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The relationship between carbon and water transport in single cells of *Chara corallina*

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Dedicated to our teacher, Professor Masashi Tazawa, on the occasion of his 65th birthday

Summary. The hydraulic resistance of the plasma membrane was measured on single internodal cells of *Chara corallina* using the method of transcellular osmosis. The hydraulic resistance of the plasma membrane of high CO_2-grown cells was significantly higher than the hydraulic resistance of the plasma membrane in low CO_2-grown cells. Therefore we tested the possibility that the "bicarbonate transport system", postulated to be present in low CO_2-grown cells, serves as a water channel that lowers the hydraulic resistance of the plasma membrane. We were unable to find any correlation between agents that inhibited the "bicarbonate transport system" and agents that increased the hydraulic resistance of low CO_2-grown cells. We did, however, find a correlation between the permeability of the cell to water and CO_2. We propose that the reduced hydraulic resistance of the plasma membrane of the low CO_2-grown cells is a function of a change in either the structural properties of the lipid bilayer or the activity of a CO_2 transport protein so that under conditions of reduced inorganic carbon, the plasma membrane becomes more permeable to CO_2, and consequently to other small molecules, including H_2O, methanol and ethanol.

Keywords: Carbon transport; *Chara corallina*; CO_2 permeability; Hydraulic resistance; Plasma membrane; Water transport.

Introduction

In higher plants, the photosynthetic strategy adopted by a species usually depends on the availability of water (Boyer 1991, Nobel 1991). Consequently three main types of light-dependent carbon metabolism occur in plants: C 3, C 4, and crassulacean acid metabolism (CAM). These three classes occur in plants subjected to low, moderate, and high levels of water stress, respectively. The integration between carbon metabolism in mesophyll cells and the regulation of water uptake at the root hairs and water loss at the guard cells depends on the coordination between cells and organs at the organismal level of organization. In the work presented here, we investigated whether a relationship exists between carbon metabolism and water permeability at the single cell level using isolated internodal cells of *Chara corallina*.

Characean cells possess the C 3 photosynthetic pathway (Tolbert and Zill 1954) and are able to carry out photosynthesis when they are grown either under low CO_2 or high CO_2 conditions. CO_2 is the preferred substrate since the photosynthetic rate is more than two to three times higher at low pH (5.5), where CO_2 is the prevalent inorganic carbon species, than at high pH (8.0) where bicarbonate is the predominant form of inorganic carbon (Lucas 1975, Mimura et al. 1993, Price et al. 1985). It is thought that characean cells favor CO_2 when it is available, but utilize bicarbonate when it is not (Lucas 1975). Given the unfavorable electrochemical gradient, a HCO_3^--transport system has been assumed to exist on the plasma membrane in order to take up this anion (Lucas 1975).

We speculated that if there is a relationship between carbon uptake and water uptake necessary for growth, then perhaps the water permeability will be higher in high CO_2-grown cells than in low CO_2-grown cells. We

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find, however, that the cells grown under low CO_2 conditions have a lower hydraulic resistance than the cells grown under high CO_2 conditions. Therefore we tested the hypothesis that the lower hydraulic resistance in low CO_2-grown cells may be a consequence of the presence of a bicarbonate uptake system that acts as a water channel. The bicarbonate uptake system potentially includes a HCO_3^--H^+ cotransporter, a H^+-ATPase and/or carbonic anhydrase (Lucas 1975, Price et al. 1985, Walker 1985, Walker et al. 1980). Indeed the bicarbonate transport protein (Band 3) of the plasma membrane of red blood cells may function as a water channel (Finkelstein 1987). However, all attempts to modify the bicarbonate uptake system had no effect on the hydraulic resistance of the plasma membrane.

Therefore we considered the possibility that physiologically-meaningful modifications in the permeability of the membrane to CO_2, a low molecular weight non-electrolyte, may result in the serendipitous alteration in the hydraulic resistance of the plasma membrane in low CO_2-grown cells. Here we present results from our investigation into the relationship between carbon metabolism and water permeability at the single cell level using isolated internodal cells of *Chara corallina* and present a novel modification to the Ferrier (1980) and Walker et al. (1980) model for carbon uptake in low CO_2-grown cells.

Materials and methods

Plant materials

Male cultures of *Chara corallina* Klein ex Willd., em. R.D.W. (= *Chara australis* R. Brown) were grown in an air-conditioned room in a soil water mixture in large plastic buckets at 299 ± 1 K (26 ± 1 °C) with a 14h:10h L:D photoperiod. During the light period, the cells were irradiated with fluorescent light (FL20, National, Tokyo, Japan) that had a photon fluence rate of approximately 100×10^{-6} mol/m$^2 \cdot$s at the surface of the bucket. Some buckets contained stagnant soil water (low CO_2-grown cells) and other buckets were bubbled with 5% CO_2 at a rate of approximately 2.7×10^{-4} m^3/s (high CO_2-grown cells). The cultures were not closed systems but were open to the environment. CO_2 bubbling in an open system does not drastically change the pH of the medium. The pH of the CO_2-bubbled media is approximately 6.5 and the pH of the low CO_2 media is between 7.2–7.3. One stagnant culture contained 2 mol/m^3 $NaHCO_3$. The pH of the culture medium of this tank was pH 8. Some cells were isolated from plants grown in stagnant cultures at 288 K (15 °C). Unless otherwise specified, the internodal cells were isolated and used immediately, although the hydraulic resistance of each cell remained constant for at least 3 h (data not shown).

Measurement of transcellular osmosis

The hydraulic resistance was measured in a double chambered apparatus designed by Tazawa and Kamiya (1965, 1966) and illustrated in Wayne and Tazawa (1988). See Dainty (1963, 1964), Dainty and Ginzburg (1964a), Osterhaut (1949), Tazawa (1972), Tazawa and Shimmen (1987), and Tazawa et al. (1987) for reviews of transcellular osmosis and membrane processes in characean cells. The cells were symmetrically placed so that the length of the cell parts in the two chambers was equal. The two chambers were physically separated by a 5×10^{-3} m Plexiglas block and a silicone seal (HVG; Toray Silicone, Tokyo, Japan). The cell parts in both chambers were bathed in unbuffered artificial pond water (APW: 0.1 mol/m^3 KCl, 1 mol/m^3 NaCl, and 0.1 mol/m^3 $CaCl_2$). The pH of this solution was 5.6. Forward transcellular osmosis was initiated by replacing the APW in chamber B with APW plus 100 mol/m^3 sorbitol (0.245 MPa at 295 K) and the quantity of water movement was recorded every 5 s. In order to eliminate any interference from the small volume changes that occur during the onset of transcellular osmosis (0–5 s) or the build-up of unstirred layers (more than 40 s after the onset of transcellular osmosis), the rate of water movement between 5 and 35 s was used to calculate the hydraulic resistance. The rate of water movement was linear during this period (Wayne and Tazawa 1988). The average hydraulic resistance of the *Chara* plasma membrane (endoosmotic and exoosmotic side) is not constant, but has a slight dependence on the magnitude of the pressure gradient (Dainty and Ginzburg 1964b, Tazawa 1972, Wayne and Tazawa 1988). Unless stated otherwise, the solutions in chamber A as well as the sorbitol solutions contained the solvents and/or agents used to treat the cells. The magnitude of water movement was measured by following the rate of movement of a column of APW in a glass capillary with microscope (Model CH-2; Olympus Corp., Tokyo, Japan) equipped with a ×10 objective lens (N.A. = 0.25) and ×10 eyepieces. The APW in the capillary tube was continuous with the solution in chamber A. The movement of the column of APW in the capillary tube was observed with dim white light that passed through several heat-absorbing filters. The photon fluence rate of the observation beam was 3×10^{-6} mol/m$^2 \cdot$s. The cells were illuminated by daylight and room light during these experiments that had a combined photon fluence rate of 10–20 $\times 10^{-6}$ mol/m$^2 \cdot$s as measured with a home-made light meter equipped with a quantum sensor (Delta-T Devices, Tokyo, Japan).

Analysis of results

The hydraulic resistance of the plasma membrane (R_H, in m$^{-1} \cdot$s$\cdot$Pa) was calculated using the following equation based on irreversible thermodynamics and described by Kamiya and Tazawa (1956),

$$R_H = S\Delta\pi/(2J_v) \quad (1)$$

where S is the surface area (in m^2) of the cell part in either chamber A or B. $\Delta\pi$ is the osmotic pressure difference in the solutions in chamber A and B, which is equivalent to the osmotic pressure of the sorbitol solution (in Pa) added to chamber B. J_v is the initial volume flow of water (in m^3/s). The calculated hydraulic resistance is the average resistance to endoosmotic and exoosmotic water flow (Dainty and Hope 1959; Kamiya and Tazawa 1956; Steudle and Tyerman 1983; Tazawa and Kamiya 1965, 1966; Wayne and Tazawa 1988, 1990).

The hydraulic conductivity of the plasma membrane (L_p, in m/s$\cdot$Pa) is obtained by taking the reciprocal of the hydraulic resistance (R_H),

$$L_p = 1/R_H. \quad (2)$$

The osmotic permeability of the plasma membrane (P_{os}, in m/s) was calculated from the hydraulic conductivity (L_p) using the following formula,

$$P_{os} = L_p RT/\bar{V}_w \quad (3)$$

where R is the gas constant (8.31 J/mol·K), T is the absolute temperature in K (set to 295 K) and $\bar{V}_w$ is the partial molar volume of water (1.806×10^{-5} m^3/mol).

Effect of light on hydraulic resistance

The effect of light on the hydraulic resistance was determined by irradiating the cells with light from a 300 W slide projector (Auto Cabin II, Tokyo, Japan). The photon fluence rate was varied between 0 and 132×10^{-6} mol/m^2·s by varying the distance between the lamp and the cell. These photon fluence rates were used because they coincide with those associated with photosynthetic activity. The saturating photon fluence rate for photosynthesis in *Chara corallina* is 100×10^{-6} mol/m^2·s (Brechignac and Lucas 1987).

