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Kinetic Response of H^+-Coupled Transport to Extracellular pH: Critical Role of Cytosolic pH as a Regulator

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Summary. H^+-coupled transport in plant and fungal cells is relatively insensitive to external pH (pH_o). H^+-coupled Cl^- transport at the plasma membrane of *Chara corallina* was studied to explore the phenomena responsible for this insensitivity. Raising pH_o from a control value of 7.5 to 9.0 results in a modest (2.5-fold) decline in J_{max} and increase in K_m. Further increase in pH_o results in a selective increase in J_{max}, in accordance with predictions from a reaction kinetic model of the transport system (Sanders, D., Hansen, U.-P., 1981. *J. Membrane Biol.* **58**:139–153). Increase in cytosolic Cl^- concentration ($[Cl^-]_c$) also results in a selective decrease in J_{max} at $pH_o = 7.5$.

Quantitative kinetic modeling of the results is not possible if it is assumed that the sole effect of pH_o is *via* mass action on the binding of external H^+ to a transport site. If, instead, the dependence of cytosolic pH (pH_c) on pH_o (Smith, F.A., 1984, *J. Exp. Bot.* **35**:1525–1536) is taken into account along with the dependence of Cl^- influx on pH_c (Sanders, D., 1980, *J. Membrane Biol.* **53**:129–141), then the observed modest changes in Michaelis parameters can be accommodated by a reaction kinetic model. The quantitative parameters of the model yield respective pK_as of the internal and external H^+-binding sites = 7.85 and 7.2, respective dissociation constants of the internal and external Cl^--binding sites = 160 and 40 μM, and an additional, kinetically transparent, H^+-binding site with a $pK_a > 8.0$. The quantitative model independently predicts the response of J_{max} and K_m to acidic conditions.

The results are discussed in terms of the general physiological requirement that fluxes through H^+-coupled transport systems are relatively insensitive to environmental variation in pH_o. It is proposed that (*i*) the weak (but finite) dependence of pH_c on pH_o, coupled with (*ii*) the strong dependence of H^+-coupled transport on pH_c are instrumental in endowing H^+-coupled transport systems with a relative insensitivity to variation in pH_o. This hypothesis might also explain why pH_c in plants and fungi is not acutely controlled with respect to variation of pH_o.

Key Words *Chara* · Cl^- · cotransport · reaction kinetic model · pH · kinetics

Introduction

Transport of a wide range of solutes across the plasma membrane of plant and fungal cells is energized via coupling to the electrochemical gradient of protons. Solutes for which gradient-coupled transport has been demonstrated include mineral ions such as Cl^- (Sanders, 1980*c*; Beilby & Walker, 1981), NO_3^- (Novacky et al., 1978; Ullrich & Novacky, 1981), SO_4^{2-} (Lass & Ullrich-Eberius, 1984), $H_2PO_4^-$ (Ullrich-Eberius et al., 1981; Ullrich-Eberius, Novacky & Bel, 1984) and K^+ (Rodriguez-Navarro, Blatt & Slayman, 1986), as well as sugars (Komor & Tanner, 1974; Slayman & Slayman, 1974; Hutchings, 1978) and amino acids (Novacky et al., 1978; Felle, 1981; Eddy, 1982; Sanders, Slayman & Pall, 1983). Unlike animal cells, for which a reasonably constant supply of Na^+ is normally available for gradient-coupled transport, plant cells live in environments that exhibit large temporal and spatial variation in the supply of H^+. Many algae, for example, live in lakes with pHs as high as 10 but are also capable of growth at neutral or even acidic pH (Hutchinson, 1975).

Potentially, growth at high pH poses a thermodynamic problem: how can sufficient energy be maintained in the protonic driving force to result in accumulation of the solute? This problem is rather easily solved if the transport system possesses a sufficiently high H^+ : solute transport stoichiometry. Effectively, the high stoichiometry results in energization of transport by the membrane potential, which in plant and fungal cells is maintained at values at least as negative as −150 mV. Thus, all H^+-coupled solute transport systems—including those for anions—examined to date possess a H^+ : solute stoichiometry consistent with net movement of *positive* charge into the cell.

Nevertheless, there remains a kinetic problem associated with H^+-coupled transport over a wide range of pH. This concerns the ability of the transport system to sustain physiological rates of transport even in conditions where supply of H^+ is limited. One trivial solution to this problem would be for a transport system to possess a very low K_m for

H^+. In other words, the pK_a of the H^+-binding site(s) is poised at a level high enough to ensure that a significant fraction of sites is occupied with H^+ even at the highest pH. However, this solution has severe kinetic consequences of its own. Consider, for example, a transport system with a proton-binding site with a $pK_a = 9.5$. When operating at an external pH equal to the cytosolic pH—say, 7.5—the principle of microscopic reversibility dictates that the highly exergonic reaction associated with H^+ binding externally must be countered by an equally large endergonic step elsewhere in the reaction cycle. The net effect would be to restrict the distribution of carrier primarily to a limited number of states, thereby inhibiting carrier recycling and severely compromising kinetic competence.

This argument is supported by observations on the response of H^+-coupled transport systems to pH_o (Schwab & Komor, 1978; Sanders, 1980*c*). Although influx declines as pH_o is raised, the dependence is less than first-order with respect to external $[H^+]$. Thus, influx is maintained over a pH_o range greater than predicted from sole consideration of proton binding to a transport site.

