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# Cl<latex>$^-$</latex> Channels in Chara [and Discussion]

M. J. Beilby, R. D. Keynes and N. A. Walker

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# $Cl^-$ channels in *Chara*

By M. J. Beilby
*Botany School, Downing Street, Cambridge CB2 3EA, U.K.*

The action potential in the cells of the freshwater alga *Chara corallina* is slower than that in the nerve by about 1000-fold. The depolarization phase is brought on by the outflow of the $Cl^-$ ions. Voltage-clamp studies show that this $Cl^-$ current can be described by the Hodgkin–Huxley equations for the $Na^+$ transient in the squid axon. The only change necessary to the form of the Hodgkin–Huxley equations is an introduction of a time delay between the stimulus and the onset of excitation.

This mathematical model of the *Chara* action potential facilitates a quantitative description of the effects of pH and temperature. While a pH shift alters various Hodgkin–Huxley parameters, temperature change influences mainly the activation and inactivation time constants but leaves the voltage-dependence of these parameters unaffected. The delays in excitation are both temperature and potential dependent.

In future some corrections to the Hodgkin–Huxley picture of the *Chara* action potential may be necessary, as recent impedance measurements suggest a change in the membrane capacitance at the time of excitation.

## Introduction

*Chara corallina* is a freshwater alga. Its large cells (1 mm in diameter and up to 20 cm in length) provide a convenient material for electrophysiological studies of the cell membrane. It is possible to insert a microelectrode into the cytoplasm between the two concentric membranes (plasmalemma and tonoplast). Electrical p.d. (potential difference) can thus be measured across a single biomembrane *in vivo*.

*Chara* can generate action potentials (a.ps) in many ways analogous to those in the nerve, but their timescale is about 1000-fold slower (see figure 1). Radioactive tracer experiments show that the principal ions involved are $Cl^-$ and $K^+$ (Gaffey & Mullins 1958; Hope & Findlay 1964).

The continuous nature of the a.p. shape makes analysis difficult. More information can be obtained by the voltage-clamp technique adapted from animal physiology (see figure 2). In these experiments the cell p.d. is controlled through negative feedback and the current necessary to maintain the clamp potential is recorded. The clamp currents at a range of potentials can be seen in figure 3. At $-130$ mV (figure 3*a*) the threshold of excitation is not yet reached and only positive current can be observed. At $-120$ mV (figure 3*b*) the excitation is triggered after a long delay and two negative current peaks appear. As the potential becomes more positive, the delay decreases, the first negative peak grows to reach a maximum at $-70$ mV (figure 3*d*), while the other diminishes and merges with the first (figure 3*d*). Above $-50$ mV a sharp positive transient dominates the clamp current (figure 3*g*).

The first negative transient responds to changes in the external $Cl^-$ concentration, while the second negative transient and the sharp positive peak vary with changes in the $Ca^{2+}$ concentration. The currents are thus tentatively identified (Beilby & Coster 1979*a*), but the reversal of the $Ca^{2+}$-dependent transient at *ca.* $-50$ mV gives the cytoplasmic $Ca^{2+}$ concentration as an



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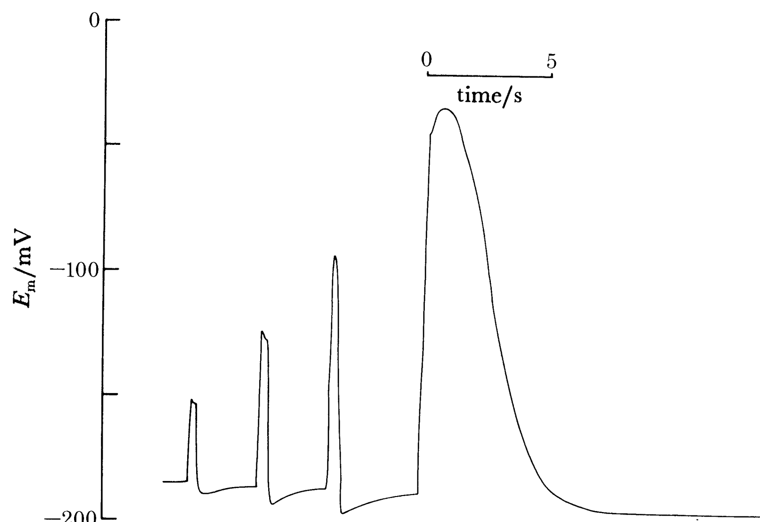


FIGURE 1. A cytoplasmic action potential triggered as the train of depolarizing pulses reaches excitation threshold.

