The plant action potential (AP) has been studied for more than half a century. The experimental system was provided mainly by the large charophyte cells, which allowed insertion of early large electrodes, manipulation of cell compartments, and inside and outside media. These early experiments were inspired by the Hodgkin and Huxley (HH) work on the squid axon and its voltage clamp techniques. Later, the patch clamping technique provided information about the ion transporters underlying the excitation transient. The initial models were also influenced by the HH picture of the animal AP. At the turn of the century, the paradigm of the charophyte AP shifted to include several chemical reactions, second messenger-activated channel, and calcium ion liberation from internal stores. Many aspects of this new model await further clarification. The role of the AP in plant movements, wound signaling, and turgor regulation is now well documented. Involvement in invasion by pathogens, chilling injury, light, and gravity sensing are under investigation.

**KEY WORDS:** Action potential, Voltage clamp, Patch clamp, Perfusion, Cytoplasmic streaming, Cl− channels, Ca2+ channels, K+ channels, Pharmacological dissection, Internal Ca2+ stores, IP3 activation, Ca2+ pump, Hodgkin Huxley model.

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**I. Introduction**

**A. Defining Features of the Action Potential**

The action potential (AP) is also referred to as excitation transient or just excitation. The AP involves rapid decrease (depolarization) of the negative membrane potential difference (PD) followed by a slower repolarization
Sufficient similarities exist between the animal and plant AP to suggest that we are looking at related phenomena:

- AP is elicited, when the membrane PD is depolarized to a definite threshold level.
- Once the threshold PD is reached, the AP form and amplitude are independent of the amplitude of the stimulus (all-or-none response; Fig. 2A).
- There is a refractory period following an AP, when a new AP cannot be stimulated (Fig. 2B).
- The AP initiated in one part of the cell propagates along the cell, sometimes several cells.
- An increase in temperature makes the AP faster (Fig. 3).

The Nobel Prize winning Hodgkin and Huxley (HH) model of the squid axon (Hodgkin and Huxley, 1952a–d) has influenced the early analysis of the plant AP. The AP in the nerve is generated by two opposing ionic fluxes: Sodium (Na\(^+\)) inflow and Potassium (K\(^+\)) outflow. The increase of the Na\(^+\) conductance is the initial response to the stimulus, which depolarizes the membrane PD. The delayed increase in K\(^+\) conductance and the spontaneous decrease in Na\(^+\) conductance repolarize the membrane back to the resting state. The mathematical model and its application to Chara AP are outlined in Section II.J.3. The HH modeling was made possible by the technique of voltage clamping, in which the membrane PD is held at a selected level by passing a current through the membrane. The AP was also fixed in space by a thin current-supplying electrode placed along the axis of the cylindrical cell. Similar technology was applied to charophytes (Section II.A.1).

However, there are also some marked differences between excitation in plant and animal cells. The time scale in plants is longer by a factor of 10\(^3\) (compare Fig. 1A and B). In plants there are two membranes, the outer plasmalemma and the inner tonoplast. Both membranes usually undergo excitation (Fig. 4A). The contributions from two membranes and the variation of ion concentration in both cytoplasmic and vacuolar compartments are the probable reasons for the shape of the plant AP being more variable (Fig. 1A). The evolutionary pressures on the animal AP are greater to keep the shape constant (Johnson et al., 2002). The plant AP peak remains at negative PDs, crossing into positive region only under unusual circumstances (Beilby and MacRobbie, 1984; Findlay, 1962). The AP peak in the squid axon usually becomes positive. (This is not apparent in Fig. 1B, as both Hodgkin and Huxley and Cole replotted APs by relabeling the negative membrane resting PD as zero and treating the AP as a positive change in PD.) The outflow of chloride ions instead of inflow of sodium ions is responsible for the
FIG. 1 Comparison of plant and nerve AP. (A) The plant AP reconstructed using HH equations (full line). The shaded area shows the variation of the AP form observed in 25 cells (Beilby and Coster, 1979b), R. P. = resting potential. (B) The axon AP data (dashed line) and HH simulation (continuous line), Cole (1968). Note the difference in time scales between (A) and (B).
depolarizing stage in plants. Calcium ion plays an important part in the AP of most charophytes (see Sections II.E.1–2).

The HH picture of the axon excitation is so good, that it dominated our approach to analysis of charophyte (and other plant) excitation for almost
half a century. It is becoming apparent that other mechanisms underlie the plant AP and their intricacies are now emerging from the shadow of the animal AP (Biskup et al., 1999; Thiel et al., 1990; Wacke and Thiel, 2001; Wacke et al., 2003).

