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Control of Cl^- Influx in *Chara* by Internal pH

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Summary. The possible regulation of Cl^- influx in *Chara* by the cytoplasmic Cl^- concentration and cytoplasmic pH was investigated using both intact and intracellularly perfused cells. In perfused cells Cl^- influx was sensitive to changes in the internal Cl^- concentration but only when the concentration was less than 1 mM.

In intact cells the metabolic inhibitors, CCCP, DCMU, and oligomycin which inhibit Cl^- influx also reduced the cytoplasmic pH. A correlation between ATP concentration and cytoplasmic pH was shown to apply when the ATP concentration was lowered using these inhibitors. The possible relationships between ATP status, cytoplasmic pH, and Cl^- influx are discussed.

Key Words *Chara* · chloride influx · pH regulation · intracellular perfusion

Introduction

In a previous paper we provided evidence that the driving force for active transport of Cl^- at the plasmalemma of *Chara* is the electrochemical potential difference of protons, $\Delta\mu_{H^+}$. The factors which regulate Cl^- influx have yet to be defined. A correlation in the whole cell between Cl^- influx and ATP concentration (Reid & Walker, 1984) requires explanation since in perfused cells ATP is not necessary for active transport of Cl^-.

The effects on Cl^- influx of pH_c [1] and pH_o have been studied by Sanders (1978, 1980*a*, *b*), Smith (1980) and Smith and MacRobbie (1981). Sanders (1980*a*, *b*) showed that the influx in perfused cells was highly sensitive to changes in pH_i and slightly sensitive to pH_o. Smith (1980) found that stimulation of Cl^- influx by amines was accompanied by an increase in pH_c by 0.15–0.25. However, Smith and MacRobbie (1981) concluded that the stimulation of Cl^- influx after Cl^- starvation could not be explained by increased pH_c. They were only able to detect changes in pH_c after starvation periods much longer than that required to produce stimulation of influx. Their results support the involvement of $[Cl^-]_c$ as proposed by Sanders (1980*a*) to account for the stimulation of influx by starvation. This proposal was supported by the finding that in perfused cells Cl^- influx was sensitive to $[Cl^-]_i$ in the range 0–10 mM (Sanders, 1980*a*).

A kinetic model for Cl^- transport in *Chara* which involves co-regulation by both pH_c and $[Cl^-]_c$ has been proposed by Sanders and Hansen (1981). This model, however, relies heavily on a small amount of data from perfused cells. In this paper we re-examine the effect of $[Cl^-]_c$ on Cl^- influx with a perfusion technique that differs from that used by Sanders (1980*a*). We also examine the effect of metabolic inhibitors on pH_c in intact cells and discuss the possible relationship between ATP concentration and pH_c.

Materials and Methods

Internodal cells of *Chara corallina* were maintained outdoors in large concrete troughs. On the day prior to experiments cells were cut, trimmed of whorl cells, and placed in a solution containing (mM): NaCl, 1; KCl, 0.2; $CaSO_4$, 0.05; plus 5 mM of a zwitterionic buffer adjusted to the required pH with NaOH.

Intracellular Perfusion

The perfusion method has been described previously (Reid & Walker, 1984) and is essentially the same as that used by Smith and Walker (1981) for studying electrogenic H^+ pumping in the *Chara* plasmalemma. Briefly, an internodal cell was mounted on a perspex block having three compartments. The two end compartments were bathed in perfusion medium (PM: mM approx – K^+, 130; EGTA, 2.5; Mg^{2+}, 1; SO_4^{2-}, 1; MES, 140; pH 7.8). The center compartment, which exposed a length of cell of 10 mm, was irrigated with flowing medium (OM: mM approx–K^+, 0.1; Cl^-, 0–0.5; Na^+, 0.2; sorbitol, 250;

[1] *Abbreviations:* CCCP – carbonyl cyanide *m*-chlorophenylhydrazone; DCMU – 3′-(3,4 dichlorophenyl)-1′,1-dimethyl urea; subscripts *i*, *c*, *v* and *o* refer to the internal phase of a perfused cell, cytoplasm, vacuole, and outside medium, respectively.

