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Excitation-Revealed Changes in Cytoplasmic Cl^- Concentration in "Cl^--Starved" *Chara* Cells

M.J. Beilby

Botany School, Downing Street, Cambridge CB2 3EA, England

Summary. The changes in the cytoplasmic Cl^- concentration, $[Cl^-]_c$, are monitored at the time of withdrawal (starvation) and subsequent replacement of Cl^- in the outside medium. The measurement technique exploits the involvement of Cl^- in *Chara* excitation. The transient clamp current due to Cl^-, I_{Cl}, is separated from other excitation transients through Hodgkin-Huxley (HH) equations, which have been adjusted to *Chara*. The I_{Cl} amplitude depends on HH parameters, $[Cl^-]_c$ and the maximum membrane conductance to Cl^-, $\overline{g_{Cl}}$. The results are discussed in terms of these quantities. I_{Cl} and $\overline{g_{Cl}}$ were found to fall after 6-10 hr of Cl^- starvation, thus supporting the hypothesis that $[Cl^-]_c$ decreases in Cl^--free medium. The best HH fit to "starved" data was obtained with $[Cl^-]_c = 3.5$ mM. The time-course for I_{Cl} decline is considerably slower than the time-course of the rise of the starvation-stimulated influx. As cells starved for periods longer than 24 hr are re-exposed to Cl^-, it is revealed that while $[Cl^-]_c$ remains low during long starvation, $\overline{g_{Cl}}$ increases to values greater than those of the normal cells. Such differences among cells starved for various lengths of time have not been detected previously.

Key words: *Chara*, voltage clamp, cytoplasm, action potential, Cl^- concentration, Cl^- transport, Cl^- starvation

After more than 10 years of research, a clear scenario for driving force and regulation of Cl^- influx into giant algal cells has yet to emerge.

The hypothesis of Smith (1970, 1972) that Cl^- influx is powered and controlled by OH^- efflux (or H^+ influx) was further developed by Smith and Walker (1976). Energetic considerations led them to adjust the stoichiometry to $2H^+/1Cl^-$. Pre-treatment of cells in a medium where Cl^- was replaced by SO_4^{2-} was found to stimulate Cl^- influx two- to fourfold (Sanders, 1978, 1980*a*). The fluxes in such "Cl^--starved" cells are of sufficient magnitude to be detected by electrophysiological methods. Experimental evidence does support the scheme of two positive charges cotransported with Cl^-. Upon re-exposure to Cl^- the resting potential (RP) was found to depolarize by 10 mV (Sanders, 1978) and voltage-clamped cells exhibited a current flowing in the direction of negative charges leaving, or positive charges entering, the cell (Beilby & Walker, 1980, 1981).

There are connections between K^+ and Cl^- influxes, but experimental evidence makes a "salt" pump unlikely (Sanders, 1980*b*; Beilby & Walker, 1981).

Studies on perfused cells of *Chara* showed that Cl^- influx is strongly inhibited by low pH_i (Sanders, 1980*b*; Sanders & Hansen, 1981). In the intact cells the role of cytoplasmic pH, pH_c, is unresolved. Reid (1980) reported an increase by 0.2 pH unit in Cl^--starved cells, while Smith (1981) found pH unchanged in cells starved for 3-6 hr and only a small increase of 0.05 pH unit after overnight starvation. The DMO method of pH measurement (Smith & Walker, 1976) was used in both cases.

Cl^- influx in the perfused cells was found to fall with increasing Cl^- concentration in the perfusion medium (Sanders, 1978, 1980*a*). Sanders proposed that the starvation effect in the intact cells can be explained in terms of changes in cytoplasmic Cl^- concentration, $[Cl^-]_c$. From Cl^- influx data he calculated a drop of $[Cl^-]_c$ from 10 to 2.7 mM in Cl^--starved cells. A direct measurement of $[Cl^-]_c$ is necessary to establish whether changes in $[Cl^-]_c$ or pH_c are responsible for the increase of Cl^- influx at the time of Cl^--starvation.

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To check the changes in $[Cl^-]_c$ experimentally, however, is difficult. There is also no agreement on $[Cl^-]_c$ in normal cells, with a wide range of quoted values. The high values (20–90 mM) are probably caused by contamination by vacuolar sap (Spanswick & Williams, 1964; Kishimoto & Tazawa, 1965; Hope, Simpson & Walker, 1966; Tazawa, Kishimoto & Kikuyama, 1974). Lower values (10–25 mM) obtained by Ag/AgCl microelectrodes (Coster, 1966; Lefevre & Gillet, 1970, 1971) seem more reasonable. Jones and Walker (1980) measured even lower $[Cl^-]_c$ of 1.8 mM by efflux analysis.

In this paper a technique is discussed, which allows an experimental estimate of changes in $[Cl^-]_c$ at a time of Cl^--starvation. This method exploits the involvement of Cl^- in *Chara* excitation. Findlay and Hope (1964) changed the outside Cl^- concentration and calculated $[Cl^-]_c$ of ~3 mM from the reversal of the clamp currents. This value is now in doubt, as they were not aware that another transient participates in *Chara* excitation.

Upon depolarization past the threshold level (~ −100 mV), there is a large transient increase in permeability to chloride (and probably calcium) ions (Beilby & Coster, 1979*a*). As Cl ions in the cytoplasm are far from equilibrium, they flow out of the cell, down their electrochemical gradient. This outflow of negative charge further depolarizes the membrane p.d. The status quo is restored by outflow of positive ions, mainly K^+.

The excitation in *Chara* is analogous to that in the nerve. The main differences are in the speed of the process and the ions involved. The excitation in charophytes is about a thousand times slower than that in the nerve. The depolarizing current in the nerve is provided by inflow of sodium ions. The excitation in the nerve was described mathematically by Hodgkin and Huxley (1952*a, b*). They separated the ionic currents responsible for excitation and formulated their behavior in the terms of ion conductances. The Hodgkin-Huxley (HH) picture can be extended to *Chara* (Beilby and Coster, 1979*b*).

The total current I_T at the time of excitation:

$$I_T = I_{Cl} + I_{Ca} + I_{ss} \qquad (1)$$

where I_{Cl} = current due to chloride ions, I_{Ca} = current due to calcium ions and I_{ss} = voltage-dependent leakage current, mainly due to potassium ions. (At higher pH, electrogenic proton pump may also be involved.) These currents were separated by curve-fitting of the voltage-clamp data.

