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Discontinuities in the Temperature Function of Transmembrane Water Transport in *Chara*: Relation to Ion Transport

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Abstract. The NMR (nuclear magnetic resonance) method of Conlon and Outhred (1972) was used to measure diffusional water permeability of the nodal cells of the green alga *Chara gymnophylla.* Two local minima at 15 and 30°C of diffusional water permeability (P_d) were observed delimiting a region of low activation energy (E_a around 20 kJ/mol) indicative of an optimal temperature region for membrane transport processes. Above and below this region water transport was of a different type with high E_a (about 70 kJ/mol). The triphasic temperature dependence of the water transport suggested a channel-mediated transport at 15–30°C and lipid matrix-mediated transport beyond this region. The K^+ channel inhibitor, tetraethylammonium as well as the Cl^- channel inhibitor, ethacrynic acid, diminished P_d in the intermediate temperature region by 54 and 40%, respectively. The sulfhydryl agent p-(chloromercuri-benzensulfonate) the water transport inhibitor in erythrocytes also known to affect K^+ transport in *Chara,* only increased P_d below 15°C. In high external potassium ('K-state') water transport minima were pronounced. The role of K^+ channels as sensors of the optimal temperature limits was further emphasized by showing a similar triphasic temperature dependence of the conductance of a single K^+ channel also known to cotransport water, which originated from cytoplasmic droplets (putatively tonoplast) of *C. gymnophylla.* The minimum of K^+ single channel conductance at around 15°C, unlike the one at 30°C, was sensitive to changes of growth temperature underlining membrane lipid involvement. The additional role of intracellular (membrane?) water in the generation of discontinuities in the above thermal functions was suggested by an Arrhenius plot of the cellular water relaxation rate which showed breaks at 13 and 29°C.

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Key words: Water transport — Temperature dependence — K^+ channels — NMR — Patch-clamp — *Chara*

Introduction

In previous investigations of the temperature dependence of water efflux and membrane resting potential in internodal cells of the freshwater alga *Chara gymnophylla,* a non-monotonic behavior was observed (Andjus, Srejić & Vučelić, 1987). This was manifested by two peaks (at 12 and 30°C) of H_2O-D_2O transmembrane exchange halftime corresponding to the two breaks (at 15 and 29°C) in the temperature function of membrane potential (Andjus et al., 1987). This pattern of temperature dependence of water exchange rate was dependent on external potassium. Thus, in accordance with the studies dealing with K^+/water coupled transport (Wayne & Tazawa, 1990; Homblé & Véry, 1992), K^+ channels were inferred as possible mediators of water transport. The role of specific water channels or aquaporins (Maurel et al., 1993), however, could not be excluded.

The present study intends to avoid the controversy of previous reports concerning the interpretation of water efflux measurements by an NMR method employing a high concentration (95–99%) of D_2O in extracellular medium (Andjus et al., 1987, 1990). To confirm the existence of discontinuities in the temperature function, the water diffusional exchange rate ($1/\tau$) through the *Chara* cell membrane was measured by employing the NMR method of Conlon and Outhred (1972) previously designed for water transport studies on erythrocytes and first applied on a plant system by Stout, Cotts and Steponkus (1977). The tentative role of ion channels in the temperature dependence of water transport (Andjus et al., 1987) was tested by the application of ion transport blockers and by comparison to patch-clamp data on the temperature dependence of the conductivity of a K^+

channel also known to cotransport water efficiently (Homblé & Véry, 1992), which was found in Characean cytoplasmic droplets putatively of tonoplast origin. A preliminary part of this study regarding NMR control measurements was previously published in Beljanski et al. (1997).

Materials and Methods

NMR measurements were done on the isolated nodes containing many nodal cells of the freshwater alga *Chara gymnophylla*. The average radius and length of a cell was 35 and 200 μm, respectively. Prior to an experiment, isolated nodes were kept in standard APW ('artificial pond water') solution (in mM: 1 NaCl, 0.1 KCl, 0.1 $CaCl_2$) overnight at 25°C. Chemical treatments of cells were done at room temperature (20–25°C). Concentrations of ion and water transport inhibitors and duration of treatment are given in the Results.

Measurements were carried out with a Bruker MSL-400 Spectrometer operating at 9.4T (400 MHz for proton). The NMR technique was employed for measuring the kinetics of water transport across the cell membrane. The procedure based on a standard NMR relaxation technique was first described by Conlon & Outhred (1972). The time of water relaxation (T_1 or T_2) inside the cells is longer than the water exchange time (τ) between the cell and the surrounding solution. If by means of relaxation agents the relaxation outside is greatly shortened ($\ll$10 msec), the relaxation of the intracellular water will be dominated by the exchange process. Thus, water molecules that are leaving the cell will lose their magnetization in contact with the external medium, and molecules that are entering the cell will mainly be without the magnetization. For ^1H T_1 relaxation measurements a standard inversion recovery pulse sequence was applied to the aqueous suspension containing 10–15% volume fraction of nodes/nodal cells (about 50 nodes in 20 μl). Two kinds of cell suspensions were prepared: (i) cells in pure APW, and (ii) cells in the relaxation agent, gadolinium diethylentriamine pentaacetic acid (GdDTPA) 100 mM (Aldrich) in APW. In the latter case, two-component relaxation was obtained: the first, and very fast (<6 msec), component originated from the magnetically labeled outer water molecules, and the second was the intracellular water signal, decaying more slowly, mainly dominated by water exchange across the membrane.