MOPS, 1; pH 7.0) at a rate of approximately 20 ml $ksec^{-1}$. To initiate perfusion, the ends of the cell were removed and a flow of PM through the cell was achieved by increasing the solution height at one end by 4–6 mm. Flow of PM through the cell continued for 50–100 sec after which the cell was left to allow dissolution of the tonoplast. Loss of the tonoplast was judged by cessation of protoplasmic streaming. The PD between an end compartment and the center was recorded continuously. When a stable PD was reached (−120 to −130 mV) the OM was replaced by OM containing ^{36}Cl. At the end of the 0.6 ksec influx period the cell was rinsed with OM for a further 0.6 ksec. Any cells whose PD "collapsed" (*see* Reid & Walker, 1984) during the influx period were rejected. At the end of the rinse period the cell and the reservoirs of PM were removed and counted for ^{36}Cl.

Two variations of the standard perfusion method were also used to measure Cl^- influx. The first was similar to the normal method except that PM was pumped through the cell at a rate of 0.3–0.5 ml $ksec^{-1}$ by means of a fine glass cone sealed over one end of the cell and connected by plastic tubing to a peristaltic pump and PM reservoir. This method was particularly useful for fixing the internal Cl^- at low concentrations because it enabled more thorough removal of the original Cl^-.

The second variation permitted influx measurements to be made on more than one cell at a time but could only be used after some certainty as to the behavior of the cells to the solution had been obtained using the normal method. These batch experiments used a silicone rubber block which was moulded to accept five cells separated from each other and with each cell traversing three separated compartments as before. The arrangement was essentially the same as that used for normal perfusion except that the OM in the center compartment was not flowing. The membrane PD was not continuously monitored, but measurements were made at the beginning and end of the influx period to check that the cell had reached a stable potential and that the potential remained stable during influx.

When it was necessary to fix the internal Cl^- concentration the cell was perfused with PM of the appropriate Cl^- concentration then the PM was removed from the end compartments and replaced with fresh PM.

Measurement of Cytoplasmic and Vacuolar pH

Cytoplasmic and vacuolar pH were measured in intact cells by distribution of ^{14}C-DMO (Walker & Smith, 1975; Raven & Smith, 1978). Batches of cells were treated in DMO-containing solutions for 3–8 hr, then rinsed, and a sample of vacuolar sap was collected by excising one end and allowing some of the sap to flow out. ^{14}C-DMO activity in aliquots of the bathing solution, in the vacuolar sap sample and the remainder of the cell was determined by scintillation counting. By assuming the cytoplasmic volume to be 5% of the cell volume (Bostrom, 1976), the DMO activity could be computed for the cytoplasm and vacuole. Cytoplasmic and vacuolar pH were then calculated using the equations.

$$\frac{C_c}{C_o}=\frac{1+10^{(pH_c-pK_c)}}{1+10^{(pH_o-pK_o)}} \quad (1)$$

and

$$\frac{C_v}{C_o}=\frac{1+10^{(pH_v-pK_v)}}{1+10^{(pH_o-pK_o)}} \quad (2)$$

C is the ^{14}C-DMO activity per unit volume, pK is the pK of DMO, and the subscripts o, c and v refer to outside solution, cytoplasm, and vacuole, respectively. A measure of the equilibration of undissociated DMO was obtained from the accumulation ratio C_v/U_o where U_o is the activity of unionized DMO per unit volume of external solution. Equilibration would give a ratio of 1. Deviation from unity could occur in the case of short loading times or if the plasmalemma or tonoplast had a significant permeability to ionized DMO. In cases where C_v/U_o was much less than unity, pH_c was also calculated by assuming equilibration of DMO at the tonoplast using the equation

$$\frac{C_c}{C_v}=\frac{1+10^{(pH_c-pK_c)}}{1+10^{(pH_v-pK_v)}}. \quad (3)$$

In these cases pH_v was estimated independently with narrow range tetra-colored indicator sticks (Merck, Darmstadt).

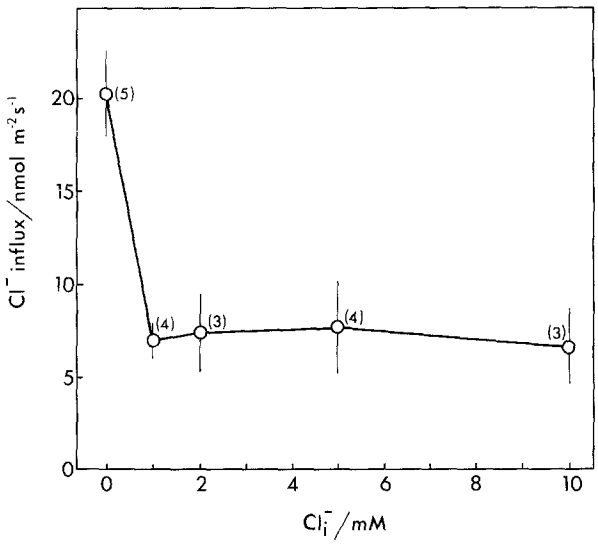

Fig. 1. The effect of variations in $[Cl^-]_i$ on the rate of Cl^- influx in perfused cells. "Batch" perfusion system. $pH_i=7.8$, $pH_o=7.0$. Error bars are SEM. Number of cells shown in parentheses

