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D_2O-induced Ion Channel Activation in *Characeae* at Low Ionic Strength

P.R. Andjus[2]*, A.A. Kataev[1], A.A. Alexandrov[1], D. Vučelić[2], G.N. Berestovsky[1]

[1]Institute of Cell Biophysics, Russian Academy of Sciences, Puschino, Moscow region, 142292, Russia
[2]Institute of General and Physical Chemistry, P.O.B. 551, Studentski trg 12, 11000 Belgrade, Yugoslavia

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Abstract. Effects of D_2O were studied on internodal cells of the freshwater alga *Nitellopsis obtusa* under plasmalemma perfusion (tonoplast-free cells) with voltage clamp, and on Ca^{2+} channels isolated from the alga and reconstituted in bilayer lipid membranes (BLM). External application of artificial pond water (APW) with D_2O as the solvent to the perfused plasmalemma preparation led to an abrupt drop of membrane resistance ($R_m = 0.12 \pm 0.03$ k$\Omega \cdot$ cm^2), thus preventing further voltage clamping. APW with 25% D_2O caused a two-step reduction of R_m: first, down to 2.0 ± 0.8 k$\Omega \cdot$ cm^2, and then further to 200 $\Omega \cdot$ cm^2, in 2 min. It was shown that in the first stage, Ca^{2+} channels are activated, and then, Ca^{2+} ions entering through them activate the Cl^- channels. The Ca^{2+} channels are activated irreversibly. If 100 mM CsCl was substituted for 200 mM sucrose (introduced for isoosmoticity), no effect of D_2O on R_m was observed. Intracellular H_2O/D_2O substitution also did not change R_m. In experiments on single Ca^{2+} channels in BLM H_2O/D_2O substitution in a solution containing 100 mM KCl (*trans* side) produced no effect on channel activity, while in 10 mM KCl, at negative voltage, the open channel probability sharply increased. This effect was irreversible. The single channel conductance was not altered after the H_2O/D_2O substitution. The discussion of the possible mechanism of D_2O action on Ca^{2+} and Cl^- channels was based on an osmotic-like stress effect and the phenomenon of higher D-bond energy compared to the H-bond.

Key words: Membrane ionic currents — Reconstituted Ca^{2+} channel — Deuterium oxide —*Nitellopsis*

* *Present address:* Scuola Internazionale Superiore di Studi Avanzati (S.I.S.S.A.), Via Beirut 2-4, 34013 Trieste, Italy

Correspondence to: P. Andjus

Introduction

The effects of H_2O/D_2O solvent substitution on action potentials have been studied in different excitable membranes (Spyropoulos & Ezzy, 1959; Stillman & Binstock, 1967; Muller-Mohussen & Albrecht-Buhler, 1969). A more detailed voltage clamp study on the giant axon (Conti & Palmieri, 1968) revealed the influence of D_2O on the characteristics of Na^+ and K^+ currents. Thus, it was shown that H_2O/D_2O substitution slowed all the velocity constants by a factor of 1.4, decreased the current amplitudes by the same magnitude, and did not change the amplitude of the action potential. Similar effects of D_2O were found for the axon of *Myxicola* (Schauf & Bullock, 1979). All these experiments were carried out in solutions with relatively high ionic strength (100–400 mM). With a few exceptions (Lindley, Hoshiko & Leb, 1964), D_2O effects were independent on the membrane side, or the possible asymmetry of the effects was not taken into account. All the mechanisms of D_2O action have been proposed for deuterated systems already in equilibrium with the D_2O solution.

Previous studies on intact cells of a *Characean* alga (Andjus Srejíc & Vučelić, 1987; Andjus & Vučelić, 1990) revealed that H_2O/D_2O substitution exerts some specific effects on the plasmalemma leading to cell excitation. The main differences from other investigations with D_2O were in experimental conditions: very low ionic strength in the external solution ("artificial pond water," APW), and the application of D_2O at one membrane side only which could give rise to stress-like effects.

Here we present a study of D_2O effects on a *Characean* cell by using the perfused plasmalemma system and the reconstituted lipid bilayer system (BLM), containing isolated Ca^{2+} channels (Lunevsky et al., 1980; Kataev, Zherelova & Berestovsky, 1984).

It was shown that the effects of H_2O/D_2O substitu-

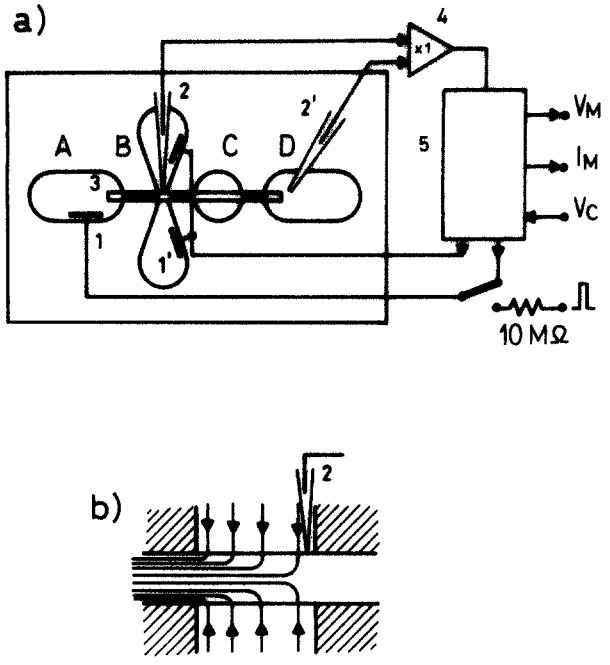

Fig. 1. Electric layout and experimental chamber for internal cell perfusion under voltage clamp conditions. (*a*) Compartments *A* and *D* were filled with the perfusion solution (PS), compartment *B* with the bathing (external) APW solution, and *C* was empty. (*1,1'*) Ag/AgCl current electrodes; (*2,2'*) voltage electrodes (glass micropipettes); (*3*) dissected internodal cell; (*4*) preamplifier probe and (*5*) voltage clamp circuit; (V_c) command input; (I_m and V_m) membrane current and voltage, respectively. (*b*) Scheme showing current flow through the cell in the working compartment (*B*).

tion depend on the ionic strength, and an asymmetry of sudden D_2O effects was revealed. This study on the subcellular level should be a first step in elucidating the mechanism underlying D_2O effects previously reported for the more complex system of an intact cell (Andjus & Vučelić, 1990).