Although some features of Characean cells and erythrocytes (the latter were used as the experimental model in Conlon & Outhred, 1972) have significant differences (e.g., presence of cell wall in the former, hence different unstirred layer effects), care has been taken to keep the main approximations of the original method based on low packed cell volume and high paramagnetic additive concentration (Conlon & Outhred, 1972). Thus, the concentration of the relaxation agent (GdDTPA) outside the cells was sufficiently high to eliminate the ''back-flux'' while the spontaneous loss of magnetization in the cell was corrected by calculating the exchange rate ($1/\tau$) of water molecules through the membrane as (Conlon & Outhred, 1972):

$$1/\tau = 1/T'_1 - 1/T_1 \quad (1)$$

where $1/T'_1$ and $1/T_1$ are the relaxation rates of intracellular water in the presence and absence of the external relaxation agent, respectively.

Upon approximating the form of a nodal cell to a cylinder (the standard shape of these cells could be characterized as a round-topped cone) the apparent diffusional membrane permeability to water (P_d) could be given by:

$$P_d = V/A \cdot 1/\tau = r/2\tau \quad (2)$$

where V, A and r are the average volume, surface area and radius. This P_d value should mainly describe the permeability of the plasma membrane which is the primary barrier to water movement in *Chara* (Kyosawa & Tazawa, 1977).

The original method of Conlon & Outhred (1972) was based on T_2 measurements which can give exchange rates lower than T_1 measurements while it is also advantageous to use T_1 measurements since much lower concentrations of the external relaxation agent are needed. To obtain meaningful $1/\tau$ values particularly in plant cells it was suggested to apply NMR T_1 measurements (Zhang & Jones, 1996). In fact, the obtained $1/\tau$ and/or P_d values at corresponding temperatures were congruent with the values obtained in other studies when the same NMR technique was applied on plant cells (Ratković & Bačić, 1980; Degani & Avron, 1982; Bačić & Ratković, 1984).

Unlike Mn^{2+} and its complexes, relaxation agents often used in studies on plants (Stout, Cotts & Steponkus, 1977; Ratković & Bačić, 1980; Bačić & Ratković, 1984), Gd-DTPA the relaxation agent frequently used in medicine, is biochemically inert and does not penetrate cell membranes, and was thus chosen as a convenient relaxation agent for a plant cell system in this study.

Although it is known that the logarithm of the dissociation constant for the relaxation agent Gd-DTPA is as low as 10^{-23} (*see* Merck Index 11,4236) toxic traces of free gadolinium were checked by means of the patch-clamp measurements (*see below*) of an easily assessable, characteristic Gd-sensitive Characean channel, the slow delayed rectifier K^+ channel (Pottosin & Andjus, 1994). No changes were observed in Gd-DTPA, 100 mM, either in the voltage dependence of macroscopic current amplitudes and deactivation kinetics (*not shown*), or in single channel conductances (78 ± 6 in control *vs.* 73 ± 4 pS in Gd-DTPA).

For each freshly prepared cell suspension T_1 and T'_1 were measured in closely spaced temperature intervals (1–2°C) from 5 to 40°C. The temperature of the samples were controlled by Bruker B-VT 1000 temperature unit within ±0.5°C.

There was no aeration of the cell sample. However, measurements of the rate of protoplasmic streaming as a viability index, revealed no significant change at the end of a control experiment.

Patch-clamp experiments were performed on inside-out membrane patches detached from cytoplasmic droplets (obtained by cell perfusion similar to the method of Bertl, 1989) from *C. gymnophylla*. The experimental bath solution was (in mM): 150 KCl, 10 HEPES-KOH, 0.5 EDTA, 2 $CaCl_2$ (0.5 mM free Ca^{2+}), pH 7.2 and the pipette solution contained (in mM): 150 KCl, 10 HEPES-KOH, 1 EGTA, 5 citric acid, 5.5 $CaCl_2$ (1.5 mM free Ca^{2+}), pH 7.2. The microelectrodes were made from borosillicate thick-wall glass capillaries (Clark Electromedical Instruments, UK) pulled on a vertical puller (Stoelting, IL). The resistance of the patch-pipettes was 10–15 MΩ. Recordings were made through an EPC9 patch-clamp amplifier (HEKA Elektronik, GmbH, Germany) connected to the Atari Mega ST-4 computer, with installed EPC9 SCREEN (HEKA) acquisition program. Data were acquired at 2 kHz, and were not additionally filtered. Single-channel recordings were analyzed by the half-amplitude threshold procedure (Colquhoun & Sigworth, 1983) implemented in the TAC software (Instrutech, NY).

The temperature in the experimental chamber was controlled by temperature sensors placed inside the bath and in the wall of the chamber holder, and regulated by a Peltier element. A temperature control unit (Luigs & Neumann, Germany) was used for measuring and changing the temperature.

The temperature-dependence data (for both NMR and patch-clamp experiments) were obtained after 15 min of equilibration at each temperature and were represented in the form of Arrhenius plots. The activation energy (E_a) was calculated from the slope of the linear portions of the plot, by fitting data to the equation

$$ln\, k = ln\, B - \frac{E_a}{RT}, \quad (3)$$

where k represents the rate constant or any other parameter that changes with temperature, B is the Arrhenius constant and R and T have their usual meanings. The activation energy estimates are presented with errors of fit. All other data are presented as mean ± SEM.