Double Labeling

Counts due to ^{36}Cl and ^{14}C-DMO during double labeling experiments could be separated by selection of the appropriate windows and amplifications on a multichannel scintillation counter. Correction for quenching was made using internal ^{36}Cl and ^{14}C standards.

Results

Effect of $[Cl^-]_c$ on Influx in Perfused Cells

Figure 1 shows the dependence of Cl^- influx into perfused cells on the concentration of Cl^- in the perfusion medium. The concentration 0 mM is only nominally so since it is difficult to guarantee removal of all Cl^- from the cytoplasm during perfusion. In any event, there would probably be some leakage of Cl^- from the chloroplasts after the ces-

Table. Effect of variation in $[Cl^-]_i$ on Cl^- influx in perfused cells ("normal" perfusion method)

$[Cl^-]_i$/mM	Influx rate/nmol m^{-2} sec^{-1}
0	42.8±4.4(8)
5	20.1±7.6(6)
10	28.7±8.1(3)

Number of cells shown in brackets. Errors are SEMs

sation of perfusion flow since this experiment was done using the "batch" perfusion system in which both OM and PM are stationary. A similar experiment using the normal perfusion method or with PM pumped through the cell gave higher influx rates (Table). In these cells OM and PM were continually mixed. It is likely that the Cl^- concentration labeled 0 in the Table would be closer to this value than that shown in Fig. 1. The reason for the difference in rates between the two methods is not apparent, but it may result from thicker unstirred layers in the batch system giving rise to a local depletion of Cl^- at the cell surface. If it is accepted that active transport of Cl^- occurs by cotransport with 1 or 2 H^+, unstirred layers adjacent to the membrane could reduce the driving force for transport.

The rate of Cl^- efflux in these cells was 1.36±0.4 nmol m^{-2} sec^{-1} so that even at $[Cl^-]_i$ of 10 mM there was a substantial net uptake of Cl^-.

Effect of pH_c on Cl^- Influx

Figure 2 shows the effect of various concentrations of CCCP on pH_c and pH_v. In the light there were significant reductions in pH_c at 5 μM CCCP. The difference in pH_c between light and dark control batches of 0.2 units was also significant ($P<0.005$ Wilcoxson rank sum). The vacuolar pH in these cells was the same in both light and dark and rose slightly in the presence of CCCP.

Figure 3 shows the effects of oligomycin, CCCP, and DCMU on pH_c and on the rate of Cl^- influx. In this experiment the two parameters were measured simultaneously on the same cells by double-labeling with ^{14}C-DMO and ^{36}Cl. The rate of Cl^- influx was measured over 1.8 ksec at the end of the DMO soaking period which was between 15 and 25 ksec. C_v/U_o was close to 0.7 for all batches and pH_c was calculated assuming DMO equilibration across the tonoplast. All three inhibitors reduced Cl^- influx but only DCMU and CCCP reduced pH_c.

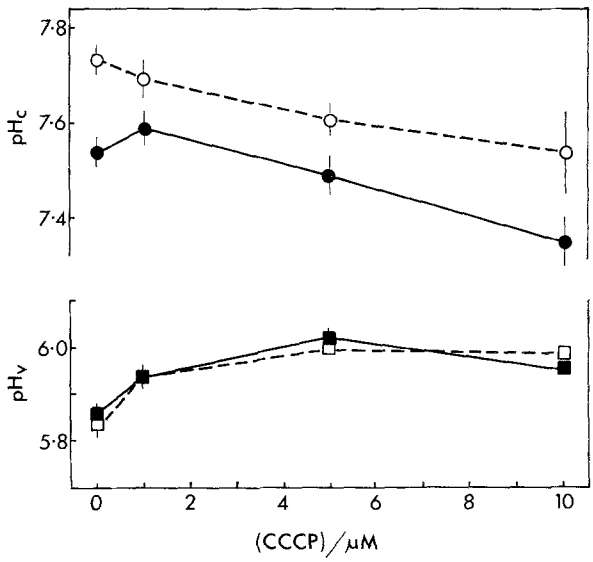

Fig. 2. Effect of CCCP on pH_c (o) and pH_v (□). Open symbols, light; filled symbols dark. Cells soaked in DMO±CCCP at pH 7.2 for 7 ksec. C_v/U_o between 1.05–1.14. Each point is mean ±SEM of 10 cells

