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Inward Membrane Current in *Chara inflata:* II. Effects of pH, Cl^--Channel Blockers and NH_4^+, and Significance for the Hyperpolarized State

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Summary. The Cl^- component of the voltage- and time-dependent inward current activated by hyperpolarizing the membrane of *Chara inflata* increases exponentially as the external pH, pH_o, is lowered from 7 with the membrane potential difference (PD) kept constant. Lanthanum and anthracene-9-carboxylic acid (A-9-C, a Cl^- channel blocker) both blocked the Cl^- component and removed the pH_o sensitivity of the inward current. Lanthanum, however, also decreased the K^+ conductance. The hyperpolarized membrane is depolarized by A-9-C in a manner similar to that caused by the removal of external Cl^-. Low external concentrations of NH_4^+ stimulated the Cl^- component of the inward current probably as a result of a change in cytoplasmic pH rather than as a result of a change in cytoplasmic $[Cl^-]$, since the effect was observed in Cl^--free solutions. The results show that the membrane PD, at hyperpolarized levels, is most likely determined by two factors: the proton extrusion pump, provided it has a reversal PD more negative than about −300 mV, and a voltage-dependent Cl^- leak.

Key Words Cl^- channels · *Chara inflata* · pH · channel blockers · NH_4^+

Introduction

When the plasma membrane of *Chara inflata* (a green alga) is hyperpolarized by a voltage clamp from a K^+-conductive state, a voltage- and time-dependent Cl^- efflux is activated which is probably via voltage-gated channels (Coleman & Walker, 1984; Tyerman et al., 1986). The membrane potential difference (PD) at which the Cl^- efflux begins to become significant in the dark is often less negative than the PD's reported for other charophytes in the light or in the hyperpolarized state (P-mode defined by Bisson and Walker, 1982). The possibility that a Cl^- efflux may be an important factor in determining the membrane PD in the hyperpolarized state has been discussed by Tyerman et al. (1986), since evidence which had been used to argue against the possibility (Kitasato, 1968) did not account for the large and perhaps independent effects of membrane PD and pH_o on the Cl^- efflux (Coster, 1969).

In its hyperpolarized state the membrane depolarizes by about 50 mV per unit decrease in pH_o from about 7 (Richards & Hope, 1974; Smith & Walker, 1976). From measured values of cytoplasmic pH and known concentrations of adenosine phosphates it has been argued that the pH_o sensitivity of the membrane PD is due to a proton pump operating near thermodynamic equilibrium and with a stoichiometry of $2H^+$ per ATP hydrolyzed (Walker & Smith, 1975). However, recent evidence indicates that the pump may not be operating near equilibrium (Smith, 1984) and that the stoichiometry may be $1H^+$/ATP (Lucas, 1982; Beilby, 1984) in which case the reversal PD of the pump would be some 300 mV more negative than the observed membrane PDs. In this case the pH_o sensitivity of the PD would be less likely to be due to the pump since passive ion fluxes must play a large role in determining the PD. The possibility that the pH_o dependence of the membrane PD is due to the Cl^- efflux is indicated from Coster (1969) who showed that the PD at which the inward current due to Cl^- became prominent (defined by Coster as the punch-through potential) depolarized by about 50 mV per unit decrease in pH_o.

In this paper we have looked again at the role of the Cl^- efflux in *Chara inflata* by investigating the electrophysiological effects on the plasma membrane of factors which change the Cl^- fluxes. These factors include agents which have been reported to block Cl^- channels and low concentrations of NH_4^+, which have been reported to stimulate the Cl^- influx (Smith & Walker, 1980).

Materials and Methods

Materials

The artificial pond water (APW) used to bathe cells in experiments had the same composition as that used by Tyerman et al. (1986). The whorl cells of *Chara inflata* used for experiments