Effect of intracellular ATP on hydraulic resistance

In order to test the effect of ATP on the hydraulic resistance of the plasma membrane we perfused tonoplast-free cells with media containing either 1 mol/m^3 ATP or hexokinase and 2-deoxyglucose (Lucas and Shimmen 1981, Mimura et al. 1993). Tonoplast-free cells were prepared using the method described by Tazawa et al. (1976). Cells perfused with 1 mol/m^3 ATP have an ATP concentration that is similar to that of intact cells (Mimura et al. 1983). ATP was depleted by perfusion with the hexokinase medium and streaming stopped.

Effect of external pH or Ca^{2+} on hydraulic resistance

In order to test the effect of pH on the hydraulic resistance, we varied the pH of the solutions from 5.5 to 11 with Good's buffers. The pH was buffered at 5.5, 7.5, 9.3, and 11 with 2 mol/m^3 MES-NaOH, MOPS-NaOH, CHES-NaOH, and CAPS-NaOH, respectively.
The external Ca^{2+} was buffered with 1 mol/m^3 EGTA-TRIS (pH 7), 2 mol/m^3 MOPS-NaOH (pH 7.5) and varying amounts of $CaCl_2$. The whole solution was then titrated to pH 7.5 with 10^3 mol/m^3 NaOH. The concentrations of each agent required for a given Ca^{2+} concentration was calculated with the aid of a computer program (Wayne 1985).

Effect of CO_2 on hydraulic resistance

In order to test the effect of CO_2 on hydraulic resistance, we compared the ability of 100 mol/m^3 sorbitol to induce a volume flow of water when mixed with freshly-opened, commercially available CO_2-saturated water (approximately 45 mol/m^3 CO_2; Old Fashioned Polar Seltzer, Polar Corp., Worcester, MA, U.S.A.) or degassed seltzer water. The seltzer water contained 0.8 mol/m^3 Ca, 0.51 mol/m^3 S, 0.35 mol/m^3 Na, 0.03 mol/m^3 K, and 0.03 mol/m^3 Mg as measured with an inductively-coupled argon plasma emission spectrograph (Model ICAP 61; Thermo Jarrel Ash Corp., Franklin, MA, U.S.A.). The pH of the solution varied between 3.4 when it was freshly opened and 4.5 after it went flat. The solution in chamber A contained only degassed seltzer water.

Measurement of reflection coefficients

Using the technique of transcellular osmosis, the volume flow of water was measured from 10 to 20 s after the addition of 100 mol/m^3 sorbitol to chamber B. Following the measurement, APW was added to chamber B. After 10 min, the volume flow of water was induced by adding 100 mol/m^3 methanol to chamber B. This procedure was repeated with each solute so that the reflection coefficients of the plasma membrane to ethanol, n-butanol, acetone and glycerol could be determined. The reflection coefficient of solute i [σ_i] is operationally defined as the ratio of the volume flow of water induced by the solute in question ($J_{v(i)}$, in m^3/s) compared to the volume flow of water induced by sorbitol (J_v, in m^3/s).

$$\sigma_i = J_{v(i)}/J_v. \tag{4}$$

The reflection coefficient of sorbitol is operationally defined as 1. This is a reasonable assumption since the volume flow of water through the plasma membrane of characean cells is identical when the volume flow is induced osmotically with mannitol or by hydrostatic pressure (Tazawa and Kiyosawa 1970). The reflection coefficient is inversely related to the permeability coefficient. The permeability coefficient of the *Chara* plasma membrane to sugar alcohols like sorbitol is in the range of 10^{-11} to 10^{-10} m/s (Kiyosawa 1993).

Measurement of membrane potential and membrane resistance

One cell at a time was placed in a Plexiglas chamber that served as a space clamp. The chamber contained two wells. The chamber in which the electrodes were immersed was 6×10^{-3} m wide and it was electrically isolated from the other by a 10^{-2} m silicone grease seal. The cells were viewed on an inverted microscope (Model IMT-2, Olympus) equipped with a ×10 (N.A. = 0.3) objective lens and ×10 eyepieces. The cells were illuminated with white light from the condenser that had a photon fluence rate of 44×10^{-6} mol/m^2·s as measured with a homemade light meter.
Membrane potentials and membrane resistances were measured using conventional microcapillary electrodes. The microcapillary electrodes were positioned with a hydraulic micromanipulator (Narishige Sci., Tokyo, Japan). The glass capillaries containing filaments were made using a microcapillary maker (Model PY-6; Narishige). The measuring electrodes were pulled (twice) with a pipette puller (Model PA-81; Narishige) and filled with 3000 mol/m^3 KCl. The reference electrodes contained 100 mol/m^3 KCl in 2% agar. The microcapillary electrodes were inserted into sereological pipettes which contained Ag/AgCl elements which originally came from commercial pH electrodes. The membrane potentials were measured with a microelectrode amplifier (Model MEZ-3R; Nihon-Koden, Tokyo, Japan) and recorded with a dual pen recorder (Matsushita Communications Co. Ltd., Japan).
In order to measure membrane resistance, a hyperpolarizing current of 2.4×10^{-8} A was applied to the cell as a 0.5 Hz square wave through Ag/AgCl electrodes in each chamber. The square wave was generated by an electronic stimulator (Model MSE-3R; Nihon-Koden) and passed to the cell through a homemade voltage-to-current converter. The specific membrane resistance was calculated by multiplying the membrane resistance by the surface area of the cell part in the 6×10^{-3} m chamber. The series resistance was approximately 0.12 MΩ. Since the series resistance was less than 0.5% of the membrane resistance, it was ignored.

Measurement of inorganic carbon uptake

The flux of carbon across the plasma membrane was determined by measuring the amount of ^{14}C (specific activity = 20.3×10^9 Bq/mol) that was incorporated from the external medium into stable photosynthetic products. Following the methods of Mimura et al. (1993), each internodal cell was placed in a glass tube (i.d. = 3.5×10^{-3} m) filled with APW plus 1 mol/m^3 $NaHCO_3$ and either 2 mol/m^3 EPPS-TRIS (pH 8.5) or 2 mol/m^3 MES-TRIS (pH 5.5) and pre-illuminated with white light from a slide projector (Cabin; photon fluence rate = 200×10^{-6} mol/m^2·s) for 10 min. Then the external medium

was replaced with a medium containing $NaH^{14}CO_3$ and the cell was illuminated again for 10 min. Inorganic carbon incorporation was terminated by methanol extraction. Each cell was removed from its tube and immediately plunged into $10^{-6}\,m^3$ of methanol : water (4 : 1). After adding acetic acid, the extract was dried overnight. Scintillation fluid was added and the radioactivity was measured with a liquid scintillation counter. Experiments were done at pH 5.5, where the external concentration of CO_2 is approximately 1 mol/m^3 and pH 8.5 where the concentration of CO_2 is approximately 0.05 mol/m^3.

Total chlorophyll (chl a and chl b) was measured by homogenizing individual cells in $10^{-6}\,m^3$ of 80% ice cold acetone, centrifuging the homogenate at 12,500 rpm in a microcentrifuge (Model CT 15D; Hitachi, Tokyo, Japan) and then reading the absorbance at 645 nm and 663 nm with a spectrophotometer (Model UV-2200; Shimadzu Inc., Kyoto, Japan). Total chlorophyll per m^2 was calculated with the following formula (Arnon 1949),

$$chl_{tot} = [0.0202(A_{645}) + 0.00803(A_{663})]/(\pi dl) \quad (5)$$

where A_{645} and A_{663} are the absorbances at 645 and 663 nm, respectively. The diameter (in m) and length (in m) of each cell are represented by d and l, respectively.

Measurement of $^{36}Cl^-$ uptake

Cl^- influx across the plasma membrane was determined by measuring the uptake of $^{36}Cl^-$ (specific activity = 19.69×10^9 Bq/mol). Each internodal cell was placed in a glass tube (i.d. = 3.5×10^{-3} m) filled with APW and pre-illuminated with white light from a slide projector (Cabin; photon fluence rate = 200×10^{-6} mol/m$^2 \cdot$ s) for 10 min. Then the external medium was replaced with a medium containing $^{36}Cl^-$ and the cell was illuminated again for 20 min. The cells were washed, blotted and placed in vials to which scintillation fluid was added. The radioactivity was measured with a liquid scintillation counter.

Measurement of cellular osmotic pressure

The cytoplasm from several cells was squeezed out and pooled. Then the cellular osmotic pressure was measured with a vapor pressure osmometer (Model 5100CXR; Wescor Inc., Logan, UT, U.S.A.).

Statistics

All results were analysed by a one-way analysis of variance, t-tests or by regression analysis using Minitab (Minitab Inc., University Park, PA, U.S.A.).

Chemicals

KCl, NaCl, $CaCl_2$, n-butanol, acetone, methanol, ethanol, MES (2-(N-morpholino) ethanesulfonic acid), MOPS (3-(N-morpholino) propanesulphonic acid), CHES (2-(cyclohexylamino) ethanesulfonic acid), EPPS (N-2-hydroxyethylpiperazine-N′-3-propane-sulphonic acid), TRIS (2-amino-2-(hydroxymethyl)-1,3-propane-diol), EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) and $NaHCO_3$ were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) was purchased from Tokyo Kasei Co. (Tokyo, Japan). Acetazolamide, cytochalasin E, DCCD (N,N′-dicyclohexylcarbodiimide), 6-ethoxyzolamide, pCMPS (p-chloromercuriphenylsulfonic acid) and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). D-sorbitol and glycerol were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Thermolysin was obtained from the Protein Research Foundation (Osaka, Japan) and Proteinase K was obtained from E. Merck (Darmstadt, Federal Republic of Germany).