Materials and Methods

Voltage Clamp on the Perfused Plasmalemma

Experiments were carried out on the *Characean* fresh-water alga *Nitellopsis obtusa* stored in APW containing (in mM): 0.1 KCl, 1.0 NaCl, 0.1 $CaCl_2$, pH 8.0–8.2 (such a high pH value was maintained by the self-regulation of stored cells). Giant internodal cells (0.6–0.8 mm in diameter and about 40–80 mm long) were isolated and fixed in the experimental chamber using vaseline (Fig. 1). The experimental chamber consisted of four compartments: the central *B*, two at each cell end (*A* and *D*) and compartment *C* which served as an air insulation to prevent electrical coupling along the cell wall. To perfuse the plasmalemma, the tonoplast had to be removed by a procedure based on Williamson (1975), Tazawa, Kikuyama and Shimmen (1976), and Kataev et al. (1984). After the cell was sealed in the chamber grooves with vaseline, it almost completely lost turgor (in 1–2 min), and both cell ends were cut off. Compartments *A* and *D* (each 2 ml in volume) were filled in succession with the Ca^{2+}-free perfusion solution (PS_{EGTA}; in mM): 1.0 EGTA, 15 KCl, 280 sucrose, 10 HEPES/KOH pH 7.2. The liquid level difference in compartments *A* and *C* determined the perfusion direction. Compartment *B* was filled with APW. The osmolarity and pH of the external medium was adjusted by addition of 200 mM sucrose and HEPES/NaOH, pH 7.2 in all experiments, except when indicated otherwise. To remove the tonoplast efficiently, the flow rate had to be increased by adding more perfusate to one of the compartments or by applying suction through a glass capillary at the opposite cell end. Tonoplast removal was followed visually with a microscope and it was considered complete when the intense extrusion of membrane vesicles came to a stop. Finally, V_m stabilized around −100 mV. Upon tonoplast removal Ca^{2+}-free perfusion could have been stopped by leveling the solutions in compartments *A* and *D*.

The exchange of external solutions in compartment *B* was performed by micropipette addition along with simultaneous suction. The perfusate was exchanged by filling different solutions in compartments *A* and *D*, thus enabling the replacement of intracellular media by changing the direction of perfusion. Constant perfusion rate (50–200 μm/sec) was maintained by successive addition of 10–30 μl doses of solution to compartments *A* or *D*.

During H_2O/D_2O exchanges ^{2}H-APW solutions were used (APW with D_2O as a solvent). Deuterium oxide utilized in these experiments was 99.8% pure (Sigma), unless otherwise stated.

The membrane was voltage-clamped using four electrodes (Smith & Walker 1981; Kataev et al., 1984) and an intracellular preamp-clamp (Dagan, Model 8500). Membrane potential (V_m) was recorded using micropipettes (tip diameter of 5–20 μm) filled with agar containing 100 mM KCl (Fig. 1). The tip of electrode 2 was placed near the cell surface on the right side of compartment *B* (close to compartment *C*). The portion of the cell placed between compartments *D* and *B* served as an extended electrode 2'. Ag/AgCl current electrodes were placed in compartments *A* and *B*. This arrangement of current and voltage electrodes and the rather narrow (2 mm) working section of the cell isolated with vaseline gaps in compartment *B* provided a quite satisfactory temporal and spatial control of membrane voltage (Lunevsky et al., 1983). It follows from general considerations that the higher the conductance of intracellular medium and the lower the conductance of the cell membrane and the external solution, the less is the relative voltage difference between two boundary points of the working cell section (Fig. 1*A*). With respect to this, we usually used external solutions of low ionic strength, and to decrease membrane current density, a relatively low concentration of the permeant anion was used (Kataev et al., 1984).

To measure the membrane conductance and current reversal potentials, rectangular and ramp-wave voltage pulses of 30 msec duration were applied. A pen recorder (Endim 620.02, Germany) and a FM tape recorder (TEAC, Japan) were used to record the signal.

All experiments were done at room temperature (20–22°C).

Measurements of Single Channel Activity

The partially purified channel-forming protein was prepared according to Grischenko, Aleksandrov and Berestovsky (1984) and modified by the addition of 1 mM protease inhibitor, phenylmethylsulfonylfluoride (Serva) in all purification steps. A heptane solution of 30 mg/ml soybean phosphatidylcholine (Type II S, Sigma) was used to form a planar lipid bilayer at a 0.2 mm wide hole in a Teflon cup. The bilayer separated 1.5 ml of a solution in the Teflon cup from 6.5 ml of a solution in an outer glass chamber. All salts were of analytical grade and their aqueous solutions were buffered with Tris-HCl to pH 7.2. Experimental temperature was 22°C.