One simple explanation, which might account for the rather broad pH optimum of H^+-coupled transport systems is that small increases in cytosolic pH (pH_c) offset inhibitory effects of raised pH_o. Indeed, preliminary reaction kinetic analysis of the behavior of H^+-glucose symport in *Chlorella* revealed that it is possible to model the rather broad pH optimum of the transport system if small changes in pH_c are assumed (Sanders et al., 1984). One aim of the present work is to test and extend this hypothesis on the H^+-Cl^- symporter of *Chara*, for which there are now reliable data on the dependence both of pH_c on pH_o (Smith, 1984*a*;*b*) and of Cl^- influx on pH_c (Sanders, 1980*c*).

The stoichiometry of H^+-Cl^- symport in *Chara* is $2H^+ : Cl^-$ (Sanders, 1980*a*; Beilby & Walker, 1981). Internally perfused (tonoplast-free) internodal cells of this genus have been employed to investigate the kinetic effects of changes in internal (equals cytosolic) concentrations of both the ionic substrates of the transport system (Sanders, 1980*a*; Sanders & Hansen, 1981). The results have been analyzed in terms of a reaction kinetic model for transport in which Cl^- binds first in strictly ordered fashion to the carrier externally, and dissociates from the carrier before H^+ internally (Sanders & Hansen, 1981; Sanders et al., 1984). None of the other three permutations of ligand-binding order is able to account for the kinetic response of transport to variation in internal ligand concentration. Furthermore, it was concluded that, in order to replicate the kinetic effects of internal pH and Cl^-, translocation of (positive) charge must occur on the form of the carrier loaded with ligands. However, the effects of pH_o on Cl^- transport kinetics have not previously been studied, and therefore, another aim of the present work was to test the ability of the reaction kinetic model to describe the kinetic response to pH_o.

Materials and Methods

Experimental

Chara corallina was rooted in river mud and grown in 60 liter tanks at room temperature in a medium initially consisting of (concentrations in mM): NaCl 1; K_2SO_4 0.2; $CaCl_2$ 0.1; HEPES-NaOH 5; pH 7.5. Illumination was provided by one 15 W "Warmwhite" fluorescent tube, and supplemented with blue light provided by a 15 W "Gro-Lux" tube. The light regime was 16 hr light/8 hr dark. The day prior to an experiment, internodal cells 3.5 to 5.5 cm in length were excised from their neighbors and bathed overnight, under illumination, either in Cl^--free medium (S-APW, composition (in mM): Na_2SO_4 0.5; K_2SO_4 0.2; $CaSO_4$ 0.5; HEPES-NaOH 5; pH 7.5), or in Cl^--containing medium (C-APW, with 1 mM NaCl replacing Na_2SO_4 in S-APW).

Cl^- influx was measured as described previously (Sanders, 1980*b*). Briefly, cells were loosely tied into batches of 10 and placed in 15-ml test tubes containing $^{36}Cl^-$ (specific activity: 1.6 GBq/mol) at the appropriate concentration. Influx solutions were buffered with HEPES (pH 7.5), TAPS (pH 8.5) or CHES (pH 9.0 and 9.5). After 10 min, the cells were removed and washed briefly in C-APW. The 10-min influx period is not of sufficient length to result in significant time-dependent changes in influx either as Cl^- fills the cytosol (cells pretreated in S-APW) or empties from it (cells pretreated in C-APW, with influx measured at low external Cl^- concentration) (Sanders, 1980*a*). Dimensions of individual cells were measured and their nodes removed before placing each one in a separate scintillation vial together with 2 ml Optiphase. Counts were recorded on an LKB 1216 RackBeta scintillation counter and quenching (maximum, 20%) was corrected for by the channels ratio method.

Preliminary experiments were performed to investigate the possibility that this batch method led to underestimation of Cl^- influx as a result of the formation of unstirred layers at low external Cl^- concentration. Cl^- influx was compared in cells suspended individually in well-stirred influx medium (five replicates at each Cl^- concentration), and in batches of 10 cells loosely tied together in stagnant medium. No significant difference between Cl^- influx for the two treatments was detected, even at the lowest concentration.

Analysis of Results

Mean influx ± SEM was calculated for each batch of 10 cells. All experiments were performed at least twice. Michaelis-Menten relationships were fitted by a nonlinear least squares algorithm (Marquardt, 1963) on an IBM-XT microcomputer.

The reaction kinetic model previously used to describe the kinetic properties of H^+-Cl^- symport in *Chara* (Sanders & Hansen, 1981; Sanders, Smith & Walker, 1985) is shown in Fig. 1. Carrier states are numbered 1 through 6, and the rate constant for the unidirectional reaction from State *i* to State *j* is designated k_{ij}. The law of mass action is assumed to apply with respect to the effects of changes in ligand concentration: variation in ligand concentration is reflected in a directly proportional change in the appropriate rate constant (e.g., k_{31} is proportional to the cyto-