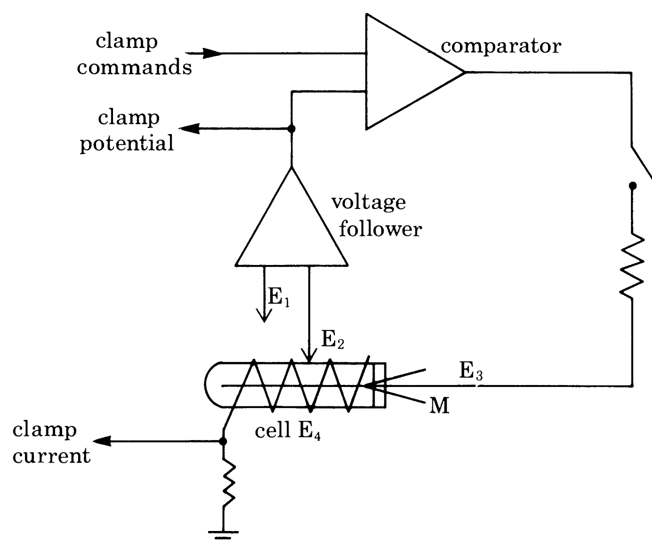


FIGURE 2. Voltage clamp circuit. The potential-measuring electrodes, $E_1$ and $E_2$, consist of glass micropipettes filled with concentrated KCl. The current-injecting electrode, $E_3$, is Pt–Ir wire threaded through a micropipette, M. $E_3$ extends throughout the length of the cell to achieve space-clamping. The current reference electrode, $E_4$, is an Ag–AgCl coil, which also serves as a cell holder.

impossibly high value of 25 mM. Tazawa *et al.* (1976), for instance, found that the free [$Ca^{2+}$] in the cytoplasm was only $10^{-7}$ M. The experiment was performed by replacing the cell sap with solutions containing the $Ca^{2+}$-chelating agent EGTA, destabilizing the tonoplast and measuring the cytoplasmic $Ca^{2+}$ bound with EGTA. Recent experiments with aequorin (Williamson & Ashley 1981, 1982) indicate a rise in $Ca^{2+}$ concentration at the time of excitation, but only to 6.7 μM. There is some evidence that the $Ca^{2+}$ channel is not totally selective and could be frequented by other cations, such as $Na^+$ and $K^+$ (Berestovskii *et al.* 1976). In this case the reversal potential cannot be used to calculate the $Ca^{2+}$ concentration in the cytoplasm.

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## Adaptation of Hodgkin–Huxley equations to *Chara*

The Hodgkin–Huxley (H.H.) equations describe the excitation in the squid axon (Hodgkin & Huxley 1952). There the ion responsible for the depolarization phase of the a.p. is $Na^+$, while $K^+$ restores the resting equilibrium. Under voltage-clamp conditions the $Na^+$ current diminishes spontaneously, but the $K^+$ current remains greater than its resting value. The

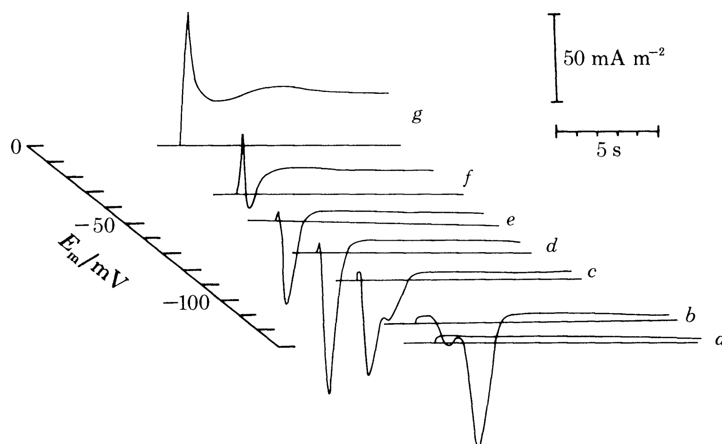


Figure 3. Ionic currents flowing across the plasmalemma at different clamp potentials.