FIG. 3  (A) The variation of the AP duration as a function of temperature (Beilby and Coster, 1976). (B) At high temperature of 37.4°C, the AP measured across both membranes of C. corallina cell shows the two superimposed responses clearly (Beilby, 1978).
A

\[ \psi_{\text{Cl}} \]

\[ \psi_{\text{Na}} \]

\[ \psi_{\text{K}} \]

\[ \psi_{\text{vc}} \]

\[ \bar{g}_{\text{vc}} \]

\[ \psi_{\text{co}} \]

\[ \bar{g}_{\text{co}} \]

Time [s]

B

Vacuolar clamp

Cytoplasmic clamp

\[ -150 \text{ mV} \]

\[ -80 \text{ mV} \]

\[ -55 \text{ mV} \]

\[ -10 \text{ mV} \]

\[ 150 \text{ mA/m}^2 \]

\[ 100 \text{ mA/m}^2 \]
B. Why Is the Study of Charophyte AP Important

Because of their large cell size, charophytes have been the subject of many electrophysiological studies, leading to an extensive body of information about them. The “green plants” or Viridiplantae are now viewed as containing two evolutionary lineages or clades, the Charophyta and the Chlorophyta (Karol et al., 2001). The charophyte clade contains the charophyte algae and embryo-phytes (land plants). This close relationship indicates that knowledge gained on the charophyte system will be applicable to a wide range of land plants.

APs play an important role in signal transmission, not only in animal tissue such as nerve and muscle, but also in plant systems. Touch-sensitive plants (Mimosa pudica) and carnivorous plants (Dionea) respond to a mechanical stimulus, which evoke APs that propagate to motor tissues, where turgor-aided movement is initiated (Pickard, 1973; Sibaoka, 1969; Simons, 1981). AP-like prolonged response to hypotonic stress enables salt-tolerant charophyte Lamprothamnium to regulate its turgor (Beilby and Shepherd, 2006). APs are also implicated in transmission of wound signals (Shimmen, 1996, 1997a,b,c). The stoppage of the cytoplasmic streaming triggered by the AP prevents leakage of cytoplasm from injured cells (Kamitsubo and Kikuyama, 1992; Kamitsubo et al., 1989). Further, Davies (1987) suggested that most plants are capable of producing APs and that these play a major role in intercellular and intracellular communication alongside hormonal and other chemical signaling. The changes in ion concentrations, turgor, and water flow may result in modified activities of enzymes in the cell wall and changes in the membranes and the cytoplasm. There is a likely role for APs in chilling injury, invasion by pathogens, and light and gravity sensing. These electrical signaling cascades await future research.

II. Historical Background (Up to Late 1990s)

A. Experimental Techniques

1. Voltage Clamp

The voltage clamp technique was adopted in charophytes independently by Findlay (1961) and Kishimoto (1961, 1964). The results became easier to
interpret, once the space-clamp was introduced and the two membranes were voltage clamped separately (Findlay, 1964b).

In four-terminal voltage clamp, one pair of electrodes is used to measure the membrane PD, while the other pair of electrodes delivers the current. The measured PD is fed into an input of a comparator operational amplifier, while the other input receives a command voltage, which can be set to a single level or a complex function, such as a ramp, bipolar staircase, or the AP shape. As long as the membrane PD is different from the command, the comparator provides current (I) in the appropriate direction to decrease the difference. Modern electronics can facilitate this negative feedback process in several ms (for more details, see Beilby, 1989; Beilby and Beilby, 1983). To fix the excitation in space (space-clamp), the current electrode was made from a thin wire, extending throughout the length of a cylindrical cell (Findlay, 1964c; for review on methodology see Beilby, 1989). To voltage clamp each membrane alone, the PD measuring electrodes are placed into cytoplasm and outside medium (plasmalemma) or cytoplasm and vacuole (tonoplast). When the voltage clamp is applied across both membranes (PD-measuring electrodes are placed in vacuole and outside medium), the PD across each membrane distributes itself according to the membrane resistance—thus neither membrane is clamped to the command PD (Findlay, 1964c).

Some researchers used more complex voltage clamp commands. Kishimoto (1972) and later other Japanese researchers employed linearly depolarizing ramp command for voltage clamp (Fig. 5A). Thiel (1995) produced “AP clamp.” This technique utilized the computer control of the command voltage for the voltage clamp circuitry. The AP was recorded and then replayed as command voltage, while a range of blocking agents was applied (Thiel, 1995; Thiel et al., 1997).