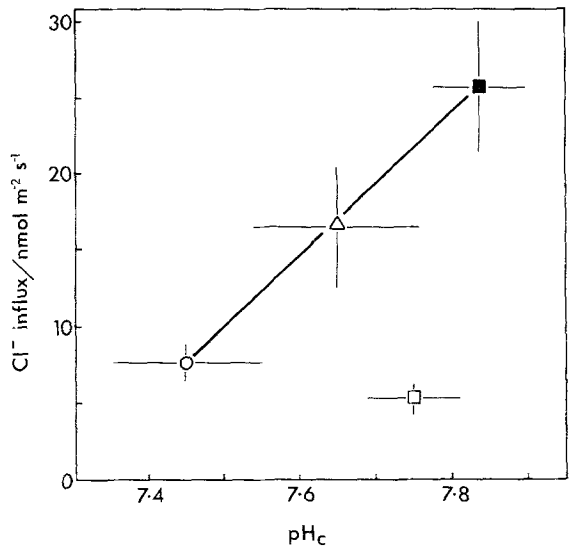

Fig. 3. Effects of inhibitors on pH_c and Cl^- influx rate. Control ■, oligomycin (5 μg ml^{-1}) □, DCMU (2 μM) △, CCCP (5 μM) ○. Conditions: light, $pH_o=7.13$. pH_c calculated by Eq. (3). Each point is the mean of 10 cells

The relationship between ATP concentration and pH_c was studied by monitoring the streaming rate, which we have previously shown to be a good indicator of cellular ATP status (Reid & Walker, 1983), while measuring pH_c. A high correlation was obtained between streaming rate and pH_c in the presence of oligomycin (Fig. 4). The streaming rate itself is not affected by changes in pH_c since changing pH_o, which alters pH_c does not change

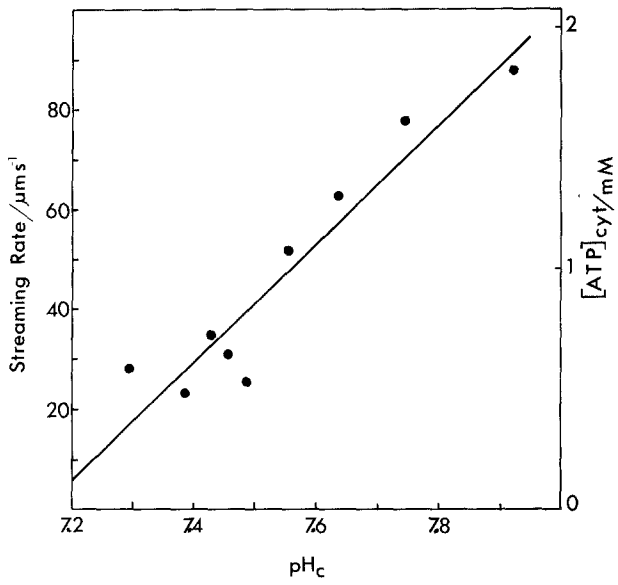

Fig. 4. Correlation between streaming rate and pH_c in the presence of oligomycin (5 μg ml^{-1}). ATP values from Reid and Walker (1983) calculated from the streaming rate. Correlation coefficient for the regression = 0.953. Each point represents one cell. Conditions: light, $pH_o = 6.7$