The chloride current I_{Cl} is given by:

$$I_{Cl} = g_{Cl}(E - E_{Cl}) \qquad (2)$$

where $g_{Cl} = \overline{g_{Cl}} m^3 h$, E = membrane potential, E_{Cl} = Nernst potential for Cl^-, $\overline{g_{Cl}}$ = maximum value for Cl^- conductance (all excitation channels open), and m, h = activation and inactivation HH parameters, that determine the fraction of excitation channels open at a particular time and potential. Their value is between 0 and 1. The changes in $[Cl^-]_c$ affect both E_{Cl} and $\overline{g_{Cl}}$. Hodgkin and Huxley (1952*b*) used an expression of the Goldman type (Goldman, 1943) relating $\bar{g}$ to the ion concentrations. The potential dependence of $\bar{g}$, which arises from this expression, was neglected by Hodgkin and Huxley. Following this treatment, $\bar{g}$ was taken to be linearly related to $[Cl^-]_c$.

The parameters m and h in *Chara* vary to some extent with temperature (Beilby & Coster, 1979*c*) and outside pH (Beilby, 1977). In the nerve drastic changes in m and h are achieved by specific drugs, but analogs to these for *Chara* have not yet been found. SO_4^{2-} medium is unlikely to change m and h values.

Thus the study of the voltage-clamp currents at the time of excitation provides information about the state of the membrane and the cytoplasmic conditions. I_{Cl} and I_{ss} have been monitored at the time of Cl^--starvation and Cl^--replacement. The results are discussed in terms of $[Cl^-]_c$, E_{Cl} and $\overline{g_{Cl}}$.

Materials and Methods

Biological Material

Very young leaf cells (3–5 mm in length) of *Chara corallina* were used. These cells are suitable for space-clamp and insertion of a cytoplasmic electrode (Beilby & Coster, 1979*a*). The culture was grown on the roof of the Botany School at the University of Cambridge. The same culture tank was used thoughout all experiments. The work was done within 6 months, starting in the winter. No consistent seasonal variations were observed. The growth rate of the cells did not change drastically over this period.

Electrophysiological Methods

The potential electrode was placed in the cytoplasm in all the experiments. Detailed descriptions of the experimental apparatus are given elsewhere (Beilby & Coster, 1979*a*). A new voltage-clamp circuit was used, a comparator based on 741 op. amp. integrated circuit (*see* Fig. 1).

Changes in conductance were measured during voltage clamp by superimposing a 5-Hz sine wave on the command voltage. The amplitude of the signal was between 10–20 mV and did not seem to affect the excitation currents. In absence of more sophisticated apparatus, this was only a rough estimate of changes in conductance, especially since it is possible that the capacitance of the plasmalemma changes at the time of excitation (Beilby & Smith, *in preparation*). For accurate measurement, a digital-impedance system, such as described by Bell, Coster and Smith (1975) would be necessary.

Solutions

Cl^-APW≡NaCl, 1.0 mM; KCl, 0.1 mM; $CaCl_2$, 0.5 mM; HEPES, 1.0 mM, NaOH to adjust to pH 7.5, 0.47 mM.

SO_4^{2-}APW≡Na_2SO_4, 0.5 mM; K_2SO_4, 0.05 mM; $CaSO_4$, 0.5 mM; HEPES, 1.0 mM, NaOH to adjust to pH 7.5, 0.47 mM.

For artificial pond water (APW) at pH 5.5, MES was substituted for HEPES. High flow rate of solution through the chamber made 1.0 mM of zwitterionic buffer sufficient to keep the pH steady.

Experimental Procedures

In all experiments the cells were cut at least a day before and soaked in appropriate medium. After insertion of the longitudinal electrode, cells were left to recover until the RP stabilized. All experiments were done at room temperature in dim light. The experiments were mostly performed at pH 7.5, with a few spot checks at pH 5.5.

1. Starvation Time-Course. The electrodes were inserted while the cell was in Cl^-APW. The potential was clamped to −80 mV at intervals of 10–30 min. (The refractory period at room temperature is estimated as less than 5 min.) Each clamp lasted ~15 sec. The potential of −80 mV was selected as I_{Cl} is at its maximum amplitude at this potential, whereas I_{Ca} is declining and can be neglected (Beilby & Coster, 1979*b*).

The cells were kept in Cl^-APW for up to 2 hr to obtain a stable I_{Cl} (*see* Fig. 2). Currents were recorded on a chart recorder. Cells which showed too much variation in I_{Cl} were discarded. SO_4^{2-}APW was then introduced into the chamber at a fast flow rate. The outside potential electrode (*see* *V*2, Fig. 1) was placed downstream from the cell, to eliminate possibility of contamination by KCl leakage from the tip. Before each clamp, Cl^-APW was introduced into the chamber through a two-way tap. When the RP decline stopped (~20 sec), the potential was clamped to

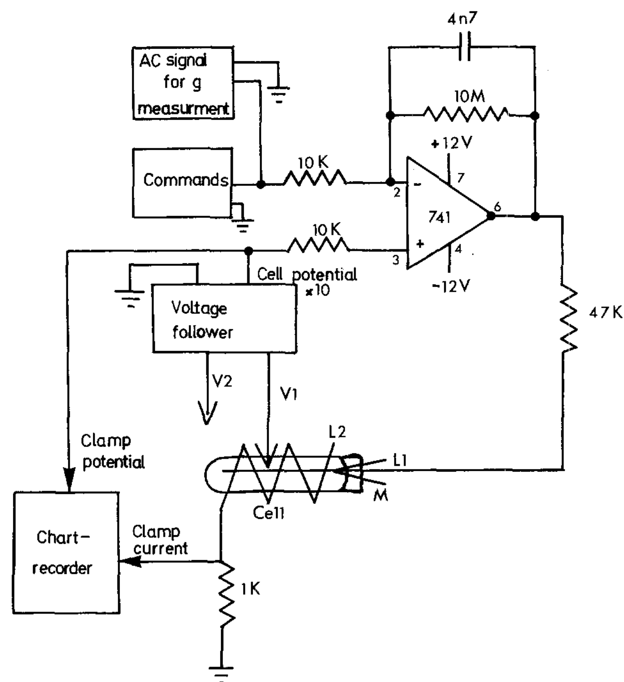

Fig. 1. The experimental apparatus. The potential-measuring electrode pair, *V*1 and *V*2, consists of glass micropipettes filled with 2 M KCl. The current-injecting electrode, *L*1, is made from Pt/Ir wire. The insertion of the wire is facilitated by puncturing of the cell wall with a glass micropipette *M* with its tip broken back. The current reference electrode *L*2 is an Ag/AgCl coil, which also serves as a cell holder. The voltage clamp circuit utilizes operational amplifier 741 with its feedback network designed to roll off the gain at high frequencies to prevent oscillations