2. Compartment Manipulation

The large size of the charophyte cells facilitates manipulation of various cell compartments. These techniques are a two-edged sword, because while much greater control can be exercised over some cell compartments, the degree of disruption of normal cell structure and function is much greater than the simple electrode insertion or even voltage clamping and the results can be misleading. The control over internal media is limited, as cells do not survive if the changes from natural sap or cytoplasm are too extreme. More details of the various techniques described later can be found in an excellent review by Shimmen et al. (1994).

a. Vacuolar Perfusion In this technique the cell is placed into a three-compartment holder, where the compartments are electrically insulated by grease. The cell ends in the two outer compartments are cut off and the cell
sap is replaced by an artificial medium. The middle compartment is left empty until the perfusion is complete to prevent plasmolysis. Subsequently it is filled by outside medium, which is made isotonic to the perfusion medium by adding sorbitol or mannitol. The cell PD is simply measured by dipping the outside electrode into the middle pool and the “inside” electrode into one of the outer pools. Such cells have no turgor pressure, but can be exposed to a series of different solutions in a short time (Tazawa et al., 1975). For the turgor pressure to develop, the ends of the cells can be ligated tightly by silk or synthetic thread. The cells can then be treated as ordinary cells and inside electrode has to be inserted into the cell. Such cells can be perfused only once and if they are left over time, they will alter the perfusion medium to be as similar as possible to the natural vacuolar sap. To retain the tonoplast, the perfusion medium must contain more than 1 mM Ca$^{2+}$ (Tazawa, 1964).

**b. Tonoplast Removal** The removal of the tonoplast can be achieved mechanically by fast flow (Williamson, 1975) or chemically by including the
Ca$^{2+}$ chelator EGTA (Tazawa et al., 1976). The tonoplast-free cells can be used in the open-vacuole mode or ligated mode. However, as most of the cell cytoplasm is washed away, the cells lack cytoplasmic crystals and organelles for efficient wound healing and electrode insertion has to be done very carefully. There are many recipes for the artificial cytoplasmic medium (Shimmen et al., 1994), which needs to contain Mg$^{2+}$ and ATP for the cell to achieve negative membrane PD, streaming, and APs.

c. Cytoplasm-Enriched Fragments  Long internodal cells (~10 cm) can be centrifuged at ~1 g for 20 min (Beilby and Shepherd, 1989; Hirono and Mitsui, 1981). The cytoplasm moves to one end of the cell. The cell is then wilted slightly and the cytoplasm-rich end is ligated and cut off (Beilby, 1989), obtaining a short (1.0–2.0 mm) cytoplasm-rich fragment. These can survive indefinitely, forming new vacuoles in a matter of hours. However, the cytoplasmic layer remains much thicker than in the intact cells and easily accessible to multiple electrode impalements.

d. Plasma-Membrane Permeabilization  This system is prepared by plasmolyzing the cell with media of high osmolarity to irreversibly detach the plasma membrane from the cell wall. The integrity of the membrane is then destroyed by exposure to ice-cold Ca$^{2+}$ chelator EGTA (Shimmen and Tazawa, 1983a). Any complex molecule that can pass through the cell wall can then access the cytoplasmic side of the tonoplast.

3. Patch Clamp

a. Tonoplast Membrane  Cytoplasmic droplets can be created spontaneously by cutting the charophyte cells. The membrane surrounding the droplets are believed to be derived from the tonoplast (Luhring, 1986; Sakano and Tazawa, 1986). The cell wall does not form and most frequently found K$^+$ channels are well characterized (e.g., see Laver, 1990). Chloride (Cl$^-$) channels were described by Tyerman and Findlay (1989), Katsuhaara and Tazawa (1992), and Beilby et al. (1999).

b. Plasma Membrane  The charophyte cells are too large for the wall to be removed by wall-digesting enzymes. Microsurgical methods of cutting the wall have been successfully applied to access the plasma membrane (Coleman, 1986; Laver, 1991; Okihara et al., 1991; Thiel et al., 1993). Both the “cell-attached” and free patch configurations have been used.
B. The Stimulus

Although the most usual stimulus is facilitated by passing a current to reduce (depolarize) the membrane PD to the threshold level, there are many other ways to achieve this. Ohkawa and Kishimoto (1975) found that if the membrane PD is made more negative (hyperpolarized) by passing an inward current, the membrane PD overshoots the previous resting level once the current is turned off. The overshoot in depolarizing direction increased with the degree of previous hyperpolarization until the excitation threshold was reached. A similar effect was observed in the squid axon, where it was called “anode break excitation” (Hodgkin and Huxley, 1952a). Findlay and Hope (1976) reviewed several methods of stimulating an AP in plants: a sudden change in temperature by \( \sim -15^\circ C \) or \( \sim +25^\circ C \) (Harvey, 1942a; Hill, 1935), irradiation with intense UV light (Harvey, 1942b), or irradiation with alpha particles (Gaffey, 1972). The last technique caused membrane PD to hyperpolarize and stimulated APs by anode break once the radiation was turned off.

