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Chloride Currents in *Chara*—A Patch-Clamp Study

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Summary. Ionic current steps were recorded with the patch-clamp technique from algal cells that had been prepared without enzyme treatment. Inward current steps with different conductance levels occurred, the lowest level being 7 pS. There were complex transitions between levels indicating either a lack of independence between single channels, or sublevels of a much larger conductance unit. The reversal potential was consistent with the permeant ion being Cl^-. Furthermore, when a different concentration of Cl^- was used in the patch electrode the reversal potential of the inward current shifted in a manner consistent with a Nernstian change in the Cl^- reversal potential. The frequency of the current steps was voltage dependent and suggestive of the hyperpolarization-activated Cl^- currents reported in voltage-clamp studies. Outward current steps, with conductances of 38 pS, were recorded when the membrane patch was depolarized by more than +120 mV. Their amplitude and frequency increased at more positive potentials. The current was probably carried by an efflux of cations through a different set of channels. The resting membrane potential, measured unambiguously without contamination from the tonoplast, was −190 ± 5 mV.

Key Words *Chara* · patch-clamp · single channels · chloride currents · algal cells · inward currents

Introduction

The presence of voltage- and time-dependent ionic currents in the membranes of plant cells has been well established with the voltage-clamp technique. This is particularly true for giant algal cells. In species of Characean algae, positive clamp steps result in inward and outward currents associated with action potentials (Findlay & Hope, 1964*b*; Lunevsky et al., 1983; Smith, 1984; Coleman & Findlay, 1985), while negative voltage-clamp steps activate an inward current that is thought to be carried by an efflux of Cl^- from the cell (Coleman & Findlay, 1985; Tyerman et al., 1986). In *Hydrodictyon africanum* an inward current can also be activated by hyperpolarizing the membrane (Findlay & Coleman, 1983), while positive voltage-clamp steps result in an outward K^+ current with an S-shaped time course which is slower than, but qualitatively similar to, the K^+ currents in the membranes of many animal cells.

The patch-clamp technique (Hamill et al., 1981) shows that, in animal cells, the transmembrane movement of ions along diffusion pathways occurs as square pulse-like steps of current. These events have been attributed to ions passing through integral membrane proteins known as channels that fluctuate between open, conducting states and closed, nonconducting states. This is also the case for the membranes of protoplasts prepared by enzyme treatment (Moran et al., 1984; Schroeder et al., 1984), though the effects of enzymes on membrane properties are not known.

This report describes the results of the application of the patch-clamp technique to the study of ionic currents in a plant cell membrane that had not been treated with enzymes. It therefore describes the occurrence and behavior of channels under physiological conditions. Inward and outward currents were recorded and these can be related to the macroscopic currents recorded from algal cells under voltage clamp.

A brief report of some of these results has appeared elsewhere (Coleman & Walker, 1984).

Materials and Methods

Young whorl tip cells, about 0.3 mm in diameter and 0.5 to 1 mm in length, from the fresh water alga *Chara australis* (R. Br.) were used. The plants were grown in tanks in the laboratory at room temperature (22 to 24°C) with a day length of 16 hr. The nodal cells from the tip of the plants, together with their leaf cells, young whorl tip cells and the preceding internodal cell were cut from the plant and stored for 5 to 15 days in artificial pond water (APW) consisting of (mmol liter^{-1}): NaCl 1.0; KCl 0.1; $CaCl_2$ 1.0; TES (N-tris(hydroxymethyl)methyl-2-aminoethane sul-