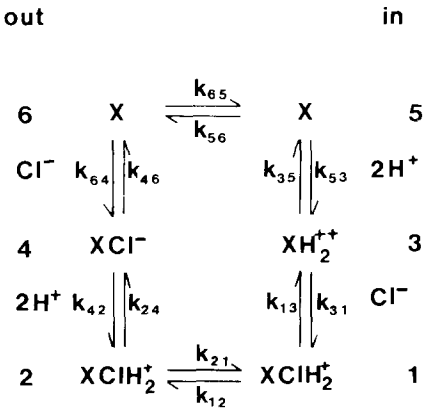

Fig. 1. First-on-first-off model for H^+-coupled Cl^- transport across the plasma membrane of *Chara* (Sanders & Hansen, 1981). Carrier is designated *X*. Individual rate constants are labeled "*k*", with subscripts indicating the numbers of the carrier states between which they operate

solic Cl^- concentration ($[Cl^-]_c$)). Rate constants from which ligand concentration has been extracted are designated k^o_{ij} (thus $k_{31} = k^o_{31}[Cl^-]_c$, etc.). In accordance with previous analysis (Sanders & Hansen, 1981; Sanders et al., 1984), and with data demonstrating very limited effects of prolonged voltage clamping on Cl^- influx (Beilby & Walker, 1981), the effects of transmembrane electrical potential on Cl^- transport are assumed to be saturating. In common with all models exhibiting a single transmembrane pathway for solute and strictly ordered ligand binding, the model in Fig. 1 obeys Michaelis-Menten kinetics for initial rate determinations of flux. The Michaelian parameters are defined in terms of the unidirectional rate constants (Sanders et al., 1984) as

$$J_{max} = N \times \frac{k_{42}k_{13}k_{35}k_{56}}{DEN} \quad (1)$$

and

$$K_m = \frac{(k_{42} + k_{46})[k_{53}k_{65}(k_{13} + k_{31}) + k_{13}k_{35}(k_{56} + k_{65})]}{k^o_{64}(DEN)}, \quad (2)$$

with

$$DEN = k_{42}[(k_{13} + k_{31})(k_{53} + k_{56}) + k_{35}(k_{13} + k_{56})] + k_{13}k_{35}k_{56}, \quad (3)$$

and N equal to the total density of carriers (units: nmol/m²). These equations were used as the basis for modeling the transport kinetic data, with ligand concentrations being made explicit where appropriate.

Results

Effects of pH_o and $[Cl^-]_c$ on Kinetics of Cl^- Influx

The effect of raised pH_o on Cl^- influx in cells pretreated in S-APW is compared in Figs. 2 and 3. At pH 7.5, the K_m is 39 μM,which is in excellent agreement with previous determinations (38 to 41 μM) made at pH_o = 5.5 on internally perfused cells (Sanders & Hansen, 1981). As pH_o is raised to 9.0, however, the K_m increases by a factor of 2.5, togther with a 2.5-fold *decrease* in J_{max}.

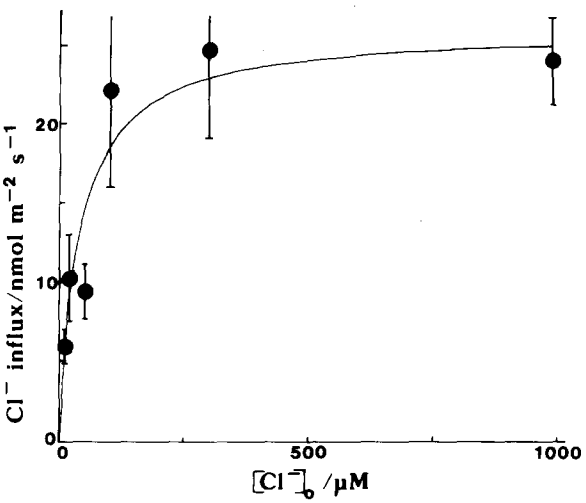

Fig. 2. Kinetics of Cl^- influx at pH_o = 7.5 in cells pretreated in S-APW (Cl^--free medium). Each point is the mean ± SE for a batch of 10 cells, with the exception of the value at 1000 μM Cl^- (20 cells). Solid line shows the fit to a Michaelis-Menten relationship, with the parameter values derived by least squares as follows: J_{max} = 25.8 ± 2.0 nmol m^{-2} sec^{-1}; K_m = 39.1 ± 12.9 μM

In principle, any given reaction kinetic model for H^+-coupled transport can exhibit a range of possible kinetic responses to pH_o, depending simply on the size-ordering of the reaction constants (Sanders et al., 1984). In the particular case of the first-on-first-off model (Fig. 1), the size-ordering of reaction constants implied by the observed decrease in J_{max} and increase in K_m as $[H^+]_o$ is lowered can be derived from Eqs. (1) to (3) as:

$$k_{13}k_{35}k_{56} \simeq [H^+]_o k^o_{42}[(k_{13} + k_{31})(k_{53} + k_{56}) + k_{35}(k_{13} + k_{56})] \quad (4)$$

and

$$k_{46} > [H^+]_o k^o_{42}. \quad (5)$$

It follows then, that as $[H^+]_o$ is lowered still further, the approximate equality in Eq. (4) will no longer hold. Inspection of Eqs. (1) to (3) shows that, with the condition

$$k_{13}k_{35}k_{56} > [H^+]_o k^o_{42}[(k_{13} + k_{31})(k_{53} + k_{56}) + k_{35}(k_{13} + k_{56})] \quad (6)$$

replacing Eq. (4), further decrease in $[H^+]_o$ should result in a selective effect on J_{max}, with no response from the K_m.

