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pH-Induced Proton Permeability Changes of Plasma Membrane Vesicles

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Abstract. In vivo studies with leaf cells of aquatic plant species such as *Elodea nuttallii* revealed the proton permeability and conductance of the plasma membrane to be strongly pH dependent. The question was posed if similar pH dependent permeability changes also occur in isolated plasma membrane vesicles. Here we report the use of acridine orange to quantify passive proton fluxes. Right-side out vesicles were exposed to pH jumps. From the decay of the applied ΔpH the proton fluxes and proton permeability coefficients (P_{H^+}) were calculated. As in the intact *Elodea* plasma membrane, the proton permeability of the vesicle membrane is pH sensitive, an effect of internal pH as well as external pH on P_{H^+} was observed. Under near symmetric conditions, i.e., zero electrical potential and zero ΔpH, P_{H^+} increased from 65×10^{-8} at pH 8.5 to 10^{-1} m/sec at pH 11 and the conductance from 13×10^{-6} to 30×10^{-4} S/m^2. At a constant pH_i of 8 and a pH_o going from 8.5 to 11, P_{H^+} increased more than tenfold from 2 to 26×10^{-6} m/sec. The calculated values of P_{H^+} were several orders of magnitude lower than those obtained from studies on intact leaves. Apparently, in plasma membrane purified vesicles the transport system responsible for the observed high proton permeability in vivo is either (partly) inactive or lost during the procedure of vesicle preparation. The residue proton permeability is in agreement with values found for liposome or planar lipid bilayer membranes, suggesting that it reflects an intrinsic permeability of the phospholipid bilayer to protons. Possible implications of these findings for transport studies on similar vesicle systems are discussed.

Key words: Proton permeability — Proton conductance — *Elodea* — Plasma membrane — Acridine orange

Introduction

In vivo experiments with a number of submerged aquatic plant species, angiosperms, e.g., *Elodea* and *Potamogeton*, as well as characeae, revealed that the proton permeability (P_{H^+}) of the plasma membrane (PM) is strongly pH dependent (Bisson & Walker, 1980; Beilby & Bisson, 1992; Beilby, Mimura & Shimmen, 1993; Miedema & Prins, 1991, 1993; Miedema, Felle & Prins, 1992). At high external pH the leaf cells of these species are in the H^+-state which is characterized by a membrane potential dominated by an H^+ permeability. The H^+-state is associated with the phenomenon of polarity and banding and plays a role in photosynthetic bicarbonate assimilation (Prins et al., 1982; Elzenga and Prins, 1989). In *Nitellopsis* the H^+-state can be induced in intact but not in perfused cells (Beilby et al., 1993). Similar results were obtained for intact and perfused *Chara* cells (Lucas & Shimmen, 1981). To see whether the H^+-state in *Elodea* is bound to the intact cell, we isolated right side out plasma membrane vesicles from *Elodea nuttallii* leaf cells. These vesicles were exposed to pH jumps and the decay of the ΔpH between vesicle content and outer medium was recorded and analyzed. Acridine orange (AO) fluorescence was used to quantify passive proton transport. Optical probes sensitive to changes in ΔpH have been applied frequently to study ATPase-mediated H^+ transport processes (Bennett & Spanswick, 1983, 1984; Palmgren, 1991; Staal et al., 1991) and H^+ transport via antiport or symport across the liposome or vesicle membrane (Deamer et al., 1972; Nichols et al., 1980; Deamer & Nichols, 1983). Because of ΔpH independent quenching it has been argued (Rottenberg & Moreno-Sanchez, 1993) that AO fluorescence can only

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be used qualitatively. To our knowledge AO has been used only once to quantify proton fluxes (Schmidt & Briskin, 1993). Although our approach was different, both studies show that AO can be used quantitatively.

The proton permeability of the phospholipid membrane itself has been determined using planar lipid bilayers (Gutknecht & Walter, 1981*a*,*b*) or liposomes (Deamer, Prince & Crofts, 1972; Deamer & Nichols, 1983; Nichols & Deamer, 1980; Nichols et al., 1980; Nozaki & Tanford, 1981). In addition, different techniques have been applied: conductivity measurements on planar lipid bilayers (Gutknecht & Walter, 1981*a*,*b*) and, in case of liposomes, measurements of pH changes using either a pH electrode (Nichols & Deamer, 1980) or ΔpH-sensitive fluorescent probes (Deamer & Nichols, 1983; Nichols et al., 1980). Recorded permeabilities differ considerably: from 10^{-11} to 10^{-6} m/sec (Forte, 1987). These differences may result from the use of different preparations and techniques. Gutknecht (1984) argued that the permeability is pH sensitive and that this probably explains the wide range of recorded values.

In contrast to studies on the phospholipid bilayer, data on intact cells or plant tissue are rather limited and, at least for plant cells, almost confined to the Characeae (Bisson & Walker, 1980; Beilby & Bisson, 1992; Beilby, Mimura & Shimmen, 1993; Yao & Bisson, 1993) and the aquatic angiosperms *Elodea* and *Potamogeton* (Miedema & Prins, 1991, 1993; Miedema, Felle & Prins, 1992).

In the present study, we address the question whether purified *Elodea* plasma membrane vesicles show a comparable (pH dependent) H^+-permeability as found in in vivo experiments. Our conclusion is twofold. Firstly, the value of P_{H^+} of the vesicle membrane is much smaller than the value found for the plasma membrane of intact leaf cells. Secondly, P_{H^+} of the vesicle membrane is still strongly pH dependent. This leads to the question how representative the vesicle membrane is for the in vivo situation. Plasma membrane vesicles are widely used to study membrane transport processes coupled to the dissipation or generation of a pH gradient. It is obvious that the results of such measurements can be influenced by a parallel passive pathway for proton movement. The consequences of both findings for the interpretation of vesicle studies are discussed.