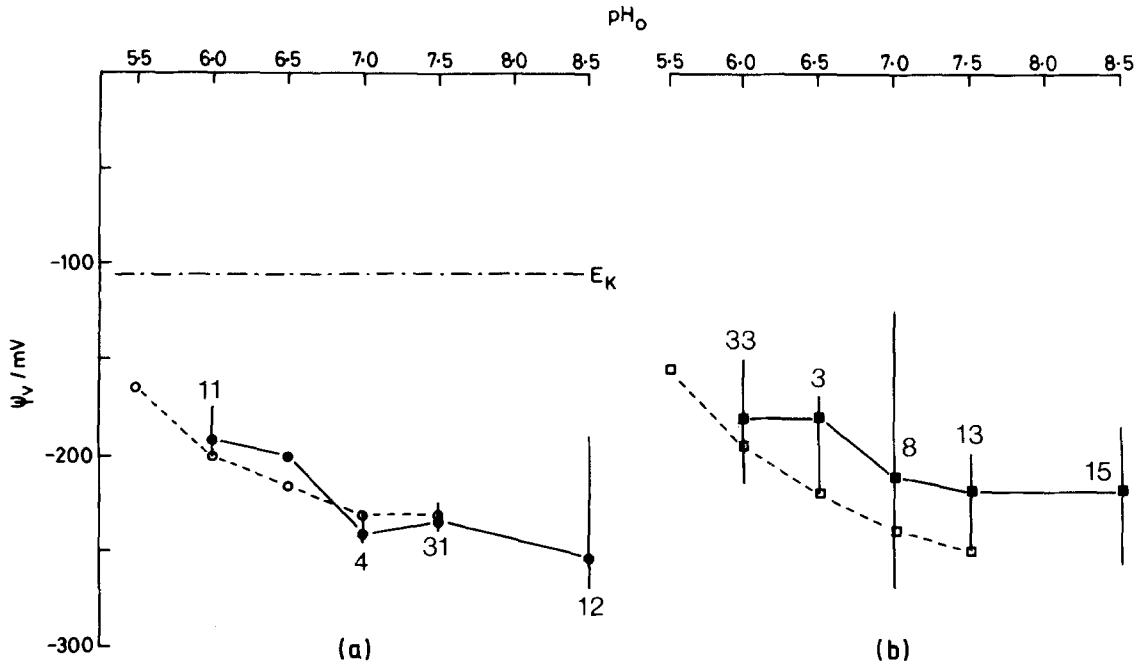

Fig. 1. (*a*) Vacuolar PD (ψ_v) as a function of pH_o for cells in the hyperpolarized state in the light (●). For one cell ψ_v was obtained over a range of pH_o (○). (*b*) Plotted as a function of pH_o are the potentials at which an inward-2 current of -50 mA m^{-2} was obtained when the membrane was hyperpolarized by voltage clamp from the K^+-conductive state (■□). The open symbols are for the same cell as in (*a*)

were taken from the same cultures used for experiments described in the previous paper.

A stock of Anthracene-9-carboxylic acid (A-9-C) (Aldrich) solution (10 mg/ml) was made up immediately before an experiment by dissolving 100 mg in 1 ml of 1 M NaOH and then diluting to 10 ml with distilled water. A concentration between 0.05 and 0.5 mM A-9-C was used in experiments. An increase in pH_o of 0.2 units in the final solution of 0.5 mM A-9-C was accounted for and a blank was tested before addition of A-9-C to the cell. Other channel blocking agents used were: tetraethylammonium chloride (TEACl), ethacrynic acid, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene (DIDS) and La^{3+}.

Methods

A full account of the methods is given in the first paper of this series (Tyerman et al., 1986). Current versus PD (*I vs.* ψ) curves were constructed from a rapid staircase of voltage pulses, a scan, superimposed on the main pulse. We used this *I vs.* ψ curve as an estimate of the instantaneous *I vs.* ψ curve. The conductance (g) was obtained from the average slope of the instantaneous *I vs.* ψ curve. This curve was usually linear in cells in APW. Data is presented as either the median and, within brackets, the range and number of observations, or the median and 95% confidence limits. In the Figures the median and range are plotted with the number of observations (n) shown adjacent to the point. For n greater than 8 the 95% confidence limits are shown.

Results

Hyperpolarized Cells

Chara inflata cells existed in either a K^+-conductive state or a hyperpolarized state, as observed in other charophytes (Bisson & Walker, 1982). The two states are characterized by properties of the conductance and the magnitude of the membrane PD. In the hyperpolarized state the PD between the vacuole and the outside (ψ_v) was usually more negative than the Nernst potential for either Na or K ions and was dependent on pH_o such that ψ_v changed from about -250 to -160 mV as pH_o was lowered from 8.5 to 5.5 (Fig. 1*a*).

The remainder of the results deals with aspects of a time- and voltage-dependent inward current which was activated by voltage-clamping cells to hyperpolarized levels from the depolarized K^+-state. This current will be referred to as the inward-2 current, as in the previous paper in this series (Tyerman et al., 1986), to distinguish it from a K^+ current which deactivated upon hyperpolarization. Unless stated otherwise the cells were provoked into the K^+-state by darkness.

Effect of pH_o on the Inward-2 Current

The inward-2 current was dependent on pH_o such that it was greater in magnitude in lower pH_o at a particular ψ. Figure 2 shows the logarithm of the steady-state inward current plotted against pH_o from voltage clamps at $\psi_v = -300$ mV. Between pH_o 7.5 and 5 the logarithm of the inward-2 current

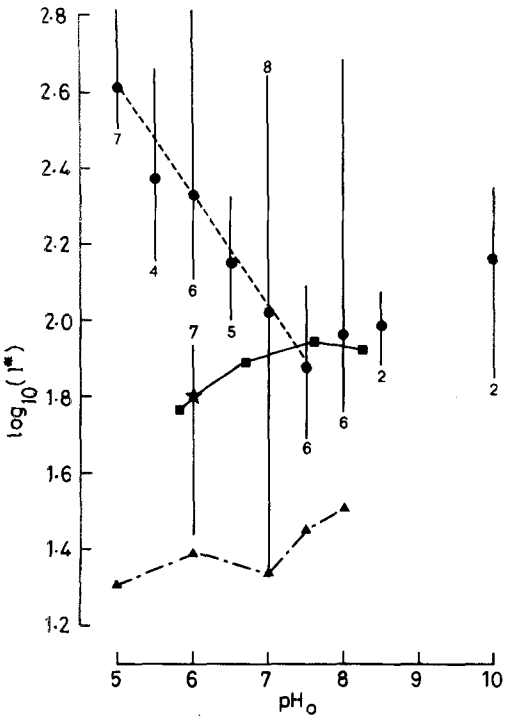

Fig. 2. Logarithm (base 10) of the inward-2 current ($I^* = -I$/mA m^{-2}) as a function of pH_o for cells clamped at −300 mV in APW (●). The median inward current remaining after A-9-C treatment is also shown from a number of cells at pH_o 6 (★) as well as the effect of A-9-C (■) and La^{3+} (▲) for two different cells over a range of pH_o