The aqueous solution of the channel-forming protein was added

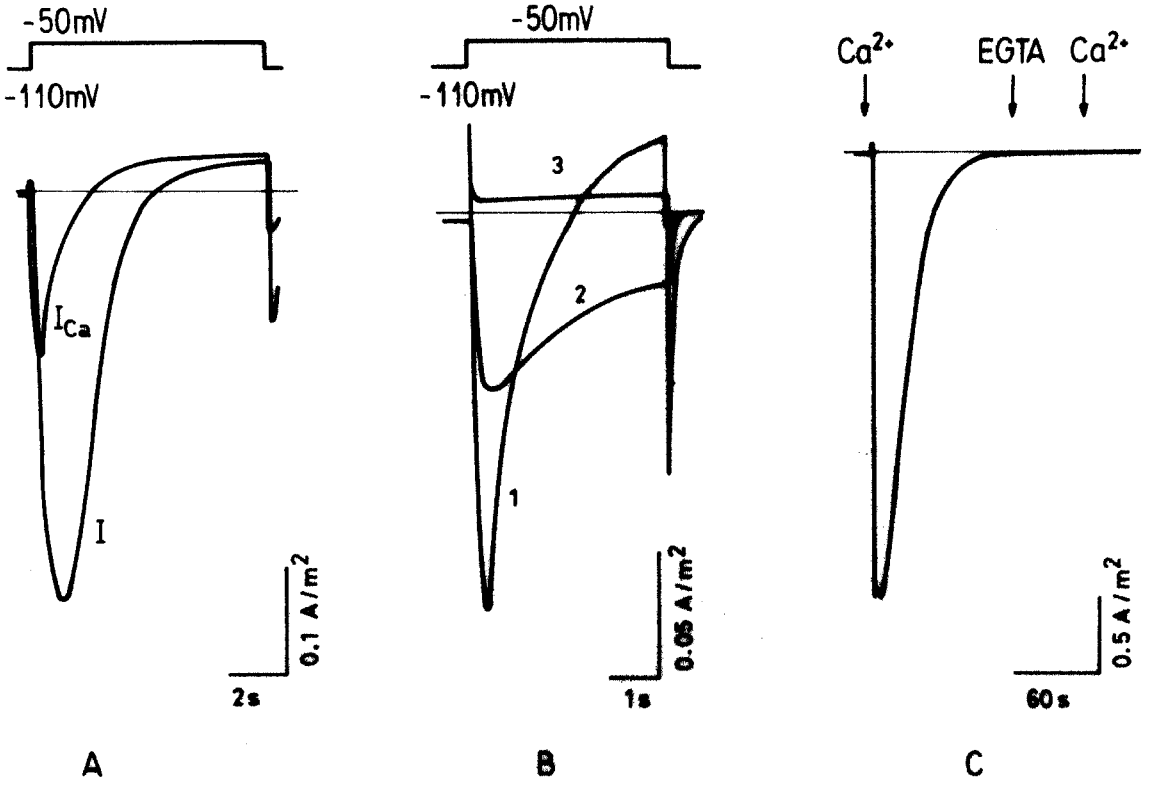

Fig. 2. Loss of electrical excitability of the cell in the course of internal perfusion. Test rectangular voltage steps (from −110 to −50 mV) were used (shown above the current traces). (*A*) The initial stage. Vacuolar sap was not substituted by *PS*. *I* is the net transient current ($I_{Ca} + I_{Cl} + I_K$) across plasmalemma and tonoplast. Upon substitution of vacuolar sap with the Ca^{2+}-free solution (PS_{EGTA}) fast inactivation (1–3 min duration) of I_{Cl} revealed the Ca^{2+}-current component (I_{Ca}). (*B*) Time course of I_{Ca} suppression with continuous perfusion (rate of flow v = 30–50 μm/sec). The Ca^{2+} current was measured at the moment of disappearance of Cl^--current (*1*), 1 min after (*2*), and 15 min after (*3*). (*C*) Activation and irreversible inactivation of Cl^- current induced by a rise of internal free Ca^{2+} concentration at the final stage of perfusion. One millimolar EGTA (Ca^{2+}-free solution) was applied at the indicated arrow (*EGTA*) and 0.5 mM Ca^{2+} was added (*Ca^{2+}*). Membrane potential was fixed at V_m = −100 mV.

to the Teflon chamber only (*cis* side). The membrane potential difference (PD) was measured as the difference between *cis*- and *trans*-side potentials. The spontaneous ion channel incorporation was registered as a current jump following channel-forming protein addition. Apparently, there was no current fluctuation without the channel-forming protein. Due to partial purity of the channel-forming protein, its exact concentration in the experimental chamber was not estimated. However, the necessary dilution of the stock solution was obtained empirically by allowing 20–30 min between two spontaneous channel incorporations. Thus, the volume of the added protein stock solution was never larger than 3% of the total volume at the *cis* side.

The selectivity of this channel type was previously characterized (Lunevsky et al., 1980, 1983; *see also* Results). According to the shift of reversal potential in the presence of different concentrations of permeant cations, it was found that this channel belongs to the type of voltage-dependent Ca^{2+} channels, as classified by Tester (1990).

In the D_2O experiments the D_2O solution, having a relatively higher specific weight due to deuterium, was added by the use of a peristaltic pump from the bottom of the outer glass chamber while an equal volume of the H_2O solution was taken from the upper part. The process of solution exchange was followed by the addition of a trace of brilliant blue to the D_2O solution. The rate of solution exchange was 10–15 ml/min. Thus, after 2 min more than three volumes of the outer chamber were pumped out, and the exchanging process was stopped.

Electrical measurements were performed with a low-noise operational amplifier (Kiethley 301) in an *I-V* converter configuration, with a current measuring resistor, $R_f = 10^9$ Ω, and the shunt capacitance C_f = 2 pF. The output signal was filtered with a low-pass four-pole Bessel filter with a cut-off frequency of 25 Hz. Traces were plotted on a chart recorder (Endim 620.02, Germany; cut-off frequency of 10 Hz) and recorded on a FM tape recorder (TEAC, Japan) for further microcomputer processing.

Results

Voltage Clamp on the Perfused Plasmalemma

Prior to experiment with D_2O, the electrical characteristics of the internodal cells of *N. obtusa* were tested in the course of internal perfusion according to Kataev et al. (1984). An example is shown in Fig. 2. At the first stage of perfusion the tonoplast was present, and electrical stimulation induced fast transient Ca^{2+} and Cl^- currents and a slow K^+ current component. Destruction of the tonoplast was caused by internal perfusion with a solution containing 1 mM EGTA (free $Ca^{2+} < 10^{-5}$ mM). The disappearance of the transient Cl^- current upon perfusion

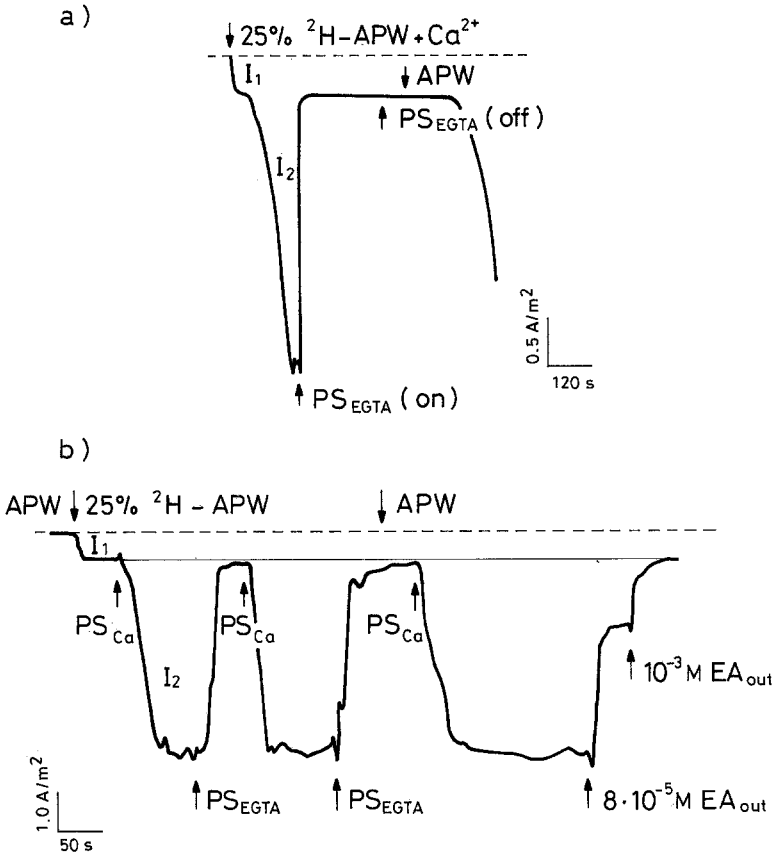

Fig. 3. The 25% D_2O-induced current components (I_1 and I_2). Top arrows indicate external solution exchange (^{2}H-APW for APW or *vice versa*). Holding potential $V_m = -100$ mV. (*a*) The effect of internal perfusion with a Ca^{2+}-free solution (PS_{EGTA}). External solutions (APW or ^{2}H-APW) contained 0.5 mM Ca^{2+}. Arrows under the current trace indicate: (PS_{EGTA} (*on*)) start of internal perfusion; (PS_{EGTA} (*off*)) its cessation. The perfusion solution was Ca^{2+} free with 1 mM EGTA. I_1 was 0.46 ± 0.08 A/m^2 ($n = 5$). (*b*) Loss of Cl^--current inactivation. (PS_{Ca}) Internal perfusion with 0.5 mM Ca^{2+}; (PS_{EGTA}) same perfusion solution as in *a*; (*EA*) externally added ethacrynic acid. APW or ^{2}H-APW contained (in mM): 0.1 KCl, 1.0 NaCl, 0.1 $CaCl_2$, 200 sucrose, 1.0 HEPES-NaOH, pH = pD = 7.2

was used as a criterion of tonoplast removal (although used in some previous studies, Mg-ATP was not added to the perfusate here since its presence only slows the run-down of the Ca^{2+}-current, but cannot prevent it; Zherelova, Kataev & Berestovsky, 1985). At this stage Ca^{2+} and Cl^- currents could not be induced by a voltage pulse, but the Cl^- current could have been stimulated by replacing the internal Ca^{2+}-free solution with one containing $>10^{-3}$ mM Ca^{2+} (Fig. 2*C*). However, this Ca^{2+}-induced generation of the Cl^- current was followed by its irreversible decay. An important property of Cl^- channels was the absence of any current generation with further changes of Ca^{2+} concentration.