Results

Hydraulic resistance of cells growing under low CO_2

Large strides in the understanding of membrane transport have been made by assuming that each internodal cell of a given species is similar to every other one. However, it is well-known that variation in physiological properties exists between characean internodal cells isolated from cultures growing in the same laboratory. The hydraulic resistance of the plasma membrane of *Chara* cells isolated from "typical" air-adapted stagnant bucket cultures varies approximately three-fold

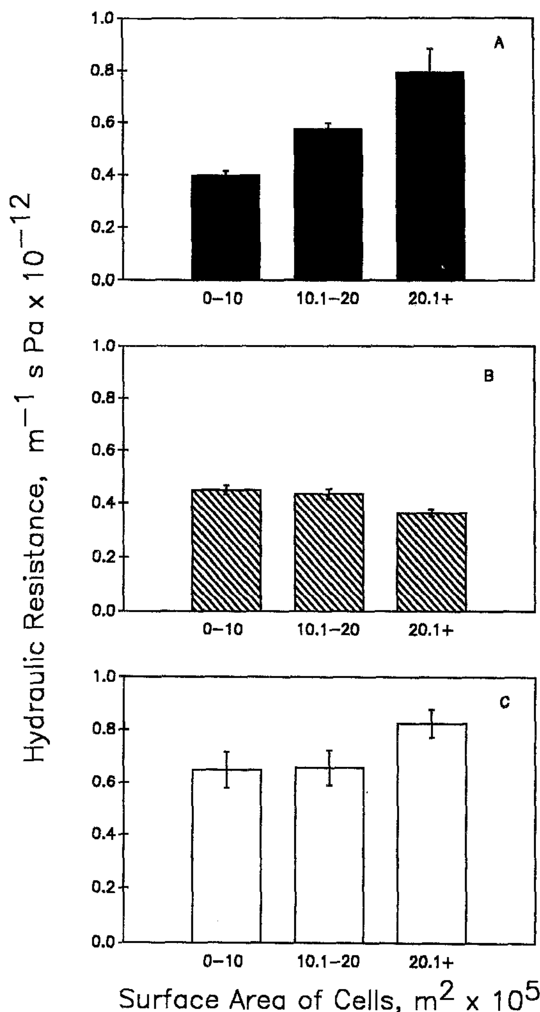

Fig. 1. The hydraulic resistance of the plasma membrane of internodal cells of *Chara*. **A** Cells growing in "typical" bucket culture under low CO_2 conditions (n = 12, 12, 9). **B** Cells growing in freshly started bucket under low CO_2 conditions (n = 7, 5, 5). **C** Cells growing in freshly started culture with CO_2 bubbling (n = 9, 5, 5)

from 0.3×10^{12} to $1 \times 10^{12} m^{-1} \cdot s \cdot Pa$. In a landmark study comparing internodal and branch cell pairs with or without an intact apex, Ding et al. (1991, 1992) have shown that the apex influences the transport properties of the cell below. In the present work we also attempted to unmask the developmental factors that contribute to the variability. When the cells are isolated from "typical" bucket cultures, the hydraulic resistance is correlated with the surface area of the cell. Cells with a small surface area have a low hydraulic resistance whereas cells with a large surface area have a high hydraulic resistance (Fig. 1 A). However, this correlation does not hold when we study cells isolated from newly started cultures where each plant has a single apical cell on the distal tip. In cells from these cultures, the hydraulic resistance is always low and independent of the surface area of the cell (Fig. 1 B).

After characterizing the variability, we became interested in determining the developmental factors that were correlated with the increased hydraulic resistance. We find that the developmental stage of the cell influences the hydraulic resistance. Small cells isolated from reproductive plants have a higher hydraulic resistance than small cells isolated from vegetative plants (Fig. 2 A). We also find that large cells have a low hydraulic resistance as long as all the apical cells are distal, however, large cells with proximal branches have a high hydraulic resistance (Fig. 2 B). The hydraulic resistance of the plasma membrane of an internodal cell is the same whether or not it is tested with or without an intact apex (Fig. 2 C).

Variability in the hydraulic resistance is not unique to typical stagnant cultures, but also occurs in cells isolated from CO_2-bubbled cultures (Fig. 1 C). In this case the hydraulic resistance varies approximately two-fold from 0.5×10^{12} to $1 \times 10^{12} m^{-1} \cdot s \cdot Pa$. Therefore, in order to limit the variation and maximize the reproducibility of the results, we only performed experiments on the first or second internodal cells that had a surface area less than $10 \times 10^{-5} m^2$ (typically 2.5×10^{-2} m long and 0.5×10^{-3} m wide, and a surface area of 3–$5 \times 10^{-5} m^2$) and were isolated from vegetative plants.

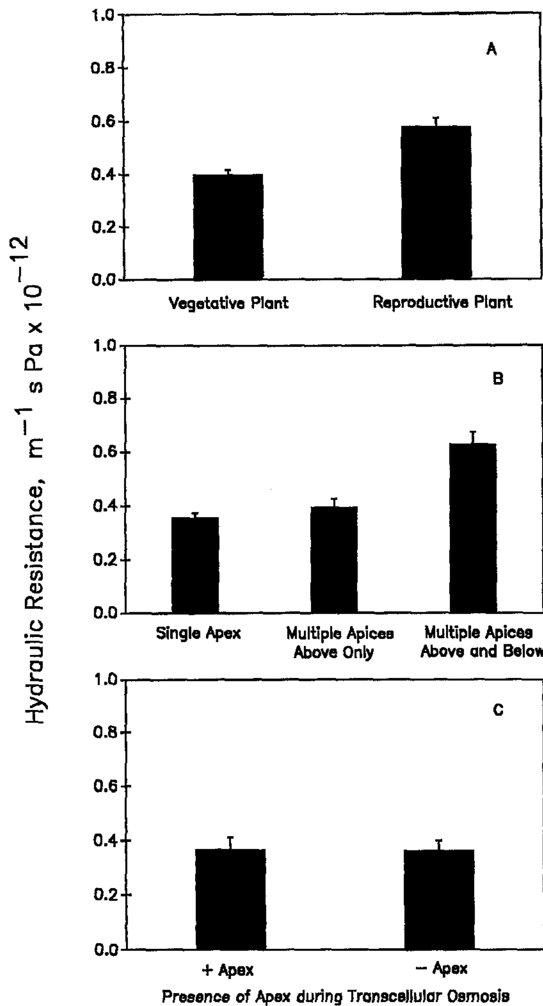

Fig. 2. The influence of developmental factors on the hydraulic resistance of the plasma membrane of *Chara* internodal cells. **A** Cells (with surface areas less than $10 \times 10^{-5} m^2$) were isolated from plants that were either vegetative or reproductive. When the plants were reproductive, the isolated cells were both subtended and supertended by antheridia-bearing branch cells (n = 7). **B** Cells (greater than $20 \times 10^{-5} m^2$) were isolated from unipolar shoots that had one apex or multipolar shoots that had more than one apex. In the case of the multipolar plants, the apices were either all above the isolated cell or both above and below (n = 3). **C** Second or third internodal cells (less than $10 \times 10^{-5} m^2$) were placed in the transcellular osmosis chamber with the apex intact. After performing transcellular osmosis on the cell, the apex was removed and the experiment was performed again. The surface area measured was that of the internodal cell placed between chambers A and B. Thus little if any water passes through the plasmodesmata under the conditions of transcellular osmosis where the turgor pressures of the two connected cells are different (n = 4)

Table 1. The hydraulic resistance (R_H, $m^{-1} \cdot s \cdot Pa \times 10^{-12}$), hydraulic conductivity (L_p, $m/s \cdot Pa \times 10^{12}$) and osmotic permeability (P_{os}, (m/s) $\times 10^6$) coefficients of the plasma membrane of *Chara corallina* grown under low CO_2 and high CO_2

Growing condition	R_H	L_p	P_{os}
Low CO_2	0.381 ± 0.006	2.625	353
High CO_2	0.565 ± 0.011	1.780	244

A t-test shows that the difference in the hydraulic resistance between low CO_2- and high CO_2-grown cells is significant at the 0.00001 level. n = 112 for low CO_2-grown cells and 73 for high CO_2-grown cells

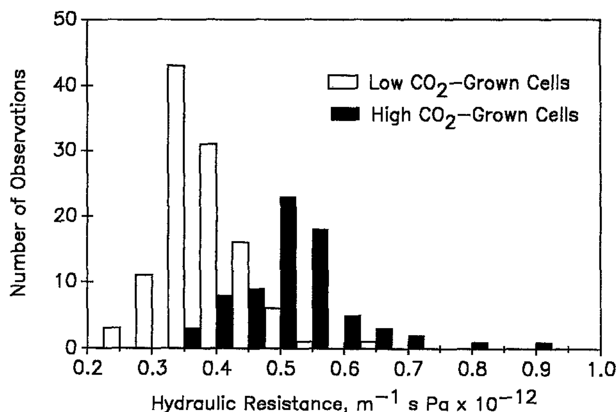

Fig. 3. The hydraulic resistance of the plasma membrane of cells grown under low CO_2 and high CO_2 conditions

Comparison of the hydraulic resistances of cells growing under low and high CO_2

Cells grown under low CO_2 conditions have a lower hydraulic resistance than cells grown under high CO_2 conditions (Fig. 3 and Table 1). The hydraulic resistances of cells grown in low CO_2 and high CO_2 are $0.381 \pm 0.006 \times 10^{12}$ and $0.565 \pm 0.011 \times 10^{12}\,m^{-1} \cdot s \cdot Pa$, respectively (Table 1). These correspond to hydraulic conductivities (L_p) of $2.6 \times 10^{-12}\,m/s \cdot Pa$ and $1.8 \times 10^{-12}\,m/s \cdot Pa$, respectively. The hydraulic conductivity can be converted to the osmotic permeability coefficient (P_{os}, in m/s) by multiplying the L_p by $RT/\bar{V}_w$ [Eq. (3)]. The osmotic permeabilities of low CO_2- and high CO_2-grown cells are 353×10^{-6} and $244 \times 10^{-6}\,m/s$, respectively.