equations describing the $Na^+$ current were borrowed for both $Cl^-$ and $Ca^{2+}$ transients in *Chara*:

$$I_{Cl^-} = g_{Cl^-}\,(E_m - E_{Cl^-}), \tag{1}$$

where $E_m$ is membrane potential, $E_{Cl^-}$ is the equilibrium potential for $Cl^-$, and $g_{Cl^-}$ is the $Cl^-$ conductance, which is both time and potential dependent:

$$g_{Cl^-} = m^3 h\,\overline{g_{Cl^-}}, \tag{2}$$

where $\overline{g_{Cl^-}}$ gives the maximum conductance, and $m$ and $h$ are dimensionless parameters varying between 0 and 1. The parameter $m$ expresses the degree of activation, the parameter $h$ the degree of inactivation of the channels. They introduce the time and potential dependence into (2):

$$m = m_\infty - (m_\infty - m_0)\exp\{(\delta_{Cl^-} - t)/\tau_m\}, \tag{3}$$

$$h = h_\infty - (h_\infty - h_0)\exp\{(\delta_{Cl^-} - t)/\tau_h\}, \tag{4}$$

where $t$ is time.

Equations (3) and (4) describe how parameters $m$ and $h$ vary at the time of the voltage clamp from their resting values $m_0$ and $h_0$, respectively, to the long time values $m_\infty$ and $h_\infty$ with time constants $\tau_m$ and $\tau_h$. The only alteration to the H.H. equations is the introduction of $\delta_{Cl^-}$, a delay before excitation begins when the potential is clamped at a level less negative than the threshold. Delays in excitation have been subsequently found in nerve by Keynes & Rojas (1975).

A similar mathematical treatment is applied to the $Ca^{2+}$ transient. (If the $Ca^{2+}$ channel is proved to be permeable to other cations, some modifications will be necessary.)



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As the excitation depolarizes the membrane p.d., the $K^+$ (and possible $Na^+$) outflow increases. The resting potential (r.p.) is thus restored after the transients have run their courses. Similarly to $I_{K^+}$ in nerve this restoring current, $I_{ss}$, lacks inactivation and is virtually time-independent (Smith & Walker 1981). As the initial rise of $I_{ss}$ (*ca.* 0.1 s) was beyond the resolution of our apparatus, the fitting of H.H. equations was not attempted. For the a.p. simulation a table of empirical values of $I_{ss}$ was compiled from the clamp data at long times (see figure 4).

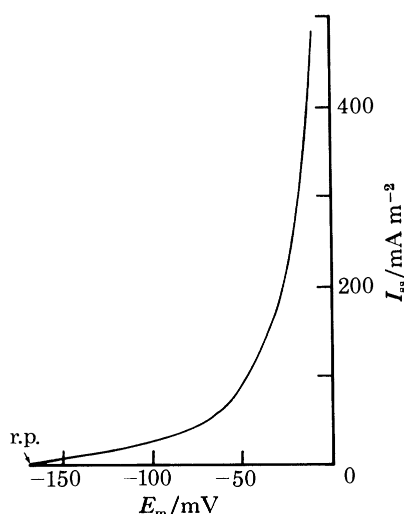


FIGURE 4. Potential dependence of the r.p.-restoring current, $I_{ss}$, in the excitable region.