Kishimoto (1968) elicited APs by mechanical stimulation. Receptor PDs, proportional to the magnitude of the stimulus, depolarized the membrane PD. When the threshold PD was reached, AP was observed. This AP was of the same form as those obtained by electrical stimulus. Kishimoto also found that a number of subthreshold stimuli added up to excite APs. Later, Shimmen (1996, 1997a, b, c) made a more detailed study of mechanostimulation. In charophytes growing in their natural environment, with rain, water currents and walking or swimming animals, the mechanical stimulus is the most likely source of excitation. Another way of mechanostimulation is to change the cell turgor via the inserted pressure probe. Zimmermann and Beckers (1978) found that both increasing and decreasing pressure stimulated APs.

Any chemical agent that causes depolarization of the membrane PD is likely to stimulate APs, unless it also affects the channels involved in the AP process. Kishimoto (1966a) exposed *Nitella flexilis* cells to 10–100 mM of NaCl or LiCl and observed periodic trains of spontaneous APs. Spontaneous APs were also obtained by adding 1-mM EDTA or 2-mM ATP.

The definite threshold PD was a source of fascination to many researchers. Changing only a few mV near the threshold PD caused a large change in the electrical characteristics of the membrane (Fig. 2A). Such phenomena can serve as illustrations of chaos and catastrophe theories (Thom, 1975). Fujita and Mizuguchi (1955), Findlay (1959), and Gaffey (1972) showed that in *Nitella* the stimulating current pulse obeyed the classical strength-duration relationship (Katz, 1937). For longer pulses the threshold PD became more negative, for shorter pulses more positive. However, there are limiting levels, in which no time extension of the wide current pulse and no amplitude
increase of the very short current pulse, produce excitation. The relationship is based on a minimum quantity of electric charge (product of stimulus current and pulse width), needed to discharge the membrane capacitance and depolarize the membrane PD to threshold level.

In voltage clamp experiments the membrane PD is usually clamped to a depolarized level for some seconds and the excitation threshold manifests itself by flow of negative (inward) current (see Fig. 4B). Kishimoto (1964, 1966b) replotted the excitation currents at different times after the start of each clamp, obtaining N-shaped current-voltage (I/V) characteristics (see Fig. 5A). Similar results were obtained by using a ramp voltage command (Kishimoto, 1972). Kishimoto observed that as the rate of the voltage ramp was varied from 150 mV/sec to 30 mV/sec, the excitation transient appeared at more positive PDs. This is known as accommodation and can be observed in nerve and muscle tissue (Hodgkin and Huxley, 1952a). Thus in case of a ramp, there is a minimum rate of depolarization to produce an AP.

C. Coupling to Streaming Stoppage

The cytoplasm of charophyte cells rotates steadily around the cell at speeds near 100 μm/s depending on the medium temperature. The streaming mechanism is now understood in terms of myosin, attached to barium sulphate crystals in the cytoplasm, which leapfrogs along the actin helix, propelling the crystal. The crystals stir the viscous flowing cytoplasm moving it along. The streaming can be detected by observing the movement of the organelles and particles present in the cytoplasmic phase, providing a beautiful and mesmerizing sight. At the time of excitation, the streaming stops suddenly and slowly recovers its original speed in 5–10 min (Kamiya, 1959). While the cells are not streaming, other APs can be stimulated. Tazawa and Kishimoto (1968) found that the viscosity of the cytoplasm does not increase at the time of the stoppage, but the motive force disappears. Tazawa and Kishimoto (1964) perfused Nitella cell vacuole with artificial sap and demonstrated that about 3-mM Ca²⁺ was necessary for normal cyclosis, but high concentration of 50 mM had an inhibitive effect. This early finding suggested the role of Ca²⁺ in cytoplasmic streaming. Beilby (1984a) observed streaming stoppage in Chara, even when Ca²⁺ in the medium was replaced by Mg²⁺. The removal of the tonoplast in the intracellular perfusion by mechanical (Williamson, 1975) or chemical (Tazawa et al., 1976) method made the cytoplasmic compartment accessible. First, this technique established the need for ATP and Mg²⁺ in millimolar concentrations. Williamson (1975) showed that in the tonoplast-free cells rise in Ca²⁺ concentration above 10⁻⁶ M retards
streaming substantially. **Tazawa et al. (1976)** disrupted the tonoplast by including calcium chelator EGTA in the perfusion medium. In such cells cytoplasmic streaming did not stop at the time of the AP. **Hayama et al. (1979)** made cytoplasm-enriched cell fragments and demonstrated that the excitation at the plasma membrane is only enough to cause streaming stoppage. They also demonstrated that tonoplast-free perfused cells could be reperfused with media without EGTA and various concentrations of Ca\(^{2+}\). High Ca\(^{2+}\) concentrations (1 mM) permanently inhibited streaming. This concentration was taken as the minimum amount for total cytoplasmic stoppage. **Tominaga and Tazawa (1981)** found that in their perfusion experiments the threshold Ca\(^{2+}\) concentration for streaming inhibition was lower at 0.5 mM. **Kikuyama and Tazawa (1983)** suggested that the perfusion technique induced changes in the motile system making it less sensitive to Ca\(^{2+}\). Later, the steady state [Ca\(^{2+}\)]\(_{cyt}\) was measured at \(\sim 200\) nM, rising to \(\sim 700\) nM at the peak of the AP (Plieth and Hansen, 1996; Williamson and Ashley, 1982). In salt-tolerant *Lamprothamnium succinctum*, the steady state [Ca\(^{2+}\)]\(_{cyt}\) was 80 nM and streaming was not inhibited at [Ca\(^{2+}\)]\(_{cyt}\) below 200 nM, but strongly inhibited above 500 nM (Okazaki et al., 2002). So clearly, the AP does involve a transient rise in [Ca\(^{2+}\)]\(_{cyt}\) and Section II.E.1 reviews the evidence on the sources of the Ca\(^{2+}\).