Effect of light on the hydraulic resistance

In order to determine whether or not the difference in the hydraulic resistances is related to the light-dependent processes of photosynthesis, we determined the effect of light on the hydraulic resistance of cells. The hydraulic resistances of both low CO_2-and high CO_2-grown cells are independent of the photon fluence rate of white light with photosynthetically active radiation (PAR) in the range of 0–132 $\times 10^{-6}\,mol/m^2 \cdot s$ (Fig. 4). The difference in the characteristic hydraulic resistances of cells grown under low and high CO_2 conditions are stable under all light conditions tested. Further evidence that the light-dependent reactions of photosynthesis are not involved in water movement comes from the observation that DCMU ($10^{-2}\,mol/m^3$, 20 min) has no effect on the hydraulic resistance (data not shown).

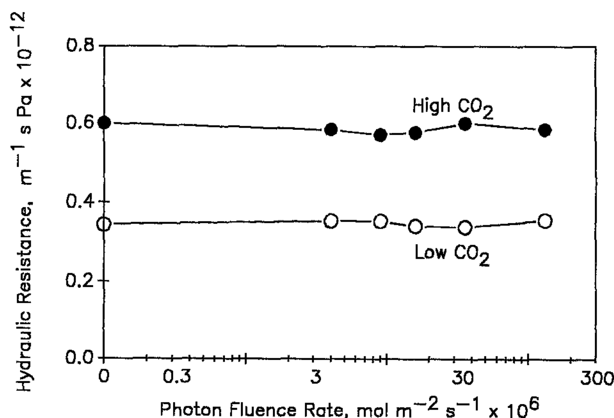

Fig. 4. The effect of light on the hydraulic resistance of the plasma membrane of *Chara corallina* (n = 3)

Effect of external pH on hydraulic resistance

Since the reduced hydraulic resistance of low CO_2-grown cells is not coupled to the light reactions of photosynthesis, perhaps a light-independent component of the bicarbonate uptake system is responsible for reducing the hydraulic resistance of the plasma membrane of the low CO_2-grown cells. Since the pH of the assay medium dramatically affects the photosynthetic rate by influencing the relative proportions of CO_2 and HCO_3^- (Lucas 1975, 1977; Mimura et al. 1993; Price and Badger 1985; Smith and Walker 1980), we measured the effect of external pH on hydraulic resistance. The hydraulic resistance of both low CO_2- and high CO_2-grown cells is only marginally influenced by the external pH (Fig. 5).

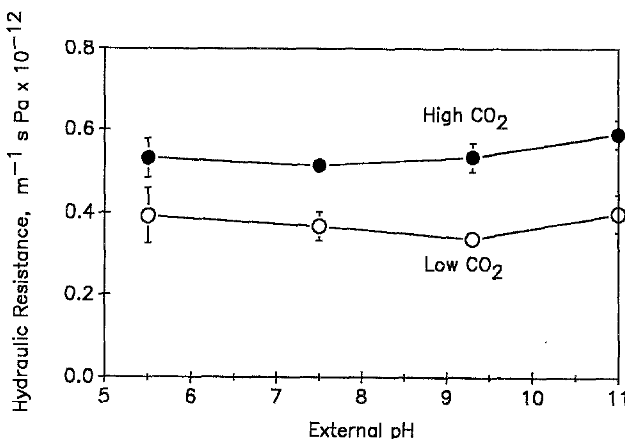

Fig. 5. The effect of external pH on hydraulic resistance of the plasma membrane of *Chara corallina*. Cells were sequentially treated with solutions of different pHs ranging from pH 5.5 to pH 11 and their hydraulic resistances were determined. The solutions contained APW plus either 5 mol/m^3 MES (pH 5.5), 5 mol/m^3 MOPS (pH 7.5), 5 mol/m^3 CHES (pH 9.3) or 5 mol/m^3 CAPS (pH 11). In all cases cells streamed vigorously (n = 3)

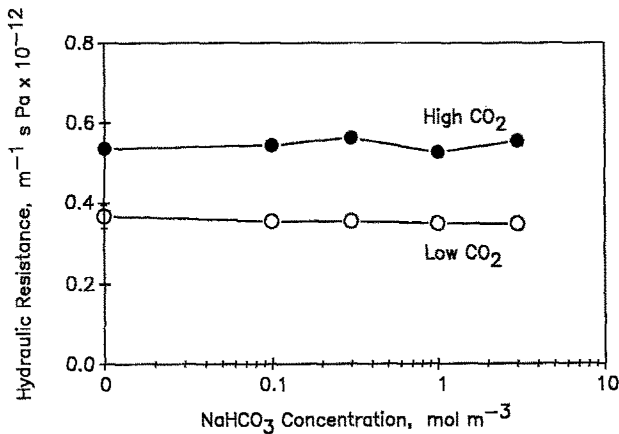

Fig. 6. The effect of external $NaHCO_3$ on the hydraulic resistance of the plasma membrane of *Chara corallina*. Cells were sequentially treated with $NaHCO_3$ from 0 to 3 mol/m^3 and their hydraulic resistances were determined. The cells were treated with each solution for 20 min. In all cases cells streamed vigorously (n = 3)

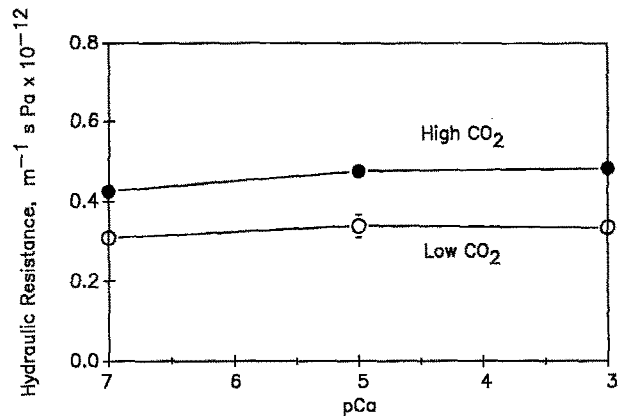

Fig. 7. The effect of external Ca^{2+} on the hydraulic resistance of the plasma membrane of *Chara corallina*. Cells were sequentially treated with Ca^{2+}-buffered solutions from pCa 3 to pCa 7 (1–10^{-4} mol/m^3) and their hydraulic resistances were determined. The cells were treated with each solution for 20 min. All solutions included 1 mol/m^3 EGTA (titrated with TRIS to pH 7), 2 mol/m^3 MOPS-NaOH (pH 7.5) and varying amounts of $CaCl_2$. The whole solution was then titrated to pH 7.5 with 10^3 mol/m^3 NaOH. The concentrations of each agent required for a given Ca^{2+} concentration was calculated with the aid of a computer program (Wayne 1985). Cells in pCa 7 solutions did not exhibit the mechanically-induced cessation of streaming response. In all cases cells streamed vigorously (n = 3). pCa = $-\log[Ca^{2+}]$

Effect of external $NaHCO_3$ on hydraulic resistance

It is reasonable to postulate that the bicarbonate transporter itself is one of the elements that functions as a water channel in low CO_2-grown cells since it may function as a water channel in red blood cells (Finkelstein 1987). The apparent uptake of bicarbonate is greater in low CO_2-grown cells than in high CO_2-grown cells (Price et al. 1985, Brechignac and Lucas 1987). The bicarbonate transporter has been analysed using typical Michaelis-Menten kinetics and bicarbonate binds to the transporter with an apparent K_m of between 0.58 and 0.71 mol/m^3 and it is saturated by approximately 2 mol/m^3 (Lucas 1975). Since bicarbonate may change the conformation of the bicarbonate transporter after binding, or compete with or facilitate the movement of water directly, we treated low CO_2-grown cells and high CO_2-grown cells with additional $NaHCO_3$ to see whether or not $NaHCO_3$ has any effect on hydraulic resistance. Low CO_2-grown cells sequentially treated with $NaHCO_3$ at concentrations from 0.1 to 3.0 mol/m^3 have the same hydraulic resistance as the untreated cells (Fig. 6). Likewise high CO_2-grown cells, are unaffected by 0.1–3.0 mol/m^3 $NaHCO_3$ and the difference in the characteristic hydraulic resistances of cells grown under low and high CO_2 conditions remains constant in the presence of $NaHCO_3$ (Fig. 6).

Effect of external Ca^{2+} on hydraulic resistance

External Ca^{2+} is essential for HCO_3^- uptake, but not for CO_2 fixation, indicating that external Ca^{2+} affects the ability of cells to utilize HCO_3^- at the plasma membrane (Lucas 1976, 1979; Lucas and Dainty 1977 a). Therefore we tested the dependence of hydraulic resistance on the external Ca^{2+} concentration. The hydraulic resistance increases slightly as the external Ca^{2+} concentration increases from 10^{-4} mol/m^3 to 1 mol/m^3. Similarly the hydraulic resistance of high CO_2-grown cells increases as the external Ca^{2+} concentration increases. The meager dependence on external Ca^{2+} contrasts with the unambiguous influence of external Ca^{2+} on other membrane properties (Mimura and Tazawa 1983, Staves and Wayne 1993, Wayne et al. 1990). The difference in the characteristic hydraulic resistances of cells grown under low and high CO_2 conditions remains constant in the presence of varying external Ca^{2+} concentrations (Fig. 7).

Effect of external monovalent cations on hydraulic resistance

Millimolar concentrations of external K^+ inhibit HCO_3^- transport in an all-or-none fashion (Lucas 1976, Lucas et al. 1978). Therefore we tested the effect of 10 mol/m^3 KCl on the hydraulic resistance of the plasma membrane of low CO_2-grown cells. We find that 10 mol/m^3 KCl has no effect on the hydraulic resistance of the plasma membrane (Table 2).