Material and Methods

Plant Material

Elodea nuttallii was collected in the surroundings of Haren, the Netherlands. The material was washed with tap water and stored for at least three days in concrete tanks with a layer of clay covered with washed sand and filled with deionized water. Light intensity was 100 μmol/$m^2 \cdot$ sec during the light period of a 12 hr light/12 hr dark regime. Temperature was 18–20°C.

Isolation of Plasma Membrane Vesicles

Right-side out oriented *Elodea* plasma membrane vesicles were isolated according to Elzenga, Staal & Prins (1989) with slight modifications. About 300 g fresh material was washed in tap water and carefully blotted dry with paper towels. The material was transferred into 500 ml extraction buffer containing 0.33 M sucrose, 25 mM BTP (Bis-TrisPropane)-MES pH 7.8, 3.0 mM EDTA, 2.5 mM DTT (DiThioThreitol) and 0.2 mM PMSF (PhenylMethylSulfonyl Fluoride). Homogenation was performed in a modified Braun(TM) homogenizer. The homogenate was filtered over 4 layers of cheese cloth and centrifuged at 10,000 × *g* for 15 min. The supernatant was centrifuged at 30,000 × *g* for 60 min. The resulting pellet was resuspended in 9 ml 0.33 M sucrose, 5 mM K_2HPO_4/KH_2PO_4 pH 7.8. The suspension was added to a phase system with a final composition of 6.2% w/v Dextran T-500, 6.2% w/v PEG 3350, 0.33 M sucrose, 3 mM KCl and 5.0 mM potassium phosphate pH 7.8. The resulting upper phases were pooled and diluted to 150 ml with 0.33 M sucrose, 1 mM EDTA-BTP pH 7.8 and centrifuged for 90 min at 40,000 × *g*. The pellets were resuspended in 0.5 ml of the same buffer. To obtain 50 mM K^+ inside, the vesicles were freeze-thawed in the same buffer supplemented with 50 mM KCl. Protein content was determined according to Bradford with the Bio-Rad protein reagent and with BSA as standard.

Fluorescence Measurement

Fluorescence of acridine orange (excitation and emission at 429 and 525 nm, respectively) was measured in 2 ml solution containing 175 mM mannitol, 14 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% w/v BSA (essentially fatty acid free), 10 mM BTP-MES (pH 8–9) or 10 mM CAPS-NaOH (pH 9.5–11) with or without 50 mM KCl and 5 μM valinomycin (1 mM stock solution in methanol). The fluorescence of acridine orange (AO) itself appeared to be pH dependent. Therefore AO was added to the different solutions in concentrations which resulted in about the same initial fluorescence. The final concentration of AO was 0.115 μM at pH 8 and 0.575 μM at pH 11. A ΔpH was established by adding plasma membrane vesicles having an internal pH of 7.8. During each recording, 7–9 μl of the vesicle suspension, corresponding with 40 μg of protein, was added to the glass cuvette. Fluorescence during formation and breakdown of the ΔpH was measured using a KONTRON · SFM 25(TM) spectrofluorometer.

Calculation of Internal pH, Fluxes and Permeability Coefficients

The pH of the weakly buffered solution inside the vesicles was always lower (initially $pH_i = 7.8$) than the pH of the buffered external medium. Addition at $t = 0$ of the vesicle suspension to the AO containing cuvette resulted in quenching of the fluorescence (F) (Fig. 1). Thereafter F recovered as a result of an H^+ efflux dissipating the applied pH gradient.

The quenching of F rather than the absolute level of F was taken as a measure of ΔpH, defined as pH_o-pH_i. Quenching, Q, was defined as $100 \times (F_\infty - F)/F_\infty$, where F is fluorescence at $t = t$ and F_∞ represents the level of fluorescence after complete dissipation of the ΔpH, that is at $t = \infty$. One might expect no quenching at $\Delta pH = 0$, thus at $t = \infty$. This apparently was not the case. At $t = \infty$, $\Delta pH = 0$ but quenching still occurred. Apparently the mere presence of vesicles caused fluorescence quenching even without a ΔpH. It may be that unspecific adsorption of AO to the outer vesicle surface played a role (Bérci & Möller, 1993). Triton(TM) makes the vesicle membrane leaky and dis-

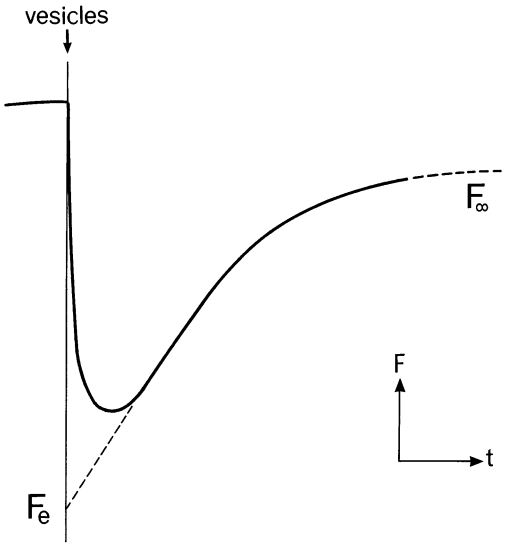

Fig. 1. Schematic representation of the changes in acridine orange (*AO*) fluorescence (*F*) upon addition of the vesicles. First, a rapid decrease of fluorescence or increase of quenching (*Q*) is seen. Depending on the permeability of the membrane to H^+, the applied pH gradient dissipates and a recovery of *F* is observed. From this the flux rate and pH_i are calculated as described in the text. Calculation of pH_i requires a relation between *Q* and ΔpH. To this end the recovery of *F* has been fitted to a single exponential curve (*see* text). Extrapolation to the moment the vesicles were added, yields *Q* as a function of initial, known ΔpH.