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Fig. 1. Inward current steps recorded from the same cell during two bursts of channel activity. The patch membrane was −10 mV from the resting potential. Open times ranged from hundreds of msec in duration to less than 1 msec. Closed times ranged from less than 1 msec during bursts of activity up to hundreds of seconds between bursts

fonic acid) 5; adjusted to pH 7.5 with NaOH. For an experiment, preparations were held in place with pins through the clear silicone rubber floor of a Perspex® recording chamber and were perfused with APW at room temperature. They were then plasmolyzed by adding sorbitol to the bathing solution through a density gradient mixer (Bock & Ling, 1954) such that the concentration of sorbitol increased linearly with time from 0 to 320 mmol liter^{-1} over 70 min. The plasmalemma of the young whorl cells usually came away from the cell wall at the tip. Fine scissors (Weck 2304) were then used to cut off the tip of the cell wall thus giving access to the plasmalemma without the use of enzymes. The cells maintained streaming during this and the subsequent recording period. The preparations were continuously perfused with APW containing 320 mmol liter^{-1} sorbitol throughout the experimental period.

Patch electrodes were pulled from micro-hematocrit tubes (Clay Adams), fire polished, coated with a silicone compound (732 RTV, Dow Corning) close to the tip and filled with solution containing (mmol liter^{-1}): tetraethylammonium-Cl, 4; choline methylsulfate, 24; sorbitol, 236; TES, 5; adjusted to pH 7.5 with tetraethylammonium-OH. In some experiments the Cl^- concentration within the patch electrode was increased from 4 to 28 mM by substituting choline-Cl for choline methylsulfate (*see* Results). The resistances of the electrodes were 25 to 40 MΩ with these solutions.

Electrical activity was measured with a Yale Mark V patch-clamp amplifier and recorded on magnetic tape. The data were subsequently replayed onto a linear chart recorder (Watanabe Linearcorder) and analyzed manually. The frequency of channel activity was defined as the mean number of "instantaneous" current steps away from the baseline per second, irrespective of the amplitude of the step. All single-channel currents were recorded in the cell-attached mode (Hamill et al., 1981). Thus inward currents refer to either the movement of cations from the patch electrode into the cell, or the efflux of anions from the cell to the patch electrode. The voltages shown correspond to the deviation of the membrane potential of the patch from the cell resting membrane potential. Thus positive potentials refer to depolarizing potentials. For some cells the membrane patch ruptured and it was possible to record the resting potential difference across the plasmalemma alone, without contamination from the tonoplast (whole-cell mode, *see* Hamill et al., 1981). All potentials, in both the cell-attached and whole-cell recording modes were corrected for liquid junction potentials at the tip of the patch electrode. For the patch electrode-cytoplasm interface, this was calculated assuming that the ionic concentrations in the cytoplasm were (mmol liter^{-1}): K^+ 115; Na^+ 3; Mg^{2+} 3; Cl^- 4; PO_4^{3-} 21 (Findlay & Hope, 1964*b*; Coster, 1966; Vorobiev, 1967; Tazawa et al., 1974; Williamson, 1975; Jones & Walker, 1980; Reid & Walker, 1983).

The statistic quoted with each mean is the standard error based on the number of preparations studied and not the number of cells or patches examined.

Results

General Observations

Seal resistances between the patch electrode and the cell membrane of greater than 1 GΩ were attainable and ranged up to 34 GΩ (19 ± 2 GΩ; n = 21).

For some 20 cells the membrane patch ruptured and it was possible to record the resting potential difference across the plasmalemma alone, without contamination from the tonoplast. After correcting for the calculated liquid junction potentials at the tip of the patch electrode, the mean value of the resting potential was −190 ± 5 mV (n = 20).

When patch-clamped recordings were made in the cell-attached mode at potentials close to the resting membrane potential, bursts of activity occurred only occasionally and consisted of inward current steps of several pA in amplitude with open times that ranged from less than 1 msec to hundreds of msec in duration (Fig. 1).

At increasingly more depolarized potentials the amplitudes of the inward current steps tended to get smaller (Fig. 2*A*). At potentials more positive than about +120 mV from the resting potential, inward current steps could no longer be discerned and outward current steps were recorded that increased in amplitude at more positive potentials (Fig. 2*A*).