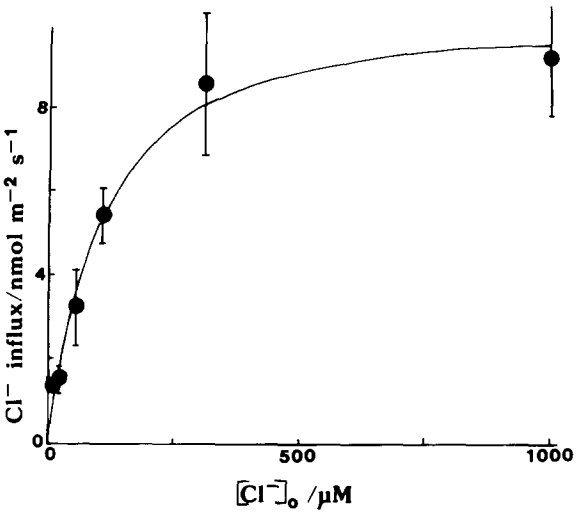

Fig. 3. Kinetics of Cl^- influx into Cl^--starved cells at $pH_o = 9.0$. Other details are as in Fig. 2. Michaelian parameters: $J_{max} = 10.6 \pm 0.5$ nmol m^{-2} sec^{-1}; $K_m = 98.1 \pm 14.0$ μM

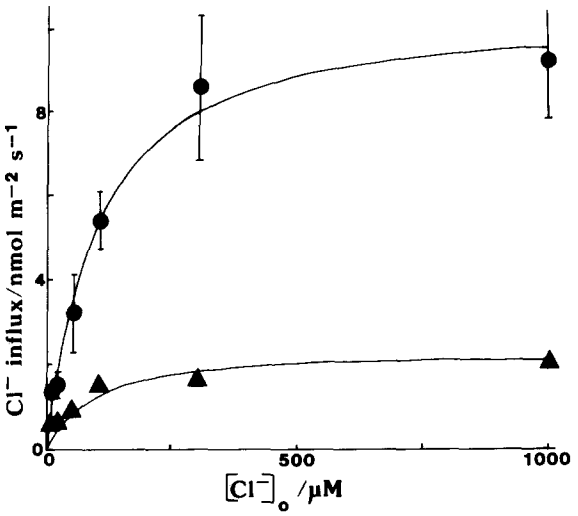

Fig. 4. Comparison of Cl^- influx kinetics between cells pretreated in S-APW and measured at $pH_o = 9.0$ (data of Fig. 3, (●)) and $pH_o = 9.5$ (▲). Data were jointly fitted to a common (least squares) value of K_m, but different values of J_{max}. Michaelian parameters: J_{max} ($pH_o = 9.0$) $= 10.5 \pm 0.4$ nmol m^{-2} sec^{-1}; J_{max} ($pH_o = 9.5$) $= 2.4 \pm 0.2$ nmol m^{-2} sec^{-1}; common $K_m = 95.7 \pm 10.4$ μM

The experimental results confirm this prediction: raising pH_o to 9.5 results in no significant change in K_m beyond that observed at pH 9.0, although J_{max} falls by a factor of 4.4. These results are displayed in Fig. 4, where the imposed constraint that the rise in pH_o to 9.5 does not change K_m clearly results in visually reasonable fits.

Cytosolic Cl^- concentration ($[Cl^-]_c$) can be increased in intact cells by pre-exposure of cells to C-APW (Sanders, 1980*a*; Beilby, 1981). This treatment raises $[Cl^-]_c$ by a factor of around 3.7 from a resting value in Cl^--starved cells of 2.7 mM. Concomitantly, Cl^- influx is inhibited (Sanders, 1980*a*). It has previously been reported that preincubation in Cl^--containing solutions results in noncompetitive inhibition of Cl^- influx at low pH_o (= 5.5; Sanders & Hansen, 1981). Figure 5 shows that at the higher $pH_o = 7.5$, this pattern is preserved: J_{max} is selectively inhibited by the factor 2.7. No significant response of K_m is observed when the two data sets are analyzed separately, and, as shown in Fig. 5, constraining the K_ms in the two conditions to a common value again results in visually reasonable fits. It is noteworthy that reaction kinetic models in which charge translocation occurs on the unloaded form of the carrier, with Cl^- leaving after H^+, do not predict this response (*see* Eqs. (A20) and (A24) in Sanders et al., 1984). The simplest size-ordering arrangement of rate constants in Eqs. (1) to (3) which gives rise to a noncompetitive inhibitory response to raised $[Cl^-]_c$ is

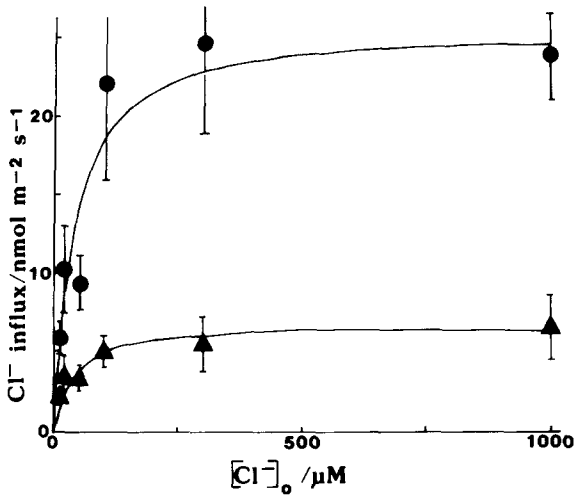

Fig. 5. Comparison of Cl^- influx kinetics between cells pretreated in S-APW (Cl^- starved, data of Fig. 2; (●)) and those pretreated in C-APW (Cl^- replete, (▲)), both measured at $pH_o = 7.5$. Data were jointly fitted to a common (least squares) value of K_m, but different values of J_{max}. Michaelian parameters: J_{max} (Cl^--starved cells) $= 25.7 \pm 1.4$ nmol m^{-2} sec^{-1}; J_{max} (Cl^--replete cells) $= 6.9 \pm 1.3$ nmol m^{-2} sec^{-1}; common $K_m = 38.4 \pm 8.9$ μM