Local minima in our temperature-dependence data were often observed. The significance ($P < 0.05$) of these discontinuities was assessed by an ANOVA test which compared the values of the data points at the minimum with the values of data points limiting the region of the minimum.

Results

Water Transport Measurements

It can be seen from Fig. 1*A* that the diffusional water permeability, P_d had two distinct local minima at about 15 and 30°C. While the local minimum at 15°C was statistically significant ($P < 0.05$ Anova) the latter minimum at 30°C although apparent, was not statistically significant ($P > 0.05$; *also see* Discussion). The Arrhenius plots of P_d are clearly triphasic (Fig. 1*B*). Between 15 and 30°C the activation energy is relatively low (E_a = 27 and 16 kJ/mol, Fig. 1*B* and as calculated from the data of Fig. 2, respectively) and in the range (although at its upper limit) of values for a diffusional mechanism through membrane channel pores (Finkelstein, 1987). Below and above that temperature region, E_a was 67 kJ/mol and 75 kJ/mol, respectively (Fig. 1*B*). The temperature dependence of the longitudinal relaxation rate of the cellular water in the absence of the relaxation agent ($1/T_1$; Eq. 1), which is influenced by the organization of intracellular and membrane water, showed breaks at about 12 and 30°C (Fig. 1*B*, inset), resembling temperatures of the discontinuities in water transport.

Membrane Transport Inhibitors

To test for the connection of membrane ion transport and water fluxes we treated nodal cells of *Chara* with various ion transport inhibitors. It is shown in Fig. 1 that the K^+ transport inhibitor tetraethylammonium, TEA, 3 mM (allowing 30 min pretreatment to obtain a steady maximal effect; Keifer & Lucas, 1982; Sokolik & Yurin, 1986) lowered the diffusional water permeability about two times in the region 15–30°C. Below 15 and above 30°C TEA had no effect, suggesting that the K^+ channels significantly contribute to water transport only in the optimal temperature region (15–30°C). To activate completely the K^+ transmembrane pathway the cells were brought into the ''K-state'' (Beilby, 1986) by adding 10 mM KCl to the external solution. Along with a general decrease of diffusional water permeability at all experimental temperatures up to 30°C, in 10 mM KCl both P_d minima became more pronounced ($P < 0.05$ for low temperature minimum; tested by Anova; *see* Materials and Methods) and broader (Fig. 2*A*, inset). Also, low temperature minimum showed certain shift (by 1–2°C) toward lower temperatures (Fig. 2*A*).

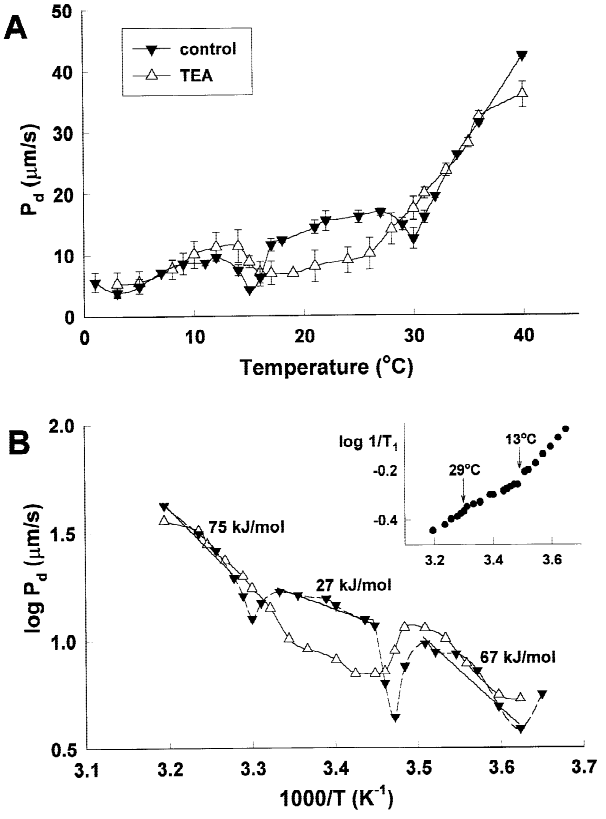

Fig. 1. Temperature dependence of diffusional water permeability (P_d) in *C. gymnophylla* nodal cells; control (closed symbols) *vs.* TEA, 3 mM, treatment (open symbols). (*A*) normal plot with standard deviations (vertical bars). Data are mean of measurements on 4 separate samples. (*B*) Arrhenius plot with activation energies indicated for the control; inset: ^1H longitudinal relaxation rate ($1/T_1$) of intracellular water in a suspension of 50 nodes.

The classical water transport inhibitor in erythrocytes and the K^+ transport modulator in *Chara*, membrane impermeable sulfhydryl agent p-(chloromercuribenzensulfonate), pCMBS, 1 mM (Lichtner, Lucas & Spanswick, 1981; Thiel, 1991) after a 60-min treatment, except for some rise of P_d below 15°C did not have an apparent effect in the whole temperature range studied (Fig. 2*B*). One trial experiment was also performed with the membrane permeable sulfhydryl agent n-ethyl maleimide (NEM), 0.1 mM which induced (after 10 min treatment) a decrease of P_d at experimental temperatures between 10 and 30°C but the local minima were not affected (*not shown*).