On the other hand, extracellular monovalent cations,

Table 2. The effect of monovalent cations on the hydraulic resistance (R_H, $m^{-1} \cdot s \cdot Pa \times 10^{-12}$) of cells grown in low CO_2

Treatment	R_H
Effect of adding 10 mol/m³ KCl[a]	
Complete medium (APW)	0.390 ± 0.004
Complete medium + 10 mol/m⁻³ KCl	0.406 ± 0.012[c]
Effect of removing monovalent cations[b]	
Complete medium (APW)	0.396 ± 0.012
Complete medium (APW) − NaCl, − KCl	0.379 ± 0.005[d]

Complete medium (APW) contained 0.1 mol/m³ $CaCl_2$, 0.1 mol/m³ KCl, and 1 mol/m³ NaCl

[a] Cells were either bathed in complete medium or in APW plus 10 mol/m³ KCl. Cells treated with APW plus 10 mol/m³ KCl were bearly streaming. [c] A t-test shows that the two treatments are not significantly different. p = 0.33, n = 3

[b] Cells were either bathed in complete medium or in 0.1 mol/m³ $CaCl_2$ only. All cells were streaming vigorously. [d] A t-test shows that the two treatments are not significantly different. p = 0.33, n = 3

Table 3. The effect of cytochalasin E on the hydraulic resistance (R_H, $m^{-1} \cdot s \cdot Pa \times 10^{-12}$)

Treatment	R_H	
Low CO_2		
Control	0.380 ± 0.012[a]	[100][e]
Cytochalasin E	0.408 ± 0.010[b]	[107]
High CO_2		
Control	0.475 ± 0.007	[100]
Cytochalasin E	0.540 ± 0.010[c, d]	[113]

Cells were treated with 0.02% DMSO and the hydraulic resistance was measured. Then the cells were treated with cytochalasin E (10 µg/ml) for 35 min and the hydraulic resistance was measured again. The sorbitol solutions contained 0.02% DMSO plus or minus CE. CE caused the streaming to stop within a few minutes, but only caused an increase in the hydraulic resistance after 35 min. n = 3

[a] t-test for low CO_2-grown control vs. high CO_2-grown control. p = 0.007

[b] t-test of control vs. CE for low CO_2-grown cells. p = 0.17

[c] t-test for low CO_2-grown, CE treated vs. high CO_2-grown, CE treated. p = 0.002

[d] t-test of high CO_2-grown control vs. CE for high CO_2-grown cells. p = 0.014

[e] In brackets, values as per cent of control

including Na^+ and K^+ may be necessary for HCO_3^- transport through various potential cotransport mechanisms, similar to those that exist in *Anabaena*, renal or pancreatic cells (Badger 1987, Grassl et al. 1987, Maren 1988, Reinhold et al. 1984). Therefore we tested whether the 0.1 mol/m³ Na^+ and K^+ that are present in the artificial pond water are necessary for the low hydraulic resistance of low CO_2-grown cells. Eliminating K^+ and Na^+ from the external medium has no effect on the hydraulic resistance (Table 2) indicating that monovalent cations are neither necessary for nor inhibitory to water movement.

Effect of cytochalasin E on hydraulic resistance

Lucas and Dainty (1977 b) showed that cytochalasins partially inhibit bicarbonate assimilation and influence the size and distribution of acid and alkaline bands. Lucas and Dainty (1977 b) propose that cytochalasins inhibit HCO_3^- uptake by diminishing the ability of the cell to exercise fine control over cytoplasmic pH. Cytochalasins also increase the total hydraulic resistance of internodal cells of *Chara corallina* by approximately 12–20% (Wayne and Tazawa 1988), indicating that the actin cytoskeleton may interact with the plasma membrane in order to regulate the transport of water. Perhaps the cytoskeleton interacts directly with the bicarbonate uptake system. Therefore we tested whether or not the low resistance of the plasma membrane of low CO_2-grown cells depends on the actin cytoskeleton by treating the cells with cytochalasin E (CE). CE (10 µg/ml, 35 min) increases the hydraulic resistance of low CO_2- and high CO_2-grown cells by 7 and 13%, respectively, indicating that while the actin cytoskeleton contributes to lowering the hydraulic resistance of the plasma membrane, it does not contribute specifically to the low hydraulic resistance of the low CO_2-grown cells (Table 3).

Effect of DCCD, a proton pump inhibitor, on the hydraulic resistance

The uptake of HCO_3^- may require the cotransport of H^+. Thus the H^+-pumping ATPase may also be involved in HCO_3^- transport. The proton pumping ATPase probably has a higher activity in low CO_2-grown cells than in high CO_2-grown cells since the membrane potential is hyperpolarized in low CO_2-grown cells (−0.196 ± 0.003 V) compared with high CO_2-grown cells (−0.152 ± 0.005 V); Table 5).

We inhibited the H^+-pumping ATPase through pharmacological means in order to determine its contribution to lowering the hydraulic resistance of low CO_2-grown cells. DCCD, applied externally to intact cells has no effect on the hydraulic resistance of the plasma membrane (data not shown).

Effect of intracellular ATP on hydraulic resistance

In order to assess further the contribution of a functional H^+-pumping ATPase in reducing the hydraulic resistance of the plasma membrane, we lowered the ATP concentration in tonoplast-free cells. This causes a drastic depolarization of the plasma membrane potential and a small increase in the electrical resistance (Lucas and Shimmen 1981). However, before we tested the effect of decreasing the ATP concentration on hydraulic resistance, we tested whether or not tonoplast-free cells containing 1 mol/m^3 ATP have the same hydraulic resistance as the intact controls. We find that the hydraulic resistance of tonoplast-free cells is the same as the hydraulic resistance of intact cells (Fig. 8). This confirms the observations of Kiyosawa and Tazawa (1977) who concluded that the tonoplast contributes negligibly to the hydraulic resistance of the cell and the plasma membrane is the major barrier to water movement.

Depleting the intracellular ATP with hexokinase and 2-deoxyglucose has no effect on the hydraulic resistance of the plasma membrane of low CO_2-grown cells, indicating that a functional ATPase, H^+ or otherwise, is not necessary for decreasing the hydraulic resistance of low CO_2-grown cells (Fig. 8).

Effect of carbonic anhydrase inhibitors on hydraulic resistance

Carbonic anhydrase catalyses the conversion of HCO_3^- to CO_2 on the cell surface of many microalgae. The CO_2 crosses the plasma membrane and is subsequently utilized as the carbon substrate for photosynthesis (Aizawa and Miyachi 1986). It is possible that this is also the mechanism used by characean cells to take up inorganic carbon (Price et al. 1985, Shiraiwa and Kikuyama 1989). Moreover, it is possible that carbonic anhydrase acts as the element in the plasma membrane that lowers the hydraulic resistance in low CO_2-grown cells. This is an especially interesting hypothesis since acetazolamide and ethoxyzolamide, two inhibitors of carbonic anhydrase in *Chara* (Price et al. 1985, Shiraiwa and Kikuyama 1989) are used medicinally as agents that modify water balance. Acetozolamide is used as a diurectic and ethoxyzolamide is used in the treatment of glaucoma (Merck Index). Moreover, Findenegg (1974) found that acetazolamide inhibits Cl^- uptake in *Scenedesmus*. However, we find that acetazolamide and ethoxyzolamide (10^{-1} mol/m^3, 20 min) have no effect on the hydraulic resistance of low CO_2-grown *Chara* cells (Fig. 9). In addition, we find that ethoxyzolamide has no effect on Cl^- uptake in either low CO_2- or high CO_2-grown cells (data not shown).

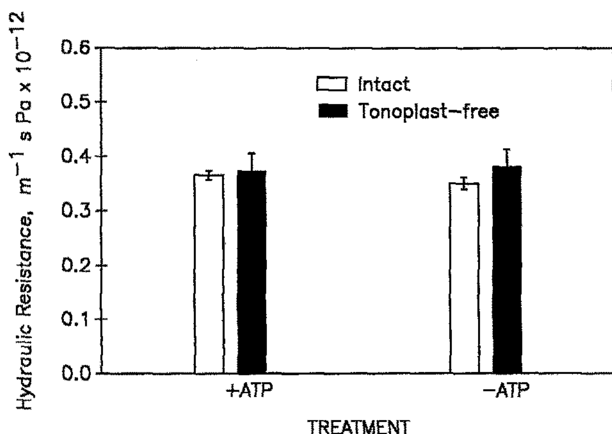

Fig. 8. The effect of cellular ATP on the hydraulic resistance of tonoplast-free cells of *Chara*. A t-test shows that there is no difference between the perfused (with ATP) and intact cells ($p = 0.82$, $n = 3$); perfused (with hexokinase) and intact cells ($p = 0.45$, $n = 3$), and perfused plus or minus ATP cells ($p = 0.87$, $n = 3$)

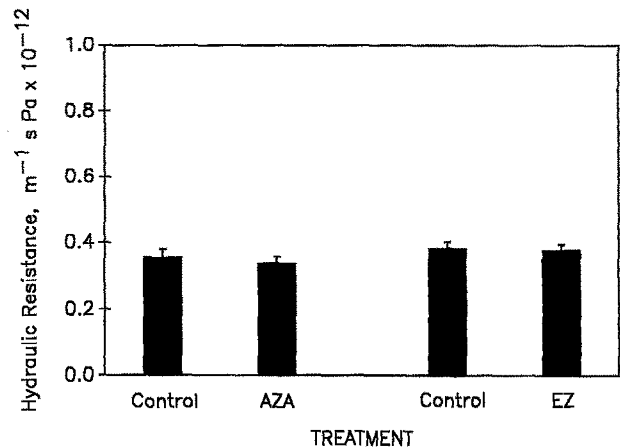

Fig. 9. The effect of carbonic anhydrase inhibitors on hydraulic resistance of the plasma membrane of *Chara corallina* (for AZA experiments, $n = 6$; for EZ experiments, $n = 3$)