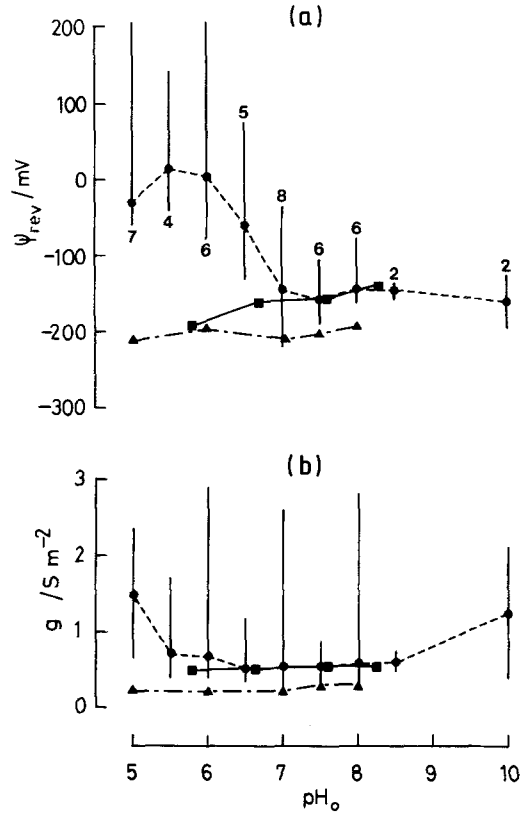

Fig. 3. (*a*) Effect of pH_o on the reversal potential (ψ_{rev}) (●) of instantaneous I *vs.* ψ_v curves obtained from scans on the steady-state inward-2 current at −300 mV. Also shown is the effect of A-9-C (■) and La^{3+} (▲) for the same two cells shown in Fig. 2. (*b*) Effect of pH_o on the conductance (g) obtained from the slope of the same I *vs.* ψ_v curves, at ψ_{rev}, described above

increased linearly with decreasing pH_o and in this range I as a function of pH_o is given by:

$$I = -B_1 \exp(-K_1\ pH_o), \quad (1)$$

where $B_1 = 12{,}270$ mA m^{-2} and $K_1 = 0.675\ pH^{-1}$.

There was also an effect of previous changes in pH_o on the magnitude of the inward-2 current. In general a larger inward-2 current would be observed in low pH_o if it was preceded by a treatment in pH_o greater than 7.5. To avoid the effects of previous pH_o treatments on the measurements, sequences of pH_o changes were varied in each experiment so that bias caused by using only one particular sequence of changes would be avoided.

From scans performed on the steady-state inward-2 current at the same ψ_v (−300 mV) in different pH_o, we obtained g and the reversal PD, the PD at which $I = 0$, as a function of pH_o (Fig. 3). For a change in pH_o from above 7 to below 6 (actual pH_o changes in time were done in various sequences) the reversal PD (Fig. 3*a*) always changed in the positive direction (10 out of 10 cells) from negative values of between −140 to −200 mV to values between −80 and 200 mV. The conductance as a function of pH_o (Fig. 3*b*) was variable between cells. Out of ten cells six showed an increase in g which correlated with the shift in reversal PD, three cells had constant g and one cell showed a decline as pH_o decreased.

We also determined as a function of pH_o, ψ_v for which the inward-2 current was 50 mA m^{-2}. This was done by bulking experiments on different cells in particular pH's and also by examining one cell in a range of pH_o values. A value of 50 mA m^{-2} was selected to obtain ψ_v (denoted by $\psi_v(50)$) because this current gave values of ψ_v similar to those obtained in the hyperpolarized state. The results of these experiments are shown in Fig. 1*b* where $\psi_v(50)$ is plotted against pH_o. It can be seen that the relationship of $\psi_v(50)$ *vs.* pH_o parallels that of the ψ_v of the hyperpolarized membrane shown in Fig. 1*a*.

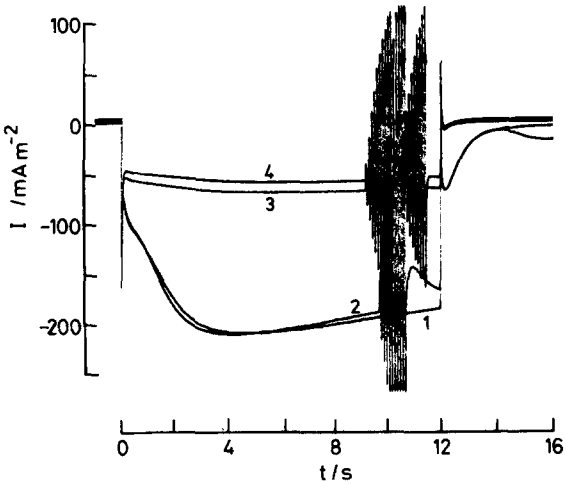

Fig. 4. Inward-2 current traces for $\psi_v = -300$ mV before (curves 1 and 2) and after (3 and 4) the addition of 0.5 mM A-9-C to the bathing solution (APW pH_o 6). Each trace was obtained at 10-min intervals. Also shown are the current responses to scans performed on the inward-2 current