The 60 min pretreatment with the Characean Cl^- current inhibitor ethacrynic acid (EA), 1 mM (Lunevsky

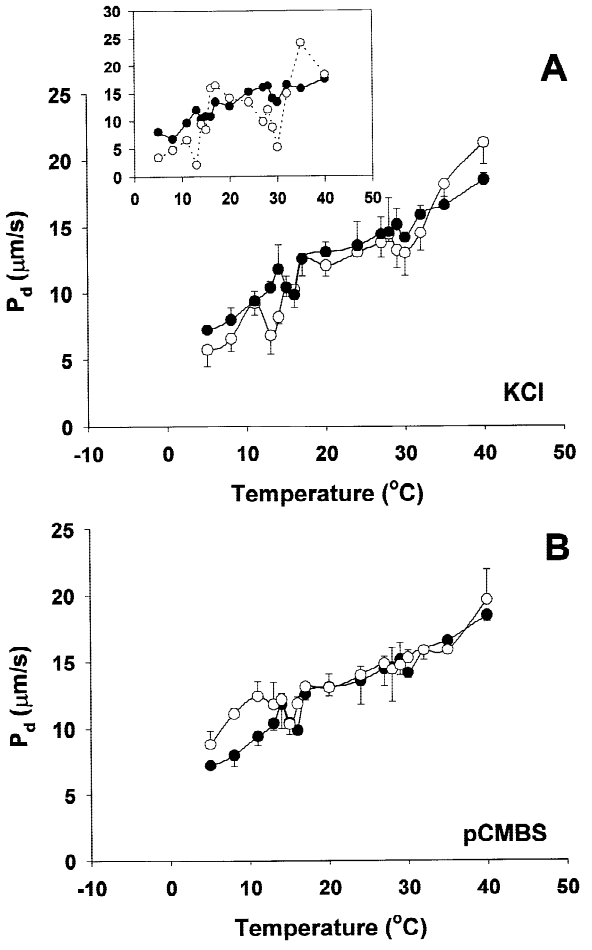

Fig. 2. Temperature dependence of diffusional water permeability (P_d) in *C. gymnophylla* nodal cells in (*A*) high external potassium (10 mM KCl) and (*B*) in PCMBS 1 mM. Closed symbols present mean data ($n = 5$) with standard error bars for the same control curve in *A* and *B*. Open symbols are mean data (with standard error bars) for $n = 5$ or $n = 3$ for experiments in high potassium (*A*) or PCMBS (*B*), respectively. The inset in *A* presents an example of a single control experiment (closed symbols) *vs.* a case of an experiment in 10 mM KCl (open symbols) with pronounced P_d minima at 15 and 30°C.

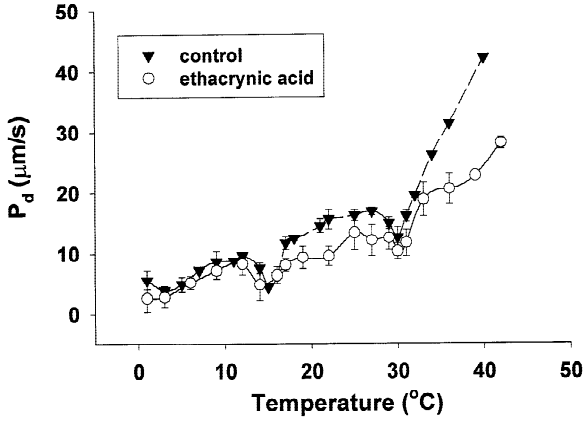

Fig. 3. Effect of ethacrynic acid (1 mM) on the temperature dependence of diffusional water permeability (P_d). The control curve (closed symbols) is the same as in Fig. 1A.

et al., 1983), lowered P_d at 15–40°C (Fig. 3) but had no apparent effect on local water transport minima.

The alleged water channel inhibitor, $HgCl_2$ 50 μM, applied for 15 min, with a subsequent wash of non-bound agent, induced a pronounced diminishing of the water transport signal, thus disabling exact evaluation of T_1' relaxation time. Values of T_1' obtained after the $HgCl_2$ pretreatment in all cases ($n = 6$) were longer than both the control T_1' value (1.20 ± 0.49 sec, $n = 11$) and T_1 correction relaxation time (2.23 ± 0.20, $n = 3$), which indicated either a block of the water transport or permeation of the relaxation agent (resulting in the observed artifactual negative rate of water transport, $1/\tau$). Even at 20°C, at the beginning of the experiment, a severe diminishing of the water signal occurred, resembling a complete block of transmembrane water efflux. If such cells were kept in the NMR tube for more than 45 min a significant shortening of the intracellular water relaxation time T'_1, was observed, indicating a loss of membrane semipermeability and the penetration of the relaxation agent, Gd-DTPA into the cells.