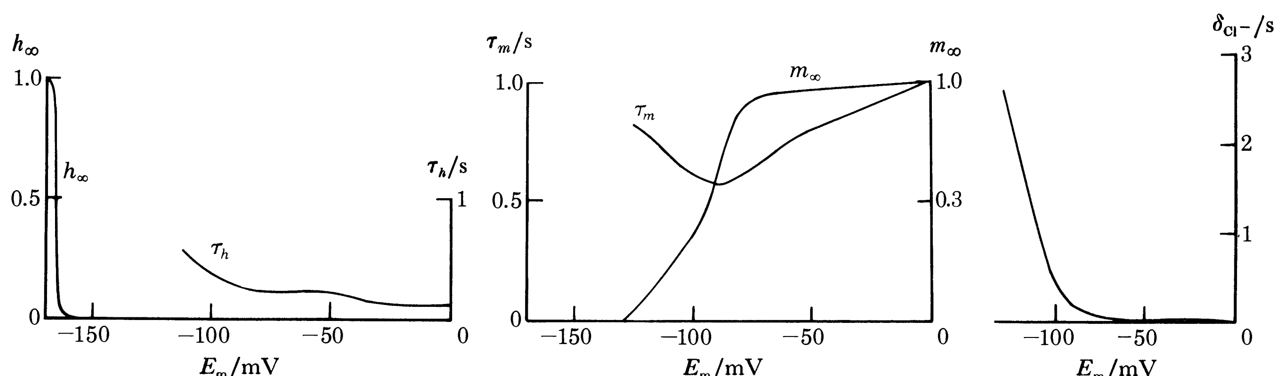


FIGURE 5. H.H. parameters for the $Cl^-$ channel. At potentials more negative than the threshold (−130 mV) the H.H. parameters cannot be obtained from a simple voltage clamp. The inactivation parameter $h_\infty$ was estimated from fitting the tail of the a.p. data.

The pharmacological dissection of the a.p., which is possible in the nerve, does not work as well in *Chara*. While there is evidence that TEA reduces $I_{ss}$ (Keifer & Lucas 1982; J. R. Smith, personal communication), the two transients cannot be selectively blocked at present. The H.H. parameters are obtained by curve-fitting the total clamp current. Fortunately, the currents are easily distinguishable near the threshold and at large depolarizations (see figure 3). The potential dependence of the H.H. parameters for $I_{Cl^-}$ is shown in figure 5.

The a.p. can be reconstructed for a space-clamped cell by numerical integration of (5):

$$dV/dt = -1/C[g_{Cl^-}(E_m - E_{Cl^-}) + g_{Ca^{2+}}(E_m - E_{Ca^{2+}}) + I_{ss}], \tag{5}$$

where $C$ is membrane capacitance.

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The $I_{Cl^-}$ and $I_{ss}$ currents are dominant at the time of a free-running a.p., as a long delay in the opening of the $Ca^{2+}$ channel means that $E_m$ has already reached $E_{Ca^{2+}}$ (Beilby & Coster 1979*b*). Thus no $Ca^{2+}$ fluxes can be observed at the time of the a.p. (Hope & Findlay 1964). The calculated a.p. (see figure 6) displays most features of the experimental data: all-or-none behaviour, a refractory period during which no further a.ps can be elicited and a shift of the a.p. peak with the changes of $[Cl^-]$ and $[Ca^{2+}]$ in the outside medium (Beilby & Coster 1979*b*).

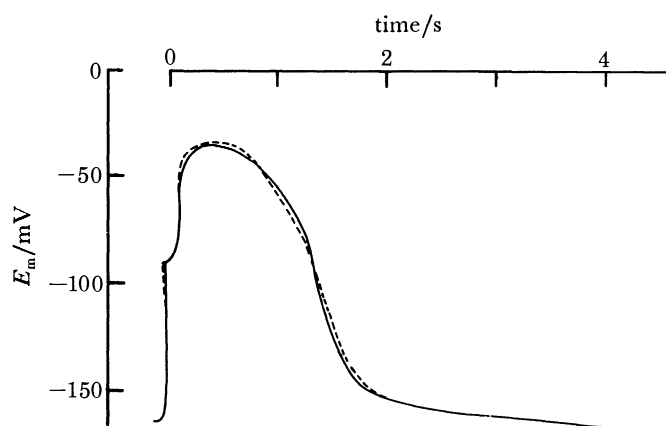


FIGURE 6. A comparison of the data (——) and the reconstructed a.p. (– – –).

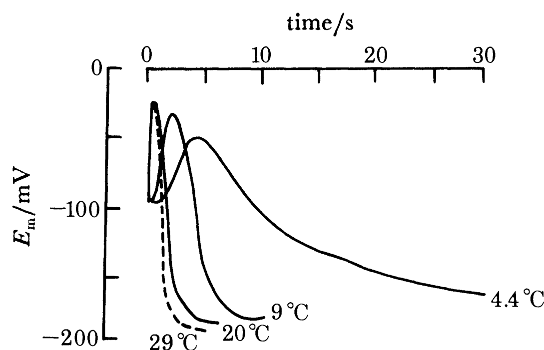


FIGURE 7. The temperature dependence of the a.p. Both the a.p. peak potential and the r.p. vary.

## THE EFFECT OF TEMPERATURE

A change in temperature affects excitation strongly (see figure 7).