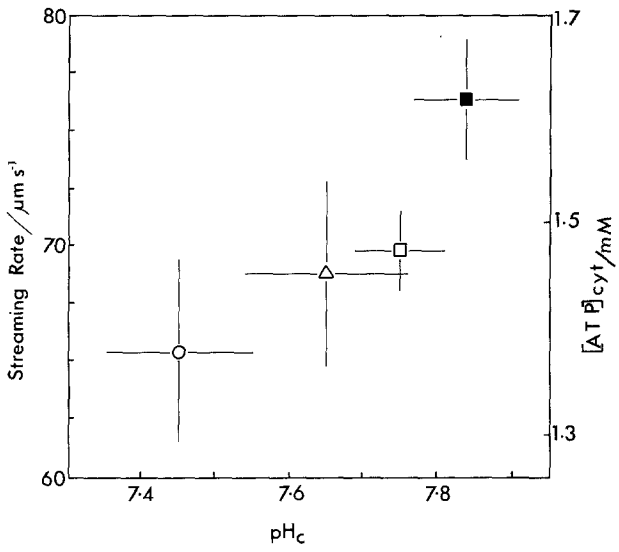

Fig. 5. Effects of inhibitors on pH_c and streaming rate. Control ■, oligomycin (5 μg ml^{-1}) □, DCMU (2 μM) △, CCCP (5 μM) ○. Same cells as for Fig. 3. ATP values calculated as in Fig. 4

the rate (R.J. Reid, *unpublished results*). In the experiment shown in Fig. 3 streaming rates were also measured and are plotted in Fig. 5. The relationship between streaming rate and pH_c is less clear from this experiment because the range of streaming rates was small. It does appear though that the slope (SR/pH_c) would not be as great as with oligomycin (Fig. 4). Possible reasons for this are presented in the Discussion.

Discussion

Regulation of Cl^- Influx by $[Cl^-]_c$

The only direct evidence that Cl^- influx can respond to the internal Cl^- concentration has come from perfused cells (Sanders, 1980*a*). The perfusion results presented here agree with the previous work insofar as they show that under some circumstances the influx can have a strong sensitivity to $[Cl^-]_i$. However, we find that the sensitivity is limited to $[Cl^-]_i$ less than 1 mM and, at least at pH_c 7.8, there is a substantial influx which is not sensitive to $[Cl^-]_i$. The magnitude of this influx is similar to that of normal cells.

The fact that a Cl^--sensitive influx exists in perfused cells would seem to suggest that in intact cells there are conditions under which such a mechanism becomes activated. This may well be the case during Cl^- starvation if $[Cl^-]_c$ is reduced sufficiently to cause a stimulation. Unfortunately, the information needed to evaluate such a proposal is lacking. There is no certainty about the actual $[Cl^-]_c$ in spite of numerous measurements and the evidence that it is reduced during starvation is only speculative. Cl^- is not an essential osmoticum in *Chara* and can be replaced as a vacuolar constituent by other electrolytes (Sanders, 1981). Cl^- is an essential ion in that it is required in micromolar concentrations for normal photosynthetic activity (Heath & Hind, 1969). It is unlikely that the starvation stimulation would be solely activated to serve this need given the relatively large amounts of Cl^- stored in the vacuole even in starved cells and which could be mobilized for chloroplastic functions. The significance of the response remains for the present unexplained.

Regulation of Cl^- Influx by pH_c

The proposal that pH_c regulates Cl^- influx is generally supported by the inhibitor results. CCCP inhibited Cl^- influx to a value at which there would be no net uptake and reduced pH_c by 0.39 units. DCMU, which eliminates the light-stimulated component of Cl^- influx (Reid & Walker, 1983), caused a drop in pH_c of 0.19 units, which compares with the difference observed between light and dark of 0.2 units (Fig. 2).

The effect of oligomycin in inhibiting Cl^- influx without significantly reducing pH_c would seem to point to a mode of action on Cl^- influx which differs from that of DCMU and CCCP. Lin and Hanson (1974) noted that oligomycin inhibited phosphate transport in corn roots disproportion-

ately to its effects on ATP concentration as compared with DNP, and suggested that the principle mechanism of oligomycin inhibition of transport was via its binding at the plasmalemma.

The effects of amines, metabolic inhibitors, darkness, and low external pH can all be accounted for in terms of changes on pH_c. After Cl^- starvation the influx may be under the control of the cytoplasmic Cl^- concentration.