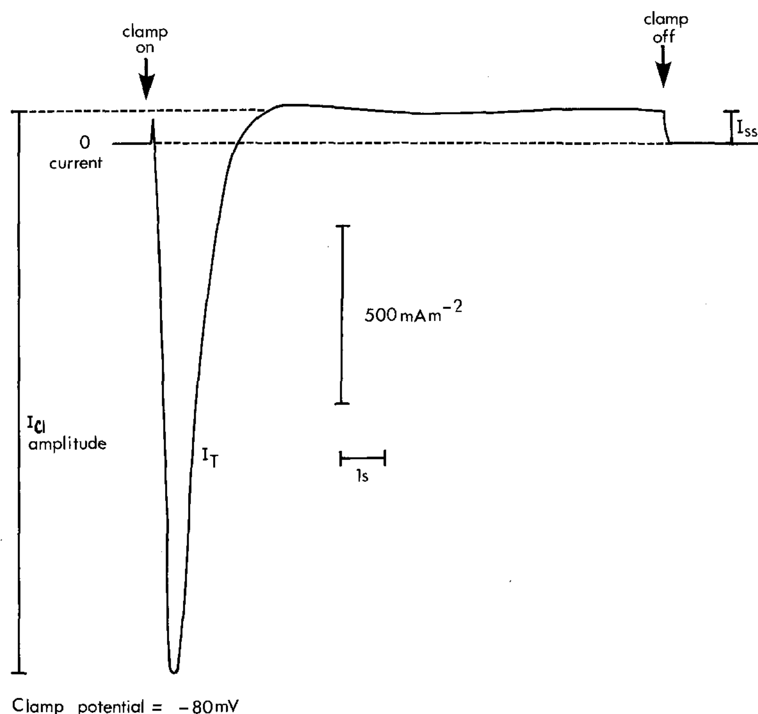

Fig. 2. Voltage clamp current I_T at −80 mV. At this potential level the current due to Ca^{2+} can be neglected. I_T then consists of a steady-state current I_{ss} and the chloride transient I_{Cl}. The amplitude of I_{Cl} can thus be estimated without HH fitting

-80 mV, current recorded and SO_4^{2-} APW returned to the chamber. I_{Cl} and I_{ss} were observed for up to 10 hr.

To ascertain that changes in clamp currents were not due to cell deterioration, control experiments were done with the cell in Cl^-APW for comparable periods.

2. Comparison of Clamp Currents Before and After Starvation. A family of clamp-currents was obtained at potential levels between -120 and 0 mV, in increments of 20 mV. (Such family of currents will from now on be referred to as a "set".) Cl^-APW was in the chamber. At least 5 min was allowed between successive clamps. Two sets of clamp currents were usually obtained from each cell to insure reproducibility.

The cell was then Cl^--starved in SO_4^{2-}APW. The potential electrodes were withdrawn during this period and reinserted after a few hours to check the currents. (No recovery period is necessary during a cytoplasmic insertion as the cell does not "gush".) When I_{Cl} reached a minimum and appeared to stay steady a set of clamp currents was obtained. A free-running action potential (AP) was also recorded before and after starvation. In some cells the conductance was also monitored at different clamp levels using the 5-Hz sine wave. A few starved cells were clamped at RP and the current induced by exposure to Cl^-APW was measured.

3. Cl^--Replacement Experiments. Cells were pre-soaked in SO_4^{2-}APW for several days. The soaking medium was changed several times a day to remove Cl^- from the efflux and from dying cells. The electrodes were inserted while the cell was in SO_4^{2-}APW. A set of clamp currents was recorded. The cell was in Cl^-APW at the time of each clamp. The exposures to Cl^- were kept as brief as possible. Then the cell was left in Cl^-APW and sets of clamp currents were recorded at $\sim$1-hr intervals (each set taking $\sim\frac{1}{2}$ hr).

In some experiments I_{Cl} at -80 mV was observed at 15-min intervals as a function of time in Cl^-APW.

4. $^{36}Cl^-$ Influx. Young leaf cells (similar to those used in electrophysiological experiments) were cut the night before the experiment and soaked in Cl^-APW.

The influx period in $^{36}Cl^-$APW was 5 min. This was followed by a 200-sec wash period in Cl^-APW. The cells were then placed on planchets, fixed with sucrose solution, dried and counted by an end-window Geiger counter.

Influxes of 5 cells were measured and then the remaining cells were placed in SO_4^{2-}APW. An influx was then measured in a single cell every 20 min for 7 hr.

5. HH Fit. HH equations were fitted to data from one cell one set of clamp currents before and one after Cl^--starvation. The calculations were done on a PET COMMODORE computer. The procedure was similar to that described by Beilby and Coster (1979*b*). Due to insufficient graphic facilities of the PET computer, the fit was very time-consuming and tedious. As the HH parameters obtained were comparable to those from Sydney cells (Beilby & Coster, 1979*b*), it was possible, using previous experience, to analyze most of the data without HH fitting (*see* Fig. 2). I_{Ca} can be neglected for potentials -80 to -40 mV. At potentials outside this range, the presence of I_{Ca} is reflected by larger error bars.

Results

Starvation Time-Course

After the cells were exposed to SO_4^{2-}APW, in some cases I_{Cl} showed a slow gradual decrease (Fig. 3*A*); in others there were oscillations before the final low value was reached (Fig. 3*B*). The time for I_{Cl} to reach a steady minimum was between 4 to 10 hr.

The controls in Cl^-APW usually showed a slight increase in I_{Cl}, which peaked after 4-5 hr, followed by a decline to initial magnitudes after $\sim$10 hr (Fig. 3*C*). If I_{Cl} was very variable in the first hour, the cell was discarded. Sometimes initially steady cells developed oscillations later (Fig. 3*D*).

The time-course was also monitored in one cell at pH 5.5. A response of the type shown in Fig. 3*A* was obtained.

Comparison of Sets of Clamp Currents Before and After Starvation

Fig. 4 shows clamp currents in one cell before (*A*) and after (*B*) 6 hr of Cl^--starvation. The negative transient currents decreased at all clamp levels in the starved cell. The zero potential level, where the I_{Ca} spike is dominant (Beilby & Coster, 1979*b*), was little changed by starvation. The summary of I_{Cl} results from several cells can be seen in Fig. 5*A*. The starved values do not show a great scatter. The larger error bars in Cl^-APW data at -100 mV and at -20 mV reflect the fact that I_{Ca} becomes more important at these potential levels, and the contribution of I_{Cl} can be accurately estimated only from the HH fit.

The steady-state current I_{ss} stayed remarkably constant in some cells for long periods of starvation (*see*, for instance Fig. 4*A*, *B*). This was taken as another assurance that the decrease in I_{Cl} was not due to general deterioration of the cell. Most cells showed a decline in I_{ss} after Cl^--starvation. To eliminate a possibility that this was due to deterioration, a cell was returned to Cl^-APW at the end of experiment and left overnight. The following morning I_{ss} returned to pre-starvation level. The summary of I_{ss} data as a function of starvation can be seen in Fig. 5*B*.

The Action Potential

Free running action potentials were measured in cells before and after Cl^--starvation (Fig. 6). The AP peak shifted to more negative levels in starved cells. The effect was not always as pronounced as in Fig. 6.