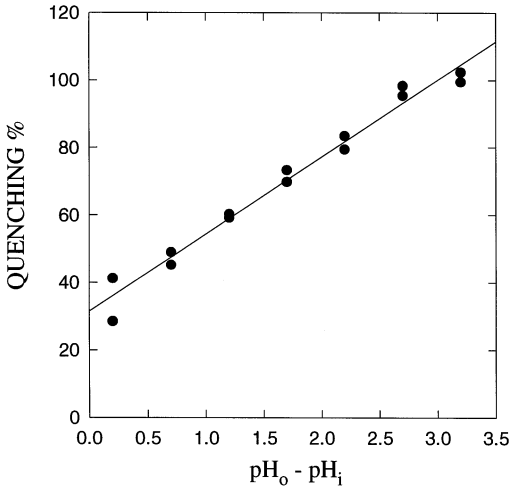

Fig. 2. Relation between ΔpH and *Q* as described in Fig. 1. Each linear fit ($Q = \alpha + \beta\Delta pH$) was based on duplicate experiments on the same membrane preparations. In the experiment shown here the intercept (α) was 22.8 ± 1.0 and the slope (β) 31.6 ± 2.0 and the regression coefficient (*rc*) 0.988. In the other 2 experiments, the respective values were 25.4 ± 1.2 (α), 31.1 ± 2.4 (β) and 0.987 (*rc*), and 25.9 ± 1.5 (α), 24.2 ± 2.9 (β) and 0.981 (*rc*).

sipates the ΔpH. Addition of Triton(TM) before the dissipation of the ΔpH thus resulted in an increase of *F* but never to the original level before the addition of vesicles. This also points to a non-specific, i.e., not dependent on ΔpH, quenching by the vesicles (Fig. 3).

Maximal quenching did not occur at $t = 0$ but was always observed 50 to 100 seconds later (Fig. 1). This apparently was due to the slow equilibration of AO. To obtain the relation between *Q* and ΔpH, and thus between *Q* and pH_i, it is necessary to derive *Q* at $t = 0$ by extrapolation of the quenching curve, assuming that the H^+ efflux starts at $t = 0$. The recovery of fluorescence was fitted using Eq. 1 (Jennings et al., 1988):

$$F = F_\infty - (F_\infty - F_e)e^{-a \cdot t} \tag{1}$$

F_e is the fluorescence calculated by extrapolation to $t = 0$, and corresponds with the initial pH_i (7.8) just after the addition of the vesicles, and ''*a*'' is the rate constant of fluorescence decay, with the dimension t^{-1}.

Similar experiments were done at different pH_os and from the fluorescence data the corresponding *Q*s at $t = 0$ were calculated. When these values were plotted as a function of initial and thus known ΔpH a linear relation was obtained. An example is given in Fig. 2. It may be surprising that the relation between ΔpH and *Q* can be described with a linear fit as at increasing ΔpH one may expect a saturating effect on *Q*. However, assuming that *Q* depends in a non-linear way on ΔpH did not improve the fit significantly (*data not shown*). Apparently within the ΔpH range used here *Q* was effectively directly proportional to ΔpH, and pH_i was calculated by linear interpolation. For each individual experiment, the interpolation was based on the initial, extrapolated value of *F*: F_e (*Q* is maximal and $\Delta pH = pH_o - 7.8$) and the final value of *F* after the total decay of ΔpH:F_∞ ($Q = 0$ and $\Delta pH = 0$). pH_i is then given by:

$$pH_i = pH_o - Q\left(\frac{F_\infty}{F_\infty - F_e}\right)\left(\frac{pH_o - 7.8}{100}\right) = pH_o - \frac{(F_\infty - F)}{(F_\infty - F_e)}(pH_o - 7.8) \tag{2}$$

The proton flux (*J*) is proportional to $\delta pH_i/\delta t$ and the internal buffer capacity (*B*), as shown in equation 3:

$$J = C\frac{\delta pH_i}{\delta t}B \tag{3}$$

The proportional coefficient (*C*) in this equation is a scaling factor, representing the ratio of total vesicle volume and total vesicle membrane area, expressed in m. The relation between total vesicle volume and added protein was estimated 2.5 μl per mg protein and from electron microscopic photographs the vesicle radius 50 nm (Elzenga, Staal & Prins, 1989). This results in a total surface of the vesicles in the assay of 6.10^{-3} m^2.

$\delta pH^+{}_i/\delta t$ was calculated using Eq. 4:

$$\frac{\delta pH_i}{\delta t} = \frac{\delta pH_i}{\delta Q}\frac{\delta Q}{\delta t} \tag{4}$$

where $\delta pH_i/\delta Q$ equals $-100F_\infty(pH_o - 7.8)/(F_\infty - F_e)$ (Eq. 2). $\delta Q/\delta t$ is given by Eq. 5:

$$\frac{\delta Q}{\delta t} = -\frac{100}{F_\infty}\frac{\delta F}{\delta t} = -\frac{100}{F_\infty}a(F_\infty - F_e)\,e^{-at} \tag{5}$$

The buffering capacity *B* (in $mol/pH \cdot m^3$) was obtained from a standard titration curve.