Inward Currents

The inward current steps occurred at a low frequency at depolarized potentials and increased in frequency as the potential was made more negative (Fig. 2*B*). This voltage dependence was statistically significant and the combined data (n = 16 patches from 13 preparations at 12 different potentials) could be described by an exponential function with an e-fold change in frequency for a 53-mV change in potential, with a regression coefficient of 0.96 and a frequency of 0.7 Hz at the resting potential.

The inward currents displayed complex transitions between various levels and examples from four different cells are shown in Fig. 3. On some occasions there was a step change in the current from the baseline to a level of relatively large amplitude and the current then returned to the baseline in

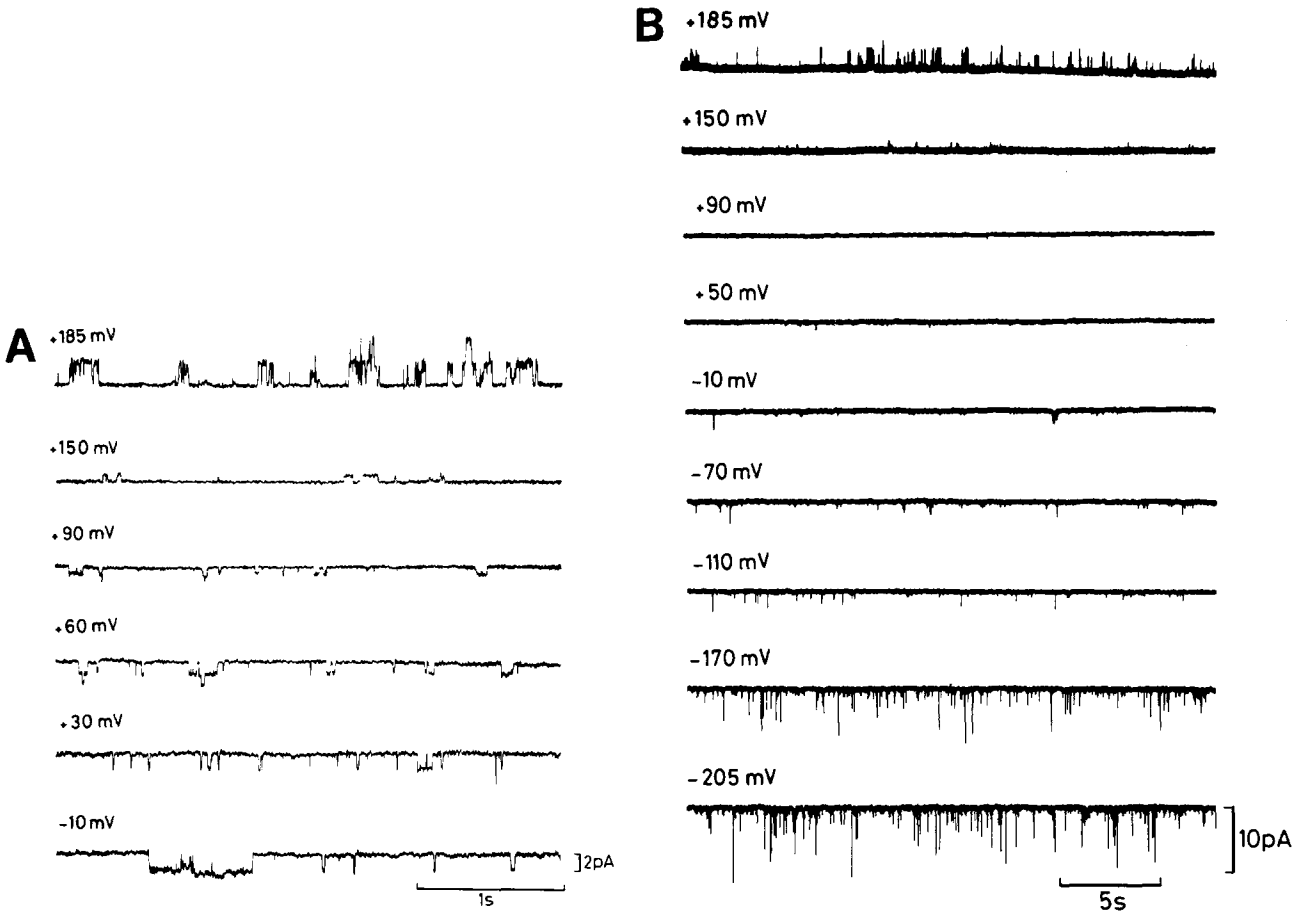

Fig. 2. Effects of membrane potential on *A*, the amplitude, and *B*, the frequency of the current steps. Positive potentials refer to depolarizations from resting potential. Records are shown for two different cells. At potentials more positive than +120 mV, outward current steps occurred which increased in amplitude and frequency at increasingly more positive potentials. At potentials more negative than +120 mV, inward current steps occurred which increased in amplitude and frequency at increasingly more negative potentials. For example, in *B*, at +50 mV, the mean frequency was 0.31 Hz, at −10 mV the frequency had increased to 1.18 Hz and at −170 mV the frequency had increased further to 10.19 Hz