Cl^--Replacement Experiments

The currents I_{Cl} and I_{ss} obtained from cells after the long exposure (>24 hr) to SO_4^{2-}APW are shown in Fig. 7*A* and *B*, respectively. Note that both I_{Cl} and

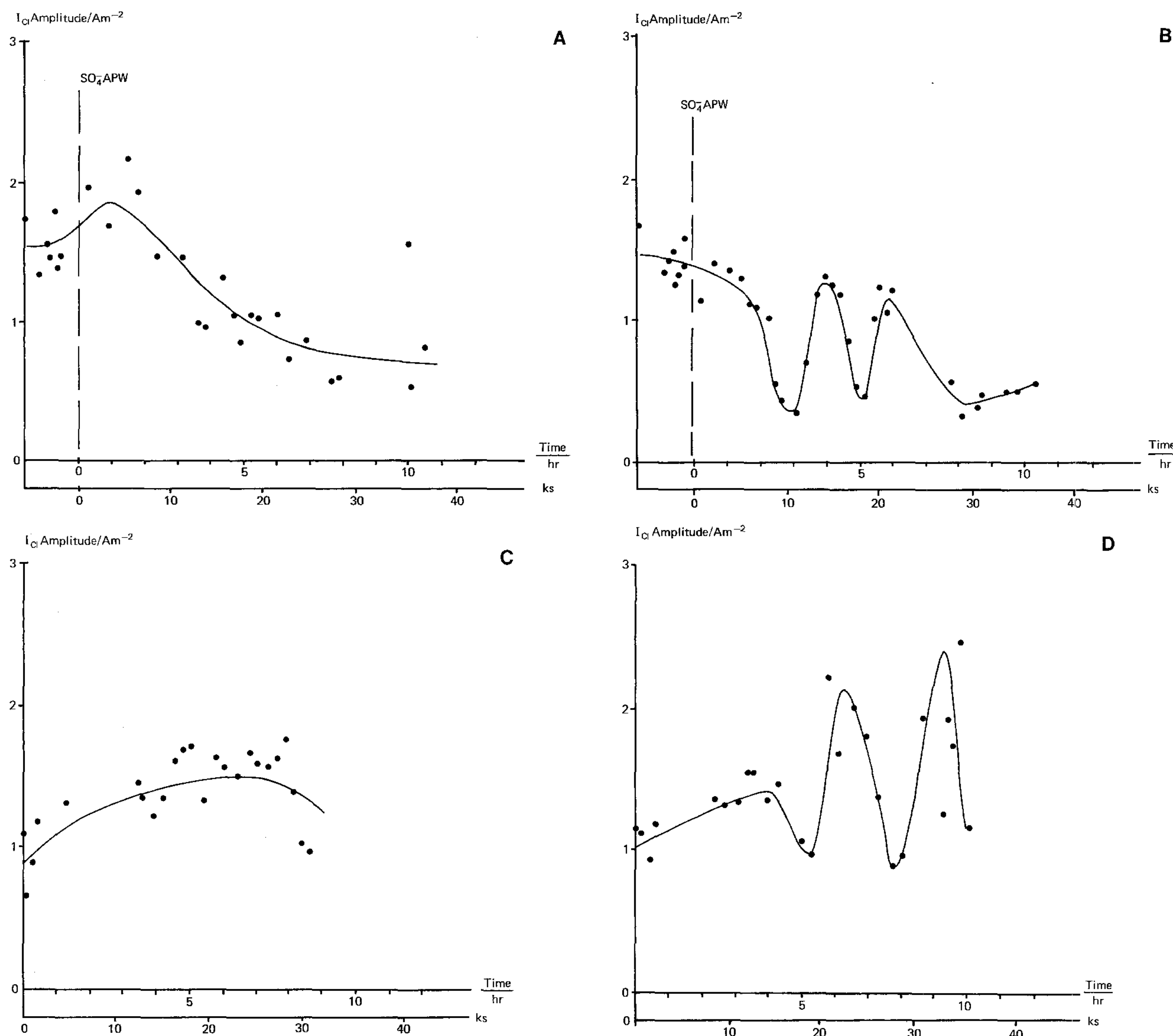

Fig. 3. (*A*) I_{Cl} amplitude as a function of the time in SO_4^{2-}APW. The Cl^--starvation commences at time=0. The behavior of I_{Cl} for more than 1 hr prior to Cl^- withdrawal is also shown. pH_o=7.5 (*B*) As in (*A*). (*C*) Control experiment: I_{Cl} amplitude in Cl^-APW over time period comparable to starvation times in (*A*) and (*B*). (*D*) As in (*C*)

I_{ss} are greater in amplitude than the ones recorded in unstarved cells (*compare* with Fig. 5*A*, *B*, closed circles). This result was unexpected, as after ~10 hr of starvation both I_{Cl} and I_{ss} amplitudes were considerably smaller than the pre-starved values (*see* Fig. 5*A*, *B*, open circles). It appears, therefore, that cells Cl^--starved for long periods (>24 hr) are in a different state than the cells starved for up to 12 hr. More long-term starvation experiments will be necessary to observe the transition from one state to another.

Exposure of cells, which have been starved for a long time, to Cl^-APW for 2-4 hr elicited no considerable changes in I_{ss} (*compare* closed and open circles in Fig. 7*B*). I_{Cl} went through a transient increase, which peaked after ~2 hr in Cl^-APW (Fig. 8). Further exposure to Cl^-APW reduced I_{Cl} to the initial magnitude.

The maximum I_{Cl}, I_{max} values for several cells are plotted in Fig. 7*A* as open circles. At −80 mV the average I_{max} is 1.6 times greater than the average initial I_{Cl} (before prolonged exposure to Cl^-APW; *see* Fig. 7*A*, closed circles).

Conductance Measurements

In the Hodgkin-Huxley equivalent circuit of the membrane (Hodgkin and Huxley, 1952*a*) the conductances due to various ionic currents at the time of excitation are added. To calculate the conduc-