$$[Cl^-]_c k_{31}^o > k_{13}. \quad (7)$$

Quantitative Kinetic Analysis

Qualitatively, the major observations reported here can be explained by the first-on-first-off model in Fig. 1, given appropriate size-ordering of the reaction constants (conditions 4 to 7). However, a good quantitative description, based on the assumption that the effects of pH_o result only in changes in k_{42},

is not possible. The problem centers around modeling the rather modest (2.5-fold) changes in J_{max} and K_m as $[H^+]_o$ changes by a factor of more than 30 from pH 7.5 to 9.0 *with the stipulation* that further increase in pH_o is selective in affecting J_{max}. Thus, while the observed change in the ratio J_{max}/K_m is a 6.25-fold decrease between 7.5 and 9.0, the lowest change in the ratio that could be achieved by modeling was by a factor of just over 30, i.e., almost proportional to the change in $[H^+]_o$. The reason for this large predicted change in J_{max}/K_m is simple to see. Taking the ratio of Eqs. (1) and (2) in conjunction with the experimentally derived condition of Eq. (5) results in the relationship

$$\frac{J_{max}}{K_m} = \frac{N[H^+]k_{42}^o k_{13}k_{35}k_{56}k_{64}}{k_{46}[(k_{53}k_{65}(k_{13} + k_{31}) + k_{13}k_{35}(k_{56} + k_{65})]}, \quad (8)$$

which shows that the J_{max}/K_m ratio has to be approximately proportional to $[H^+]_o$.

Nevertheless, a good description of the data can be obtained by taking into account two additional observations. First, it is now well established that pH_c shows a slight dependence on pH_o in *Chara* (Smith, 1984*a*;*b*), as is the case in other plant and fungal cells (Komor & Tanner, 1974; Smith & Raven, 1979; Sanders & Slayman, 1982). In *Chara*, Smith's (1984*b*) data can be used to derive the relationship

$$pH_c = 0.262pH_o + 6.068 \quad (9)$$

for values of pH_o above 6.0. [Below $pH_o = 6$, pH_c is not detectably pH_o dependent.] The second observation is that Cl^- influx in *Chara* is an acutely sensitive function of pH_c (Sanders, 1980*c*; Reid & Walker, 1984). Indeed, Cl^- influx titrates as though 2 H^+ were dissociating from sites with pK_as = 7.85. The following empirical relationship can then be specified for the first-on-first-off model:

$$\frac{k_{53}^o}{k_{35}} = \frac{k_{53}}{[H^+]_c^2 k_{35}} = 5.01 \times 10^{15}\ \text{M}^{-2}. \quad (10)$$

Figures 6 and 7 show, for both Cl^--starved and -replete cells, the results of modeling the kinetic response of Cl^- influx to raised pH_o when Eqs. (9) and (10) are taken into account. Reasonable descriptions of the data can, in fact, be obtained by taking the relative values of all ligand-insensitive rate constants (k_{13}, k_{24}, k_{35}, k_{46}, k_{56}, k_{65}) as unity. The two essential specifications, in addition to those embodied by Eqs. (9) and (10) turn out to be

$$\frac{k_{42}^o}{k_{24}} = \frac{k_{42}}{[H^+]_o k_{24}} = 1.58 \times 10^7\ \text{M}^{-1} \quad (11)$$