After tonoplast removal the membrane voltage stabilized at a constant level (−100 to −120 mV), and membrane resistance was 10 ± 2 kΩ · cm^2. At this moment the perfusion was stopped and the experiments with H_2O/D_2O substitution began. When the voltage was clamped at the electrically unexcitable membrane, the external substitution of APW by ^{2}H-APW led to an abrupt drop of membrane resistance which made voltage clamp unattainable. Therefore, to analyze the D_2O-induced current, a 25% ^{2}H-APW (APW with 25% D_2O as the solvent) was used instead of ^{2}H-APW. Upon this solvent substitution, membrane resistance (R_m) decreased in two steps. During the initial step, R_m decreased to 2.0 ± 0.8 kΩ · cm^2 (I_1 in Fig. 3*a* and *b*). Instantaneous voltage-current curves (IVCC; Fig. 4), obtained with 30 msec ramp voltage pulses (dV/dt = 5 V/sec), revealed that reversal potential, $V_r = -37 \pm 2$ mV ($n = 11$), for the first current component is close to the value for the Ca^{2+} current in the plasmalemma (*see* Table). In the next 1–3 min a further R_m decrease was observed ($R_m < 200$ Ω · cm^2), and a corresponding current component of >0.5 mA cm^{-2} was detected (I_2 in Fig. 3). IVCC of this current component, which becomes 5–10 times larger compared to the first, revealed that its $V_r = 40 \pm 2$ mV ($n = 9$) is close to the value for the Cl^- current. Due to the significant contribution of the first current component, the exact value of V_r for the second current component could not be obtained. Thus, ethacrynic acid (EA), a specific blocker of Cl^- channels (Lunevsky et al., 1983), was used. As shown in Fig. 3*b*, the second current component was fully blocked by EA. For further discrimination between the two current components, the external concentration of $CaCl_2$ was changed from 0.1 to 0.5 mM, thus inducing a change in V_r for the first current component as expected for the Ca^{2+} current (Table).

If D_2O induced the Cl^- current by an increase in

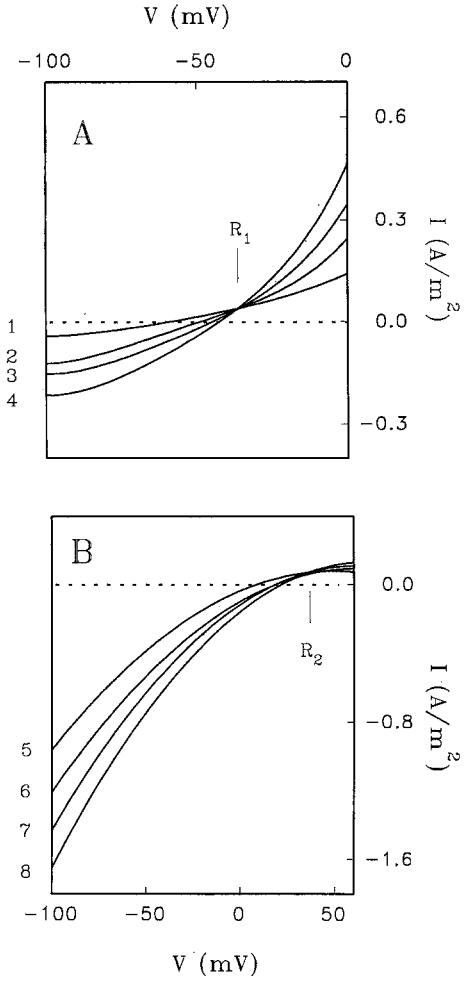

Fig. 4. Example of instant voltage current curves (IVCCs) obtained during 25% D_2O-induced excitation. IVCCs for the first (*A*) and second (*B*) current component (I_1 and I_2 in Fig. 3, respectively) were obtained by ramp-wave voltage pulses: (*A*) 100 mV, 30 msec, current through membrane capacity, $I_{R1} = C\ (\partial V/\partial t) = 0.046$ A/m^2, and (*B*) 180 mV, 30 msec, $I_{R2} = 0.075$ A/m^2. Ramp pulses were applied at 10 (*1*), 20 (*2*), 35 (*3*), 55 (*4*), 535 (*5*), 600 (*6*), 630 (*7*), and 650 (*8*) sec after 25% D_2O application. R_1 and R_2 indicate reversal potentials for the two current components. Perfusion medium was (in mM): 15 KCl, 3 EGTA, 280 sucrose, 10 HEPES-KOH (pH = pD 7.2). There was no streaming of the perfusion solution during measurement.

internal free Ca^{2+} (resembling excitation in the intact cell; Lunevsky et al., 1983) the removal of internal Ca^{2+} would cause the disappearance of the second current component. The results of Fig. 3*a* are consistent with this prediction. Namely, if the plasmalemma was again internally perfused with the Ca^{2+}-free solution (PS_{EGTA}), the second current component vanished along with an increase in R_m up to the value of 2.0 ± 0.5 k$\Omega \cdot$ cm^2, typical for the first D_2O-induced current component. If this perfusion was stopped again, the second current component was restored within 1–3 min. The contribution of K^+ channels may be negligible if Cs^+ replaces Na^+ and K^+ in APW. Such a replacement, however, did not change the D_2O effect (*not shown*). As mentioned earlier, all experiments with H_2O/D_2O substitution on animal cells were carried out in solutions with relatively high ionic strength. In our experiments with *Characean* cells, APW of low ionic strength was used but by applying Cs^+ ions, impermeable for K^+ channels, the ionic strength in the external solution was increased. If D_2O was used to substitute H_2O in the external solution containing APW with 100 mM CsCl instead of 200 mM sucrose (having the same osmolarity; Fig. 5), there was no change in R_m (at least in 20 performed experiments). The Cl^- current, in this case directly activated by an increase of Ca^{2+} in the perfusate (Fig. 5), showed irreversible inactivation, resembling the control (Fig. 2). In contrast to the perfused cell in H_2O, the Cl^- current in ^{2}H-APW did not inactivate (Fig. 3*b*). Returning APW in the outside chamber at any step after 2–3 min of exposure to ^{2}H-APW did not restore the initial membrane state. Moreover, the activation process followed the same pattern seen during the permanent external presence of ^{2}H-APW (Fig. 3). The Table shows the similar origin of the first current component induced by D_2O and the Ca^{2+} current in the plasmalemma, while the experiments with Ca^{2+} stimulation and EA (Fig. 3*b*) indicated the Cl^- channel origin of the second current component.