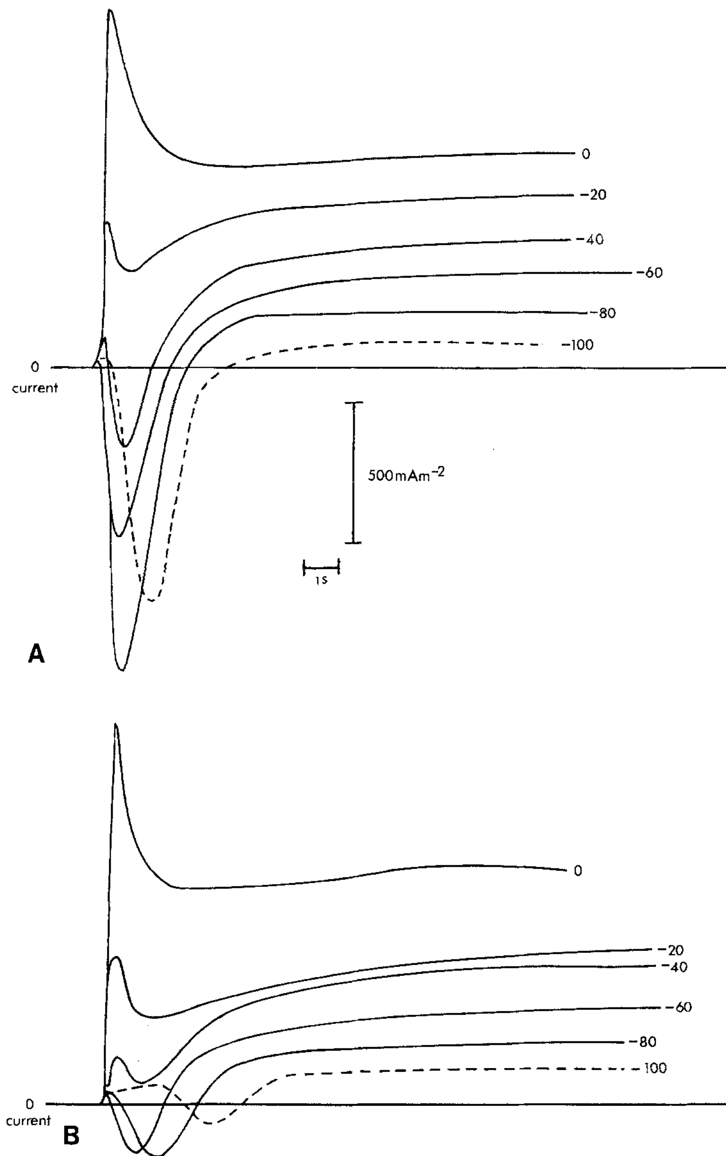

Fig. 4. Set of voltage-clamp currents at potential levels indicated next to each curve. (*A*) Before Cl^--starvation. (*B*) After 6 hr of SO_4^{2-}APW

tance as I_{Clamp}/V_{Clamp}, however, will lead to underestimation, as I_{Cl}, I_{Ca} and I_{ss} oppose each other at most potential levels. A better estimate of g can be obtained by superimposing a small AC signal on the command voltage. Fig. 9 shows such conductance measurement in a cell before (*a*) and after (*b*) starvation. The change in conductance in the potential range -40 to -80 mV measured in four cells was by a factor of 2.6 ± 0.6. The changes in conductance were only observed after I_{Cl} and I_{ss} decreased during Cl^--starvation.

HH Fitting

The HH parameters for both $I_{Cl}(a)$ and $I_{Ca}(b)$ are displayed in Table 1. The Cambridge cells show greater values for all the time constants (τ_m, τ_l, τ_h and τ_k) over the whole potential range, thus making both I_{Cl} and I_{Ca} slower than those in Sydney cells (compare with Beilby and Coster, 1979*b*). This slowing down of the transient currents is consistent with the temperature dependence of the excitation phenomenon (Beilby & Coster, 1979*c*). The Cambridge room temperature in the winter is 15–18 °C as compared to Sydney's 20–23 °C. The greatest increase is shown by τ_m and this is also consistent with the pH-dependence of this time constant (Beilby, 1977).

HH parameters obtained from the data in Cl^-APW were used to fit the Cl^--starved clamp currents of the same cell. No consistent change in any of the parameters was required, except for the delay in I_{Ca}, $D2$, which seemed to decrease in the

Fig. 5. Statistical analysis of the Cl^--starvation effect on (*a*) I_{Cl}, (*b*) I_{SS}. The points indicate the mean ($\pm$SE) with a number of cells given in brackets next to each point. In both (*A*) and (*B*) the closed circles show pre-starvation values, the open circles values after 6–10 hr of SO_4^{2-} APW

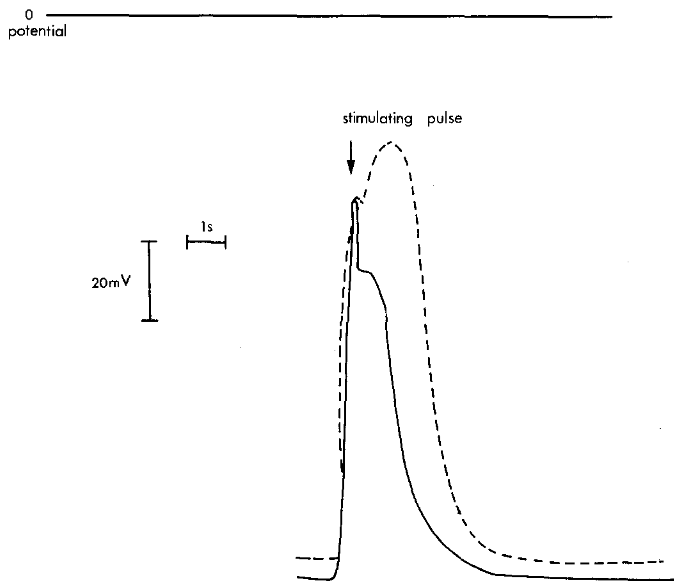

Fig. 6. Action potential before Cl^--starvation (------) and after 6 hr of SO_4^{2-}APW (———). Note that the resting potential remained unchanged during Cl^--starvation