Effect of Channel Blocking Agents

To attempt to further identify the major ions carrying the inward-2 current we treated *Chara inflata* cells with various chemicals known to block Cl^- channels in animal cells. These included DIDS, A-9-C, ethacrynic acid and cadmium. Ethacrynic acid has been reported to block the Cl^- component of the action potential in some charophytes (Lunevsky et al., 1983). However, only A-9-C at concentrations between 0.1 and 0.5 mM reduced the inward-2 current in *Chara inflata*. It also considerably reduced the peak of the action potential in *Chara corallina*, and in *Chara inflata* when action potentials could be evoked initially (Tyerman & Findlay, *unpublished results*). The action of A-9-C was slow at concentrations less than 0.1 mM taking an hour or more for an effect to be observed. With concentrations of A-9-C between 0.2 and 0.5 mM, significant inhibition occurred within 10 to 20 min. Results of an experiment on a cell in pH_o 6.0 are shown in Fig. 4 where the current traces obtained at 10-min intervals are shown before and after the addition of 0.5 mM A-9-C. After 20 min the inward current reached a steady lower level and the inward current tails were reduced. After removal of the A-9-C the inward-2 current recovered over a period of about 30 min to close to its initial level.

In pH_o 6 at -300 mV the inward current was reduced by the addition of A-9-C from a median value of -170 mA m^{-2} (-102, -271, $n = 8$) to -64 mA m^{-2} (-28, -87, $n = 8$) (Fig. 2). Scans were performed on the inward-2 current before and after the application of A-9-C and the resulting instantaneous I *vs.* ψ_v curves showed that g had decreased from 0.69 S m^{-2} (0.48, 1.09, $n = 8$) to 0.41 S m^{-2} (0.17, 0.63, $n = 8$), and that the reversal PD had shifted in the negative direction from -44 mV (55, -136, $n = 8$) to -157 mV (-185, -123, $n = 8$) (Fig. 3). The instantaneous curves before and after inhibition intersected on average at about the electrochemical equilibrium potential for Cl^-, E_{Cl}. Note that because the holding PD was some 420 mV more negative than E_{Cl} a reduction in conductance of only 40% resulted in the inward current being reduced by about 60%.

In pH_o 6, A-9-C caused the reversal PD to shift to values similar to those obtained in high pH_o without A-9-C present (Fig. 3). In fact inhibition of the inward-2 current could only be observed at pH_o less than about 7. In the presence of A-9-C at -300 mV the reversal PD of instantaneous I *vs.* ψ_v curves on the remaining inward current was independent of pH_o or decreased slightly as pH_o decreased. An example of this result is shown in Fig. 3.

Addition of A-9-C to cells which were hyperpolarized in the light at pH_o 6 caused a slow depolarization to E_K with time courses similar to those observed when cells were put in the dark or when external Cl^- was removed (Fig. 5). Generally darkness resulted in a more rapid depolarization than observed with A-9-C or Cl^--free solutions. It was difficult to compare the rates of depolarization since there were large differences between cells and it was not possible to apply all three treatments on one cell. The examples in Fig. 5 were obtained at different values of pH_o. Although this affected the original PD in the hyperpolarized state it did not affect the characteristics of the time course. It is interesting to note that for the three treatments an initial hyperpolarization was often observed before the cells depolarized.

The lanthanum ion, which has been reported to act similarly to an elevated external calcium concentration (Takata et al., 1966), has been shown to decrease cation permeability in *Chara corallina* (Keifer & Spanswick, 1978), and from the results of Beilby (1984) it appeared to reduce the inward-2 current. In *Chara inflata*, La^{3+} at a concentration of 0.1 mM caused a slow and irreversible inhibition of the inward-2 current. Figure 6*a* shows the steady-state I *vs.* ψ_v curves obtained before and after La^{3+} treatment. Note that although the inward-2 current was considerably reduced with La^{3+} there was still a tendency for the curve to increase in slope as the PD was taken more negative. Such a result was also obtained with A-9-C (Fig. 6*b*). With La^{3+}, however, the outward current was also inhibited indicating that the K^+ conductance was reduced. The level of