a staircase-like manner (second trace from the top). In other cases the current increased in amplitude in a staircase-like manner and then returned to the baseline via a single, large step (trace 3). Other current steps occurred whose amplitudes appeared to be multiples of the amplitudes of the smaller current steps (traces 1 and 4). Of the total number of inwardly directed current steps (single- and multi-level transitions) for the cells of Fig. 3, at the potentials shown, the proportions of "instantaneous" multi-level transitions were, from top to bottom, 35, 27, 42 and 22%. Such behavior was typical for the inward current steps (but not for the outward currents, *see below*) and is highly improbable for independent channels.

The transitions between the various levels for the inward current suggest that the ion or ions carrying the currents are the same for the various levels and therefore have the same reversal potential. The current-voltage data were interpreted accordingly and the lines of best fit for the mean amplitudes of the inward current steps were calculated such that the intercepts on the voltage axis were forced to a common value. These lines were not statistically different from ordinary lines of best fit not forced through a common point. Examples for two cells are shown in Fig. 4. Overall, the conductance levels were 7 ± 1 pS (n = 9), 16 ± 1 pS (n = 9), 28 ± 3 pS (n = 7) and 44 ± 12 pS (n = 2).

The reversal potential for the inward current was +169 ± 12 mV (n = 9) from the resting potential, that is −21 ± 13 mV, in terms of the absolute membrane potential. This is in agreement with the value of −19 ± 9 mV obtained from five of these cells for which both the resting membrane potential and the reversal potential were determined. The variability in the values of the reversal potential most likely resulted from the determination of the