Fitting the H.H. parameters at a range of temperatures yields an interesting result: the time constants decrease with increasing temperature but the functional relation is the same at all potentials. This potential-independence of the enthalpies, $\Delta H^*(m)$ and $\Delta H^*(h)$, for the activation and inactivation processes, respectively, suggests that no charge (other than the clamp currents) crosses the membrane at the time of the activation and inactivation of the gates. The Arrhenius plots give $\Delta H^*(m)$ as 60 kJ $mol^{-1}$ and $\Delta H^*(h)$ as 40 kJ $mol^{-1}$ (Beilby & Coster 1979*c*). The values of the enthalpies for both the $Ca^{2+}$ and the $Cl^-$ channels are very close. $\Delta H^*(I_{ss})$ is 44 kJ $mol^{-1}$ (15–20 °C) and 60 kJ $mol^{-1}$ below 15 °C (Beilby & Coster 1976). As the enthalpies are similar for the processes controlling the rising and the falling phase of the a.p., the form is temperature-invariant (Beilby & Coster 1976).

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$\Delta H^*$ values are comparable with those found for the animal a.p. In the squid axon Hodgkin & Huxley (1952) calculated $\Delta H^*$ for the $m$ and $h$ of the $Na^+$ channel and for $n$ of the $K^+$ channel as 78 kJ $mol^{-1}$. In the *Xenopus* node, the enthalpy for the $m$ gate is 49 kJ $mol^{-1}$ and that for the $h$ and $n$ gates is 78 kJ $mol^{-1}$ (Frankenhaeuser & Moore 1963).

The delays before the onset of excitation are both temperature and potential dependent. Plotting $\Delta H^*(\delta_{Cl^-})$ and $\Delta H^*(\delta_{Ca^{2+}})$ against potential yields energies per volt corresponding to

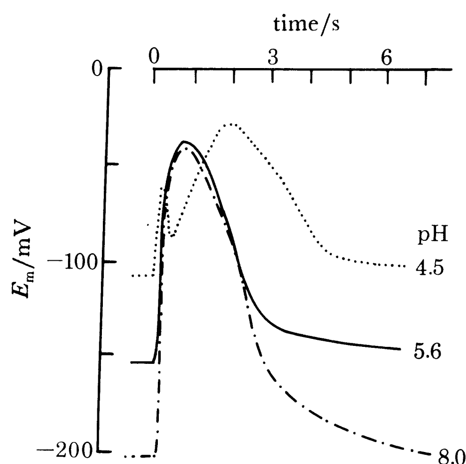


FIGURE 8. The pH dependence of the a.p. The a.p. peak remains relatively constant, while the r.p. varies.

$4F$ (where $F$ is the Faraday constant) for the $Cl^-$ channel and $10F$ for the $Ca^{2+}$ channel (Beilby & Coster 1979*c*). Thus a transformation of the channels into a precursor state may be necessary before the excitation can begin. This process seems to require a movement of 4 charges per molecule for the $Cl^-$ channel and 10 charges per molecule for the $Ca^{2+}$ channel in a direction normal to the membrane.

## pH DEPENDENCE

The behaviour of *Chara* excitation was studied as a function of pH in the limited range of 4.5 to 8. It is now known (Bisson & Walker 1980) that *Chara* can withstand a pH of up to 11 and still be excitable (M. A. Bisson, personal communication).

pH 4.5 represents the lower limit at which the r.p. of *Chara* declines to *ca.* −100 mV, and excitation disappears after the cell is held at this pH for longer than about an hour. The a.ps at pH 4.5, 5.6 and 8 are compared in figure 8.

Clamp currents at different depolarizations are displayed in figure 9. The data were fitted with H.H. equations, but the results were scattered and more work is necessary. However, it is possible to establish some trends. There is little change between pH 5.6 and 8: the delays decrease with increasing pH and the $\overline{g_{Cl^-}}$ and $\overline{g_{Ca^{2+}}}$ increase. The latter can, perhaps, be interpreted in terms of new channels opening in the membrane. At pH 4.5 the time constants for both the transients increase. At more positive clamp potentials the differences diminish (see figure 9).