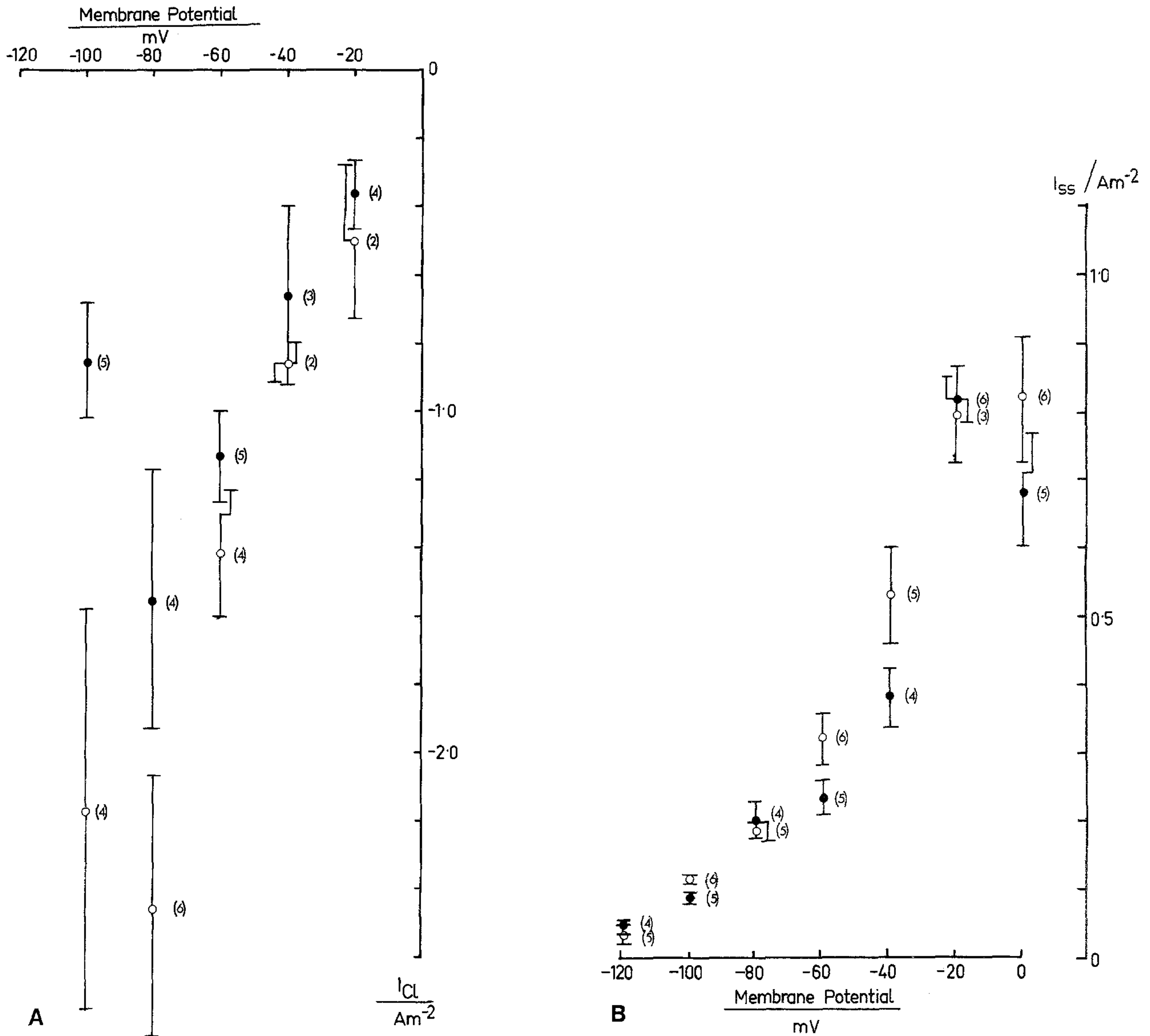

Fig. 7. Statistical analysis of the Cl^--replacement effect on (*A*) I_{Cl}, (*B*) I_{SS}. The closed circles show values after long exposure (>24 hr) to SO_4^{2-} APW. The open circles indicate values after the subsequent exposure to Cl^- APW for ~1.5 hr (*A*) and 2–4 hr (*B*). The data are processed as in Fig. 5

Cl^--starved data. It will be necessary to fit more data to determine if the change is significant. The fitting process involved decreasing the value of $[Cl]_c$ (and proportionally $\overline{g_{Cl}}$) from 10 mM until the best fit was obtained at 3.5 mM. The value of $\overline{g_{Cl}}$ was decreased accordingly from $20\,Sm^{-2}$ to $7\,Sm^{-2}$ giving the ratio $\overline{g_{Cl}}(u)/\overline{g_{Cl}}(s) = 2.86$ (u = unstarved, s = starved). The behavior of $I_{Cl}(u)/I_{Cl}(s)$ over the potential range is displayed in Table 2. The middle column shows how much of this ratio is due to $[Cl^-]_c$ change.

For most of the data, I_{Cl} was approximated as I_{Cl} amplitude as shown in Fig. 2. The summary of $I_{Cl}(u)/I_{Cl}(s)$, obtained in this manner, is presented on the right side of Table 2.

$^{36}Cl^-$ *Influx*

The Cl^- influx was found to increase from 20 nmol $m^{-2}\,sec^{-1}$ to ~40 nmol $m^{-2}\,sec^{-1}$ after ~100 min (6 ksec) of SO_4^{2-}APW. The starved data showed a large scatter. Similar variability was observed by

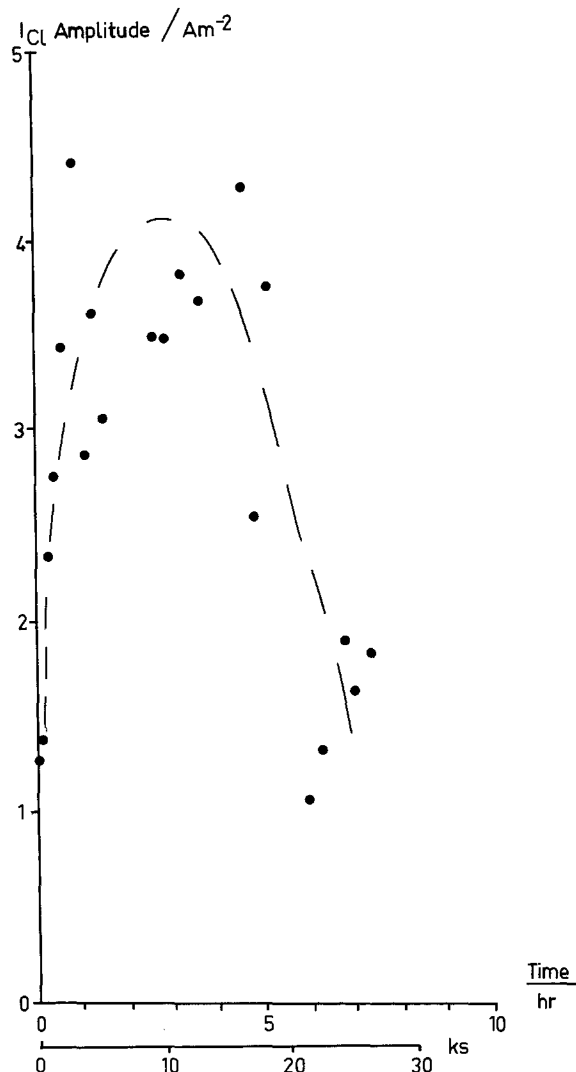

Fig. 8. Effect of Cl^--replacement on I_{Cl} amplitude in a cell exposed to SO_4^{2-}APW for a long period (>24 hr) prior to the experiment. The Cl^-APW was introduced into the chamber at time = 0