To calculate permeability coefficients from the flux data it is necessary to ascribe the measured flux specifically to H^+ to OH^- or to

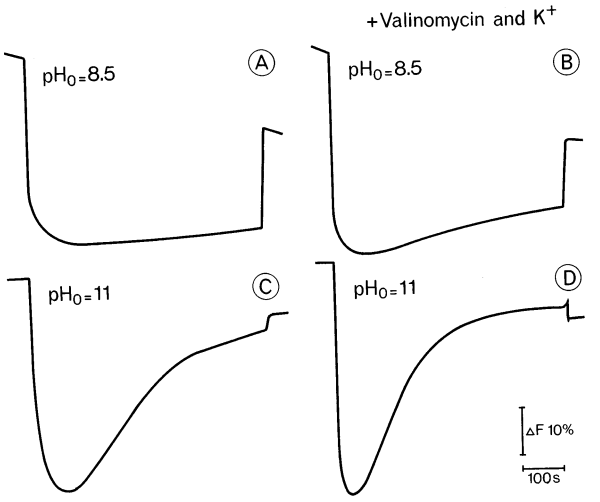

Fig. 3. Fluorescence time courses at $pH_o = 8.5$ and 11, in the absence (*A* and *C*) or presence, inside and outside, of 5 μM Valinomycin and 50 mM K^+ (*B* and *D*). At the end Triton(TM) was added to dissipate the remaining ΔpH. This resulted generally in a further increase of *F*, but never to its original level in the absence of vesicles. In (*D*) addition of Triton(TM) resulted in a slight decrease of *F*. This was probably due to a dilution effect in combination with a ΔpH already close to zero.

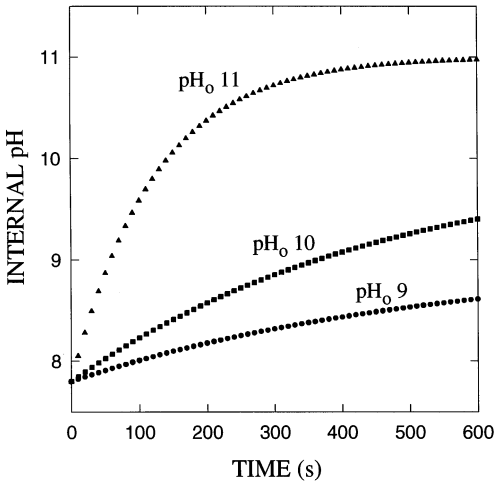

Fig. 4. Time courses of pH_i at $pH_o = 9$, 10 and 11.

a combination of both ion species. The permeability coefficient (*P*) is defined as the ratio between flux (*J*) and driving force. The membrane potential was clamped at zero mV by the addition of 5 μM valinomycin and symmetrical 50 mM K^+. Raising the valinomycin concentration up to 25 μM did not increase the recovery rate showing that 5 μM was enough to clamp the potential at zero. Under these conditions, *J* is given by equation 6:

$$J = J_{H^+} + J_{OH^-} = P_{H^+}(H_i^+ - H_o^+) + P_{OH^-}(OH_o^- - OH_i^-) \quad (6)$$

For $P_{OH^-} = 0$ this equation changes into 7:

$$J = J_{H^+} = P_{H^+}(H_o^+ - H_i^+) \quad (7)$$

While the value found for the permeability depends on the assumption of what ion carries the flux, H^+ or OH^-, the value found for the conductance does not (*see* Appendix). The conductance, *G*, is given by the current carried by *J* divided by the driving force, $E - E_{H^+}$, where *E* is the membrane potential and E_{H^+} the equilibrium or Nernst potential for H^+. The current is given by the product of *J* and *F*, the Faraday number and as *E* is clamped at zero mV, *G* is given by Eq. 8:

$$G = \frac{JF}{-E_H} \quad (8)$$

Results

The data presented here are based on duplicate measurements on three different batches of plasma membrane vesicles. Fluorescence measurements were performed at external pH_os, varying from 8.0 to 11.0, in steps of 0.5 pH units. The initial pH_i was always 7.8. Examples of original fluorescence recordings are shown in Fig. 3, for pH_o = 8.5 (*A* and *B*) and $pH_o = 11$ (*C* and *D*) and in the absence (*A* and *C*) and presence of valinomycin and K^+ (*B* and *D*). Two things are obvious: the rate of fluorescence recovery is higher at high external pH, and the presence of valinomycin and K^+ increases the rate of fluorescence recovery. In the absence of K^+, valinomycin had no such effect, indicating that valinomycin itself does not introduce a significant permeability for H^+ or OH^+. The combined effects of valinomycin and K^+ also demonstrate the tightness of the vesicles. All further calculations described below are based on data obtained in the presence of valinomycin and K^+.

Figure 4 shows the course of pH_i in time at a pH_o of respectively 9, 10 and 11, while the calculated fluxes at an external pH of 10, 10.5 and 11 are given in Fig. 5. Between pH_o 8.5 and 10.0, not shown here, the fluxes decreased monotonously in time. At more alkaline pH, a shoulder ($pH_o = 10.5$) or even a temporal increase ($pH_o = 11$) of the flux was observed. As only pH_i changed while pH_o remained constant during the course of a single experiment, this shows an effect of pH_i on *P*. Apparently the pH_i induced increase of P_{H^+} caused a temporary increase of J_{H^+}, despite the declining driving force (ΔpH). Figure 6 shows the calculated P_{H^+} and P_{OH^-} as a function of pH_i, at a pH_o of 8.5 (*A*) and 11 (*B*), assuming that the flux is the result of the exclusive movement of either H^+ or OH^-. At a pH_o of 8.5 P_{H^+} increased about 50%, from 42 to 65 × 10^{-8} m/sec, when pH_i increased from 7.8 to 8.5. On the other hand, if the flux was entirely ascribed to OH^-, P_{OH^-} slightly declined from 22 to 17 × 10^{-10} m/sec under these conditions. At a pH_o of 11 the calculated changes in *P* were more drastic. As pH_i increased further to 11, P_{H^+} increased more than four orders of magnitude to 10^{-1} m/sec. As was seen at pH_o 8.5, compared to the sensitivity of P_{H^+} to pH_i, the value of P_{OH^-} appeared to be relatively constant over the entire range of pH_i and was in the order of 10^{-10} to 10^{-9} m/sec.