**D. Resolving Responses at Plasmalemma and Tonoplast**

Plant cells have a cytoplasmic layer sandwiched between plasmalemma and tonoplast. Even in giant charophyte cells, the cytoplasm is only about 10-\(\mu\)m thick and opinions vary on how difficult it is to place an electrode there. If the cell is stimulated, the excitation occurs at both membranes. **Findlay and Hope (1964a)** were the first to record the excitation transients simultaneously at both membranes in *Chara* (Fig. 4A). When the AP is measured between the vacuole and the outside medium, the two transients superimpose on each other. At high temperature, it is possible to resolve the peaks of the transient at each membrane (Fig. 3B). Findlay and Hope (1964a) also measured the resting resistance of both membranes as 12.1 ± 2.5 k\(\Omega\).cm\(^2\) and 1.1 ± 0.1 k\(\Omega\).cm\(^2\) for plasmalemma and tonoplast, respectively. The resistances fell to about 300 \(\Omega\).cm\(^2\) for both membranes. Similar excitation responses at both membrane were measured in a brackish charophyte *Nitellopsis obtusa* (Findlay, 1970).

However, as greater variety of charophytes became the subject of electrophysiological studies, several trends emerged. **Kikuyama (1986b)** found that *N. axilliformis* exhibited negative-going PD transient across the tonoplast. In *N. flexilis*, the plasmalemma and tonoplast transients can be resolved even
when the data are gathered by inserting the inside microelectrode into the vacuole (Kikuyama and Shimmen, 1997; Shimmen and Nishikawa, 1988). The tonoplast excitation could only be observed when \([\text{Ca}^{2+}]_{\text{cyt}}\) increased. The \([\text{Ca}^{2+}]_{\text{cyt}}\) was monitored by injecting aequorin into the cytoplasm. The timing of the rise in \([\text{Ca}^{2+}]_{\text{cyt}}\) was closely correlated with the plasmalemma AP, but the plasmalemma AP was not affected by lack of \(\text{Ca}^{2+}\) in the outside medium.

Findlay (1970) compared the excitation currents measured by voltage-clamping the plasmalemma alone and both membranes in series. A comparison at several PD levels is shown in Fig. 4B. Beilby (1990) made a detailed comparison of the data measured with the PD-measuring electrode in the vacuole and cytoplasm, with the short segment of the cell compartment-clamped or short cell space-clamped with a thin wire electrode. For most steady state currents, the plasmalemma resistance is much greater than that of the tonoplast. Thus most of the clamp PD will be across plasmalemma and the vacuole/outside data will be a good approximation of the plasmalemma characteristics. However, at the peak of the transient current, the plasmalemma may be equally or more conductive than the tonoplast and that is why the current transients are different with the electrode placement (Fig. 4B). Note particularly two peaks at PDs near the threshold level and the sharp positive peak near 0 PD in the plasmalemma-only clamp series.