In another type of experiment the change in transmembrane potential difference (no voltage clamping) caused by external ^{2}H-APW application was examined as a function of external Ca^{2+} concentration. Prior to these measurements, the Cl^- current was blocked by continuous perfusion with EGTA. Figure 6 shows that V_m was dependent on external Ca^{2+} in a manner similar to that found for V_r of Ca^{2+} channels (*see* Table).

The perfused plasmalemma was also tested for the effects of H_2O/D_2O substitution at the internal side. A complete internal substitution of solvents in a Ca^{2+}-free solution did not cause any change in membrane resistance.

Single Ca^{2+} Channel Activity

Figure 7 shows an example of the single Ca^{2+} channel activity at different ionic concentrations also used in the experiments on the perfused cell. Thus, obtained mean V_r values are summarized in the Table. The channel activity at V = const. is shown in Fig. 8. K^+ was chosen to be the permeated ion since it is known to give (Lunevsky et al., 1980) the highest current through the channel relative to other alkali and rare-earth cations (except Rb^+). The ability to pass monovalent cations in the absence of divalent cations was also shown to be common to many Ca^{2+} channels in animal cells (Tsien et al., 1987).

Table. Reversal potentials (in mV) for the single channel current in BLMs, Ca^{2+} current in perfused plasmalemma[a] and the first current component induced by H_2O/D_2O substitution

Outside (*trans*-side) concentration (in mM)	2 Na^+ 0.1 Ca^{2+}	2 Na^+ 0.5 Ca^{2+}	2 Na^+ 1.0 Ca^{2+}	2 Na^+ 5.0 Ca^{2+}
Single channel[b]	−35 ± 3 (4)	−23 ± 2 (6)	−15 ± 2 (4)	
Ca^{2+} current in perfused plasmalemma[c]	−36 ± 3 (8)	−22 ± 2 (5)	−11 ± 1 (3)	+12 ± 2 (3)
First current component induced by H_2O/D_2O substitution[c]	−37 ± 2 (11)	−21 ± 3 (6)	−12 ± 2 (3)	+10 ± 3 (4)

[a] Measurements were performed when the transient Ca^{2+} current was significant. For example, *see* Fig. 2*A*.
[b] Inside (*cis-side*) solution was 30 mM KCl.
[c] In all experiments inside solution contained 25 mM K^+ as the permeant cation, and $[Ca^{2+}] < 10^{-8}$ M.
Data are presented as mean ± SD, with the number of experiments in parentheses.

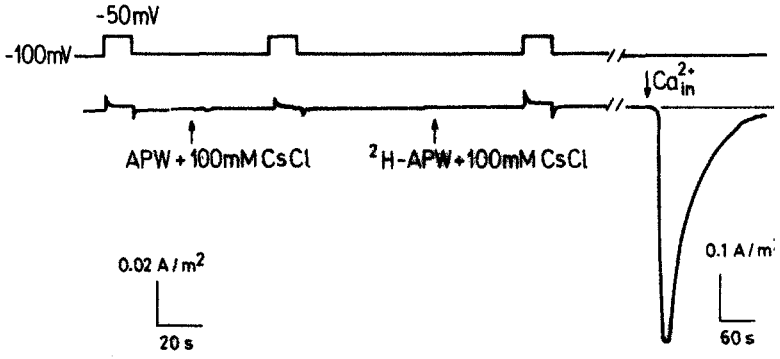

Fig. 5. The effect of D_2O on membrane current in the presence of 100 mM CsCl. Arrows indicate the addition of 100 mM CsCl to external APW or substitution of external medium by ^{2}H-APW containing 100 mM CsCl. Also shown is the current recording after the substitution of the internal Ca^{2+}-free solution by a solution containing 0.5 mM Ca^{2+}. Upper traces show the command voltage steps.

To obtain a linear *I-V* relation, identical salt solutions were used at both membrane sides. At potentials more negative than −80 mV the channels were closed (Fig. 8; *H_2O*). In the range of potentials between −80 and −20 mV close-open transitions were detected as a function of potential, and above −20 mV the channels were permanently opened. Similar channel kinetic behavior was observed in symmetrical 10 and 100 mM KCl solutions, while the conductance of the open state was increased from 30 ± 5 to 210 ± 20 pS in 10 and 100 mM KCl, respectively.

Substitution of H_2O with D_2O in a solution of 100 mM KCl at the *trans* side produced no effect on channel activity. However, when this operation was performed with symmetrical 10 mM KCl solutions, it caused a significant effect on Ca^{2+} channel gating; an example of this is shown in Fig. 8. At potentials more negative than −80 mV where the channels in H_2O were mainly closed (open channel probability was close to 0.1), those in D_2O had an open state probability of about 0.8. However, at these negative potentials, in D_2O, an increase of current noise at the channel open state was detected compared to more positive potentials. The single channel conductance was not altered after H_2O/D_2O substitution. This effect of H_2O/D_2O substitution was irreversible: when 10 mM KCl in H_2O was returned at the *trans* side, the initial voltage dependence was not restored (*not shown*).

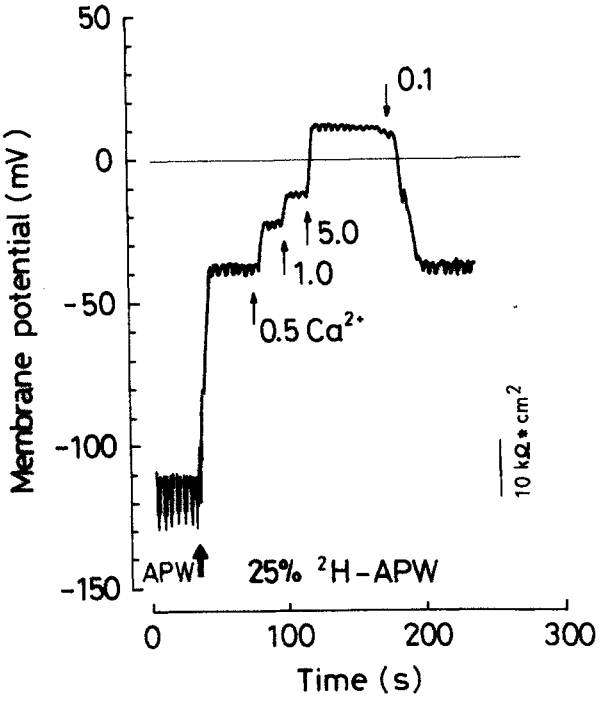

Fig. 6. Change of membrane potential and resistance during the replacement of external APW by 25% ^{2}H-APW and subsequent addition of different Ca^{2+} concentrations (shown in mM by the arrows). The results of this experiment are summarized in the Table.