Temperature Dependence of K^+ Channel Conductance

The K^+ channel from cytoplasmic droplets was chosen for patch-clamp exploration since patching the plasma membrane of *C. gymnophylla* poses serious technical problems (due to the presence of the cell wall and cortical cells). The choice of this K^+ channel was further justified by its ability to efficiently cotransport water (Homblé & Véry, 1992). This was the most prominent channel observed in the excised inside-out membrane patches of cytoplasmic droplets, each patch containing 3–5 K^+ selective channels or approx. 1 channel per 1 μm^2. The selectivity of channels for K^+ over different cations was studied in bi-ionic systems composed of 100 mM KCl at one side and 100 mM *X*Cl (where $X = Na^+$, K^+, Rb^+, or Cs^+) at the other side of the patch. These conditions were used to obtain the selectivity series: $K^+ > Rb^+ \gg Na^+, Cs^+$ and the relative permeabilities (P_X/P_K): $P_{Rb} = 0.40$, $P_{Na} = 0.01$, $P_{Cs} \approx 0$. In the symmetrical 100 mM KCl solution at room temperature (approximately 20°C), the *I/V* dependence for K^+ channel open state is S-shaped with a linear portion having a slope of about 130 pS (*not shown*).

The effect of temperature on the single K^+-channel conductance (G_K) is shown in Fig. 4. A triphasic behavior with significant local minima at 14 and 28°C ($P < 0.05$, Anova) resembling water transport temperature dependence is clearly visible (Fig. 4*B*). As with water transport, the region between the minima was optimal for

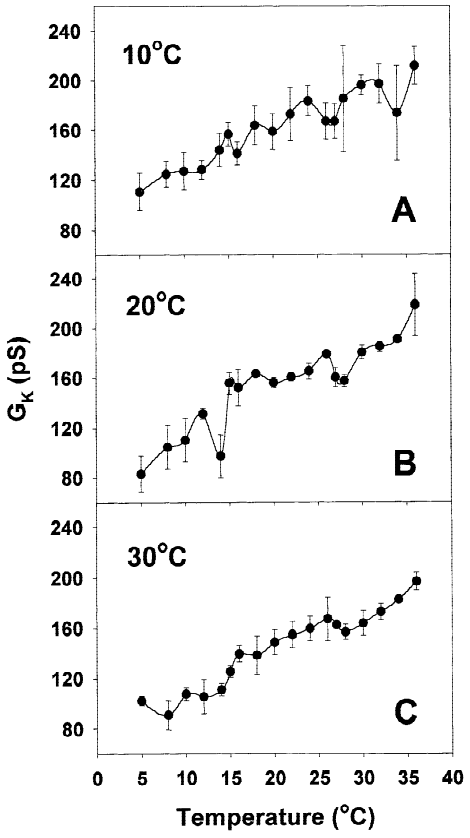

Fig. 4. Temperature dependence of the conductance (G_K) of the single K^+ channel obtained from *C. gymnophylla* algae grown at three different temperatures: (*A*) 10°C, (*B*) 20°C and (*C*) 30°C. Inside-out membrane patches were detached from cytoplasmic droplets. Holding potential was 50 mV. Data present mean values with standard error bars (n = 3–4).

transport of potassium since the activation energy was the lowest in this region (5.3 ± 3.1 kJ/mol).

When algae were grown for three weeks in aquaria at two extreme temperatures 10 or 30°C the thermal behavior of G_K changed. The growth at 10°C abolished both temperature minima (Fig. 4*A*). In addition, the E_a in the 15–30°C region was 20.5 ± 4.7 kJ/mol, about four times larger as compared to plants grown at 20°C (5.3 ± 3.1 kJ/mol). At the growth temperature of 30°C both minima were broadened (Fig. 4*C*) and E_a (for 15–30°C) increased to 13.5 ± 2.3 kJ/mol as compared to plants adapted at 20°C. In general, both cases of temperature adaptation caused a smaller difference in activation energies between the three characteristic temperature regions. This resulted in closely linear and comparable temperature dependence curves lacking the statistically significant local minima observed for plants grown at 20°C.

Discussion

Measurements of water diffusional permeation showed that local minima in P_d at 15 and 30°C delimited a temperature region with low activation energy indicating a transport mechanism close to water self-diffusion. Thus, at these optimal temperatures a significant portion of transmembrane water flux was probably channel mediated. Moreover, the calculated apparent P_d value of $1.5 \cdot 10^{-3}$ cm/sec at 22°C is an order of magnitude lower than the osmotic water permeability ($P_f \approx 1 \cdot 10^{-2}$ cm/sec) obtained by the method of transcellular osmosis on Characean internodal cells (Wayne & Tazawa, 1990). The inequality $P_f/P_d > 1$ points to the presence of ''water pores'' (Finkelstein, 1987). This relation, however, can be taken only as an indication of the presence of transmembrane water pathways since the value of P_d from nodal cells was compared to the P_f value from internodal cells of another Characean species.

At this point it must be mentioned that the low temperature minima as opposed to higher temperature minima (Figs. 1*A* and 2*A*), were statistically significant (Anova test). Though apparent, higher temperature minima were not statistically justified, probably due to the uncontrolled sporadic shifts in the real temperature of the NMR sample which caused overlapping of minima obtained in separate experiments, and thus added to the error of P_d estimates in the rather narrow minimum region.