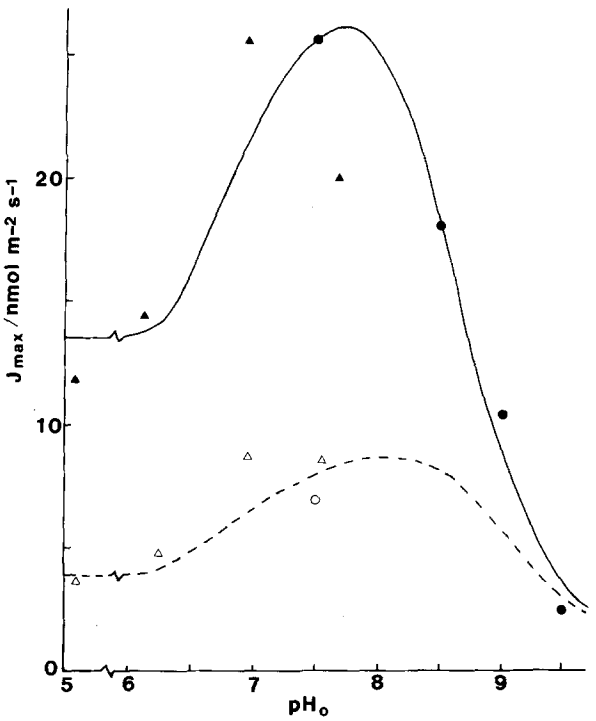

Fig. 6. Response of J_{max} to pH_o according to the first-on-first-off model for H^+-coupled Cl^- transport (Fig. 1) in Cl^--starved (S-APW-pretreated) cells (solid line) and Cl^--replete (C-APW-pretreated) cells (dashed line). Equation (1) was used for the calculations with the values of the rate constants (*see* Materials and Methods and Fig. 1 for nomenclature) all held at unity with the exception of: k_{42} (*see* Eq. (11)); k_{31} (*see* Eq. (12): the value is 17 sec^{-1} for Cl^--starved cells and 62.9 sec^{-1} for Cl^--replete cells); k_{53} (adjusted in accordance with Eqs. (9) and (10)). N, the total carrier density, which enters Eq. (1) purely as a scaling factor, was taken as 770 nmol m^{-2}. Symbols represent observed values of J_{max} and are coded for as follows: (●): Cl^--starved cells, from values reported in Figs. 2–4, together with an additional determination (*original data not shown*) at pH_o = 8.5; (▲): Cl^--starved cells, influxes measured at 1 mM $[Cl^-]_o$, data of Smith and MacRobbie (1981); open circle (○): Cl^--replete cells, from value reported in Fig. 5; (△): Cl^--replete cells, influxes measured at $[Cl^-]_o$ = 1 mM, data of Smith and MacRobbie (1981). The data of Smith and MacRobbie (1981) have been (down)scaled by the factor 1.34

and

$$\frac{k_{31}^o}{k_{13}} = \frac{k_{31}}{[Cl^-]_c k_{13}} = 6.29 \times 10^3\ \text{M}^{-1} \quad (12)$$

implying a pK_a = 7.2 for binding of external H^+, and a dissociation constant for cytosolic Cl^- = 0.16 mM. This latter value is in accord with a previously implied (but undetermined) value in the sub-mM range (Sanders & Hansen, 1981). In addition, for scaling purposes, the value of N has been taken as 770 nmol/m^2, and the dissociation constant for external Cl^- has been defined by the relationship

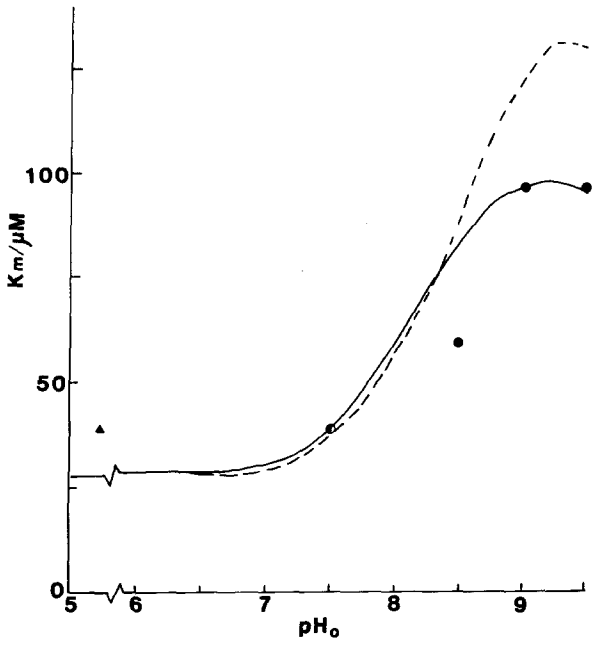

Fig. 7. Response of K_m to variation of pH_o according to Eq. (2) for both Cl^--starved cells (solid line) and Cl^--replete cells (dashed line). Calculations performed as for Fig. 6, with the additional stipulation specified in Eq. (13), which serves as a scaling factor (*cf.* Eq. (2)). Symbols represent observed values of K_m, and are coded as follows: (●): K_m values from data of Figs. 2 and 4 for Cl^--starved cells, with an additional determination (*original data not shown*) at pH_o = 8.5; open circle superposed on filled circle: from data of Fig. 5 for Cl^--replete cells, pH_o = 7.5; (▲): determined with internally perfused cells at low pH_o (Sanders & Hansen, 1981)

$$\frac{k^o_{64}}{k_{46}} = \frac{k_{64}}{[Cl^-]_o k_{46}} = 2.53 \times 10^4\ M^{-1} \qquad (13)$$

as 39.5 μM.

By substituting Eqs. (9) through (13) into Eqs. (1) through (3), the threefold decline of J_{max} in Cl^--replete cells can now clearly be replicated, as can the rather gentle decline in J_{max} above pH_o = 7.5 (Fig. 6). Furthermore, the model predicts a peak in J_{max} in the pH range 7 to 8, with decline on the more acid side. This latter phenomenon is confirmed by the experimental data of Smith & MacRobbie (1981) (triangles on Fig. 6), which was obtained at 1 mM Cl^-, i.e., a concentration giving approximately J_{max} rates, and has been observed in many other studies (Smith & Walker, 1976; Jayasuria, 1975; Sanders, 1980*a*; Lucas, Keifer & Pesacreta, 1986). The rise, and then plateauing, of K_m as pH_o is raised can also be replicated (Fig. 7). Note that stabilization of K_m below pH_o = 7.5 is predicted, and that this is also reasonably in accord with previous results (triangle in Fig. 7; Sanders & Hansen, 1981).