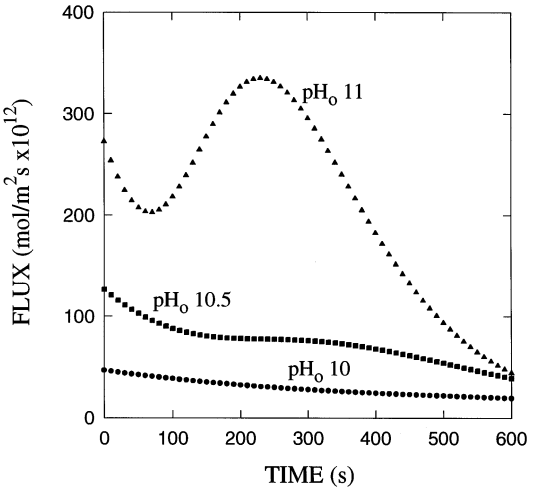

Fig. 5. Examples of calculated fluxes (J) at pH_o = 10, 10.5 and 11.0. Note the shoulder at pH_o 10.5 and the temporary raise of J at pH_o 11.0. As the driving force (ΔpH) declines in time, this increase reflects an increase of P. Fluxes are expressed in mol/m^2 · sec.

The values calculated for G are not sensitive for the choice of either H^+ or OH^- (*see* Appendix). The values obtained for G at pH_o = 8.5 and 11 are shown in Fig. 7*A* and *B*, respectively. At pH_o 8.5, G showed an almost constant value of 13×10^{-6} S/m^2 over the whole range of pH_i from 7.8 to 8.5. At pH_o 11, G was rather constant below pH_i 9.5, but showed a tenfold increase at more alkaline pH, from 3×10^{-4} for pH_i = 9.5 to 31×10^{-4} S/m^2 for pH_i = 11.

While P_{H^+} and P_{OH^-} are affected by pH_i, pH_o alone also has a direct effect on P, as can be seen in Fig. 8. Here we calculated P_{H^+}, P_{OH^-} and G for pH_i = 8.0 and for pH_o ranging from 8.5 till 11.0. A value of 8 was chosen for pH_i as it comes close to a typical cytosolic pH under conditions of a rather alkaline external pH (Reid & Smith, 1988). Over the pH_o range 8.5–11, P_{H^+} increased form 2 to 26×10^{-6} m/sec (Fig. 8*A*), while P_{OH^-} decreased by about the same factor, from 22 to 3×10^{-10} m/sec (Fig. 8*B*). The calculated value of G (Fig. 8*C*) is almost insensitive to pH_o below 10. However, a three- to fourfold increase of G is observed during the increase of pH_o from 10 to 11, resulting in a conductance of 15×10^{-5} S/m^2 for pH_o = 11.

The assumption that either H^+ or OH^- is transported probably is a simplification. It may well be that both ion species participate in the total flux. This would imply that changes in the calculated values for P are apparent, i.e., reflecting a shift in the contribution of each ion species to the total flux. Figure 9*A*, *B* and *C* shows the flux data at respectively pH_o = 8, 9 and 10 fitted to Eq. 6, assuming that both ions contribute (P_{H^+} and $P_{OH^-} > 0$) and that both permeabilities are pH insensitive. For comparison the curves obtained while assuming that only one ion species carries the flux (P_{H^+} or P_{OH^-} = 0), are also given. It is clear that the observed data are better described assuming a combined flux of H^+ and OH^- instead of a flux carried exclusively by H^+ or OH^-. The calculated permeabilities were $P_{H^+} = 1.4 \times 10^{-7}$ and $P_{OH^-} = 2.5 \times 10^{-9}$ for pH_o = 8, $P_{H^+} = 6.5 \times 10^{-7}$ and $P_{OH^-} = 9.8 \times 10^{-10}$ for pH_o = 9, and for pH_o = 10 $P_{H^+} = 1.5 \times 10^{-6}$ and $P_{OH^-} = 2.8 \times 10^{-10}$. However, it is clear that it is still not possible to fit the data accurately. Only at pH_o 8, Eq. 6 seems to be able to describe the data. This, however, reflects the very limited pH_i range (7.8 to 8.0) rather than an actual independence of P on pH. Apparently, the observed fluxes can only be described assuming a pH-dependent proton and/or hydroxyl permeability of the vesicle membrane.

Discussion

The correlation between a transmembrane ΔpH and fluorescence quenching of AO, and other amino acridines, is well documented. Nevertheless there is no consensus regarding the precise nature of the underlying mechanism. One view is that there is an accumulation of AO in the acidic compartment, implying actual passage of the membrane by AO (Palmgren, 1991). Alternatively, it has been proposed that quenching results form electrostatic interactions between the positively charged AO and negative surface charges (Kraayenhof, 1977, 1980; Kraayenhof, Sterk & Wong Fong Sang, 1993; Bérci & Möller, 1993). Binding of AO to the membrane surface might also lead to ΔpH-independent fluorescence quenching. Because of this it has been argued that AO fluorescence quenching can only be used for qualitative studies (Rottenberg & Moreno-Sanchez, 1993). The present results show that despite the uncertainties regarding the underlying mechanism, AO fluorescence quenching can be used for precise quantitative measurements, provided some mathematical transformations are applied. Calculations of the ΔpH should be based on an interpolation of the fluorescence signal between its two extreme values at zero ($t = \infty$) and initial ΔpH (= maximal at t = 0).