Removal of the tonoplast in the cell perfusion (see Section II.A.2) also isolated the plasmalemma response. Although this technique answered several questions, it also introduced new puzzles. By washing out the intricate cytoplasmic phase with its many organelles, the system was irreversibly altered. Shimmen et al. (1976) found that the shape of the AP in tonoplast-free cells became rectangular and the duration varied from more than a minute to a fraction of a minute in repeated stimulations by an outward (depolarizing) current (Fig. 6A). No refractory period was observed. Beilby et al. (1993) measured membrane currents, while the membrane PD was clamped at levels more positive than excitation threshold in cells of tonoplast-free *Nitellopsis obtusa*. The currents also exhibited very slow inactivation compared to the intact cells (Fig. 6B and C). As the \(\text{K}^+\) concentration in the medium was increased from 0.1 mM in artificial pond water (APW) to between 3–10 mM, it was possible to shift the membrane PD between two stable states by a short depolarizing or hyperpolarizing pulse (see Fig. 6A). Often the increase in medium \(\text{K}^+\) caused the shift to depolarized level, while \(\text{K}^+\) being replaced by \(\text{Ca}^{2+}\) repolarized the membrane PD.

Coster et al. (1974) inserted two microelectrodes into the *Chara* cytoplasm, one filled with 2-M KCl, the other with various dilute solutions of KCl or NaCl. The variation of the PD between the two electrodes with KCl or NaCl concentration was consistent with the cytoplasm behaving as a Donnan phase with 0.1–0.2 M negative fixed charges. At the time of excitation the
Donnan potential disappeared, suggesting a loss of fixed negative charges. How do these fixed charges arise? Do they play a role in electroneutrality at the time of AP? This interesting direction in research needs to be continued.

E. Resolving Individual Transporters

1. Calcium Channels

What ion flows produce the AP? The peak of the AP is found at small negative PDs (Fig. 1A). Thus Na\(^+\) was eliminated early, as the equilibrium (Nernst) PD was too negative. Both Cl\(^-\) and Ca\(^{2+}\) have positive equilibrium PDs.

FIG. 6  Excitation in tonoplast free cells. (A) The record of PD in APW shows the prolonged AP, with decreasing duration upon repeated stimulation. Upon addition of 2-mM K\(_2\)SO\(_4\), the membrane PD could be manipulated between two levels by hyperpolarizing and depolarizing pulse, respectively (Shimmen et al., 1976). Comparison of the excitation clamp currents in *Nitellopsis obtusa* (Beilby et al., 1993), (B) perfused tonoplast-free ligated cell, and (C) intact cell. The cells were voltage clamped at their resting PD, then to a PD level indicated (upward arrow) on the scale and finally back to the preclamp resting PD (downward arrow).
The involvement of Ca\textsuperscript{2+} was obvious from quite early experiments. Osterhout and Hill (1933) performed experiments with \textit{Nitella} and reported loss of excitability (anesthesia), when medium was replaced with distilled water. The choice of this ion was also attractive from an historical point of view, as only animals more advanced than Coelenterates have Na\textsuperscript{+} based AP. The more ancient AP mechanism is Ca\textsuperscript{2+} based (Hille, 1992; Wayne, 1994). The Australian researchers, Hope (1961a,b) and Findlay (1961, 1962), correlated the increased amplitude of the AP spike to greater concentration of Ca\textsuperscript{2+} in the medium. They suggested that an inflow of Ca\textsuperscript{2+} carried the inward current of the depolarizing phase of the AP. However, they were unable to observe any changes in \textsuperscript{45}Ca\textsuperscript{2+} influx (Hope and Findlay, 1964) and so correctly assumed that Cl\textsuperscript{−} is the main depolarizing ion, and that Ca\textsuperscript{2+} is needed for activation. Interestingly, if Ca\textsuperscript{2+} was the depolarizing ion, it might not have been detected, as only in later years the difficulty with wall bound Ca\textsuperscript{2+} became known (Reid and Smith, 1992). Fifteen years later, Hayama \textit{et al.} (1979) did detect Ca\textsuperscript{2+} influx, but not large enough to account for the AP transient or the streaming stoppage. So, Ca\textsuperscript{2+} influx is involved in many types of charophytes, but mainly to increase the cytoplasmic concentration, which in turn opens the Cl\textsuperscript{−} channels on both plasmalemma and tonoplast and K\textsuperscript{+} channels on the tonoplast. As most charophytes need Ca\textsuperscript{2+} or (Sr\textsuperscript{2+}) in the medium to be excitable, it is a reasonable assumption that at least some of the Ca\textsuperscript{2+} comes from the outside through Ca\textsuperscript{2+} selective channels. Beilby and Coster (1979a), while voltage clamping \textit{Chara}, observed two transient currents, when the membrane PD was clamped just a little more positive than the excitation threshold (Fig. 4B). They thought that the second transient could be due to Ca\textsuperscript{2+} flow, but this timing was not right for the scheme of Ca\textsuperscript{2+} -activation of Cl\textsuperscript{−} channels. Lunevsky \textit{et al.} (1983), voltage clamping the plasmalemma of \textit{Nitellopsis}, inhibited the large transient current by Cl\textsuperscript{−} channel blocker, ethacrynic acid (see Fig. 7B). A small prompt transient was revealed at the beginning of the excitation. This transient seemed a better candidate for the Ca\textsuperscript{2+} inflow. However, the channel that passes the first transient current was found not to be selective. An increase in concentration of K\textsuperscript{+} and Na\textsuperscript{+} in the medium affects the first transient, but as these are low in the normal APW, Ca\textsuperscript{2+} is then the preferred ion (Lunevsky \textit{et al.}, 1983).