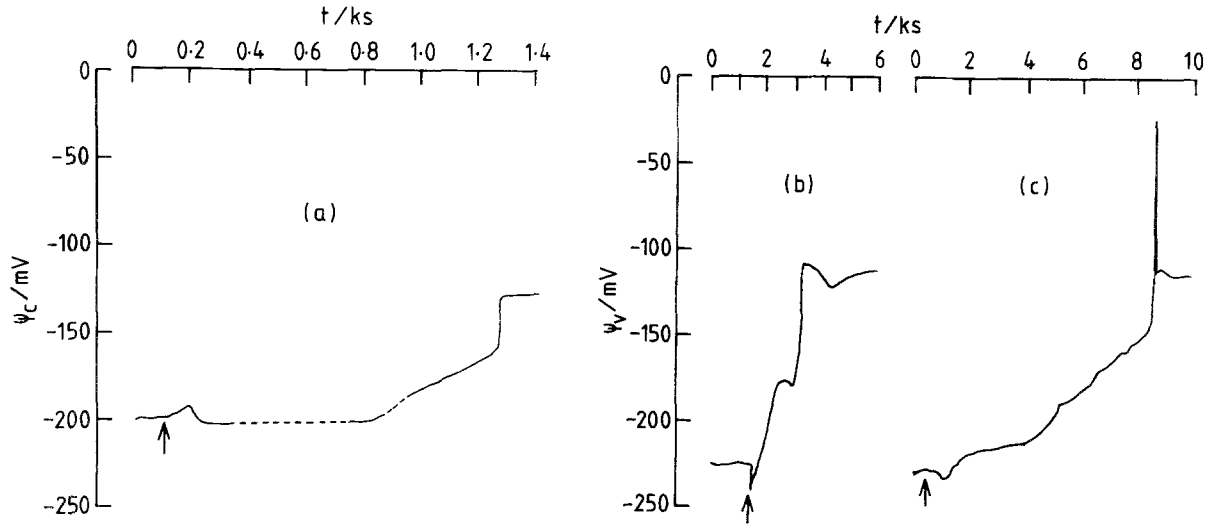

Fig. 5. Time courses of depolarization caused by (*a*) the addition of 0.5 mM A-9-C to APW pH_o 6, (*b*) darkness or (*c*) replacing external Cl^- with SO_4^{2-}. In (*a*) ψ_c was recorded and for periods indicated (----) the recording became erratic. ψ_v was 30 mV more positive than ψ_c during the time course. For (*b*) and (*c*) the time courses were obtained on the one cell (APW pH_o 7.5)

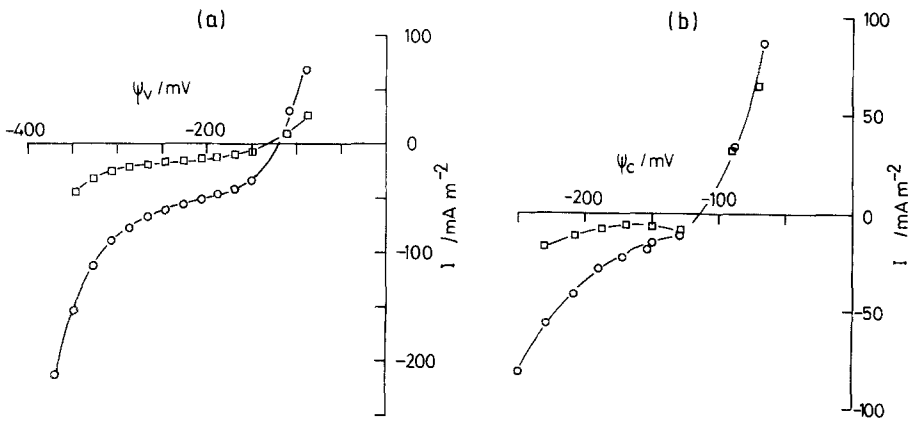

Fig. 6. Steady-state *I vs.* ψ curves obtained (*a*) before (○) and after (□) the addition of 0.1 mM La^{3+} or (*b*) 0.5 mM A-9-C to the bathing solution (APW pH_o 6). Results are from two different cells

inhibition caused by La^{3+} was usually greater than that observed with A-9-C and the reversal PD of the instantaneous *I vs.* ψ_v curves on the remaining inward current at −300 mV ranged between −180 and −200 mV in pH_o 6 (Fig. 3). As for A-9-C the reversal PD in the presence of La^{3+} was independent of pH_o (Fig. 3). Unlike A-9-C, however, La^{3+} had little effect on the hyperpolarized PD in the light. Other reputed K^+-channel blockers such as TEA^+ and Cs^+ had no effect on the inward-2 current. Nitrate, which accompanied the La^{3+} in our experiments, did not affect the inward-2 current in *Chara inflata* as it has been reported to do in *Aplysia* neurones (Chesnoy-Marchais, 1983).

In some treatments it was necessary for an inhibitor or channel blocker to be dissolved in ethanol so that the solute could be carried into the aqueous phase. However, it was found that 0.1% ethanol inhibited the inward-2 current at −300 mV to about the same extent as A-9-C. Alcohols also inhibit anion transport in red blood cells (Fortes, 1977).

Effect of Low Concentrations of Ammonium

An ammonium concentration of either 50 or 100 μM stimulated the inward-2 current at −300 mV (pH_o 6.0) in four out of five cells by factors of between 1.36 and 1.94 after 10 to 20 min. The cell for which no change was observed had an exceptionally large inward-2 current to begin with. Larger inward current tails were associated with inward-2 current stimulated by NH_4^+ but the overall shape of the activation curves was usually unaltered. The inward-2 current was also increased by NH_4^+ in Cl^--free solutions but not in the presence of 0.5 mM A-9-C. After

Table. Effect of NH_4^+ on the inward-2 current[a]

Cell	Control		plus NH_4^+		Conditions[b]
	g (S m^{-2})	rev PD (mV)	g (S m^{-2})	rev PD (mV)	
6784	0.44	−173	0.24	54	1
	0.41	−133			
	0.42	−173	0.29	−46	
11784	0.33	−73	0.28	81	1
			0.25	116	
	0.31	−88	0.33	87	1,3
			0.34	57	4
	0.42	−67			
	0.45	−97	0.35	−7	2
12784	0.55	−90	0.43	46	2,3
	0.52	−110	0.46	−69	2
13784	0.59	−69	0.55	55	1,3

[a] The conductance (g) and reversal PD (rev PD) were obtained from scans on the inward-2 current at ψ_o = −300 mV. The control was obtained immediately before the addition of NH_4^+ or 10 min after it was removed. All cells were in darkness in APW pH_o 6 and unless indicated otherwise the plus NH_4^+ values were obtained 10 min after the addition of NH_4^+.