Proton fluxes observed in vivo with intact leaves of the aquatic angiosperm *Potamogeton* were in the order of 1 μmol/m^2 · sec (Miedema & Prins, 1992), three to four orders of magnitude higher than the flux rates reported here. The cause of this is unclear. In intact *Chara* cells P_{H^+}, 10 m/sec, and G, 1–10 S/m^2, (Beilby & Bisson, 1992) were also five to seven orders of magnitude higher than values reported for the phospholipid bilayer from liposomes ($P = 10^{-6}$ m/sec) or planar lipid bilayers ($G = 10^{-5}$ to 10^{-4} S/m^2). This indicates an H^+ permeability inherent to the phospholipid bilayer plus an additional H^+ permeability in the intact *Chara* plasma membrane. In *Elodea* it may also be that conductances for H^+ or OH^-, or activating factors thereof, normally present in the intact plasma membrane, are (partly) lost during iso-

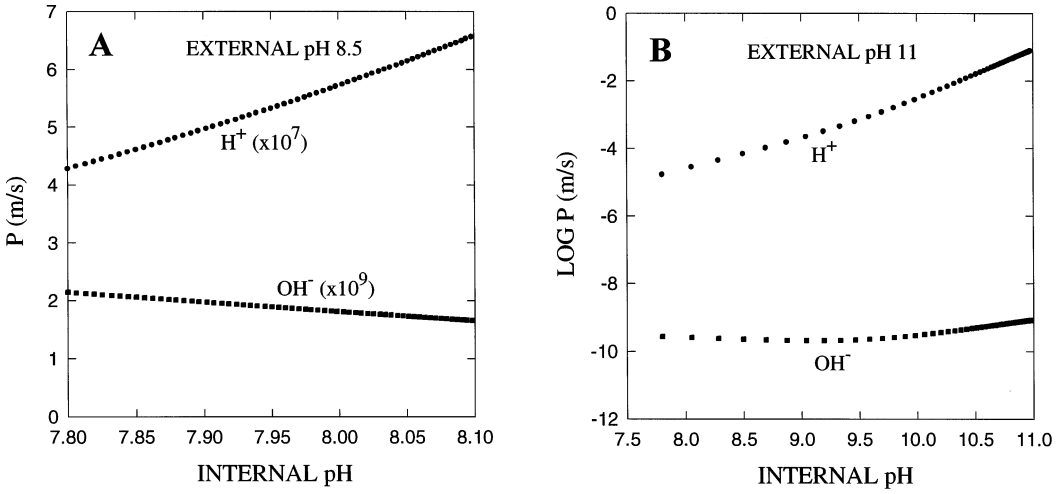

Fig. 6. Calculated permeability for $pH_o = 8.5$ (*A*) and (*B*), assuming that the flux results of either H^+ or OH^- movement. At $pH_o = 8.5$, P_{H^+} increased with increasing pH_i from 42 to 65×10^{-8} m/sec, while P_{OH^-} remained constant, circa 20×10^{-10} m/sec. At $pH_o = 11.0$, the pH-dependence of P_{H^+} was more pronounced, four orders of magnitude with a final value of 10^{-1} m/sec, and is therefore plotted logarithmically. P_{OH^-} was again much less affected and was circa 10^{-10} m/sec.

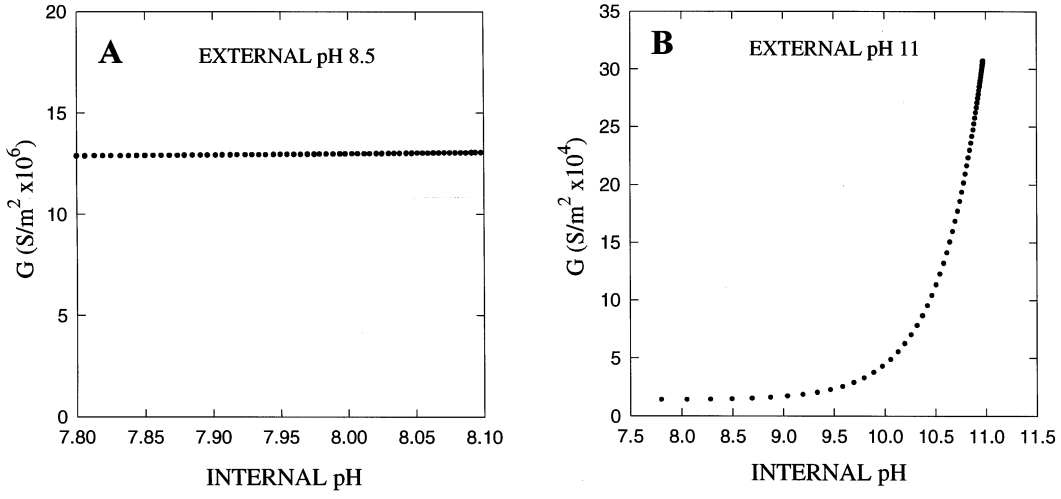

Fig. 7. Conductivities calculated from the data given in Fig. 6. Conductances were calculated according to Eq. 8. At $pH_o = 8.5$ (*A*), *G* was almost constant, 13×10^{-6} *S*/m^2. At $pH_o = 11$ (*B*), a very significant exponential increase in *G* was seen at pH_is higher than pH 9.5. Under symmetrical conditions, $pH_i = pH_o = 11$, *G* reached its highest value in the present experiments: 30×10^{-4} S/m^2.

lation of the vesicles. This agrees with conclusions from perfusion studies on *Nitellopsis* (Beilby et al., 1993) and *Chara* cells (Lucas & Shimmen (1981). In these cells the H^+-state was lost by this perfusion. In whole-cell patch-clamp experiments with *Elodea* protoplasts the chord conductance was about 3×10^{-2} S/m^2 at zero electrical potential, in symmetrical K^+ and in the absence of ATP (Miedema, 1992). This value is much lower than generally associated with the H^+-state in intact cells and comes close to the value found for the perfused *Nitellopsis obtusa* cells. The protoplast in a whole-cell patch-clamp experiment is comparable with a perfused Characean internodal cell as the cytosol exchanges with the pipette content. Washout of a conductance activating factor is well known in patch-clamp experiments. This leads to the question if the observed effect of pH on permeability and conductance has consequences for patch-clamp whole cell or single channel measurements. In plant cells a low estimate of the whole cell pump conductance is around 10^{-2}/m^2 (*see* e.g., Becker et al., 1993) Even this low estimated value is still ten times higher than the maximum conductance of 31×10^{-4} S/m^2 at symmetrical pH 11 calculated from the present data. Thus under most conditions the contribution of the here described H^+/OH^- conductance may probably be ignored in whole cell patch clamp experiments. Based on the