According to Figs. 1 and 3 it is evident that the discontinuities at 13–16°C and 28–31°C separate three distinct water transport processes with different temperature dependence: (i) low temperature region with high E_a, insensitive to ion transport inhibitors; (ii) intermediate temperature region (corresponding to the optimal range of habitat temperatures) with low E_a, sensitive to TEA and EA and hence ion transport-dependent; and (iii) high temperature region with the same high E_a as in (i), but sensitive to EA. In a recent study of water and solute membrane transport in *Chara corallina* (Hertel & Steudle, 1997) an E_a of 17 kJ/mol was obtained for P_d between 10 and 35°C which is almost identical to 16 kJ/mol calculated from the data of Fig. 2 for the intermediate temperature range. According to its high E_a and insensitivity to TEA and EA (Figs. 1 and 3), the lower-temperature transport process was probably only mediated by membrane lipid pathways. The transport process at highest temperatures, although also with a high E_a, may have still been influenced by ion transport pathways since it was inhibited by the Cl^- channel blocker EA. It is worth noting that the curvatures (especially at extreme temperature regions) as well as the minima in the Arrhenius plot presented in Fig. 1*B* indicate that the calculated E_a values can only be regarded as approximations obtained by an empirical method (Iwaya-Inoue, Sakaguchi & Kaku, 1989) in order to compare these data with those from previous studies. The experiments with adaptation of algae to extreme temperatures demonstrated an apparent change in the shape of the tempera-

ture dependence of G_K. This behavior is typical for a thermal adaptation in membrane lipid synthesis (Nishida & Murata, 1996) and points to the role of membrane lipids in ion and water transport.

The triphasic Arrhenius plot of water membrane transport (Fig. 1*B*) is reminiscent of the phase separation model for the influence of lipid phase transitions on transport functions introduced by Thilo, Trauble & Overath (1977). According to this model the transport proteins behave like membrane-embedded probe molecules which sense both the beginning and the end of the fluid-ordered lipid transition. The observed minima in the temperature dependence of P_d may be a manifestation of a more complex phase separation of membrane lipids and of subsequent partitioning of transport proteins (water and ion channels). Moreover, in a previous study employing differential scanning calorimetry two thermal transitions have been revealed in the membrane lipid fraction from *C. gymnophylla* (Beljanski et al., 1997). The characteristic temperatures (15–20°C, and 30–35°C) of these transitions were close to the ones for the discontinuities in membrane transport. The fact that TEA diminished water transport exactly between these characteristic temperatures which were also bordering the region of minimal E_a for G_K, indicates that the state of membrane lipids in this thermal region is optimal for the function of K^+ channels (Thilo et al., 1977). The state of membrane lipids above and below this thermal region should be more in favor of the transport through the lipid bilayer (as justified by a higher E_a). Thus, temperature could modulate the interplay between two parallel membrane transport arrays (proteinaceous channels *vs.* lipid arrays) in accordance with the composite model of the Characean plasma membrane which was proposed by Henzler & Steudle (1995).

Regarding the nature of ''water pores'' it was shown that the K^+ transport inhibitor TEA had a significant effect on diffusional water permeability, P_d, in the intermediate region (15–30°C) inhibiting 54% of the water transmembrane flux at 22°C. This could be taken as a quantitative estimate of K^+ channel contribution to water transport. In addition, Cl^- transport inhibitor EA and the sulfhydryl reagent NEM also lowered P_d in this region (about 40% inhibition in both cases), thus in the intermediate temperature region a large part (54 + 40%) of water transmembrane flux occurs through ion channels. It was apparent that the only agent that diminished water transport at optimal temperatures to the level of P_d minima was the selective K^+ channel blocker TEA. Unselective sulfhydryl agents, pCMBS and NEM are chemically diverse sulfhydryl agents that are also known to affect K^+ transport in *Chara* (Lichtner et al., 1981; Thiel, 1991) and both induced complementary effects on water transport in *C. gymnophylla.* Thus, pCMBS only affected membrane water transport at low temperatures (below 15°C) by increasing P_d, while the less polar NEM caused a significant decrease of diffusional water permeability at higher temperatures in a wide range (10–30°C). The effect of NEM resembled the action of TEA and EA and thus could have occurred through elimination of diverse water transporting ion channels at temperatures of their optimal intramembraneous organization. pCMBS, however, did not affect the same proteins (the same K^+ channels?) but could have acted upon structural membrane proteins affecting their interaction with membrane lipids. Such a modification of protein-lipid interaction could cause a rise of water permeability at low temperatures where transmembrane pathways should be mainly lipid mediated (*see above*). The K^+ channel modifiers, especially the selective blocker TEA, already had an effect on water transport at low external potassium (at standard 0.1 mM KCl in APW) when potassium channels should mostly be closed. This could mean that even a small population of opened K^+ channels contributes significantly to water transport. In fact, Homblé and Véry (1992) have demonstrated that 29 water molecules pass with one K^+ ion through the TEA-sensitive large-conductance K^+ channel in *Chara.* Such high water-ion transport coupling could explain the significant role of K^+ channels in water transport even at low external K^+ concentration. When the K^+ channels were activated by high external potassium (''K-state''), however, the P_d minima became more pronounced and a general decrease of P_d occurred. Assuming that K^+/H_2O cotransport occurs at least partly in single-file mode (Homblé & Véry, 1992) a rise in external K^+ concentration could cause a decrease of the driving force for the net K^+/H_2O outward coupling. Experiments in the K-state along with TEA data suggest that K^+ channels could cotransport water and thus be responsible for optimal water transport and the generation of water transport temperature minima. Since these minima are also present in K^+ channel conductance (Fig. 4*B*) and are delimiting the optimal temperature region, the K^+ channel may serve as the functional sensor of thermal limits of this region.