[b] Conditions: 1 = 100 μM NH_4^+; 2 = 50 μM NH_4^+; 3 = Cl^--free; 4 = 20 min later.

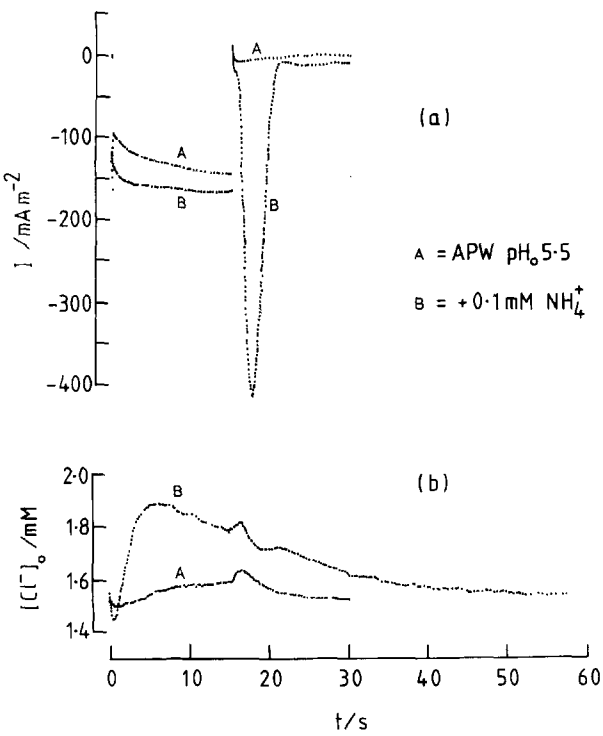

Fig. 7. (*a*) Inward-2 current responses and (*b*) corresponding extracellular Cl^- responses before and after the addition of 100 μM NH_4^+ to the bathing solution (APW pH_o 6)

the removal of the NH_4^+ the inward-2 current declined towards the previous levels within 10 to 20 min.

From scans taken on the inward-2 current before and after the application of NH_4^+ we obtained the change in reversal PD and conductance associated with the stimulation. The Table presents these data for four cells under different conditions. The NH_4^+ stimulation was associated with either no change in the conductance or more commonly with a decrease while the null potential shifted by a large amount in a positive direction. It was found that the inward-2 current stimulated by NH_4^+ was associated with an increase in Cl^- efflux (Fig. 7) measured by a Cl^- electrode (Tyerman et al., 1986). Note that in Fig. 7 an action potential occurred when the potential was returned to the resting level. This was reflected by a slight rise during the decay of $[Cl^-]_o$. An action potential often occurred where a current tail would normally be seen when the inward-2 current was stimulated, whether by NH_4^+, lowering pH_o or the removal of A-9-C.

Discussion

Inhibitors and Stimulators of the Inward Current

Anthracene-9-COOH, which has been reported to block the Cl^- conductance in muscle (Bryant & Morales-Aguilera, 1971), reduced the inward-2 current in *Chara inflata* by an amount depending on the pH_o. The auxin analog, 2,4-dichlorphenoxyacetic acid, which has a structure similar to A-9-C, also blocks the Cl^- conductance in mammalian skeletal muscle (Rüdel & Senges, 1972). There is good evidence that in *Chara inflata* only the Cl^- component was blocked by A-9-C, since the I *vs.* ψ curves before and after A-9-C addition intersected at E_{Cl} and with A-9-C the reversal PD in low pH_o shifted to levels observed in high pH_o where Cl^- was a small component of the inward current. The inward-2 current was blocked by A-9-C in Cl^--free solutions indicating that the effect was not an indirect result of reducing cytoplasmic $[Cl^-]$ by blocking the Cl^- influx. The possibility that A-9-C may have directly changed cytoplasmic pH was also discounted because its pK_a of 3.65 was well below the normal range of pH_o used in our experiments.

Anthracene-9-COOH also caused cells to depolarize to E_K from the hyperpolarized state, similar to the depolarization caused by the removal of external Cl^-. The removal of external Cl^- which would stop the Cl^-/H^+ symport, has been argued to

reduce the activity of the H^+ efflux pump due to an increase in cytoplasmic pH (Spanswick, 1980). The removal of external Cl^- and the addition of A-9-C may have the same effect on the hyperpolarized membrane if A-9-C directly blocked the Cl^- efflux thereby slowing the Cl^-/H^+ symporter due to a rise in cytoplasmic [Cl^-] (Sanders & Hansen, 1981). It is also possible that the Cl^- efflux is a reversal of the Cl^- influx mechanism in which case A-9-C may block Cl^- influx and efflux simultaneously.

Lanthanum blocked the inward-2 current but did not cause a depolarization to E_K from the hyperpolarized state. Unlike A-9-C, La^{3+} also blocked the K^+ conductance which may have prevented a depolarization to E_K. It is interesting that La^{3+}, considered as a general blocker of cation conductances, should also block an anion efflux. However, Ca^{2+} has also been found to inhibit the inward-2 current in *Chara inflata* (Findlay, Tyerman & Paterson, *unpublished data*) so La^{3+} may be acting as a "super Ca^{2+}" in *Chara inflata* consistent with its proposed mode of action (Takata et al., 1966).