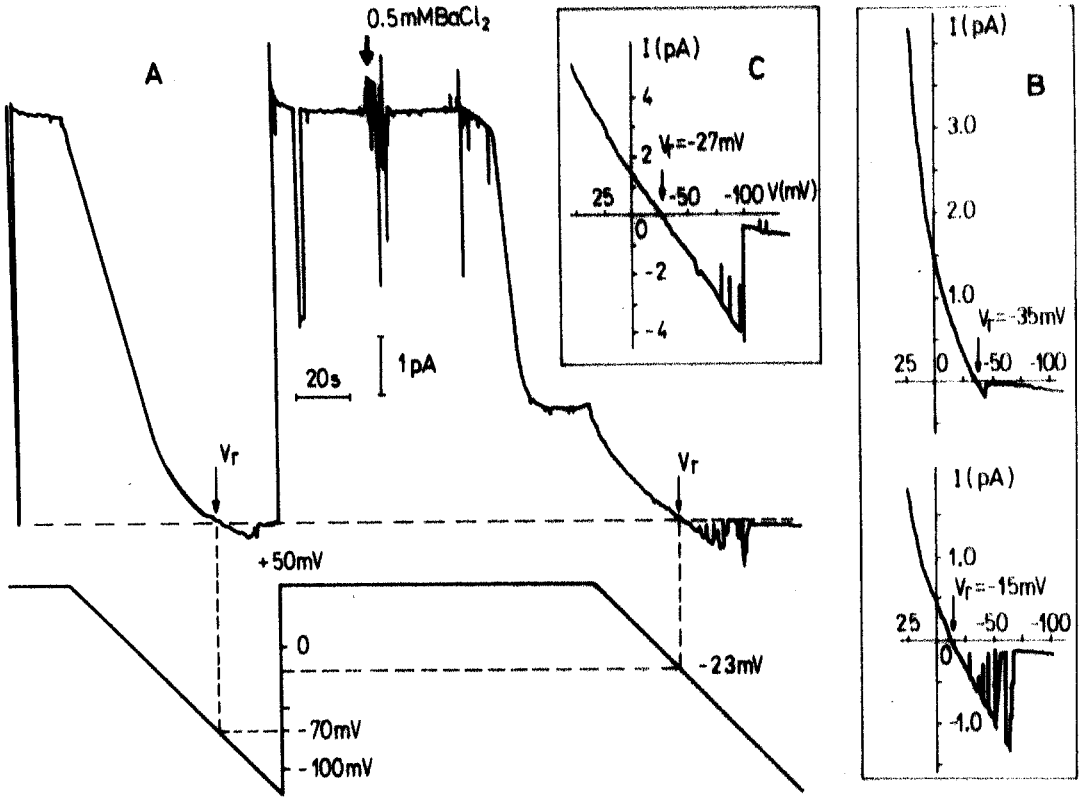

Fig. 7. An example of single channel recording in different bath solutions. (*A*) Upper trace: solutions, *cis* side: 30 mM KCl; *trans* side: 2 mM NaCl. $V_m = 50$ mV; single channel current was 7 pA, $V_r = -70$ mV. V_r value is obtained as zero current voltage using a ramp-wave voltage pulse ($dV/dt = 2.5$ mV/sec, $I_c = C(dV/dt) \approx 2 \times 10^{-13}$ A. The command voltage is shown at the bottom. $BaCl_2$ (0.5 mM) is added at the *trans* side as indicated by arrow. During stirring, I_m decreased to 1.8 pA, while $V_r \approx -20$ mV. Insets: (*B*) Last phase of the same experiments but with 0.1 mM $BaCl_2$ (upper curve) and 1.0 mM $BaCl_2$ (lower curve). V_r values are shown in the Table. (*C*) Example of a single channel recording in $BaCl_2$. At the *cis* side, solution contains 50 mM, and at the *trans* side 5 mM $BaCl_2$. The estimated value of V_r was -27 ± 2 mV ($n = 8$).

Discussion

Effects of D_2O on Ca^{2+} Channels in the Perfused Plasma Membrane

The application of D_2O at one membrane side could induce not only an isotope effect due to H/D exchange but also a solvent substitution effect resembling an osmotic shock, since it is known that membrane permeability for D_2O is less than for H_2O (Collander, 1954; Dainty & Ginzburg, 1964). The early work of Brooks (1937) showed that D_2O application to *Nitella* cells caused a transient change of cell turgor. First, turgor decreased (≈15 sec) due to an outflow of H_2O, followed by a second phase (≈2–3 min) during which turgor restored due to equilibration of D_2O and H_2O at both membrane sides. The range of time for the duration of the above processes was the same as for the electrical effects observed in this paper. Large osmotic volume flows could produce irreversible changes in the channel structure, resulting in channel opening. However, this process could not happen in experiments at higher salt concentrations. By applying 100 mM CsCl (instead of 200 mM sucrose) at the external side of the membrane, it was expected that the magnitude of the outward H_2O volume flow would not change. It is also important to stress that our previous studies on *N. obtusa* (Lunevsky et al., 1980, 1983) have shown that Cs^+ does not block the Ca^{2+} channel in *Nitellopsis* and can pass through it with a 0.75 permeability ratio to K^+. However, no effects similar to the cases at lower salt concentration could be observed. This result was also confirmed on single Ca^{2+} channels by changing the ionic strength with KCl (*see* Results and Fig. 8). Therefore, it was concluded that the low ionic strength is the necessary condition for the sudden effects of H_2O/D_2O substitution.

During H_2O/D_2O substitution, due to the fast transmembrane diffusion of D_2O, only a short-time period has to pass before the external and internal unstirred layers at the membrane become equal in D_2O content. This short time should be independent of the membrane side at which D_2O is added and could not be easily calculated due to the occurrence of a high osmotic flow. According to Brooks (1937) this time period may be estimated to be