Effect of pCMPS on hydraulic resistance

Since pCMPS has been shown to increase the hydraulic resistance of the plasma membrane in *Nitellopsis* by inhibiting proteinaceous water channels (Wayne and Tazawa 1990), we tested whether or not the pCMPS-reacting proteins are specifically responsible for lowering the hydraulic resistance of low CO_2-grown cells. We find that pCMPS (1 mol/m^3, 1 h), which is relatively ineffective in inhibiting carbon uptake (Lucas and Alexander 1981), is effective in increasing the hydraulic

Table 4. The effect of pCMPS on the hydraulic resistance (R_H, $m^{-1} \cdot s \cdot Pa \times 10^{-12}$) of internodal cells of *Chara corallina* grown under low CO_2 and high CO_2 conditions

Treatment	R_H	
Low CO_2		
Control	0.383 ± 0.003[a]	[100][e]
pCMPS	0.523 ± 0.025[b]	[135]
High CO_2		
Control	0.555 ± 0.025	[100]
pCMPS	0.660 ± 0.024[c, d]	[119]

Cells were treated with APW and the hydraulic resistance was measured. Then the cells were treated with pCMPS (1 mol/m^3) for 60 min and the hydraulic resistance was measured again. The sorbitol solutions were made with or without pCMPS. pCMPS has no effect on the streaming rate and all the cells streamed vigorously. For low CO_2-grown cells, n = 3; for high CO_2-grown cells, n = 4
[a] t-test for low CO_2-grown control vs. high CO_2-grown control. p = 0.007
[b] t-test of control vs. pCMPS for low CO_2-grown cells. p = 0.032
[c] t-test for low CO_2-grown, pCMPS treated vs. high CO_2-grown, pCMPS treated. p = 0.018
[d] t-test of control vs. pCMPS for high CO_2-grown cells. p = 0.034
[e] In brackets, values as per cent of control

resistance of both low CO_2- and high CO_2-grown *Chara* cells by 35 and 18%, respectively (Table 4). These data indicate that the pCMPS-sensitive water channels contribute to lowering the hydraulic resistance of low CO_2-grown cells. The greater activity of pCMPS-sensitive channels, however, does not solely account for the lower hydraulic resistance of low CO_2-grown cells.

Effect of protease treatment on hydraulic resistance

It is likely that proteins contribute to the lowering of the hydraulic resistance of the plasma membrane of both low CO_2- and high CO_2-grown cells since pCMPS and CE increase the hydraulic resistance. In order to characterize further the proteins responsible for lowering the hydraulic resistance of low CO_2-grown cells, we treated the cells with trypsin (0.1%, 20 min), proteinase K (0.1%, 20 min) or thermolysin (0.1%, 20 min). However, neither trypsin, proteinase K nor thermolysin have any effect on the hydraulic resistance of the plasma membrane of low CO_2-grown cells (data not shown).

Relationship between hydraulic resistance and electrical resistance

Since it has been postulated that the HCO_3^--uptake system may be associated with the movements of various ions, including H^+, OH^-, and Cl^-, it may be possible to detect a relationship between hydraulic resistance and electrical resistance. In contrast to the dependence of hydraulic resistance on the surface area of cells grown in typical bulk cultures, there is no correlation between the specific electrical resistance of the membrane and its surface area. In order to determine more thoroughly the relationship between hydraulic resistance and specific electrical resistance, we measured the hydraulic resistance and electrical resistance of the plasma membrane of the same cells. We did not find any correlation between the hydraulic resistance and the specific electrical resistance of the same cell, indicating that all the channels providing the low resistance pathway for water probably do not contribute to a low resistance pathway for highly permeant ions (Fig. 10 A).

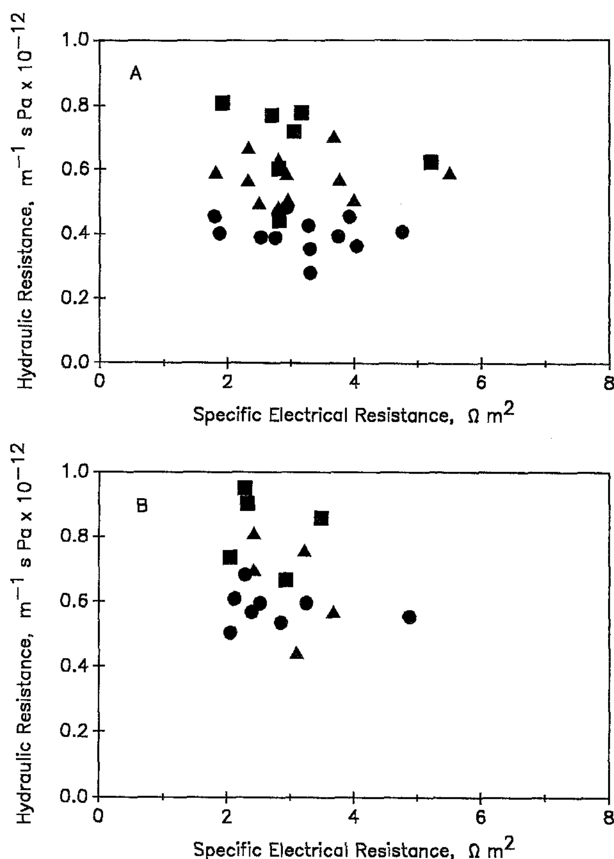

Fig. 10. The hydraulic resistance and specific electrical resistance of the plasma membrane of *Chara corallina* measured in the same cells. **A** Low CO_2-grown cells. **B** High CO_2-grown cells. Cells with a surface area ● between 0 and $10 \times 10^{-5} m^2$, ▲ between 10.1 and $20 \times 10^{-5} m^2$, ■ between 20.1 and $35 \times 10^{-5} m^2$

We also measured the hydraulic resistance and electrical resistance of the plasma membrane of the same cells grown under high CO_2 conditions. Similarly, we

Table 5. The membrane potential and specific electrical resistance of cells *Chara corallina* grown under low CO_2 and high CO_2 conditions

	Membrane potential (V)	Electrical resistance (Ω m^2)
Low CO_2		
All cells	−0.186 ± 0.003 (100)	2.77 ± 0.11 (100)
Small cells	−0.196 ± 0.003 (51)	2.70 ± 0.12 (51)
High CO_2		
All cells	− 0.154 ± 0.005 (19)	2.77 ± 0.16 (19)
Small cells	− 0.152 ± 0.005 (8)	2.80 ± 0.33 (8)

Small cells include only cells with surface areas less than $10 \times 10^{-5}\,m^2$. Number of cells is given in parentheses. The external solution contained unbuffered APW (0.1 mol/m^3 KCl, 1 mol/m^3 NaCl, and 0.1 mol/m^3 $CaCl_2$)

find that the hydraulic resistances of these cells are not correlated with their specific electrical resistance (Fig. 10 B). A further indication that the water channels are not necessarily synonymous with ion channels comes from the observation that while the hydraulic resistance of low CO_2-grown cells is lower than the hydraulic resistance of high CO_2-grown cells, the specific electrical resistances of the two types of cells are similar (Table 5).

Measurement of $^{36}Cl^-$ uptake in low CO_2- and high CO_2-grown cells

The low hydraulic resistance of low CO_2-grown cells compared to high CO_2-grown cells is not a result of a general increase in membrane permeability. The $^{36}Cl^-$ influx, although active, is lower in low CO_2-grown cells than in high CO_2-grown cells. The inwardly-directed fluxes are $80 \pm 8 \times 10^{-9}\,mol/m^2 \cdot s$ (n = 5) and $110 \pm 18 \times 10^{-9}\,mol/m^2 \cdot s$ (n = 4), respectively (p = 0.17). The fluxes we measured in freshly isolated internodal cells are relatively high and similar to those found in aged Cl^--starved cells (Keifer et al. 1982, Lucas et al. 1986, Sanders 1980).

Table 6. The osmotic concentration and osmotic pressure of cells of *Chara corallina* grown under low CO_2 and high CO_2 conditions

	Osmotic concentration (mol/m^3)	Osmotic pressure (MPa)
Low CO_2	240.2 ± 3.5	0.590
High CO_2	244.8 ± 2.3[a]	0.600

The cytoplasm and cell sap were expressed from cells with surface areas less than $10 \times 10^{-5}\,m^2$ and pooled. The osmotic concentration was measured with a vapor pressure osmometer. Each mean is a result of 5 independent measurements. The osmotic pressure (π) at 295 K was calculated from the osmotic concentration (c, in mol/m^3) using the van't Hof equation: $\pi = R\mathrm{Tc}$, where R is the universal gas constant (8.31 J/mol · K) and T is the temperature (in K)

[a] A t-test for high CO_2-grown cells vs. low CO_2-grown cells shows that p = 0.31. n = 5

Measurement of cellular osmotic pressure in low CO_2- and high CO_2-grown cells

Since the hydraulic resistance depends on the cellular osmotic pressure (Kiyosawa and Tazawa 1972, 1973), we determined the osmotic pressure of the low CO_2- and high CO_2-grown cells. The osmotic pressure of cells grown in both low and high CO_2 is approximately 0.6 MPa (at 295 K), indicating that the difference in hydraulic resistance is not a consequence of a difference in cellular osmotic pressure (Table 6).