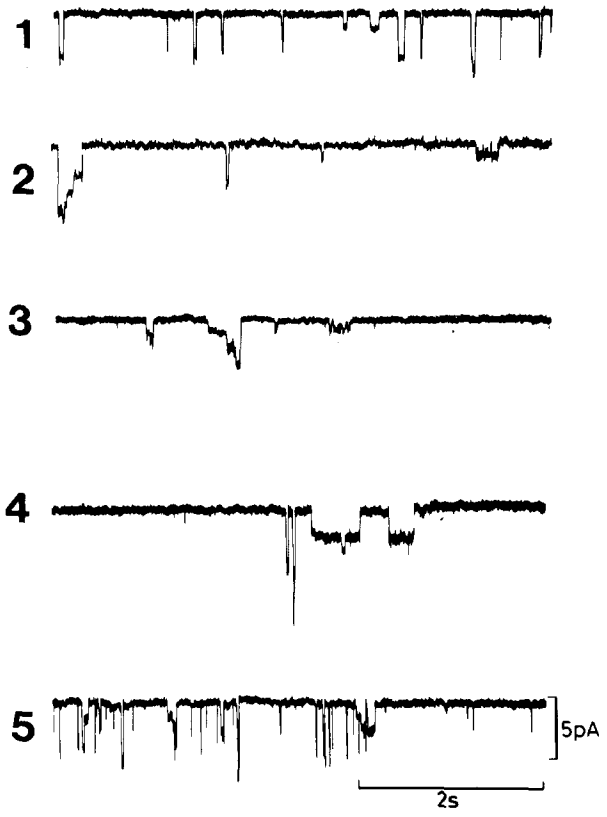

Fig. 3. Examples from four different cells of the complex transitions between current levels recorded during bursts of activity. The patch potentials were, from top to bottom, +90, +120, +42, +40 and +42 mV, relative to the resting potential. Traces 3 and 5 were from the same patch. These results indicate that a number of conductance levels can open simultaneously, shut simultaneously, or both. The total number of inwardly directed current steps which occurred, the total recording time at that potential and the mean frequency were, from top to bottom, 78 events/201 sec (0.39 Hz), 51 events/208 sec (0.25 Hz), 324 events/131 sec (2.48 Hz) and 57 events/100 sec (0.57 Hz)

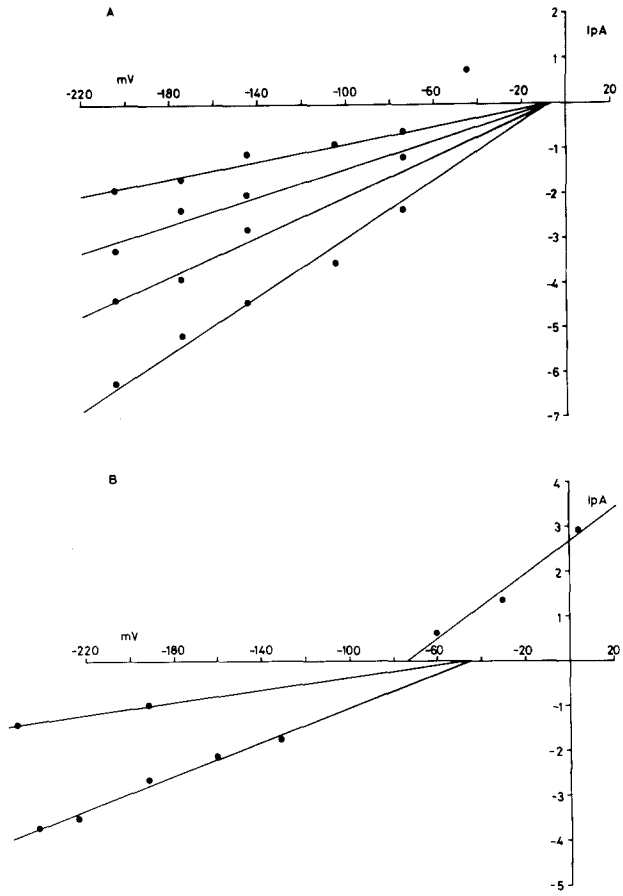

Fig. 4. Current-voltage relations for inward and outward current steps recorded from two cells. The voltage axes are labeled in terms of the absolute resting potential difference across the plasmalemma since, for these cells, the patch membrane ruptured after the current steps had been recorded and it was possible to determine the resting potential directly, after adjusting for liquid junction potentials. *A*. The slope conductances for the inward current steps were 9, 15, 22 and 32 pS and the equilibrium potential was −9 mV. *B*. For the inward current steps the slope conductances were 7 and 19 pS and the equilibrium potential was −43 mV. The outward current steps had a slope conductance of 36 pS and an equilibrium potential of −74 mV

values from somewhat long extrapolations of lines with small slopes, necessitated by the low frequency and small amplitude of current steps at depolarized potentials. A value of about −20 mV suggests that the inward current was carried by the efflux of Cl^- from the cells and corresponds to a concentration of Cl^- in the cytoplasm of 2 ± 1 mM, which is within the range of some earlier estimates (1 to 10 mM, Findlay & Hope, 1964*b*; Coster 1966; Jones & Walker, 1980). The concentration of Cl^- in the electrode was increased from 4 to 28 mM by substituting choline-Cl for choline methylsulfate. The reversal potential changed significantly from −19 ± 9 mV to −58 ± 14 mV (n = 5), consistent with the expected Nernstian shift (to −68 mV) in the Cl^- reversal potential.