The case for regulation of Cl^- influx by pH_c is strong but as noted by Smith and MacRobbie (1981) the correlation between changes in pH_c and Cl^- influx in intact cells is not conclusive evidence of causality. The demonstration by Sanders (1980*b*) that Cl^- influx in perfused cells is sensitive to changes in pH_c makes the argument more convincing because it shows that a mechanism does exist for changing influx by variation in pH_c. In addition, the sensitivity of influx to pH_c in intact cells closely corresponds to that of perfused cells. If the data from Fig. 3 are compared with the data from perfused cells (Sanders, 1980*b*) over the same range of pH_c the two sets of results are indistinguishable. In Fig. 6 the percentage change in Cl^- influx is plotted against change in pH_c or pH_i; the two sets of data can be fitted by a single line. For comparison, the line fitted by Smith (1980) to the data that he obtained by increasing pH_c by addition of amines is also shown. This line implies a greater change in influx for a given change in pH_c than the other results. This may be caused by an interaction between amine and Cl^- fluxes or alternatively the greater slope may be attributed to a higher pH sensitivity of influx above pH_c 7.8 since the data covers the range pH 7.6 to 8.2 whereas the perfusion and inhibitor results are in the range 7.4 to 7.8. The latter explanation is supported by the finding that in perfused cells the effect of changes in pH_i becomes less as pH_i decreases (Sanders, 1980*b*), although this has only been investigated at pH_i below 7.8.

It is clear that application of metabolic inhibitors does change pH_c but it is not at all clear how this occurs. Since the inhibitors used here all act to reduce ATP production, their effect on pH_c is presumably mediated by a drop in ATP concentration or in ΔG_{ATP}. If this is true then it follows that pH_c must be very sensitive indeed to the concentration of ATP. Changes in ATP concentration that are associated with relatively large inhibitions of Cl^- influx and reductions in pH_c are often very small. For example, DCMU and darkness, both of which reduce pH_c by about 0.2 units (Figs. 2 and 3) cause a drop in ATP concentration of less than 10% (*see* Reid & Walker, 1983). Regulation

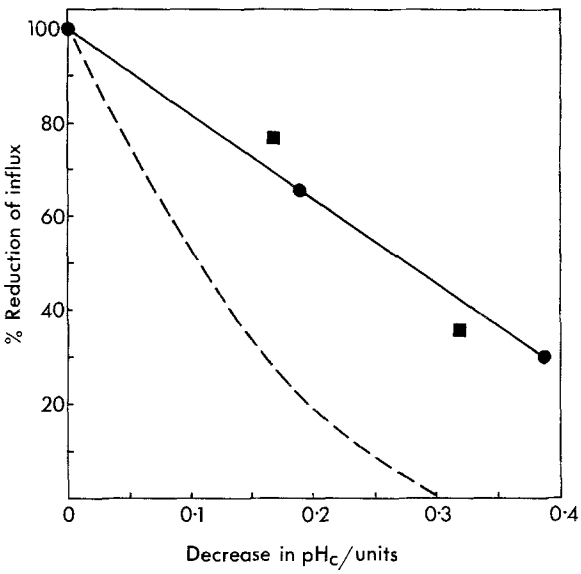

Fig. 6. Relationship between decrease in Cl^- influx and decrease in pH_i or pH_c. ■ data from perfused cells (Sanders, 1980*b*) in the range pH_i 7.4–7, ● data from Fig. 3 (oligomycin omitted) in the range pH_c 7.4–7.8, --- data from intact cells (Smith, 1980) in the range pH_c 7.6–8.2 approx

of pH_c was considered by Smith and Raven (1979) to result from a balance between metabolism and proton pumping across the boundary membranes. Whether the sensitivity of pH to the concentration of ATP is conferred by the response of proton pumps or of metabolism, or both is not easy to answer at present given our rather meager understanding of the energetics of proton pumping ATPases. Smith and Walker (1981) thought that on basis of their own work with perfused cells the plasmalemma proton pump in *Chara* would operate near reversible equilibrium and therefore respond to changes in ΔG_{ATP}. Hopefully, further refinements of the perfusion technique will provide an answer to this problem.

In summary, there is a considerable body of evidence both from intact and perfused cells that under most circumstances Cl^- influx is regulated by pH_c. A Cl^--sensitive influx can be demonstrated in perfused cells when $[Cl^-]_i$ is less than 1 mM and may be activated during Cl^- starvation. The magnitude of the Cl^--insensitive influx in perfused cells is comparable to the Cl^- influx of normal cells. Changes in pH_c are correlated with changes in ATP concentration; if this relationship is causal then the cellular ATP status is an important determinant of membrane transport rates. Regardless of the nature of the relationship between ATP concentration and pH_c the results demonstrate that in the use of metabolic inhibitors to study any membrane tranport process it is essential

to take proper account of the possible direct or indirect effects caused by changes in intracellular pH.

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