Some comparisons can be made with the animal world. In the node of Ranvier of *Rana pipiens* $g_{Na^+}$ decreases below pH 7 (see, for example, Hille 1968). In the nerve the effects of pH

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are less pronounced at more depolarized clamp levels (Woodhull 1973) and the timescale ($\tau_m$) increases at low pH (see, for example, Hille 1968; Ehrenstein & Fishman 1971; Drouin & The 1969). The H.H. parameters mainly affected in the nerve are $m_\infty$ and $h_\infty$, which shift along the potential axis. This last effect is not observed in *Chara*. However, considering the fitting difficulties, small shifts would probably go undetected.

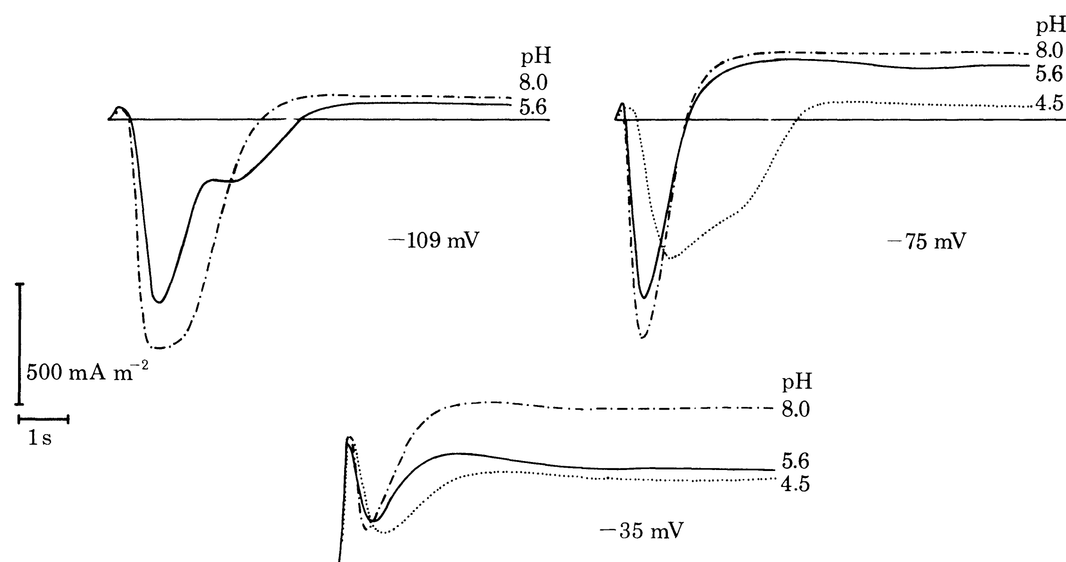


FIGURE 9. A comparison of the clamp currents at pH 8, 5.6 and 4.5 at three depolarizations. At −109 mV the cell was inexcitable at pH 4.5. The data were obtained from a single cell.

The pH dependence of the excitation channels can be explained in terms of the surface charge on the membrane (see, for example, Drouin & The 1969). An alternative view is an f.e.t. (field-effect transistor) model of the excitation channel by Coster & Smith (1977*a*). This model predicts a decrease of excitation currents at low pH and an increase of $\tau_m$, and a decrease of response to pH at greater depolarizations. The calculations have been performed for the $Na^+$ channel (negative core with a positive gate). It will be necessary to repeat the calculations for a positive core with a negative gate to simulate the $Cl^-$ channel.

## IMPEDANCE MEASUREMENTS

An apparatus has just been completed to measure the conductance changes accurately at the time of excitation. A small sinusoidal signal is superimposed on the clamp commands (see figure 10*a*). The baselines are then stripped from the total clamp current and the current sine wave and the potential sine wave juxtaposed. The phase difference as well as the amplitude can thus be obtained. Such a process yields the conductance, $g$, and the capacitance, $C$, of the membrane. Coster & Smith (1977*b*) and Smith & Coster (1980) showed that the $g$–$C$ values of the *Chara* plasmalemma are 'visible' at a frequency range of 1–100 Hz. A frequency of 5 Hz was chosen for present experiments.