Outward Current Steps

The outward current differed from the inward current in a number of ways. Small changes in potential resulted in relatively large changes in the amplitudes of the current steps and this can be seen in Fig. 2*A* and Fig. 4*B*. Figure 2*B* shows that outward current steps increased in frequency at potentials increasingly more positive, and did so with a relatively strong voltage dependence. Changing the potential from +150 mV to +185 mV from the resting potential resulted in the mean frequency increasing fourfold, from 1.8 ± 0.4 to 7.2 ± 2.5 Hz (n = 5). The

conductance change underlying the outward current was 38 ± 5 pS (n = 5). The reversal potential was +115 ± 5 mV from the resting potential and −75 ± 7 mV in absolute terms, using the corrected mean resting membrane potential. With 28 mM Cl^- in the patch electrode, the reversal potential was not changed, with values of +118 ± 18 mV (n = 5) from the resting potential, and −82 ± 11 mV (n = 4) in absolute terms for cells in which both the resting potential and reversal potentials were determined. This indicates that the outward current is carried by ions other than Cl^-.

Discussion

Inward Current Steps

At the resting membrane potential of *Chara australis* an inward current occurs in which ions pass through the membrane via voltage-dependent channels that are qualitatively similar to those that occur in the membrane of animal cells. This inward current is most likely carried by the efflux of Cl^- since its extrapolated reversal potential gives a reasonable prediction for the cytoplasmic concentration of that ion and the reversal potential shifted in a manner consistent with the expected Nernstian shift in the Cl^- reversal potential. Na^+, K^+ or Ca^{2+} can be excluded from consideration because they were absent from the solution in the patch electrode. At a pH of 7.5 the possibility of H^+ being involved would be very low since the permeability of the membrane to H^+ only becomes significant for values of pH greater than 10 (Bisson & Walker, 1980).

The voltage dependence of the inward current steps is in qualitative agreement with Cl^- flux studies (Coster & Hope, 1968), which revealed a large increase in the efflux of Cl^- as the membrane was hyperpolarized, and current-clamp studies (Coster, 1965, 1969), which showed that the conductance of the membrane increased considerably as the membrane was hyperpolarized. More recent studies of giant algal cells under voltage clamp have revealed an inward current which was activated by hyperpolarization of the membrane (Ohkawa & Kishimoto, 1977; Findlay & Coleman, 1983; Coleman & Findlay, 1985; Tyerman et al., 1986). Chloride was identified as a major charge carrier of the current and the peak value of the current had an exponential dependence on voltage with an e-fold increase in the current for about every 30 mV increase in the membrane potential (Tyerman et al., 1986). This is a greater voltage dependence than that of the frequency of current steps observed in the present study (*see* Results). This difference is not surprising since whole-cell currents depend not only on channel frequency, but also on channel open times and the driving force. Furthermore, the voltage-dependence of the whole-cell currents was determined for the peak values of the current, before any significant inactivation had occurred, whereas there may have been some inactivation of the channels in the present study since the membrane was usually kept at the same potential for some minutes at a time. The plasmolysis of the cells in the present study and the possibility of other transport systems contributing to the whole-cell currents are other factors which may account for the different voltage dependences of the whole-cell and patch-clamp currents. Although the whole-cell currents and the frequency of channel activity did not have the same quantitative voltage dependence, both currents are activated by hyperpolarization of the membrane and are sensitive to the Cl^- concentration. These similarities suggest that the inward current steps recorded in the present study correspond with the hyperpolarization-activated Cl^- currents recorded under whole-cell voltage clamp.