Measurement of reflection coefficients of the plasma membrane

After failing to find any mechanism that relates the uptake of bicarbonate with the reduced hydraulic resistance of low CO_2-grown cells, we considered the possibility that the plasma membrane of low CO_2-grown cells may be more permeable to small nonelectrolytes in general, compared to high CO_2-grown cells. It is generally acknowledged that the nature of the lipid bilayer determines the permeabilities of nonelectrolytes (McElhaney 1985). Therefore we measured the reflection coefficients of a number of nonelectrolytes, including methanol, ethanol, n-butanol, acetone and

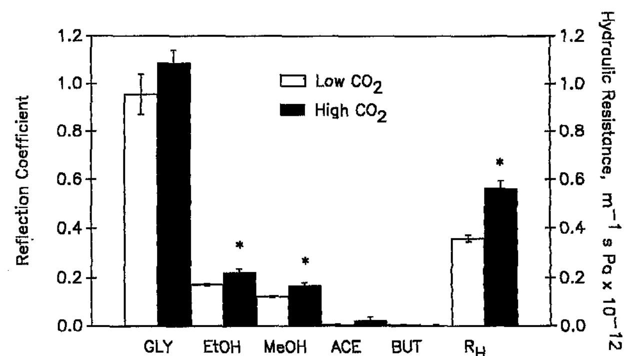

Fig. 11. The reflection coefficients of the plasma membrane of low CO_2- and high CO_2-grown cells. Cells were sequentially treated with 100 mol/m^3 of each nonelectrolyte and the volume flow of water was measured between 10 and 20 s after the addition of the nonelectrolyte (n = 6). The levels of significance (p) are 0.54, 0.01, 0.03, 0.70, and 0.57 for glycerol, methanol, ethanol, butanol, and acetone, respectively. The level of significance for the hydraulic conductivity (R_H) is 0.001

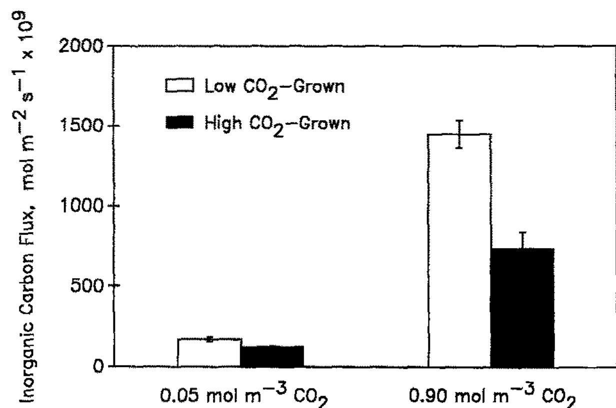

Fig. 12. The inorganic carbon flux of low CO_2- and high CO_2-grown cells at two different concentrations of CO_2. Inorganic carbon uptake was deduced from the amount of ^{14}C incorporated into photosynthetic products per unit surface area of cells during a 10 min time period. The total inorganic carbon concentration was 1 mol/m^3 in both cases. A t-test shows that the carbon fluxes in the low CO_2- and high CO_2-grown cells are statistically different at 0.9 mol/m^3 CO_2 ($p = 0.006$, $n = 4$, but not at 0.05 mol/m^3 CO_2 ($p = 0.065$, $n = 4$). In both low CO_2- and high CO_2-grown cells, the inorganic carbon flux is greater at 0.9 mol/m^3 than at 0.05 mol/m^3 ($p = 0.001$ and 0.013, respectively)

glycerol using the transcellular osmosis method (Dainty and Ginzburg 1964c, Staves et al. 1992). We find that the reflection coefficients of the plasma membrane for methanol and ethanol are significantly smaller in the low CO_2-grown cells than in the high CO_2-grown cells, indicating that the permeabilities of the membrane to small nonelectrolytes are different in the two cell types and may account for the difference in hydraulic resistance (Fig. 11).

Measurement of the inorganic carbon flux across the plasma membrane

Next we determined whether or not the permeability of the plasma membrane to CO_2 is different in low CO_2- and high CO_2-grown cells. The flux of inorganic carbon across the plasma membrane is greater in low CO_2-grown cells than in high CO_2-grown cells (Fig. 12). If diffusion of CO_2 across the plasma membrane is considered to be the limiting factor in inorganic carbon fixation (Mimura et al. 1993), then the apparent permeability coefficients of the plasma membrane for CO_2 (P_{app}, in m/s) can be calculated from the following equation:

$$P_{app} = -J_{CO2}/[CO_{2(i)} - CO_{2(o)}] \qquad (6)$$

where J_{CO2} is the measured flux of CO_2 (in mol/m^2/s), $CO_{2(i)}$ is the internal concentration of CO_2 (in mol/m^3) and is assumed to be 0 here, and $CO_{2(o)}$ is the external concentration of CO_2 (in mol/m^3).

The apparent permeability coefficients for low CO_2- and high CO_2-grown cells are 2.5×10^{-6} m/s and 1.65×10^{-6} m/s, respectively. In actuality, the apparent permeability coefficient (P_{app}, in m/s) is a function of the true membrane permeability coefficient (P_m, in m/s) and the permeability coefficient of the unstirred layers (P_u, in m/s) according to the following equation:

$$1/P_{app} = (1/P_m) + (1/P_u) \qquad (7)$$

where P_u is equal to D_{CO2}/δ, and D_{CO2} is the aqueous diffusion coefficient of CO_2 (1.94×10^{-9} m^2/s at 298 K) and δ (in m) is the thickness of the unstirred layer (Hill and Whitingham 1955). If P_m has an infinitely high value, then $(1/P_m)$ approaches zero and P_{app} is equal to P_u. In this case, the thicknesses of the unstirred layer of low CO_2- and high CO_2-grown cells can be calculated to be 776×10^{-6} m and 1176×10^{-6} m, respectively. If the thicknesses of the unstirred layers are actually equal to the maximal thicknesses calculated above, then P_m would be infinitely larger than the P_{app}. However, while the actual thicknesses of the unstirred layers are unknown, the thicknesses of the unstirred layers (sensu strictu) should be identical for the cells grown under the two conditions. In actuality, the apparent thickness of the unstirred layer is due to a true unstirred layer as well as an unstirrable layer within the cell wall space which is a function of the cell wall thickness. We have minimized the effect of the unstirrable layer by using young cells that have a wall thickness of $0.8–1 \times 10^{-6}$ m compared to the typically-used older cell that has a $5–10 \times 10^{-6}$ m thick wall (Walker 1985). Thus, if the unstirred layers are assumed to be equivalent in the cells grown under the two conditions and equal to 100×10^{-6} m, then P_u would be equal to $(1.94 \times 10^{-9}$ m^2/s$)/(100 \times 10^{-6}$ m$)$,
1.94×10^{-5} m/s, and the plasma membrane permeability coefficient to CO_2 would be about 10% larger than the apparent permeability coefficient.

The difference in the flux of inorganic carbon into low CO_2- and high CO_2-grown cells is not due to a difference in chlorophyll content. The amount of chlorophyll per unit area (in mg chl/m^2) is 199.4 ± 18.8 and 201.8 ± 18.4 ($p = 0.93$, $n = 5$) for low CO_2- and high CO_2-grown cells, respectively.

Effect of CO_2 on hydraulic resistance

Relatively long-term treatments with CO_2 increase the hydraulic resistance of various plants cells, although

the mechanism of inhibition is unknown (Chang and Loomis 1945, Glinka and Reinhold 1962). It is possible that CO_2 inhibits water movement by competing for the same channels. In order to determine whether or not CO_2 passes through the same channels as water, we investigated whether or not CO_2 inhibits water movement in low CO_2-grown *Chara* cells. We find that CO_2 immediately increases the hydraulic resistance of the plasma membrane by 17%, indicating that CO_2 and water may pass through the same channels. The hydraulic resistance of low CO_2-grown cells tested in the absence and presence of 45 mol/m^3 CO_2 is $0.408 \pm 0.015 \times 10^{12}$ and $0.477 \pm 0.020 \times 10^{12}$ $m^{-1} \cdot s \cdot Pa$, respectively ($p = 0.014$, $n = 10$). Using 45 mol/m^3 CO_2 alone, we were unable to induce any water movement and conclude that the reflection coefficient for CO_2 is approximately zero.

Determination of the hydraulic resistance of the plasma membrane in cold-acclimated cells

Since the lipid composition of membranes may vary in plants acclimated to low temperatures (288 K) compared to those growing at ambient temperatures (299 K; Kasamo et al. 1992, Lynch and Steponkus 1987), we measured the hydraulic conductivity of low CO_2-grown cells, grown at the two temperatures.

Prior to doing the experiment, we assumed that the lipid bilayer in low temperature-grown cells would have shorter and less saturated fatty acids compared with the ambient temperature-grown cells in order to keep the membrane "more fluid" and thus we predicted that the hydraulic resistance might be lower in low temperature-grown cells compared with ambient temperature-grown cells. However, we find that the hydraulic resistance of the plasma membrane measured at room temperature is high in cells grown at 288 K ($0.639 \pm 0.037 \times 10^{12} m^{-1} \cdot s \cdot Pa$, $n = 4$) compared with cells grown at 299 K. While many factors may affect the hydraulic resistance, perhaps the high hydraulic resistance of low temperature-grown cells is related, in part, to the fact that the CO_2 concentration in the medium is markedly dependent on temperature and is 35% higher at 288 K than at 299 K. The hydraulic resistance of low temperature-grown cells is also sensitive to pCMPS (Table 7).

Determination of the hydraulic resistance of the plasma membrane in cells grown in 2 mol/m^3 $NaHCO_3$

In order to test whether it was the CO_2 in the bulk solution per se or the increase in the inorganic carbon concentration ($\approx$ 2 mol/m^3) that resulted in the greater hydraulic resistance of high CO_2-grown cells compared to low CO_2-grown cells, we supplemented one stagnant culture of cells with 2 mol/m^3 $NaHCO_3$. The hydraulic resistance of the plasma membrane of these cells was as high as the high CO_2-grown cells ($0.527 \pm 0.023 \times 10^{12} m^{-1} \cdot s \cdot Pa$, $n = 4$), indicating that the hydraulic resistance may depend on the availability of inorganic carbon in the bulk solution.