In the H.H. equivalent circuit of the membrane the excitation conductances are modelled in parallel (Hodgkin & Huxley 1952). During the voltage clamp the long time conductance,

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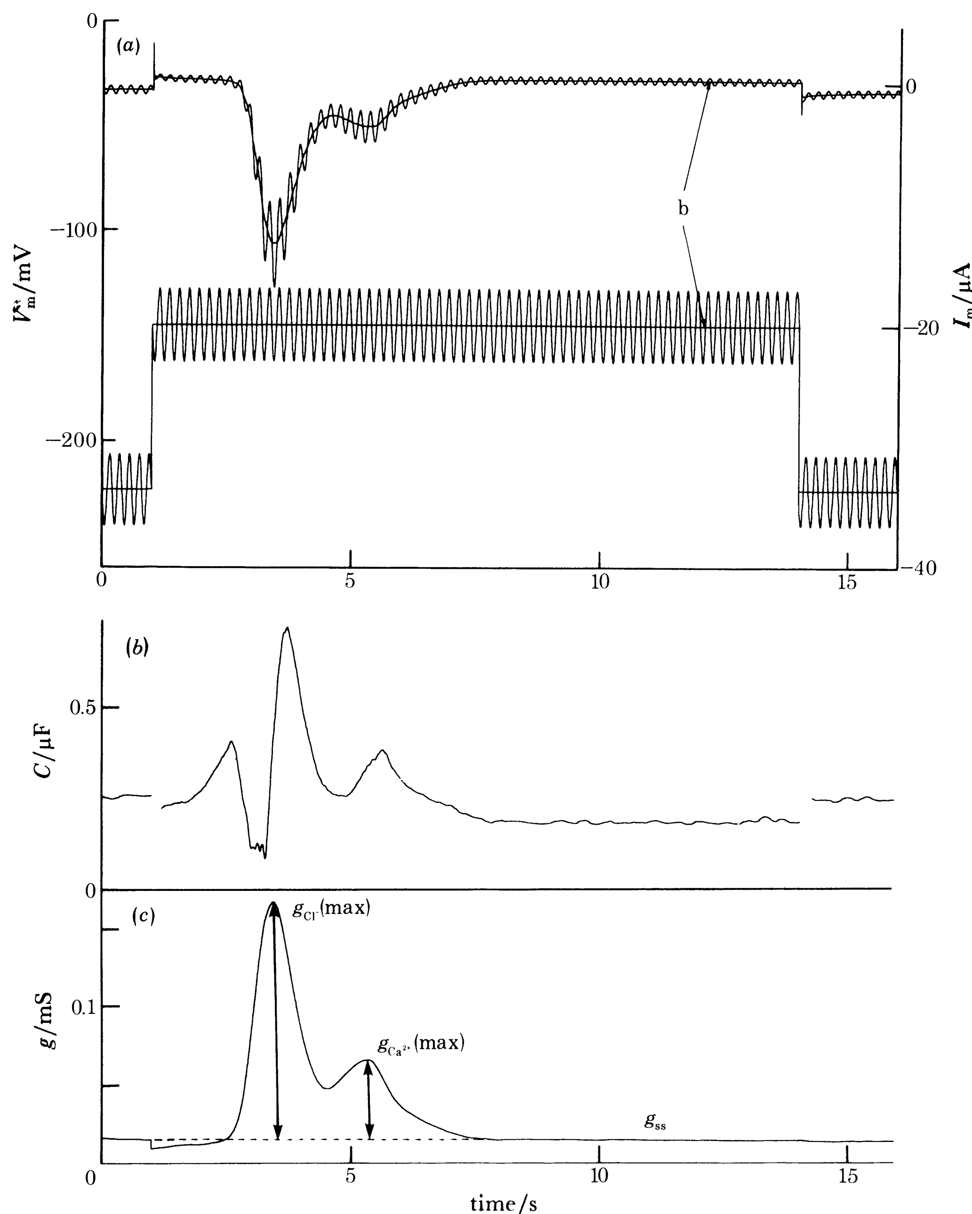


FIGURE 10. (*a*) Clamp potential (lower trace) and the current response (upper trace) with 5 Hz sine (amplitude 20 mV) modulating the clamp command. The cell was clamped at r.p. for the first five cycles, then to −140 mV and finally back to r.p. The baselines, b, were fitted to the data by an averaging procedure. The cell area was 17 mm². (*b*) Capacitance and (*c*) conductance calculated from the above data. The estimates of $g_{Cl^-}$ (max), $g_{Ca^{2+}}$ (max) and $g_{ss}$ are also shown.