The channels carrying the inward current appear to have complex kinetics, with varying numbers of conductance levels being able to open or shut in virtual synchrony. Similar behavior has also been reported for channels extracted from Characean cells and inserted into bilayer membranes (Volkova et al., 1981), for K^+ (Kazachenko & Geletyuk, 1984) and Cl^- (Geletyuk & Kazachenko, 1985) currents in molluscan neurones and Cl^- currents in pyramidal cells from the hippocampus of the rat (Gibb, 1985). One possible explanation is that the current steps of different amplitudes result from transitions between subconductance levels of a larger conductance channel. Another interpretation is that a number of small conductance channels tend to aggregate in a stable organization and can open and shut in virtual synchrony.

An interesting aspect of the results is that inward currents associated with the action potential were not detected when the membrane patch was depolarized. Although it cannot be ruled out that very small currents were buried in the noise, this observation seems to agree with earlier studies which showed that action potentials did not occur in Ca^{2+}-free solution (Hope, 1961; Findlay & Hope, 1964*a*,*b*) since this ion was absent from the solution in the patch pipette.

Outward Current Steps

Compared with the inward current steps, the outward current steps had a more negative reversal potential that was not altered by changing the con-

centration of Cl^-, a greater conductance and a frequency of channel activity that had a greater voltage dependence. It therefore seems likely that the outward current is not the reversal of the inward current but is probably carried by an efflux of cations. This outward current may correspond to the outward current that dominates voltage-clamp currents as the potential is shifted towards 0 mV (Findlay & Hope, 1964*b*; Lunevsky et al., 1983).

Resting Potential

The values of the resting membrane potential determined in this study were within the range of values obtained with intracellular electrodes (Beilby & Coster, 1979; Smith & Walker, 1981; Findlay & Coleman, 1983; Coleman & Findlay, 1985; Tyerman et al., 1986). Furthermore, they support the observations that, in *Chara australis,* plasmolysis does not affect the resting membrane potential significantly (Smith & Walker, 1981).

This study has shown that it is feasible to patch clamp giant algal cells under physiological conditions, without the use of enzymes. Hyperpolarization of the membrane tends to activate Cl^- channels with a low conductance and complex kinetics. Depolarization of the membrane activates an outward current with a greater conductance and the frequency of current steps has a much stronger voltage dependence.

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References

Beilby, M.J., Coster, H.G. 1979. The action potential in *Chara corallina*. II. Two activation-inactivation transients in voltage clamps of the plasmalemma. *Aust. J. Plant Physiol.* **6**:323–335

Bisson, M.A., Walker, N.A. 1980. The *Chara* plasmalemma at high pH. Electrical measurements show rapid specific passive uniport of H^+ or OH^-. *J. Membrane Biol.* **56**:1–7

Bock, R.M., Ling, N.S. 1954. Devices for gradient elution in chromatography. *Anal. Chem.* **26**:1543–1546

Coleman, H.A., Findlay, G.P. 1985. Ion channels in the membrane of *Chara inflata*. *J. Membrane Biol.* **83**:109–118

Coleman, H.A., Walker, N.A. 1984. Patch-clamp recording from a plant cell. *Proc. Aust. Physiol. Pharmacol. Soc.* **15**:196P

Coster, H.G. 1965. A quantitative analysis of the voltage-current relationships of fixed charge membranes and the associated property of "punch-through." *Biophys. J.* **5**:669–686

Coster, H.G. 1966. Chloride in cells of *Chara australis*. *Aust. J. Biol. Sci.* **19**:545–554

Coster, H.G. 1969. The role of pH in the punch-through effect in the electrical characteristics of *Chara australis*. *Aust. J. Biol. Sci.* **22**:365–374

Coster, H.G., Hope, A.B. 1968. Ionic relations of cells of *Chara australis*. *Aust. J. Biol. Sci.* **21**:243–254

Findlay, G.P., Coleman, H.A. 1983. Potassium channels in the membrane of *Hydrodictyon africanum*. *J. Membrane Biol.* **75**:241–251

