possibility of a pH change during the solution exchange. This change, caused either by the influence of the cell sample or by the difference between pD and pH (pD = pH + 0.4; Glasoe & Long, 1960), did not exceed 0.8 pH units. This difference, when occurring within the neutral pH range of the unbuffered experimental solutions, should not cause significant changes in membrane transport (Kitasato, 1968). Moreover, control experiments in which extracellular K^+ was followed after increasing the pH of the buffered medium by 0.5, showed no appreciable potassium leakage.

Measurements of extracellular Cl^- during D_2O treatment revealed that 10 min upon D_2O application the extracellular concentration of chloride also rose by 23.4 ± 3.3 µmol per cm^3 of cell volume.

After D_2O exposure, an almost instantaneous cessation of protoplasmic streaming has been observed followed by an early recovery process (Fig. 3, bottom). However, the cyclosis recovery is only partial and dependent on time and temperature. Usually, the recovery process is accomplished in 10 min. The degree of recovery increases with temperature from a small percentage up to a maximum of about 40% (Fig. 3, top). The activation energy for cyclosis can be directly calculated from Einstein's diffusion equation

$$D = D_o e^{-E/RT} = v_c RT \qquad (1)$$

where D is the diffusion coefficient; D_o is the self-diffusion coefficient; E is the activation energy; v_c is the cyclosis velocity; R is the gas constant; and T is the absolute temperature. In cells completely adapted to D_2O (after several hours) cyclosis, besides having a substantially reduced velocity, also showed a higher activation energy as compared to the control in APW (41 and 31 kJ/mol, respectively, as estimated from Eq. (1) for 20 and 30°C).

Discussion

D_2O-Induced Potential Spike

D_2O can affect the cell membrane as well as the cell interior. The primary membrane effect is the excitation manifested by a potential spike which resembled an action potential (Fig. 1). It is known that the action potentials in giant algal cells may be elicited with electrical as well as with mechanical stimuli (Hope & Walker, 1975). In the presently described case the excitation could have been caused by an osmotic-like effect ("osmotic stress"). It was previously shown on Characean cells that the action potential can be generated as a response to osmotic stress (Zimmerman & Beckers, 1978). D_2O has a lower chemical potential than H_2O, thus making a solution of salts in D_2O hyperosmotic towards the corresponding H_2O solution. A hyperosmotic effect of D_2O with an induced H_2O efflux in cells of *Nitella* was already discovered very early by Brooks (1937). Such a net efflux of H_2O could also occur if the membrane had a different permeability for D_2O as compared to H_2O. However, in previous studies on *C. gymnophylla* (Andjus, Srejić & Vučelić 1987), no difference between the diffusional membrane permeabilities for H_2O and D_2O could have been revealed in the temperature range of 19 to 29°C. The hypothesis of the osmotic stress is in accordance with the temperature dependence of the PD change. Namely, the chemical PD between H_2O and D_2O becomes significantly smaller with rising temperature and, consequently, the D_2O-induced osmotic stress should be less expressed. Thus, if it is assumed that the D_2O-induced potential spike is not an all-or-none phenomenon (it has not been unambiguously shown that the electrically induced action potential in *Chara* resembles an all-or-none process), its size should fall with rising temperature as justified by experiment (Fig. 1*a–c*).

Although the hypothesis of osmotic stress as the cause of D_2O-elicited action potentials seems probable, other options cannot be rejected. These are: lower ion activity coefficients in D_2O than in H_2O; relatively lower ion diffusion coefficients due to higher viscosity of D_2O as compared to H_2O; or the abrupt blockage of the electrogenic pump.

The pH/pD ratio can be ruled out as a possible source of cell excitation during solution exchange, since it was observed that in internally perfused Characean cells action potentials, followed by a 10-fold drop of membrane resistance, can be elicited by the H_2O/D_2O exchange of buffered (pH = pD = 7.2) solutions (*unpublished*).