Table 7. The effect of pCMPS on the hydraulic resistance (R_H, $m^{-1} \cdot s \cdot Pa \times 10^{-12}$) of internodal cells of *Chara corallina* grown at low temperature (288 K)

Treatment	R_H	
Control	0.640 ± 0.038	[100][b]
pCMPS	0.853 ± 0.074[a]	[133]

Cells were treated with APW and the hydraulic resistance was measured. Then the cells were treated with pCMPS (1 mol/m^3) for 60 min and the hydraulic resistance was measured again. The sorbitol solutions contained plus or minus pCMPS. pCMPS has no effect on the streaming rate and all the cells streamed vigorously. All experiments were done at room temperature 293–294 K

[a] A t-test of control vs. pCMPS for low temperature-grown cells. $p = 0.05$. $n = 4$

[b] In brackets, values as per cent of control

Discussion

The plasma membrane functions to separate the protoplasm from the external environment and must prevent promiscuous movement while allowing the transport of selected substances. Water is essential for almost every process that takes place in the cytoplasm and consequently the membrane must be permeable to water. There is no doubt that various proteins act as water channels in the membranes of plant and animal cells (Benga 1989; Maurel et al. 1993; Nielsen et al. 1993; Rygol et al. 1992; Solomon 1989; Verbavatz et al. 1993; Verkman 1992; Wayne and Tazawa 1988, 1990; Zhang et al. 1993). Maurel et al. (1993) and Verkman (1992) propose that a class of proteinaceous channels called aquaporins (Chrispeels pers. comm.) function specifically for the transport of water. There is still a question, however, as to what proportion of water is transported through the protein pathway versus the lipid pathway.

While proteinaceous water channels may have a high conductance for water, they may account for only a small proportion of the membrane area, whereas lipids may or may not have a high conductance, but always

account for a great proportion of the membrane surface area. Thus in discerning the pathway of water movement across a given membrane we must account for these two components as well as the possibility that the interface of certain proteins and lipids provide a low resistance pathway for water. Presumably the ratio of water movement through proteins, lipids and the lipid/protein interfaces will be different for each membrane and must be tested. Moreover, since protein function can be dependent on the boundary lipids (Kasamo 1990), a given protein may act as a water channel in one lipid environment but not in another.

At the onset of this work we believed that the hydraulic resistance of the plasma membrane may be under strong selective pressure since water is essential for intracellular reactions, passes through proteins, and its permeability is regulated by various physiological stimuli. Indeed, the fact that the osmotic permeability coefficient ($\approx 10^{-4}$ m/s) is about one hundred times greater than the permeability coefficients of highly permeant ions ($\approx 10^{-6}$ m/s) also supports the thesis that there are specific water-permeable channels. However, in the process of carrying out the present experiments, we have begun to consider the possibility that water channels may be multifunctional and the hydraulic resistance of the plasma membrane may be contingent upon selection pressures for the transport of other substances. Support of this antithesis comes from the observation that the hydraulic resistance of the plasma membrane varies over three orders of magnitude between that of *Chlamydomonas* and that of *Chara* even though cells from both organisms grow under similar aquatic conditions (Raven 1984).

As a consequence of the results obtained in the present work, we began to consider the possibility that the permeability of the plasma membrane to water may reflect, in part, the permeability of the membrane to CO_2. It is usually assumed that CO_2 traverses the plasma membrane of *Chara* through the lipid bilayer, although the possibility of a CO_2 transport protein has not been eliminated.

Our interpretation that the hydraulic resistance is dependent on the permeability of the membrane to CO_2 depends on the finite (i.e., limiting) permeability of the membrane to CO_2. Heretofore, it has been assumed that the permeability of cytoplasmic membranes of *Chara* to CO_2 is high and hence does not limit photosynthesis since the CO_2 permeability coefficient of a lecithin/cholesterol/n-decane black lipid membrane is 3.5×10^{-3} m/s (Gutknecht et al. 1977). In fact the resistance to CO_2 uptake was considered to be due exclusively to an unstirred layer (Walker et al. 1980). However, Gimmler et al. (1990) and Zenvirth and Kaplan (1981) have shown that the permeability coefficient of plasma membranes to CO_2 is 100–10,000 times lower than previously thought and is between 0.1 and 11×10^{-6} m/s. We find that the permeability of the plasma membrane of *Chara* to CO_2 also falls within this range. Therefore the resistance of the plasma membrane to CO_2 must be taken into consideration when modeling the transport of inorganic carbon. The permeability of the membrane to CO_2 could be regulated by either changes in the lipid composition of the bilayer or by a change in activity or amount of a putative CO_2 transport protein.

Heretofore we believed that all lipid bilayers, including both black lipid membranes (BLMs) and bilayers in the plasma membrane are similar and that any measurement of transport properties of any BLM will closely resemble the properties of a natural lipid bilayer. We now realize that the permeability coefficient of black lipid membranes to nonelectrolytes does vary as a consequence of differences in the lipid composition (Cass and Finkelstein 1967, Graziani and Livne 1972, Holz and Finkelstein 1970, Jain 1972). Moreover, in the past the lipid bilayer has been considered to act as a Newtonian fluid, where diffusion obeys Stokes' law and is linearly dependent on molecular size. However, analyses by Stein (1986) reveal that the lipid bilayer should be considered to be a non-Newtonian fluid where movement is nonlinearly related to size and shape of permeants. Thus the ability of a solute to permeate the membrane depends on the tortuosity which is a function of "the collisional probability of the solute with the macromolecular network of the membrane" (Ginzburg and Katchalsky 1963). Variation in the lipid head groups or fatty acid tails may cause a change in the polymeric structure of the bilayer which will allow certain molecules to reptate through faster relative to others. Considering the lipid bilayer to be a polymer rather than a fluid allows one to visualize easily how different lipid configurations can influence transport selectivity.

The majority of studies on membrane biology have focused on the contribution of integral membrane proteins in regulating membrane processes. However, the ability of extrinsic membrane proteins (e.g. the membrane skeleton) and membrane lipids to modulate some membrane properties has not been ruled out (Cullis et al. 1983, Gimmler et al. 1990). Lipids have been shown to have "channel-like" properties similar to more typical proteinaceous ionic channels (Meissner

pers. comm., Woodbury 1989). Moreover, the variation in physicochemical properties among membrane lipids is becoming recognized as a factor that may also influence intracellular transport as well as membrane transport (Van Deenen 1969). For example myosin-I, an actin-activated mechanochemical ATPase, is only capable of binding to anionic phospholipids, including phosphatidylserine, phosphatidylinositol phosphate, phosphatidylinositol-4,5-bisphosphate and phosphatidylglycerol (Adams and Pollard 1989, Hayden et al. 1990, Miyata et al. 1989, Zot et al. 1992).

The lipids in a biological membrane are not randomly arranged, but exist in domains (Aloia 1983, Jain 1983). A given domain in a lipid monolayer may be considered to be a two-dimensional macromolecule with many functional groups, including choline, inositol, ethanolamine and serine. similar to the structure of proteins, the structure of such a domain, as measured by its physical attributes such as viscosity, can be influenced by hydrophobic agents, temperature, hydrostatic and osmotic pressure, membrane potential, pH and $[Ca^{2+}]$ (Shinitzky 1984). A change in the structure of a microdomain may affect its channel-like activities. In order for a lipid bilayer to act as a "channel", there must be "communication" across the bilayer, and each monolayer must be functionally coupled to make a low resistance transport pathway.

Our data on water permeability have led us to the hypothesis that the permeability of the plasma membrane to CO_2 can be regulated by the carbon concentration in the growing medium and this may be one possible mechanism aquatic cells use to increase their ability to utilize dissolved inorganic carbon. When cells are grown under low CO_2 conditions, the plasma membrane permeability to CO_2 may increase as a result of changes in the lipid bilayer and/or the addition of CO_2 transport proteins. The additional low resistance pathway to CO_2 is also permeable to other small polar nonelectrolytes like methanol, ethanol and water. We believe that this is the reason that the hydraulic resistance decreases in low CO_2-grown plants.

This interpretation assumes that CO_2 is at least one of the inorganic carbon species transported across the plasma membrane of low CO_2-grown cells. This is consistent with the model of Ferrier (1980) and Walker et al. (1980) where either CO_2 or HCO_3^- (depending on the pH of the bulk medium) diffuses through the bulk solution and the extracellular matrix to the periplasmic space. A plasma membrane-localized H^+ pumping ATPase acidifies the periplasmic space so that CO_2 is formed from HCO_3^- and H^+. This is supported by the observations that the H^+-ATPase as well as carbon assimilation require ATP (Mimura et al. 1993) and Ca^{2+} (Staves et al. in prep.) and are inhibited by N-ethyl maleimide (Tsutsui and Ohkawa 1993) and pH buffers (Price et al. 1985). The production of CO_2 in the periplasmic space may be accelerated in a reaction catalysed by carbonic anhydrase since inhibitors of carbonic anhydrase inhibit carbon assimilation at low CO_2 concentrations. Moreover, short term experiments measuring the fixation of $^{14}CO_2$ or $H^{14}CO_3^-$ indicate that CO_2 is the inorganic carbon species that is absorbed by *Chara* (Shiraiwa and Kikuyama 1989). Upon its formation, CO_2 diffuses across the plasma membrane where it is trapped in an alkaline cytosol and stroma by conversion to HCO_3^-. The permeability of the plasma membrane to CO_2 depends on the concentration of inorganic carbon in the growth medium. The CO_2 that passes across the plasma membrane moves through channels created by proteins, lipids or a combination of both and is eventually fixed by ribulose bisphosphate carboxylase/oxygenase (Yeoh et al. 1981). The regulation of the permeability of the plasma membrane to CO_2 may affect its hydraulic resistance.

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