Shimmen and Tazawa (1980), using tonoplast-free \textit{Chara} cells, found that the AP peak was not sensitive to internal Cl\textsuperscript{−} concentration. Thus excitation occurred with very low Cl\textsuperscript{−} inside the cell or with EGTA, which prevented Ca\textsuperscript{2+} concentration inside the cell to rise (Fig. 5A). Kikuyama \textit{et al.} (1984) could not detect Cl\textsuperscript{−} efflux from tonoplast-free cells containing abundant Cl\textsuperscript{−}. Mimura and Tazawa (1983) found that if \textit{Nitellopsis} cells were reperfused with media containing varying concentrations of Ca\textsuperscript{2+}, membrane PD depolarized and membrane resistance decreased, but Cl\textsuperscript{−} efflux did not increase. Shina and Tazawa (1987) tested Ca\textsuperscript{2+} and Cl\textsuperscript{−} channel inhibitors (see Section II.F.3 and
the results were consistent with Ca\(^{2+}\) carrying the excitation current. The Japanese researchers suggested that under some circumstances Ca\(^{2+}\) inflow alone is responsible for the AP in tonoplast-free cells.

Using the patch clamp technique, is it possible to detect Ca\(^{2+}\) channels in the plasmalemma? The answer is not simple. Thiel et al. (1993) employed cell-attached configuration and found nonselective low conductance (4 pS) channel, which could conduct Ca\(^{2+}\). However, the activity of these channels did not correlate with the activity of the Cl\(^{-}\) channels. Thiel et al. (1997) suggested that the cytoplasmic side of the plasma membrane contains stores for Ca\(^{2+}\), which are replenished by the non-selective channels. A release of Ca\(^{2+}\) is favored by depolarizing the membrane PD. The cytoplasmic Ca\(^{2+}\) concentration rises locally and Cl\(^{-}\) channels in the vicinity are activated. The AP is a superposition of such stochastic microscopic events, the voltage dependence of the Cl\(^{-}\) channel (as modeled by Beilby and Coster, 1979b) being an illusion. The refractory period correlates with the time needed to refill the internal stores.

FIG. 7  (A) The dependence of the tonoplast AP in *Nitella pulchella* on Cl\(^{-}\) concentration of the vacuolar sap. The PD measuring electrode was in the vacuole. (a) Cell containing the natural cell sap. Single arrow shows the plasmalemma AP, double arrow the tonoplast AP. (b) The natural cell sap was replaced with an artificial medium lacking Cl\(^{-}\). The AP reversed in a negative (downward) direction (Kikuyama and Tazawa, 1976). (B) Inhibition of the excitation Cl\(^{-}\) clamp current (curve 1) by ethacrynic acid (curves 2–5 taken in 10 min intervals) in *Nitellopsis obtusa*. The current through cation channels is revealed and can be explored at different clamp PDs (Lunevsky et al., 1983).
This picture was supported by elegant experiments of Plieth et al. (1998) with Mn\(^{2+}\)-induced quenching of fluorescent Ca\(^{2+}\) indicator fura-dextran. The addition of Mn\(^{2+}\) to the external medium or injection into the vacuole did not cause fluorescence quenching at the time of AP. As it was expected that Mn\(^{2+}\) can permeate through the Ca\(^{2+}\) channels, the result suggested that Ca\(^{2+}\) increase at the time of AP was due to release from internal stores. This hypothesis was further supported by preincubating Chara cells in high Mn\(^{2+}\) medium (25–30 mM). Such cells exhibited transient quenching of fura fluorescence at the time of AP in Mn\(^{2+}\)-free solutions. It was assumed that Mn\(^{2+}\) was taken up into the internal stores and released at the time of AP. Plieth et al. (1998) suggest that the Ca\(^{2+}\) store might be the endoplasmic reticulum (ER). The importance of Ca\(^{2+}\)-filled internal stores for normal AP to occur can explain several observations from other experiments. Williamson and Ashley (1982) observed slow regeneration of Ca\(^{2+}\) rise at the time of excitation after transfer of cells from Mg\(^{2+}\)- to a Ca\(^{2+}\)-containing medium. The Ca\(^{2+}\) channel blocker La\(^{3+}\) takes several hours to abolish excitation (Beilby, 1984b; Tsutsui et al., 1986, 1987). These effects can be explained by the need to fill or to empty the Ca\(^{2+}\) internal stores.