Findlay, G.P., Hope, A.B. 1964*a*. Ionic relations of cells of *Chara australis*. VII. The separate electrical characteristics of the plasmalemma and tonoplast. *Aust. J. Biol. Sci.* **17**:62–77

Findlay, G.P., Hope, A.B. 1964*b*. Ionic relations of cells of *Chara australis*. IX. Analysis of transient membrane currents. *Aust. J. Biol. Sci.* **17**:400–411

Geletyuk, V.I., Kazachenko, V.N. 1985. Single Cl^- channels in molluscan neurones: Multiplicity of the conductance states. *J. Membrane Biol.* **86**:9–15

Gibb, A.J. 1985. Characteristics of a high conductance chloride channel observed in pyramidal cells dissociated from adult rat hippocampus. *Proc. Aust. Physiol. Pharmacol. Soc.* **16**:205P

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100

Hope, A.B. 1961. Ionic relations of cells of *Chara australis*. V. The action potential. *Aust. J. Biol. Sci.* **14**:312–322

Jones, S., Walker, N.A. 1980. Chloride compartmentation in *Chara corallina* by efflux analysis. *In:* Plant Membrane Transport: Current Conceptual Issues. R.M. Spanswick, W.J. Lucas, and J. Dainty, editors. pp. 583–584. Elsevier-North Holland Biomedical, Amsterdam

Kazachenko, V.N., Geletyuk, V.I. 1984. The potential-dependent K^+ channel in molluscan neurones is organized in a cluster of elementary channels. *Biochim. Biophys. Acta* **773**:132–142

Lunevsky, V.Z., Zherelova, O.M., Vostrikov, I.Y., Berestovsky, G.N. 1983. Excitation of *Characeae* cell membranes as a result of activation of calcium and chloride channels. *J. Membrane Biol.* **72**:43–58

Moran, N., Ehrenstein, G., Iwasa, K., Bare, C., Mischke, C. 1984. Ion channels in plasmalemma of wheat protoplasts. *Science* **226**:835–838

Ohkawa, T., Kishimoto, U. 1977. Breakdown phenomena in the *Chara* membrane. *Plant Cell Physiol.* **18**:67–80

Reid, R.J., Walker, N.A. 1983. Adenylate concentrations in *Chara:* Variability, effects of inhibitors and relationship to protoplasmic streaming. *Aust. J. Plant Physiol.* **10**:373–383

Schroeder, J.I., Hedrich, R., Fernandez, J.M. 1984. Potassium-selective single channels in guard cell protoplasts of *Vicia faba*. *Nature (London)* **312**:361–362

Smith, P.T. 1984. Electrical evidence from perfused and intact cells for voltage-dependent K^+ channels in the plasmalemma of *Chara australis*. *Aust. J. Plant Physiol.* **11**:303–318

Smith, P.T., Walker, N.A. 1981. Studies on the perfused plasmalemma of *Chara corallina*: I. Current-voltage curves: ATP and potassium dependence. *J. Membrane Biol.* **60**:223–236

Tazawa, M., Kishimoto, U., Kikuyama, M. 1974. Potassium, sodium and chloride in the protoplasm of *Characeae*. *Plant Cell Physiol.* **15**:103–110

Tyerman, S.D., Findlay, G.P., Paterson, G.J. 1986. Inward membrane current in *Chara inflata*. I. A voltage and time-dependent Cl^- component. *J. Membrane Biol. (in press)*

Volkova, S.P., Lunevskii, V.Z., Spiridonov, N.A., Vionokurov, M.G., Berestovskii, G.N. 1981. Chemical composition of calcium channels of Characean algal cells. *Biophysics* **25**:556–561

Vorobiev, L.N. 1967. Potassium ion activity in the cytoplasm and the vacuole of cells of *Chara* and *Griffithsia*. *Nature (London)* **6**:1325–1327

Williamson, R.E. 1975. Cytoplasmic streaming in *Chara:* A cell model activated by ATP and inhibited by cytochalasin B. *J. Cell Sci.* **17**:655–668

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