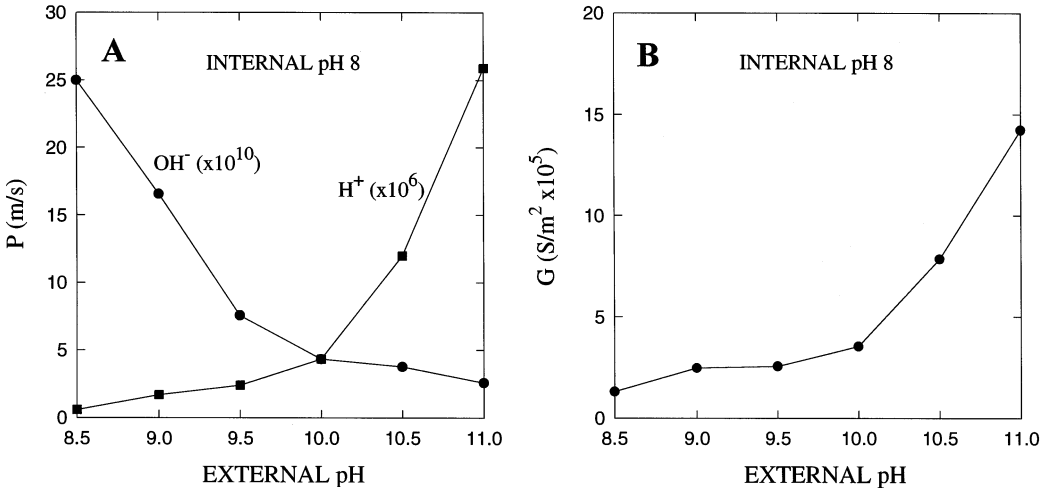

Fig. 8. Permeability coefficients (*A*) for H^+ and OH^- and conductance (*B*) at constant pH_i of 8 and varying pH_o. From pH_o = 8.5 to 11.0 P_{H^+} increased tenfold to 26 × 10^{-6} m/sec, while P_{OH^-} decreased to 3 × 10^{-10} m/sec. For pH_o < 10, *G* was rather insensitive to pH_o, circa 3 × 10^{-5} S/m^2. From pH_o = 10 to 11 *G* increased about fourfold, from 4 to 15 × 10^{-5} S/m^2.