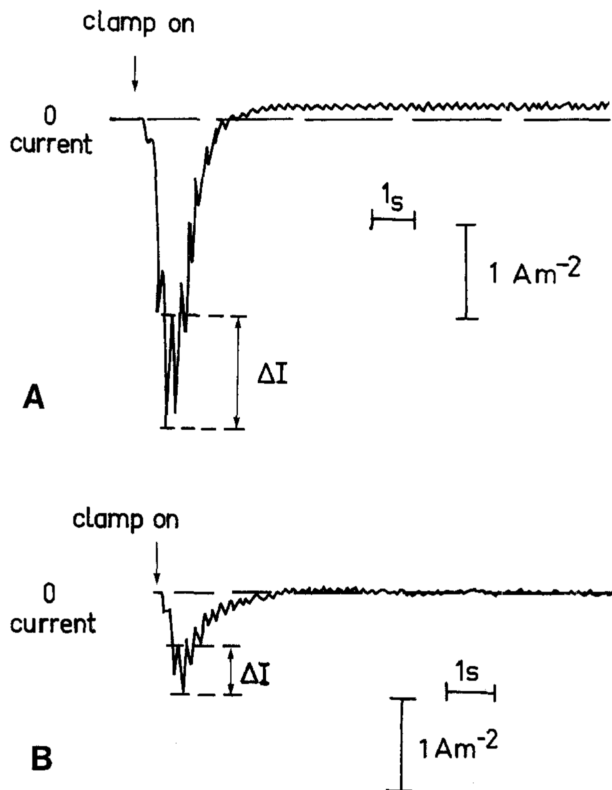

Fig. 9. Conductance measurement at clamp potential of −80 mV using perturbation of the command voltage by 5-Hz sine wave. (*A*) Before starvation. (*B*) After 8 hr of SO_4^{2-}APW. The conductance at the peak of the transient current can be calculated as $g = \Delta I/\Delta V$, with $\Delta V \approx 20$ mV in both (*a*) and (*b*). The ratio $g(u)/g(s) = 2.4$

Sanders (1978, 1980*a*) and Smith *(personal communication)*.

Discussion

The amplitude of I_{Cl} fell following a period of Cl^--starvation. The time to reach a steady low value varied from 6–10 hr. In some cells I_{Cl} declined steadily, in others oscillations could be observed. It is not clear if the oscillations resulted from the starvation process, as some controls also oscillated.

The best Hodgkin-Huxley fit to the starved data was obtained by reducing $[Cl^-]_c$ from 10 to 3.5 mM. The maximum conductance $\overline{g_{Cl}}$ was also decreased by a factor of 2.86. This value is in good agreement with the change in conductance measured by the sine-wave perturbation method:

$$\frac{g(u)}{g(s)} = 2.6 \pm 0.6.$$

This conductance, however, is a sum of g_{Cl} and g_{Ca}. Although I_{Ca} is small in potential range −80 to −50 mV, g_{Ca} can be large (Beilby & Coster, 1979*b*).

Most of the data, where I_{Cl} was estimated without full HH fit, showed a Cl^--starvation decrease of I_{Cl} by a factor 2–7 over the potential range −20 to −100 mV (*see* right side of Table 2). In the potential range −40 to −80 mV, where the estimate of I_{Cl} is most accurate, the $I_{Cl}(u)/I_{Cl}(s)$ ratio is lower than that of the HH fit (*see* left column of Table 2). This might indicate that on average the drop in $[Cl^-]_c$ is not as much as in the HH fitted data.

Cells starved for periods greater than 24 hr, however, showed I_{Cl} amplitudes *greater* than unstarved cells. No indication of the shift to such state was

Table 1. Hodgkin-Huxley Parameters: (*a*) I_{Cl}

Membrane potential (mV)	m_∞		τ_m sec		τ_h sec		$D1$ sec	
	u	*s*	*u*	*s*	*u*	*s*	*u*	*s*
0	1.0	1.0	1.8	1.8	0.2	0.2	0	0
−20	0.99	0.99	1.9	1.9	0.4	0.42	0	0
−40	0.98	0.98	1.72	1.68	0.33	0.33	0	0
−60	0.96	0.96	1.48	1.39	0.28	0.33	0	0
−80	0.8	0.8	1.32	1.36	0.38	0.38	0.05	0.19
−100	0.41	0.41	0.86	1.15	0.52	0.52	0.48	1.08

s = Cl⁻-starved; u = unstarved; $\overline{g_{Cl}}(u) = 20\,\mathrm{Sm^{-2}}$; $\overline{g_{Cl}}(s) = 7\,\mathrm{Sm^{-2}}$; $[Cl^-]_c(u) = 10\,\mathrm{mM}$; $[Cl^-]_c(s) = 3.5\,\mathrm{mM}$.
Time and potential dependence of activation and inactivation parameters for I_{Cl} and I_{Ca}:

$m = m_\infty - (m_\infty - m_o)\exp[(D1 - t)/\tau_m]$
$h = h_\infty - (h_\infty - h_o)\exp[(D1 - t)/\tau_h]$
$l = l_\infty - (l_\infty - l_o)\exp[(D2 - t)/\tau_l]$
$k = k_\infty - (k_\infty - k_o)\exp[(D2 - t)/\tau_k]$

where t = time, $m_o = l_o = 0$, $h_o = k_o = 1$, $h_\infty = k_\infty = 0$.

(*b*) I_{Ca}

Membrane potential (mV)	l_∞		τ_l sec		τ_k sec		$D2$ sec	
	u	*s*	*u*	*s*	*u*	*s*	*u*	*s*
0	1.0	1.0	0.55	0.55	0.15	0.14	0	0
−20	0.98	0.98	0.8	0.8	0.17	0.15	0.15	0.05
−40	0.98	0.98	0.6	0.55	0.115	0.115	0.5	0.3
−60	0.79	0.79	0.8	0.8	0.25	0.3	1.1	0.55
−80	0.8	0.8	1.27	1.27	0.3	0.27	1.5	1.0
−100	0.62	0.62	1.8	1.7	0.35	0.35	3.0	2.0

$\overline{g_{Ca}} = 12\,\mathrm{Sm^{-2}}$; $[Ca^{2+}]_c = 25\,\mathrm{mM}$.

Table 2. $I_{Cl}(u)/I_{Cl}(s)$

HH fit of one cell:			I_{Cl} amplitude estimates:
$[Cl^-]_c(u) = 10\,\mathrm{mM}$; $[Cl^-]_c(s) = 3.5\,\mathrm{mM}$	$\dfrac{\overline{g_{Cl}}(u)}{\overline{g_{Cl}}(s)} = 2.86$		$[Cl^-]_c(s)$, $[Cl^-]_c(u)$ unknown
Membrane potential (mV)	$I_{Cl}(u)/I_{Cl}(s)$	$\dfrac{(E - E_{Cl}(u))}{(E - E_{Cl}(s))}$	$I_{Cl}(u)/I_{Cl}(s) + \mathrm{SE}$
−20	5.4	1.87	7.3 ± 3.0 (4)
−40	4.4	1.54	2.8 ± 0.3 (6)
−60	3.9	1.38	2.4 ± 0.2 (8)
−80	3.7	1.3	2.5 ± 0.2 (8)
−100	3.6	1.25	4.2 ± 1.3 (5)

observed during starvation time course experiments. Presumably, it occurs after more than 12 hr in SO_4^{2-}APW.