Discussion

Critical Evaluation of the Kinetic Modeling Approach

Practical value in the above kinetic model for H^+-coupled transport rests upon the validity of its underlying assumptions. Foremost among these assumptions is that pH_o effects on Cl^- flux solely concern transported protons. Although there is no absolute guarantee on this point, two important predictions of the model are supportive. First, that J_{max} tends to fall and K_m to rise over the pH_o range 7.5 to 9.0 *requires* that further increase in pH_o results only in a decrease in J_{max}. This prediction is borne out by the results at pH 9.5. Second, although the quantitative features of the model were derived to describe results at $pH_o \geqq 7.5$, it naturally and accurately predicts the declining J_{max} and steady K_m at lower pHs (*see* Figs. 6 and 7). Furthermore, the fact remains that the most obvious manner in which to describe the well-known bell-shaped dependence of Cl^- influx on pH_o is to take account of all the available experimental data, including the dependence of pH_c on pH_o and of Cl^- influx on pH_c.

A second critical factor relates to the thermodynamic properties of the quantitative model. As it stands, the model is in accord with the principle of microscopic reversibility. Consider the situation in which the system is in equilibrium with respect to the transported ions, and let the internal binding reactions be poised at equilibrium. For $[Cl^-]$ = 160 μM on each side of the membrane (k_{13}/k_{31}) = 1: Eq. (12)), the external Cl^--binding reaction will be displaced by a factor of 4 (= k_{64}/k_{46}: Eq. (13)) in the forward direction with respect to Cl^- influx. However, the external H^+-binding reaction will be displaced by an almost equal factor (k_{24}/k_{42} = 4.5: Eq. (11)) in the backward direction.

Nevertheless, no consideration has so far been given to the fact that whereas two H^+ are transported by the transport system, the kinetic data indicate binding of only one H^+ externally. The most obvious explanation for the kinetic silence of the second proton is that the pK_a for its binding is somewhat higher than that for binding of the first proton. This disparity in pK_as for the two external binding sites need not be large, if it is assumed that transport fails to take place unless *both* protons are bound. Thus, were the second site to have a pK_a raised one unit in comparison with the first, the kinetic response of transport to changed internal pH would be effectively manifested only by titration of the first site. The potential disruption of microscopic reversibility engendered by the higher pK_a of the second H^+-binding site could be compensated

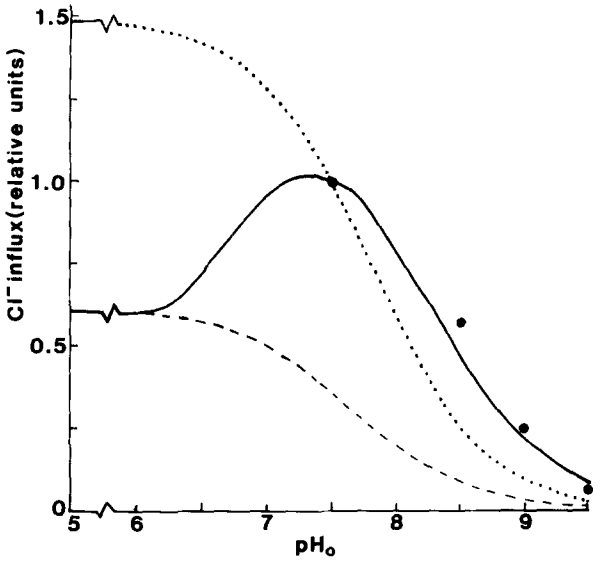

Fig. 8. Dependence of model-derived Cl^- influx at $[Cl^-]_o$ = 50 μM for Cl^--starved cells. Solid line: pH_c allowed to vary with pH_o according to Eq. (9), with other reaction kinetic parameters as for Figs. 6 and 7; dashed line: as for solid line, except that pH_c constrained to the value pertaining at $pH_o < 6.0$ (pH_c = 7.70; k_{53} = 2.02: from data of Smith, 1984*b*); dotted line: as for solid line, but pH_c constrained to value of pH_c pertaining at pH_o = 7.5 (= 8.03: k_{53} = 0.437: from data of Smith, 1984*b*). Points: influx calculated for parameter values given in the legends to Figs. 2–4, with an additional point for data obtained at pH 8.5 (*see* Figs. 6 and 7)

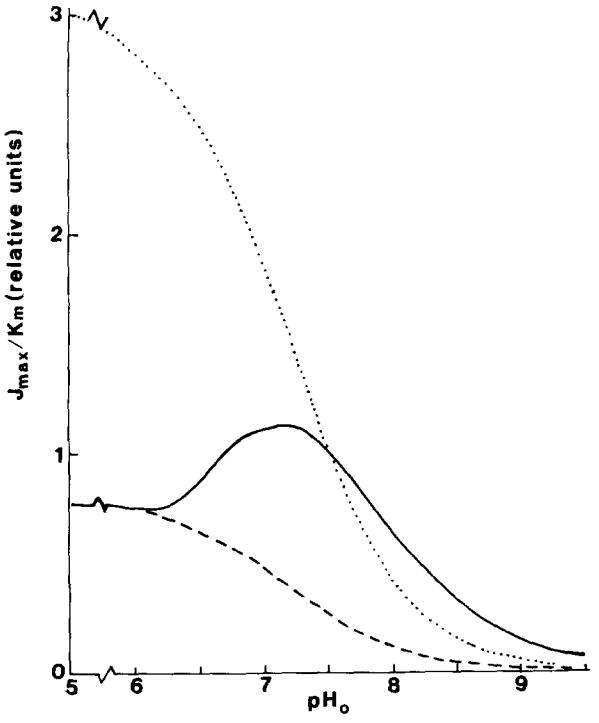

Fig. 9. Variation of the ratio J_{max}/K_m with pH_o. All parameter values correspond to those used in Fig. 8: solid line: pH_c allowed to vary according to Eqs. (9) and (10); dashed line: pH_c constrained to value obtaining at $pH_o < 6.0$; dotted line: pH_c constrained to value obtaining at pH_o = 7.5

for by proposing that the transmembrane reaction of the loaded carrier, measured at zero electrical driving force, is slightly endergonic (2 kJ/mol) in the direction of influx.