The shift in reversal PD of instantaneous *I vs.* ψ curves from about -120 up to -200 mV when the membrane was hyperpolarized in the presence of La^{3+} or A-9-C may indicate an apparent shift in electrochemical equilibrium potential of the transported ion due to accumulation/depletion adjacent to the membrane. The inward currents and conductances were small under these conditions and it appeared that the shift in reversal PD went more negative with smaller conductances (comparing A-9-C and La^{3+} results). If the shift in reversal PD was caused by accumulation/depletion of an ion its concentration in the vacuole must be low. This may suggest that H^+ is involved except that the reversal PD was not dependent on pH_o in the manner expected (Fig. 3). The shift in reversal PD to more negative potentials as the membrane was hyperpolarized is consistent with the notion that an outward current with a reversal PD more negative than -300 mV may be activated by hyperpolarizing the membrane. This component of the net inward current which is also discussed in Tyerman et al. (1986) warrants further investigation.

The effect of NH_4^+ was to increase the Cl^- conductance as indicated by the increase in inward current and Cl^- efflux at constant ψ_v. The stimulation of the inward current by NH_4^+ in Cl^--free solutions suggests a direct effect on Cl^- efflux rather than an indirect stimulation caused by a stimulated Cl^- influx. The instantaneous *I vs.* ψ_v curves showed a shift in reversal PD towards E_{Cl} but interestingly the conductance measured from these curves decreased (Table). Thus the conductance of the non-Cl^- components of the inward current was decreased by NH_4^+. If the long-term effect of NH_4^+ is via a change in cytoplasmic pH then the direction of the change appears to not only stimulate Cl^- influx (Smith & Walker, 1980) but also to stimulate Cl^- efflux and inhibit another as yet unidentified ion flux.

The Cl^- Current as a Function of pH_o and ψ

It was shown by Tyerman et al. (1986) that the inward-2 current which was exponentially dependent on ψ was due to the efflux of Cl^-. An increase in efflux was also shown in that paper to account for the increase in inward-2 current when pH_o was lowered at constant ψ. In this paper it was shown that for pH_o less than 7 the inward-2 current was an exponential function of pH_o at constant ψ_v. That this was due to an increasing Cl^- efflux was supported by the increase in reversal PD as pH_o was lowered and from A-9-C treatment which removed the component of inward-2 current dependent on pH_o.

Tyerman et al. (1986) have shown that the inward-2 current, which will be taken as a first approximation to the Cl^- current (I_{Cl}), was a function of ψ given by the following equation:

$$I_{Cl} = -B_2 \exp(-K_2 \psi_c) \qquad (2)$$

where the median values of B_2 and K_2 were 4.66×10^{-2} mA m^{-2} and 3.18×10^{-2} mV^{-1}, respectively. It was found that for decreasing pH_o, K_2 was constant while B_2 increased. In terms of a Cl^- channel explanation, this means that decreasing pH_o had the effect of increasing the number of operative channels rather than affecting the voltage dependence of a channel. One could surmise that the channel gate has to be protonated on the outer surface of the membrane before it can operate since the power relationship between I_{Cl} and the concentration of H^+ at constant PD suggests an acid/base reaction. Other explanations as to the mode of action of pH include: i) negative surface charge in the vicinity of the voltage sensitive part of the channel is neutralized by protonation (Hille, 1968), ii) pH has an effect on the internal free energy of opening, a voltage-independent part of opening (Labarca et al., 1980).

Although it has been shown that the Cl^- efflux is exponentially dependent on ψ and pH_o, it is difficult to arrive at quantitative information due to problems in defining the non-Cl^- components and in compatibility between cytoplasmic and vacuolar

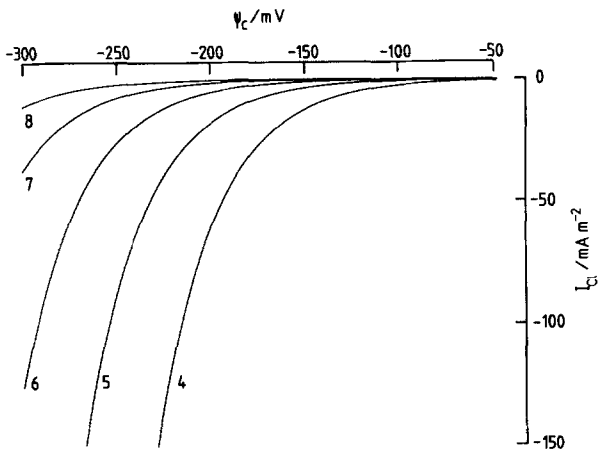

Fig. 8. Estimation of the chloride inward current from Eq. (4) as a function of ψ_c at five pH_o (values indicated against corresponding curve)