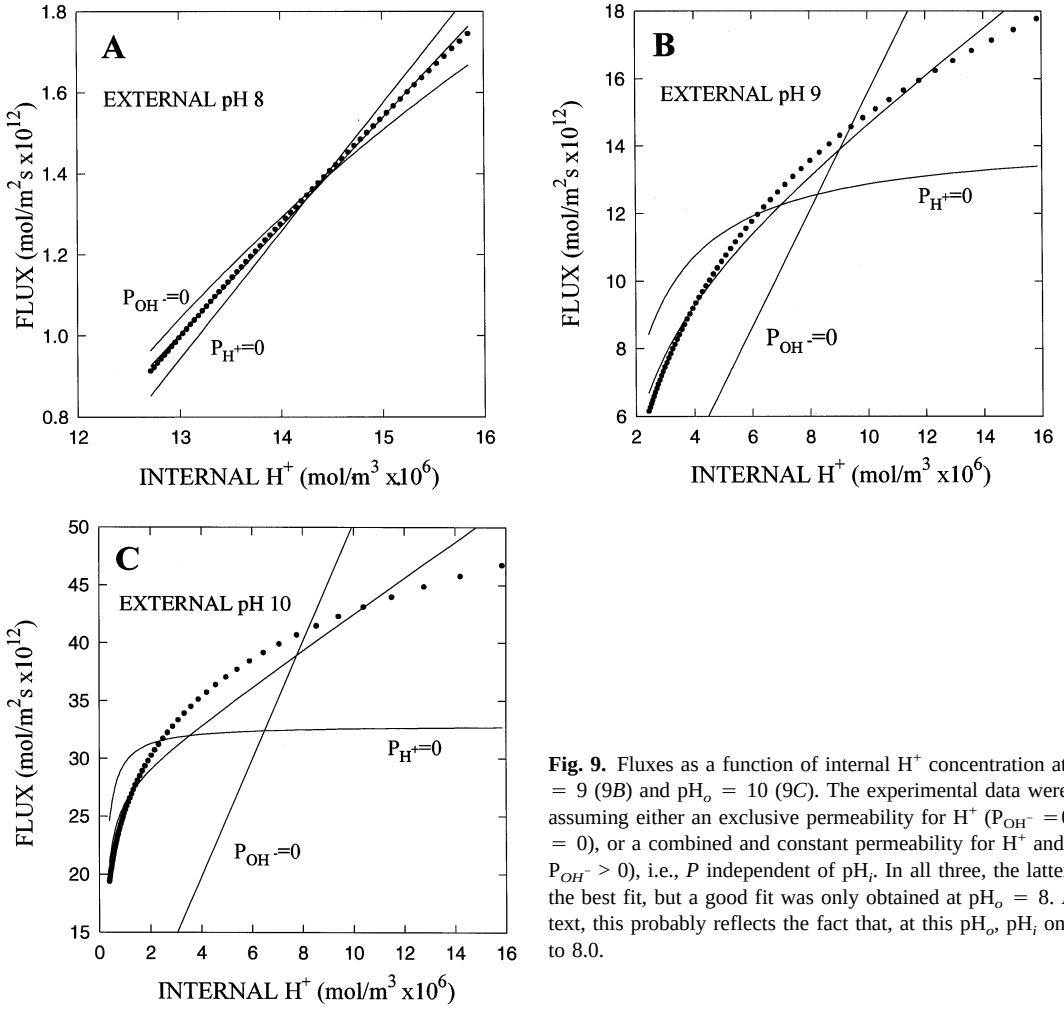

Fig. 9. Fluxes as a function of internal H^+ concentration at pH_o = 8 (9*A*), pH_o = 9 (9*B*) and pH_o = 10 (9*C*). The experimental data were fitted to Eq. 6, assuming either an exclusive permeability for H^+ (P_{OH^-} = 0) or for OH^- (P_{H^+} = 0), or a combined and constant permeability for H^+ and OH^- (P_{H^+} > 0 and P_{OH^-} > 0), i.e., *P* independent of pH_i. In all three, the latter conditions rendered the best fit, but a good fit was only obtained at pH_o = 8. As discussed in the text, this probably reflects the fact that, at this pH_o, pH_i only changed from 7.8 to 8.0.

value of 31×10^{-4} S/m^2 and assuming a membrane surface for an isolated patch of 25 μm^2 the contribution to the total conductance of the patch would only be around 10^{-4} pS and thus again insignificant as compared to normal single channel conductances.

If it is assumed that in the vesicles all H^+ or OH^- conducting activity involved in the H^+-state is lost, the observed conductance changes probably reflect a conductive pathway that is a property of the bilayer itself. P_{H^+} and G calculated for the *Elodea* vesicles are comparable with values reported for liposomes, $P = 10^{-6}$ m/sec, around neutral pH (Nichols & Deamer, 1980, Nichols et al., 1980) and even more with measurements on planar lipid bilayers, $G = 10^{-5}$–10^{-4} S/m^2 at pH 1.6 and 10.5, respectively (Gutknecht, 1984). In the literature, we could find only one cell type showing a P_{H^+} value of the plasma membrane comparable to the *Elodea* vesicles or liposomes. P_{H^+} of the cell membrane of *Dunaliella acidophila* under conditions for optimal growth, i.e., pH_o 1, has been estimated to be 10^{-9} m/sec (Remis, Treffny & Gimmler, 1994).

From Fig. 9, it is obvious that a combined permeability of H^+ and OH^- using pH-insensitive values of P_{H^+} and P_{OH^-} does not suffice to describe the observed flux data adequately. An accurate fit of the data apparently requires P to be dependent on pH. Therefore, we studied the effect of introducing a pH dependent P_{H^+} and P_{OH^-} in Eq. 6. For this, it was necessary to obtain a relation between pH and both permeabilities. It was supposed that protonation of a putative H^+ or OH^- transporter T determines the value of P. The Henderson-Hasselbach relation gives the ratio of protonated (HT) and dissociated transporter (T^-) as a function of pH and pK of the protonation site: $HT/T^- = 10^{pK-pH}$. Furthermore we assumed that P was proportional to either HT/T^- or T^-/HT. However, this procedure did not result in a better fit of the experimental data (*results not shown*).

Whatever the exact mechanism of the pH dependent H^+ permeability, it is clear that pH induced proton permeability changes occur in isolated plasma membrane vesicles and that these changes affect proton fluxes. Reversibly, as the permeability is pH sensitive, proton fluxes affect the permeability by changing the pH, resulting in a positive feedback mechanism.

The present results can have consequences for transport studies using similar vesicle systems, with regard to the pH-sensitive proton permeability. Inside-out vesicles are frequently used to monitor H^+-ATPase activity. As long as the initial rate of quenching is used, as is usually done in these experiments, the results will probably not be affected, provided the initial internal pH equals the outside pH (Bennett & Spanswick, 1983). This may be different for studies based on the dissipation of a preexisting transmembrane H^+ gradient (Barkla, Zingarelli, Blumwald & Smith, 1995). Here there is the possibility of pH-induced changes in P_{H^+} of the plasma membrane (and tonoplast membrane?).

The present data also question the assumption that the vesicle membrane is representative for the plasma membrane in vivo. The *Elodea* plasma membrane vesicles were prepared in a way similar to those used by others from different tissue (Palmgren, 1991; Staal et al., 1991). Future research should establish whether discrepancies in behavior between the intact and the vesicle membrane observed here with *Elodea* also occur in other tissues of other species.

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Appendix

Relation between P_{H^+}, P_{OH^-} and G

In vesicles with an acidic interior and in the absence of an electric potential across the membrane, the ratio of P_{H^+} and P_{OH^-} is given by:

$$\frac{P_{OH^-}}{P_{H^+}} = \frac{H_i^+ - H_o^+}{OH_o^- - OH_i^-} = \frac{H_o^+ H_i^+}{K_w} \tag{9}$$

or, expressed in terms of pH_o and pH_i:

$$\frac{P_{OH^-}}{P_{H^+}} = 10^{14-pH_o-pH_i} \tag{10}$$

This equation implies that both permeability coefficients are identical only under the restriction of $pH_o + pH_i = 14$. As can be seen from Eqs. 6 and 8:

$$G \sim P_{H^+}(H_i - H_o) + P_{OH^-}(OH_o - OH_i) \tag{11}$$

Using the relation between P_{H^+} and P_{OH^-} (Eq. 9) and writing $OH^- = K_w/H^+$ results in expression 12:

$$G \sim P_{H^+}(H_o^+ - H_i^+) + P_{H^+}(H_o^+ - H_i^+) = 2P_{H^+}(H_o^+ - H_i^+) \tag{12}$$

This expression reflects the fact that H^+ and OH^- equally contribute to the total conductance, irrespective of the ratio of their permeability coefficients.