K^+ Leakage

The second main D_2O effect on the membrane level is the leakage of about one-third of intracellular K^+. This differs from the results on animal cell membranes which show a reduction of sodium efflux by about one-third (Keynes, 1965; Landowne, 1987). A lower Na^+ efflux in the latter case (Landowne, 1987) was ascribed to a general decay of the (Na^+ + K^+)-ATPase activity and an increase of the apparent ATPase affinity towards potassium. By analogy, a similar hypothesis could be posed for plant cell membranes. In the majority of plants as well as in Characean cells the function of an electrogenic H^+-ATPase is essential for membrane transport of K^+. D_2O can cause the drop of the H^+-ATPase activity, probably due to the rise of apparent affinity towards the D^+ ion, thus causing the decay of intracellular K^+ and a new resting PD. However, a relatively old result (*see* Hope & Walker, 1975 for references) could make the simple and general ATPase hypothesis more complex. In giant algal cells it has been shown that during an electrically evoked action potential there is a transient rise of passive potassium efflux. Thus, it may be confirmed that the membrane excitation in general (including D_2O-elicited excitation) causes a large potassium efflux.

The observed abruptness of the induced K^+ efflux (Fig. 2) points to the activation of potassium transmembrane pathways which is a process also known to occur during membrane excitation and which initiated here the K^+ leakage. These K^+ pathways may not be just K-channels but, even more probable, channels which are more selective for other ions, e.g., Cl or Ca channels.

However, it cannot be neglected that there may be no direct connection between the electrically induced K^+ efflux and the D_2O-elicited K^+ leak, although a potential spike is generated in both cases. Recent studies have shown that D_2O irreversibly activates chloride channels in the *Characean* plasma membrane (P.R. Andjus, *unpublished*) and measurements of extracellular chloride presented here have revealed a D_2O-induced Cl^- loss. Chloride could thus be the accompanying ion to the K^+ efflux, and since in these cells the equilibrium potential for Cl^- is more positive than for K^+, Cl^- efflux could, thus, induce membrane depolarization and further K^+ loss.

Protoplasmic Streaming in D_2O

Intracellular D_2O effects are the consequence of the early events triggered at the membrane and of the inflow of D_2O into the cytoplasm. This study re-

vealed that the membrane excitation induced by D_2O, similarly to the one induced with other stimuli (Kishimoto & Akabori, 1959; Williamson & Ashley, 1982), caused the transient cessation of cyclosis. At lower temperatures this effect is instantaneous and pronounced (Fig. 3), which is in accordance with larger amplitudes of the D_2O-elicited potential spikes. The recovery of protoplasmic streaming is a complex process which includes the post-excitation recovery and the process of adaptation to D_2O which has penetrated into the cytoplasm (exchange half time at 25°C is around 20 sec; Andjus et al., 1987). Both processes show a complex dependence on temperature and, thus, the curves in Fig. 3 (bottom) cannot be unambiguously resolved.

Upon penetration of D_2O into the cell the cyclosis activation energy increased significantly. This increase is the consequence of a higher viscosity of the electrolyte with D_2O as compared to H_2O (Katz & Crespi, 1970). Due to higher viscosity, the diffusion coefficient of protons and deuterons in D_2O falls by a factor of 1.51 (Roberts & Northey, 1974). If it is assumed that the activation energies increase by the same factor, then the value for cyclosis in D_2O should be $E = 47$ kJ/mol which is close to the value of 41 kJ/mol obtained from experimental data.

Conclusion

Although all details of the mechanism of D_2O action are still unresolved the following basic sequence of events can be envisaged: (*i*) D_2O induces immediate membrane excitation characterized by a potential spike which resembled an action potential with an incomplete repolarization phase. (*ii*) As a consequence of membrane excitation protoplasmic streaming is stopped. (*iii*) Potassium transmembrane pathways (potassium or other membrane channels accessible to K^+) are activated leading to an abrupt K^+ efflux. This is also a process characteristic of membrane excitation. The induced K^+ outflow along with the reduction in the electrogenic pump activity causes the loss of approximately one-third of intracellular K^+. (*iv*) Finally, the slowest process in this sequence of events is the recovery of cyclosis which leads to a state of slower protoplasmic streaming with a higher activation energy.

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