$g_{ss}$, can be subtracted from the total $g$ and $g_{Cl^-}$ and $g_{Ca^{2+}}$ measured (at the clamp levels where the two transients are easily separated, such as in figure 10*c*). By fitting the H.H. equations to the current baseline, the accuracy of the fit can be verified. Only preliminary measurements are available at present and the program for fitting H.H. equations to the current baseline is still being developed. The conductances (figure 10*c*) are of comparable magnitude with those calculated from H.H. parameters fitted to Cambridge *Chara* cells (Beilby 1981). However, the

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amplitude of the excitable transients varies considerably from cell to cell and direct comparison between $g$(measured) and $g$(H.H.) fitted to the current baseline will be more informative.

The impedance measurement technique revealed some unexpected features in the behaviour of $g$ and $C$ at the time of voltage clamp in the excitable region. During the delay, the total membrane conductance drops (see figure 10*c*). This result contradicts our temperature measurements which indicated a transfer of charge at the time of $\delta_{Cl^-}$ and $\delta_{Ca^{2+}}$. It was therefore expected that the conductance before the transients would be greater than the conductance at long times due to $I_{ss}$ only. As $I_{ss}$ remains constant before and after the transients (see the baseline in figure 10*a*), the $g$ decrease is probably due to membrane changes before the excitation transients. A further study will be necessary.

In the H.H. model the membrane capacitance is considered to be constant during excitation. In *Chara* the measured capacitance seems to vary, increasing during the delay, going through a sharp minimum as the conductance flares up, and increasing again after the $g$ peak (see figure 10*b*). Similar behaviour can be observed at most clamp potential levels, but as the currents are more prompt the baseline calculation is not so reliable (higher frequencies or lower temperatures may be necessary). The $C$ minimum is sometimes positive, sometimes at zero or even negative (inductive). However, at small $C$ values our measurements of the phase angle are not very accurate. The capacitance changes in the range indicated by the measurements (5–50 mF $m^{-2}$) do not considerably alter the calculated a.p. shape (Beilby 1977), although (5) may become undefined for short times when $C = 0$.

Capacitance shifts are thought to reflect structural changes in the membrane (see, for example, Coster *et al.* 1969; Coster 1973) and may improve our understanding of the excitation process.

A successful fitting of the H.H. equations to *Chara* seemed to indicate that the action potential mechanism was present in the membrane before the evolutionary paths of plants and animals parted. Do the capacitance changes occur at the time of an animal a.p., or does this phenomenon highlight the difference between plant and animal excitation process?

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## *Discussion*

R. D. Keynes, F.R.S. (*Physiological Laboratory, Cambridge, U.K.*). In fitting the Hodgkin–Huxley equations to an inflected rise in ionic current, the time-shift $\delta_{Cl^-}$ that Dr Beilby has employed is interchangeable with an alteration of the power to which $m$ is raised. May I ask whether she has evidence that the time-shift was the same for $m$ and $h$, and whether she has tried fitting the data with a power greater than three?

M. J. Beilby. The same time-shift $\delta_{Cl^-}$ was employed for both $m$ and $h$. This was done for mathematical simplicity and in the absence of experimental evidence pointing either way. It is not possible at present to selectively block the $m$ and $h$ processes in *Chara*.

At negative clamp potentials near the excitation threshold $\delta$ is very long (several seconds) and would require a rather high power of $m$ to achieve a good fit. At more positive potentials, on the other hand, the delay disappears and the rise-time of the transient current is very prompt. It seemed to me that $m$ raised to a high power would not accommodate these extremes. I shall verify these thoughts numerically when my Cambridge computer system software is completed.

N. A. Walker (*School of Biological Sciences, University of Sydney, Australia*). In answer to the question about the function of the action potential in charophyte plants, I should like to suggest that:

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(i) in nature it will usually be set off by mechanical injury to the plasmalemma, which will cause depolarization,

(ii) it does not by any means always propagate to neighbouring internodal cells but does normally propagate throughout the cell in which it arises,

(iii) its normal result is to stop protoplasmic streaming for several minutes, by raising the cytoplasmic concentration of $Ca^{2+}$ (this works either directly on the streaming mechanism or via some systems (Williamson & Ashley 1982)),

(iv) both intracellular and intercellular transport depend on protoplasmic streaming.

The function of the action potential then is to reduce the rate of these processes until the mechanical damage to the plasmalemma is repaired. This will serve to isolate and reduce the effects of damage on solute concentrations in the cytoplasm.



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