The discrepancy of the effect of pCMBS and TEA (which both should affect K^+ channels), however, offers an alternative membrane pathway: the aquaporins or water channels (Maurel et al., 1993). As inferred from our data these specific water transporting channels should not be affected by pCMBS and could mediate a coupled transport of water with K^+ ions. Water transport through these channels may also manifest minima with temperature and dependence on external K^+. This hypothesis, however, must assume that these water channels are indeed affected by TEA. In fact, aquaporins may form aggregates of four subunits each having a phenylalanine residue in the external E loop or the hemichannel (Agre et al., 1993; Höfte et al., 1992) and could thus form a binding site for TEA similar to the one described for the

Shaker K^+ channel (Heginbotham & MacKinnon, 1992). To reveal this interesting hypothesis and this yet unknown function of TEA, additional experiments would be needed. It should be mentioned that in a recent study on *Chara corallina* hydraulic conductivity (Schütz & Tyerman, 1997) mercurial-sensitive water channels were described that were not affected by pCMBS. Potassium channel blockers, however, also did not have an effect. This discrepancy with the present study may be due to different water transport phenomena (hydraulic conductivity *vs.* diffusional permeability) studied by diverse methods (pressure probe *vs.* NMR). In our experiments, $HgCl_2$ induced an apparent loss of semipermeability which may be due to a nonselective and invasive effect of mercurials, like provoking membrane depolarization, as observed by Schütz & Tyerman (1997).

It is worth noting that the patch clamp data on K^+ channels were obtained on cytoplasmic droplets which are presumably of tonoplast origin (Sakano & Tazawa, 1986) while the water transport data were mainly related to the plasma membrane (*see* Materials and Methods). Nevertheless, the occurrence of minima of G_K at the same characteristic temperatures for the plasmalemmal water transport (around 15 and 30°C) is striking and points to similar phase transitions and/or a general protein-lipid interaction in the two membranes. It was previously indicated that Characean droplets have similar electrophysiological properties to those of the plasmalemma (Inoue, Ishida & Kobatake, 1973; Homblé, Ferrier & Dainty, 1987). Thus, tonoplast K^+ channel counterparts in the plasma membrane (Tester, 1990) could have a significant role in shaping of water transport temperature dependence.

The breaks in the temperature dependence of cellular water T_1 relaxation (Fig. 1*B*, inset) at temperatures close to those for local minima of P_d and G_K, point to the role of membrane-associated water in these membrane transport functions and offers another general mechanism of interaction with channel proteins that could occur in plasmalemma as well as in tonoplast. Furthermore, the discontinuities in Arrhenius plots of I_K were shifted by 2–3°C to higher temperatures when H_2O was substituted by D_2O (*not shown*). This shift in transition temperatures for D_2O relative to H_2O was reported for water layers at interfaces (Timmermans & Bodson, 1937) and it was also observed for transmembrane water efflux in *Chara* internodal cells (Andjus et al., 1987; 1990).

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References

Agre, R.P., Preston, G.M., Smith, B.L., Jung, J.S., Raina, S., Moon, C., Guggino, W.B., Nielsen, S. 1993. Aquaporin CHIP: the archetypal molecular water channel. *Am. J. Physiol.* **265:**F463–F476

Andjus, P.R., Srejić, R., Vučelić, D. 1987. The temperature peaks of cell membrane permeability. *In:* Structure, Dynamics and Function of Biomolecules. A. Ehrenberg, R. Rigler, A. Graslund, L. Nilsson, editors. pp. 299–302. Springer-Verlag, Berlin

Andjus, P.R., Vučelić, D., Kataev, A.A., Pottosin, I.I., Alexandrov, A.A., Berestovsky, G.N. 1990. D_2O/H_2O exchange at the Characean cell membrane: transport mechanisms and the solvent isotope effect. *Studia biophysica* **138:**105–114

Bačić, G., Ratković, S. 1984. Water exchange in plant tissue studied by proton NMR in the presence of paramagnetic centers. *Biophys. J.* **45:**767–776

Beilby, M.J. 1986. Potassium channels and different states of *Chara* plasmalemma. *J. Membrane Biol.* **89:**241–249

Beljanski, M.V., Andjus, P.R., Hadži-Pavlović, A., Srejić, R., Vučelić, D. 1997. Differential scanning calorimetry of the plasma membrane-enriched fraction in *Chara. Plant Sci.* **125:**171–176

Bertl, A. 1989. Current-voltage relationships of a sodium-sensitive potassium current in the tonoplast of *Chara corallina. J. Membrane Biol.* **109:**9–19

Colquhoun, D., Sigworth, F.L. 1983. Fitting and statistical analysis of single channel records. *In:* Single Channel Recording. B. Sakmann, E. Neher, editors. pp. 191–263. Plenum Press, New York

Conlon, T., Outhred, R. 1972. Water diffusion permeability of erythrocytes using an NMR technique. *Biochim. Biophys. Acta* **288:**354–361

Degani, H., Avron, M. 1982. The diffusional water permeability in the halotolerant alga *Dunaliella* as measured by nuclear magnetic resonance. *Biochim. Biophys. Acta* **690:**174–177

Finkelstein, A. 1987. Water Movement Through Lipid Bilayers, Pores, and Plasma Membranes. Theory and Reality. Wiley-Interscience Publication. 228 pp. New York