Role of pH_c in Determining Response of Transport to pH_o

Analysis of the kinetic results has shown that only by taking account of the pH_o-dependence of pH_c can the response of Cl^- influx to variation in pH_o be successfully modeled. It is, therefore, of interest to examine the putative response of Cl^- influx to pH_o in the event that pH_c were acutely controlled around a single value independently of the prevailing pH_o. Results of this consideration are displayed in Figs. 8 and 9.

Figure 8 displays the model-derived dependence of Cl^- transport on pH_o with $[Cl^-]_o$ = 50 μM. The solid line represents the anticipated response taking pH_c changes into account, whereas the dashed line shows the relationship obtained when pH_c is constrained to the value observed at pH_o = 6 and below. The consequences of tight control of pH_c are clear: at $pH_o > 7.5$, Cl^- influx is reduced by a factor of at least three in comparison with the flux which takes the small rise in pH_c into account. The obvious advantages of this situation are that physiological rates of transport are maintained at much higher pH_o than would otherwise be possible.

Similar conclusions can be drawn from inspection of Fig. 9, which displays the pH_o-dependence of J_{max}/K_m. (The J_{max}/K_m ratio can be visualized as proportional to the flux at low $[Cl^-]_o$, e.g., 10 μM.) Again the dashed line shows the response with pH_c constrained to a value of 7.70 at all pH_o, with the solid line constructed on the basis of the observed dependence of pH_c on pH_o. The range of catalytic competence of the carrier is extended by more than 1 to 2 pH units above pH_o = 6.

General support for the notion that pH_c has a role in regulation of the response of Cl^- influx to pH_o is available from studies of Cl^- influx on internally perfused cells. There, the large effective cytosolic volume and high buffer capacity of the experimental solutions can counter the tendency of pH_c to vary with pH_o. Thus, in perfused *Chara* cells, Cl^- influx rises steadily as pH_o decreases, with no evidence of the typical bell-shaped response observed in intact cells (Sanders, 1980*c*).

Given these results, it might be asked why pH_c could not simply be maintained at a consistently higher value, which is nevertheless independent of pH_o. This would then enable the catalytic capacity of the carrier to be exploited to full advantage, without attendant transinhibition from cytosolic protons. Results of modeling this state of affairs is also displayed in Figs. 8 and 9, in which the dotted line shows the response curves generated by constraining pH_c to the constant value (= 8.03) obtaining at pH_o = 7.5. Although the model in which pH_c is allowed to vary with pH_o still generates a rightward shift of the flux *vs.* pH_o relationships in alkaline conditions, the change is not large. Instead, the predominant effect of constraining pH_c to a relatively high value is to enhance the flux in more acidic conditions.

At this juncture, it is advantageous to consider the physiological function of Cl^- transport. Cl^- is accumulated in plants principally to fulfil the role of a metabolically cheap vacuolar osmoticum (Sanders, 1984) thereby generating cell turgor and endowing the cells with structural stability. Presumably, therefore, it is physiologically advantageous that the Cl^- transport proceeds at a rate commensurate with the general demands of cell growth, and is relatively insensitive to prevailing environmental conditions. Thus, although maintenance of a high pH or its evolutionary alternative—a low pK_a for the cytosolic H^+-binding sites—would both ensure maximal rates of transport for any given pH_o, it is evident from the dotted curves in Figs. 8 and 9 that Cl^- influx is nevertheless a very sensitive function of pH_o. This lack of control over Cl^- influx in the face of environmental perturbation is potentially incompatible with the general requirement that influx be tuned to the demands of cell growth.

The corollary of this argument is that the continued decline of pH_c below pH_o = 7.5 is as important to the control of Cl^- influx as the increase of pH_c in more alkaline conditions externally. The net result (*see* Fig. 9) is that Cl^- influx is maintained within a factor of 3 of its value at pH_o = 5 over a wide pH_o range to values in excess of 8.5. By contrast, regardless of the *fixed* value of pH_c, influx is very sensitive to pH_o and cannot be sustained to within a factor of 3 of the value at pH_o = 5 beyond pH_o = 7.5.

General Implications

In plant and fungal cells, pH_c, although clearly controlled, is not a perfectly invariant function of pH_o, even in the steady state (Komor & Tanner, 1974; Smith & Raven, 1979; Sanders & Slayman, 1982; Smith, 1984*a*;*b*). We propose here that this limited dependence of pH_c on pH_o can be visualized as serving a regulatory function in allowing H^+-coupled transport to proceed relatively insensitively with respect to spatial and temporal variation in pH_o. The primary additional requirement for such insensitivity is that the internal H^+-binding site of the coupled transport system possesses a pK_a at, or slightly above, the prevailing value of pH_c, thereby rendering transport rate sensitive to small variation in pH_c. It is noteworthy, then, that those H^+-coupled transport systems of plant and fungi for which data are available are all acutely sensitive to pH_c (Komor, Schwab & Tanner, 1979; Sanders, 1980*b*; Ballarin-Denti et al., 1984).

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