When Cl⁻ was supplied to such cells, I_{Cl} increased by a factor of 0.9 to 2.5 (over the potential range −20 to −100 mV). This increase reached a maximum after ~1.5 hr in Cl⁻APW and then the I_{Cl} declined to its initial value (before prolonged exposure to Cl⁻APW).

I_{ss} exhibited a decline after 12 hr of starvation, but increased to slightly greater than unstarved level in cells that were starved for more than 24 hr.

The young leaf cells showed influx stimulation in SO_4^{2-}APW comparable in magnitude and time-course to those observed by Sanders (1978, 1980*a*) in axial internodes. Cl⁻-starved leaf cells voltage-clamped to the RP exhibited currents in the range 50–100 nmol m^{-2} sec^{-1} (for monovalent ion) upon exposure to Cl⁻APW. These values compare well with saturated currents observed by Beilby and Walker (1981) in axial internodes of *Chara*. It seems reasonable, therefore, to assume that the Cl⁻ influx mechanism is the same in the axial internodes and young leaf cells of *Chara*.

The results support the hypothesis of Sanders (1978, 1980*a*), that the cytoplasmic concentration drops to about one-third of its normal value after Cl⁻-starvation. The time-course of $[Cl^-]_c$ decrease is much slower than the time-course of Cl⁻ influx increase (Sanders, 1980*a*). Further, Sanders found that in perfused cells stimulation of Cl⁻ influx did not occur until $[Cl^-]_c$ had fallen below 3 mM (Sanders, 1978; Sanders & Hansen, 1981). These results were obtained at $pH_i = 7.45$, which would correspond to pH_c in intact cells with $pH_o = 5.5$. With $pH_i = 7.79$ (pH_c for cells in medium of 7.5 pH) the K_I for the inhibition of Cl⁻ influx by rising Cl_i was increased. At this pH_i Cl⁻ influx was more sensitive to small drops in $[Cl^-]_c$; the fall of $[Cl^-]_c$ from 10 to 5 mM (Sanders, 1978) produced starvation-stimulated influx comparable to that observed in $^{36}Cl^-$ influx experiment. Thus, on the basis of Sanders' data, to stimulate the influx to half of its final value, $[Cl^-]_c$ would only need to drop to ~7.5 mM. This would produce ~30% decrease in I_{Cl}. If the oscillations in Fig. 3*b* reflect changes in $[Cl^-]_c$, it would still take 2 hr to reach this reduction of I_{Cl}, as opposed to ~50 min for $^{36}Cl^-$ influx to reach half its final magnitude. On the other hand, the small decrease in I_{Cl} could be hidden in the scatter. It is, therefore, not possible to say at this stage whether the $[Cl^-]_c$ change provides a signal for the Cl⁻ influx to rise. The oscillations in $[Cl^-]_c$ would certainly furnish an explanation for the large scatter in the influx data seen by both Sanders and Smith.

A spot check carried out at pH_o 5.5 revealed a similar time-course in I_{Cl} decline as at pH_o 7.5. If the data of Sanders and Hansen (1981) can be extrapolated to intact cells, it is unlikely that the $[Cl^-]_c$ drop provides a control signal at this pH_o.

Thus, although the $[Cl^-]_c$ change may be a control signal under some circumstances, there must be other cues for the cell to raise its influx upon exposure to Cl^--free medium.

The unexpected return of I_{Cl} to greater than unstarved values in cells treated in SO_4^{2-}APW for longer than 24 hr could mean: (1) that cytoplasmic Cl^- is replenished from the vacuole, or (2) that there is a rise in $\overline{g_{Cl}}$. The second possibility seems more likely, as it explains the transient increase in I_{Cl} upon re-exposure of such cells to Cl^-APW. If $\overline{g_{Cl}}$ rises to values greater than normal in cells after long treatment in SO_4^{2-}APW, while $[Cl^-]_c$ remains at low level of ~3 mM, then the combination of high $\overline{g_{Cl}}$ and refilling cytoplasm upon exposure to Cl^- would produce very large I_{Cl}. In Cl^-APW, $\overline{g_{Cl}}$ is eventually restored to its normal level, and so is I_{Cl}. The time-course for I_{Cl} to reach a maximum after exposure to Cl^-APW follows Sanders' time-course for the decline of the Cl^- influx (and the presumed refilling of cytoplasm with Cl^-). The maximum I_{Cl} was greater by factors of 0.9 to 2.5 over the potential range −20 to −100 mV, which is close to the estimate of 1.2–1.87 from HH fit (factor due to change in $[Cl^-]_c$ only, Table 2).

The decline in I_{ss} in short-starved cells agrees with Sanders' observations that K^+ influx decreases together with Cl^- influx (Sanders, 1980*b*). It would be interesting to observe whether K^+ influx returns to normal after long exposures to SO_4^{2-}APW.

The shift of the AP peak to more negative values reflects the decrease in both $\overline{g_{Cl}}$ and $[Cl^-]_c$. With a more sophisticated computer system, it would be possible to stimulate the AP using "starved" HH parameters and compare the result with the data. This procedure provides a check on accuracy of the HH fit (*see* Beilby & Coster, 1979*b*).

In the perfused cells (Shimmen & Tazawa, 1980), the AP peak is independent of $[Cl^-]_i$ over a wide range of concentrations (0 to ~30 mM). However, Shimmen and Tazawa (1980) also found that the inner surface of the plasmalemma is not very anion-selective. Thus in the cells with SO_4^{2-} perfusion medium, for instance, $I_{SO_4^{2-}}$ might maintain excitation in absence of Cl^-. Findlay and Hope (1964) showed that in the external medium Cl^- can also be replaced by NO_3^- or Br^-. In the present experiments all the AP's and the clamp-currents were obtained in Cl^-APW to avoid the possibility of an addition of $I_{SO_4^{2-}}$ to I_T at the time of excitation. However, it is also possible that another anion from the cytoplasm (e.g. malate) might flow through the Cl^- excitation channels of Cl^--starved cells. The method is thus not selective to Cl^- and an overestimate of I_{Cl} (and consequently an underestimate of the decrease in $[Cl^-]_c$) might result. Intracellular perfusion might provide an answer to this problem.

At pH 7.5, the H^+ pump is fully turned on (Richards & Hope, 1974). As data on potential dependence of the pump is scarce, it is difficult to determine whether changes in pump conductance contribute to I_T at the time of excitation. Kishimoto, Kami-ike and Takeuchi (1980) calculated conductance changes due to the pump at the time of excitation and found them much smaller than the conductances across the excitation pathways.

The present method could be improved by accurate impedance measurements, which would test the assumptions that $\overline{g_{Cl}}$ is proportional to $[Cl^-]_c$ and independent of potential.

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