data. The latter difficulty arises because I as a function of ψ ($I(\psi)$) was determined from cytoplasmic data while I as a function of pH_o ($I(pH_o)$) was determined from vacuolar data. However, for an initial semiquantitative approximation to I_{Cl} as a function of pH_o and ψ these problems can be resolved as follows: (i) It will be assumed that the Cl^- component is completely blocked by A-9-C, giving a general leak of -64 mA m^{-2} at -300 mV. This leak can be taken as being constant with pH_o in our initial approximation (*see* Fig. 2). Thus by subtracting 64 mA m^{-2} from the median value of inward-2 current at each pH_o an estimate of I_{Cl} can be obtained as a function of pH_o which was fitted by the following equation (correlation coefficient = 0.97):

$$I_{Cl} = -B_3 \exp(-K_3\ pH_o) \tag{3}$$

where $B_3 = 171{,}174$ mA m^{-2} and $K_3 = 1.22\ pH_o^{-1}$. (ii) Our estimate of $I_{Cl}(pH_o)$ (Eq. 3) determined from vacuolar clamps will be assumed to be a close approximation to I_{Cl} for the plasmalemma. This is justified from the data of Tyerman et al. (1986) who found that the steady-state inward-2 current from the vacuole and cytoplasm were nearly identical functions of PD and that differences in $I(\psi)$ between cells were much larger than possible differences between vacuolar and cytoplasmic clamps. (iii) The two empirical equations (Eqs. 2 and 3) for the steady-state Cl^- current do not match, that is, $I_{Cl}(\psi)$ gives a current of -648 mA m^{-2} at -300 mV and pH_o 6, while $I_{Cl}(pH_o)$ at pH_o 6 and -300 mV gives -113 mA m^{-2}. It would be fortuitous if they did exactly match since the range of values for $I(\psi_c)$ was very large (Tyerman et al., 1986). Using the experimental finding of Tyerman et al. (1986) that the rate constant of $I_{Cl}(\psi_c)$ was constant with pH_o and thus that the rate constant of $I_{Cl}(pH_o)$ was also constant with ψ_c, we can offset $I_{Cl}(\psi_c)$ to match $I_{Cl}(pH_o)$ by changing A_2 (Eq. 2). We have offset $I_{Cl}(\psi_c)$ since it may have generally larger values as a result of not accounting for all of the non-Cl^- leak. The modified function is also still within the large range of observed values for $I_{Cl}(\psi_c)$ (Tyerman et al., 1985).

With the assumptions and arguments listed above, the steady-state inward current due to Cl^- can be approximated by the following equation.

$$I_{Cl} = -B \exp(-K_3\ pH_o - K_2\ \psi_c), \tag{4}$$

where $B = 12.3$ mA m^{-2}, and K_2 and K_3 have the same values as in Eqs. (2) and (3). Using Eq. (4), I_{Cl} as a function of ψ_c at five different pH_o has been plotted in Fig. 8.

Relevance to the Hyperpolarized State

The estimates of the Cl^- current as a function of PD given in Fig. 8 have been derived from data on cells in the dark and indicate that a Cl^- efflux of about 55 nmol m^{-2} sec^{-1} would be obtained at PD's of the hyperpolarized membrane. This flux is about 10-fold larger than Cl^- effluxes in the light but similar to maximum rates observed in the dark (Sanders, 1980). The overestimation of I_{Cl} by Eq. (4) could be accounted for by the pre-exponent constant B being smaller in the light than in the dark, while it is likely that the rate constants are the same. Data from Hope et al. (1966) show that this is indeed the case since they show that plots of the logarithm of Cl^- efflux as a function of potential in the light and dark are parallel. This is similar to the effect of pH_o. Therefore Eq. (4) would be relevant to the hyperpolarized membrane only in that it approximates the PD and pH_o dependence of the Cl^- efflux but not the actual magnitude of the efflux.

There is some evidence that the stoichiometry of the proton efflux pump in *Chara* is one proton per ATP hydrolyzed (Beilby, 1984) in which case in the ψ range of -150 to -250 mV the pump may be acting as a constant current source as in *Neurospora* (Slayman & Gradmann, 1975). If it were, our data provide a reason why the membrane depolarizes as pH_o is lowered and why the Cl^- efflux has been observed to be constant in different pH_o (Kitasato, 1968; Smith & Walker, 1976). Equation (4) predicts that for a one-unit decrease in pH_o the Cl^- efflux would rise by a factor of 3.4 if the potential was clamped, whereas the Cl^- influx may well remain about the same (Smith & Walker, 1976; Beilby & Walker, 1981). To obtain a net current of zero the membrane would depolarize by 38 mV irrespective of the actual value of the pump current, provided

the pump current remained the same. This is close to the observed depolarization for hyperpolarized cells in the light. That the Cl^- efflux determines the PD of the hyperpolarized membrane as a function of pH_o is further supported by the close correlation observed between ψ_v for the hyperpolarized membrane and the voltage-clamped $\psi_v(50)$ as a function of pH_o (Fig. 1). The function of the proposed influence of the Cl^- efflux over the pH_o sensitivity of the hyperpolarized membrane is unclear except that the changes in PD as a function of pH_o result in the electrochemical equilibrium potential for protons remaining approximately constant, as noted by previous authors (Walker & Smith, 1975).

Finally we would like to point out the similarity between the voltage dependence of the Cl^- efflux in *Chara inflata* and that observed and predicted in *Acetabularia* where the Cl^- efflux appears to be a reversal of the Cl^- electrogenic pump (Mummert et al., 1981). It was also suggested by Hope et al. (1966) that the Cl^- efflux may occur via a reversal of the Cl^- influx mechanism.

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