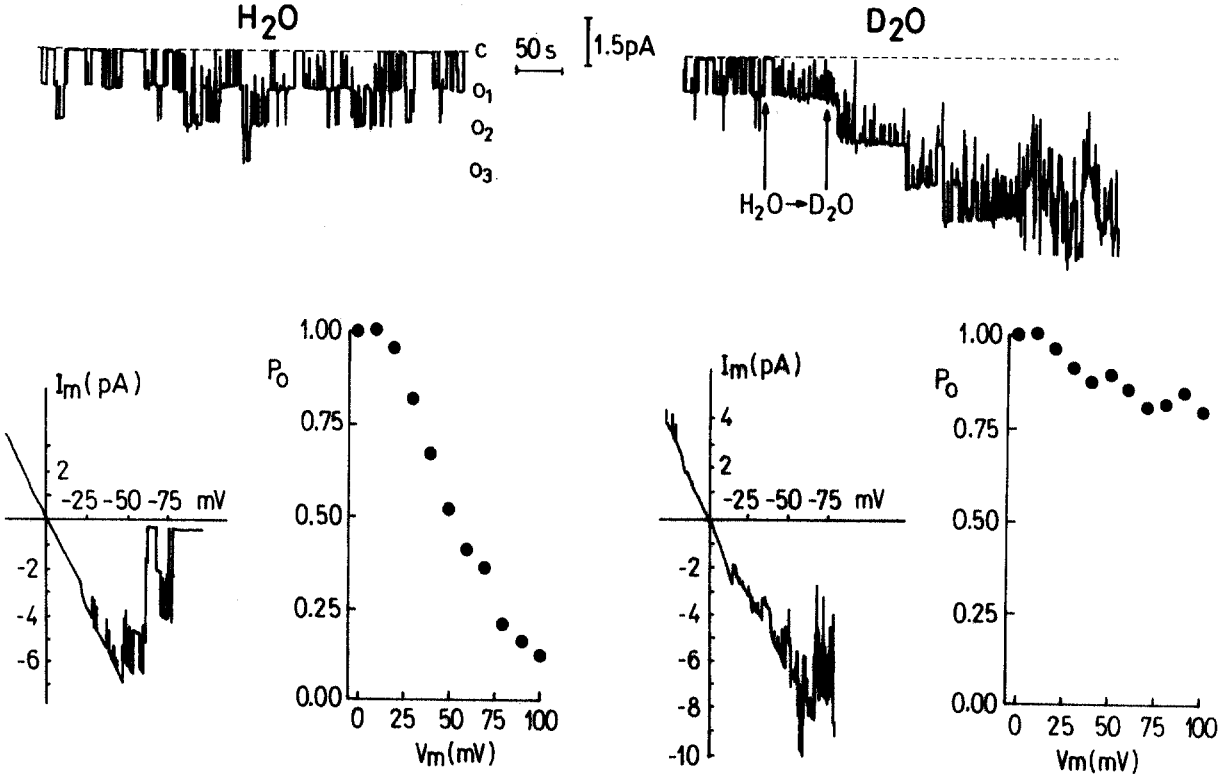

Fig. 8. Example of the current recording obtained from a membrane containing four single channels in symmetric 10 mM KCl. (*H_2O*) Single channel activity measured at $V_m = -50$ mV (*top*); *I/V* curve of the single channel obtained by ramp pulse ($dV/dt = 2.5$ mV/sec); axes shown with the correction to I_c current ($I_c = C(dV/dt) = 2 \times 10^{-13}$A) (*bottom, left panel*); probability of the open state as a function of V_m (averaged over 10 *I/V* curves) (*bottom, right panel*). Open probability values were derived by taking the mean current measured at each V_m relative to the maximal current at this potential ($P_o = I(V)/I_{max}(V)$. (*D_2O*). Same characteristics after H_2O/D_2O substitution at the *trans* side. The time course of substitution is shown by arrows at the upper trace.

not more than 2–3 min. However, introduction of D_2O inside the cell did not produce any effect similar to D_2O outside. Furthermore, when H_2O was substituted by D_2O at the external side after several minutes of internal perfusion with the D_2O-containing solution, no effect was observed. It is not clear what change in channel conformation could be induced by this asymmetric condition (H_2O inside, D_2O outside) at the early stage of solvent exchange. This change should not only be functionally significant but also irreversible, since returning H_2O in the outside chamber did not restore the initial channel inactive state.

Effects of H_2O/D_2O Substitution on the Ca^{2+} Channel Reconstituted in BLM

According to the control experiments (Fig. 7), it could be expected that reconstituted Ca^{2+} channels resemble the Ca^{2+} channels in the plasmalemma. The comparison of the values of V_r in the Table confirm this assumption.

Using the simple BLM model system, we could reproduce most of the effects of H_2O/D_2O substitution on Ca^{2+} channels in the perfused plasmalemma. First, we found that H_2O/D_2O substitution at the *trans* side leads to Ca^{2+} channel opening only at low ionic strengths. There were no effects on channel gating in 100 mM KCl compared to 10 mM KCl. Solvent substitution at the *cis* side caused no significant effect (*not shown*), resembling the experiment on the perfused cell. The experiments with single channels pointed out that the single channel conductance was unchangeable after H_2O/D_2O substitution. The results of these experiments discard the possible role of other membrane constituents from the discussion on mediation of the D_2O-induced effects on channel kinetics.

Effects of D_2O on Chloride Channels

The effect of H_2O/D_2O substitution on Cl^- channels is found to be a result of Ca^{2+}-channel activation. The delay in Cl^--current generation which resulted from the inward flow of Ca^{2+} ions could be attributed to the slow increase of Ca^{2+} concentration in the internal unstirred layer. The internal free Ca^{2+} concentration was adjusted to 10^{-5} mM.

The delay in Cl^--current generation should be equal to the time which is necessary for a rise in free internal

Ca^{2+} concentration up to 10^{-3} mM which is known to be the threshold concentration for Cl^--channel activation (Kataev et al., 1984). For calculation of this time, *see* Appendix. The principal mechanism of Cl^--current activation was confirmed by using internal perfusion with a Ca^{2+}-free solution after D_2O excitation, which abolished the Cl^- current (Fig. 3*a*).

The properties of the Cl^- current in D_2O-treated membranes differed from those found in the control. The major difference was the lack of Cl^--channel inactivation (Fig. 3*b*). Moreover, Cl^- current was repeatedly turned on and off by the change in internal free Ca^{2+} concentration. In contrast, this operation could be performed only once in the H_2O control. These data also showed that the effects of D_2O were irreversible. The significant effect of H_2O/D_2O substitution regarding the abolishing of Cl^--current inactivation was also evident. However, with 100 mM CsCl in APW there was no difference between the Cl^- current in the control (Fig. 2) and after H_2O/D_2O substitution (Fig. 5).

The Mechanism of D_2O Action on Membrane Ion Channels

The effects of D_2O on Ca^{2+} and Cl^- channels were found to share two important characteristics: both took place only at low ionic strength and after external D_2O application. In a few minutes after D_2O application the ion channel activity was insensitive to further changes of external solution (Fig. 3). This points to an irreversible stress-like effect of D_2O. The stress-like nature of the D_2O effect should underlie the difference between the results of this and of other studies (Spyropoulos & Ezzy, 1959), which revealed only moderate effects due to prior equilibration to D_2O.

A hypothesis of the D_2O stress effect can be based on the phenomenon of a higher D-bond energy relative to the H-bond (Singh & Wood, 1969; Bass & Moore, 1973; Lewin, 1974; Karasz & Gajnos, 1976). Having this fact in mind, the absence of a D_2O effect at high ionic strength could be explained. Namely, electrostatic interactions share most of the H-bond energy, and at large electrolyte concentrations the charges of the groups related to the H-bond will be screened and the isotopic effect will be negligible. The role of H-bonds in stabilizing the protein structure was mainly shown on soluble globular proteins (Calvin, Hermans & Scheraga, 1959; Hermans & Scheraga, 1959; Scheraga, 1960). A different effect may appear with ion channels as integral membrane proteins. In these functional proteins only the local protein regions, protruding from the membrane, could have been subjected to a fast H/D exchange. Thus, the balance of the stabilizing forces in a native structure could have been altered. These asymmetric local perturbations of protein structure should underlie the main difference from the data on soluble globular proteins which have the whole surface equally exposed to the solvent.