Heginbotham, L., MacKinnon, R. 1992. The aromatic binding site for tetraethylammonium ion on potassium channels. *Neuron* **8:**483–491

Henzler, T., Steudle, E. 1995. Reversible closing of water channels in *Chara* internodes provides evidence for a composite transport model of the plasma-membrane. *J. Exp. Bot.* **46:**199–209

Hertel, A., Steudle, E. 1997. The function of water channels in *Chara:* The temperature dependence of water and solute flows provides evidence for composite membrane transport and for slippage of small organic solutes across water channels. *Planta* **202:**324–335

Höfte, H., Hubbard, L., Reizer, J., Ludevid, D., Herman, E.M., Chrispeels, M.J. 1992. Vegetative and seed-specific forms of tonoplast intrinsic protein in the vacuolar membrane of *Arabidopsis thaliana. Plant Physiol.* **99:**561–570

Homblé, F., Ferrier, J.M., Dainty, J. 1987. Voltage-dependent K^+ channel in protoplasmic droplets of *Chara corallina.* A single channel patch clamp study. *Plant Physiol.*. **83:**53–57

Homblé, F., Véry, A.A. 1992. Coupling of water and potassium ions in K^+ channels of the tonoplast of *Chara. Biophys. J.* **63:**996–999

Inoue, I., Ishida, N., Kobatake, Y. 1973. Studies of the excitable membrane formed on the surface of the protoplasmic drops isolated from *Nitella.* IV. Excitability of the drop membrane in various compositions of the external salt solution. *Biochim. Biophys. Acta* **330:**27–38

Iwaya-Inoue, M., Sakaguchi, K., Kaku, S. 1989. Statistical studies using AIC method to decide the question of ''break'' or ''straight'' in Arrhenius plots of water proton NMR relaxation times in chill-

ing-sensitive *Vigna* and insensitive *Pisum* seedlings. *Plant Cell Physiol.* **30:**309–316

Keifer, D.W., Lucas, W.J. 1982. Potassium channels in *Chara corallina*. Control and interaction with the electrogenic H^+ pump. *Plant Physiol.* **69:**781–788

Kyosawa, K., Tazawa, M. 1977. Hydraulic conductivity of tonoplast free *Chara* cells. *J. Membrane Biol.* **37:**157–166

Lichtner, F.T., Lucas, W.J., Spanswick, R.M. 1981. Effects of sulfhydryl reagents on the biophysical properties of the plasmalemma of *Chara corallina*. *Plant Physiol.* **68:**899–904

Lunevsky, V.Z., Zherelova, O.M., Vostrikov, I.Y., Berstovsky, G.N. 1983. Excitation of Characeae cell membranes as a result of activation of calcium and chloride channels. *J. Membrane Biol.* **72:**43–58

Maurel, C., Reizer, J., Schroeder, J.I., Chrispeels, M.J. 1993. The vacuolar membrane protein γ-TIP creates water specific channels in *Xenopus* oocytes. *EMBO J.* **12:**2241–2247

Nishida, I., Murata, N. 1996. Chilling sensitivity in plants and Cyanobacteria: the crucial contribution of membrane lipids. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **47:**541–568

Pottosin, I.I., Andjus, P.R. 1994. Depolarization-activated K^+ channel in *Chara* droplets. *Plant Physiol.* **106:**313–319

Ratković, S., Bačić, G. 1980. Water exchange in Nitella cells: A PMR study in the presence of paramagnetic Mn^{2+} ions. *Bioelectrochem. Bioenerg.* **7:**405–412

Sakano, K., Tazawa, M. 1986. Tonoplast origin of the envelope membrane of cytoplasmic droplets prepared from *Chara* internodal cells. Protoplasma **131:**247–249

Schütz, K., Tyerman, S.D. 1997. Water channels in *Chara corallina*. *J. Exp. Bot.* **48:**1511–1518

Sokolik, A.I., Yurin, V.M. 1986. Potassium channels in plasmalemma of *Nitella* cells at rest. *J. Membrane Biol.* **89:**9–22

Stout, D.G., Cotts, R.M., Steponkus, P.L. 1977. The diffusional water permeability of *Elodea* leaf cells as measured by nuclear magnetic resonance. *Can. J. Bot.* **55:**1623–1631

Tester, M. 1990. Plant ion channels: whole-cell and single-channel studies. *New Phytol.* **114:**305–340

Thiel, G. 1991. p-CMBS modifies extrafacial sulfhydryl groups at the *Chara* plasma membrane: activation of Ca^{2+} influx and inhibition of two different K^+ currents. *Bot. Acta* **104:**345–354

Thilo, L., Trauble, H., Overath, P. 1977. Mechanistic interpretation of the influence of lipid phase transitions on transport functions. *Biochemistry* **16:**1283–1290

Timmermans, J., Bodson, H. 1937. La tension superficielle de l'eau et celle de l'eau lourde. *Compt. Rend.* **204:**1804–1807

Wayne, R., Tazawa, M. 1990. Nature of the water channels in the internodal cells of *Nitellopsis*. *J. Membrane Biol.* **116:**31–39

Zhang, W.H., Jones, G.P. 1996. Water permeability in wheat root protoplasts determined from nuclear magnetic resonance relaxation times. *Plant Sci.* **118:**97–105