After a few minutes the uneven distribution of H and D at both membrane sides was not significant due to fast D_2O penetration. However, this short period will be sufficient for the creation of a new equilibrium conformation of the channel-forming protein, which was insensitive to further solution substitutions. The new conformation will also have a different gating mechanism compared to the native state. Although tentative, the above hypothesis could explain all the presented experimental results. The importance of ionic strength in experiments on D_2O effects in other systems should also be stressed. It can now be proposed that the studies of H_2O/D_2O substitution on ion currents in excitable cells would give rather different results if done at low ionic strength.

The D_2O effect presented here shows some similarities to the salt-stress effect induced by 100 mM NaCl (but not with isotonic sorbitol) in *N. obtusa,* characterized by depolarization and R_m decrease (Katsuhara & Tazawa, 1986). This effect was significantly reduced by adding 10 mM $CaCl_2$ which may have been a tolerance phenomenon also induced by screening of charges having a role here in Na^+ transmembrane transport.

Conclusions

The experimental results herein point to a tentative chain of events which occur upon H_2O/D_2O substitution:

(i) An osmotic-like stress effect, evidenced by the same time dependence of the electrical changes as in the D_2O-induced turgor change in *Nitella* (Brooks, 1937).

(ii) Appearance of a Ca^{2+}-dependent inward current component, i.e., opening of Ca^{2+} channels, is the first reaction to a sudden transmembrane H_2O/D_2O chemical potential gradient. This could point to an osmoregulatory role of Ca^{2+} channels which could be sensing the osmotic pressure difference. Such channels sensitive to osmotic and/or hydrostatic pressure difference have recently been discovered in plant cell membranes (Tester, 1990; Alexandre & Lassalles, 1991; Cosgrove & Hedrich, 1991).

(iii) D_2O causes an irreversible opening of the Ca^{2+} channel, probably by inducing the open channel conformation with deuterium(s) substituted for hydrogen(s) and stabilizing it by subsequently being buried inside the protein and unaccessible to the outer solution for reverse exchange. Thus, there is no inactivation of the first Ca^{2+} current component, even after returning the H_2O solution.

(iv) The inflow of Ca^{2+} ions through the open channel activates the Cl^- channel as indicated by the appearance of the second EA-sensitive current component. The delay in Cl^--current generation was close to the calculated time necessary for the buildup of the threshold

internal Ca^{2+} concentration. The indirect effect of D_2O on the Cl^- channel was shown by abolishing the Cl^- current in external D_2O with Ca^{2+}-free internal perfusion. However, D_2O abolished the Cl^- current inactivation.

It was shown that the above effects do not appear at high ionic strength. Screening of H-bond groups in the channel protein was suggested as a possible explanation, but further experimental evidence is needed.

The effect occurs only when D_2O is exchanged for H_2O externally. The mechanistic explanation for this asymmetric condition is not known.

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Appendix

After APW/25% ^{2}H-APW substitution in the outside solution, a Ca^{2+} current was generated. Concentration of Ca^{2+} ($[Ca^{2+}]$) in APW was 0.1 mM and the mean value of the Ca^{2+} flux was $J_{Ca} \approx 30$ μA/cm^2. The perfused solution contained approx. 1 mM EGTA with free $Ca^{2+} < 10^{-5}$ mM. When the perfusion was stopped, Ca^{2+} ions passed through the membrane to the inside solution. Due to the presence of EGTA, the initial concentration of internal free Ca^{2+} was insignificant. However, free Ca^{2+} would increase after the concentrations of total Ca^{2+} and EGTA become nearly equal ($EGTA^{2-} + Ca^{2+} \rightarrow EGTA - Ca$). According to Kataev et al. (1984) the Cl^- current appears already at free $[Ca^{2+}] \geqslant 10^{-3}$–$10^{-2}$ mM. Such a small value could be achieved when total $[Ca^{2+}]$ reaches the EGTA concentration. The time necessary for this process could be estimated by the following calculation.

The experimental cell could be approximated by a cylinder with radius $a = 4 \times 10^{-2}$cm. If this cylinder initially has a uniform concentration of free Ca^{2+}, if $C_o \approx 0$, and if there is a constant rate of diffusion of substance F_o per unit area of membrane surface, the equation for such a diffusion would be:

$$\frac{\partial C}{\partial t} = \frac{\partial}{r\partial r}\left(r\frac{\partial C}{\partial r}\right) \quad 0 \leqslant r \leqslant a \qquad 0 < t < \infty \tag{A1}$$

and the initial conditions are:

$$D\frac{\partial C}{\partial r} = F_o \qquad r = a \qquad 0 < t < \infty$$

$$C(r,o) = 0 \qquad 0 \leqslant r \leqslant a.$$

the solution of this equation (Crank, 1956) is:

$$C = \frac{F_o a}{D}\left\{\frac{2Dt}{a^2} + \frac{r^2}{2a^2} - \frac{1}{4} - 2\sum_{n=1}^{\infty}\frac{J_o\left(\mu_n \frac{r}{a}\right)}{\mu_n^2 J_o(\mu_n)} \cdot \exp\left(-\frac{D t \mu_n^2}{a^2}\right)\right\} \tag{A2}$$

where μ_n denotes the positive roots of $J_1(\mu) = 0$, and J_o and J_1 are the Bessel functions of the first kind for the zero and first order, respectively. For the above experimental case:

$$F_o = \frac{I}{zeN_A} \approx 2 \times 10^{-10}\frac{\text{mol}}{\text{sec cm}^2},$$

since: $I = 30\ \mu\text{A/cm}^2$, $z = 2$, $e = 1.6 \times 10^{-19}$C, N_A (Avogadro's number) $= 6 \cdot 10^{23}\ \text{mol}^{-1}$.

If $a = 4 \cdot 10^{-2}$ cm; $C = 1$ mM, and $D = 1.3 \cdot 10^{-5}\ \text{cm}^2/\text{sec}$, it can be easily calculated that

$$\frac{DC}{F_o a} = 1.6$$

If we take into account only a thin layer of approx. 10^{-3} cm near the membrane surface, then $(r)/(a) = 0.98$ and $J_o(\mu_n) \approx J_o((r)/(a)\ \mu_n)$. According to Crank (1956), for the above values of $(DC)/(F_o a)$ and $(r)/(a)$, it follows that $(Dt)/(a^2) > 0.3$, and thus, the sum $\Sigma_{n=1}^{\infty}(1)/(\mu_n^2)\ \exp\ (-(Dt\mu_n^2)/(a^2)$ should be negligibly small and the diffusion Eq. (A2) should reduce to:

$$C \approx \frac{F_o a}{D}\left(\frac{2Dt}{a^2} + 0.2\right) \tag{A3}$$

Solving this equation for t with the values of parameters from the above experimental case gives $t \approx 100$ sec. This time interval is in good agreement with the experimentally obtained value for the delay of the Cl^- current (Fig. 3).