2. Chloride Channels

Gaffey and Mullins (1958), Mullins (1962), and Hope and Findlay (1964) loaded cells with \(^{36}\)Cl\(^-\) and found efflux from excited cells 100 times greater (\(\sim 1 \times 10^{-6}\) mol m\(^{-2}\) s\(^{-1}\)) than that in the resting cells. Mailman and Mullins (1966) improved time resolution by using a Ag/AgCl electrode. Later the increased Cl\(^-\) efflux at the time of excitation was measured using other methods (Haapnen and Skoglund, 1967; Kikuyama, 1986a,b, 1987; Oda, 1976; for other references see Shimmen et al., 1994). In fact, this seemed to be one of the favorite experiments during the 1960s, 1970s and 1980s. Using the Goldman equation, Wayne (1994) estimates the rise of Cl\(^-\) permeability about 100\(\times\)at the time of the AP.

The experiments with tonoplast-free cells provided a wealth of data, but also some confusion, as under some circumstances, the Ca\(^{2+}\) influx seems to be wholly responsible for excitation (see previous section on Ca\(^{2+}\)). Shiina and Tazawa (1988), using Nitellopsis, measured time-independent increase in Cl\(^-\) efflux upon increasing Ca\(^{2+}\) in the perfusion medium to pCa 5.4 (\(\sim 4.0\) \(\mu\)M). They concluded, that in experiments of Mimura and Tazawa (1983), EGTA concentration in the perfusion medium prevented Ca\(^{2+}\) increase and Cl\(^-\) channel activation. Mimura and Shimmen (1994), using Chara, and Kataev et al. (1984), using Nitellopsis, found Ca\(^{2+}\)-dependent membrane PD depolarization accompanied by transient efflux of Cl\(^-\). The peak membrane PD depolarization saturated at pCa of \(\sim 4\). The peak Cl\(^-\) efflux increased more gradually with Ca\(^{2+}\) concentration and saturated at pCa of \(\sim 3.0\). Kataev
et al. (1984) suggested that Cl\textsuperscript{−} channels may be inactivated by the continuous exposure to high Ca\textsuperscript{2+}, which might act on the cytoskeleton. They argued that at the time of AP, the exposure to high Ca\textsuperscript{2+} is too short for this inhibition to occur and the Cl\textsuperscript{−} current is simply inactivated by Ca\textsuperscript{2+}-concentration decrease.

The patch clamp technique was employed to find Cl\textsuperscript{−} channels that underlie the AP. Several types of Cl\textsuperscript{−} channels have been observed in charophyte plasmalemma (Coleman, 1986; Laver, 1991; McCulloch et al., 1997), but their properties did not make them obvious candidates for the depolarizing phase of the AP. Okihara et al. (1991, 1993) found two types of Cl\textsuperscript{−} channels in the inside-out patches from the Chara plasmalemma. One of these, type A, was quite selective for Cl\textsuperscript{−} and required 1-μM Ca\textsuperscript{2+} for optimum activation. Higher or lower Ca\textsuperscript{2+} concentration caused channel inactivation. The voltage dependence exhibited inward rectification and greater open probability at PDs above −160 mV.

Thiel and coworkers (Homann and Thiel, 1994; Thiel, 1995; Thiel et al., 1993, 1997) used cell-attached configuration and also observed two types of Cl\textsuperscript{−} channels with unitary conductances of 15 and 38 pS, respectively, with 100-mM Cl\textsuperscript{−} in the pipette. The advantage of cell-attached mode was the ability to check whether the cell was undergoing excitation. Both types of channels had a very low open probability in nonexcited cells. The open probability increased transiently at the time of an AP and up to ~100 Cl\textsuperscript{−} channels per patch would activate within 1–2 s. However (see previous section), the patch clamp data did not show depolarization-activated Ca\textsuperscript{2+} channels at the beginning of the AP. Thiel et al. (1997) concluded that while the depolarization of the membrane PD favors excitation, it is not sufficient for Cl-channel activation. Extracellular Ca\textsuperscript{2+} also favors excitation, but there are no visible patch clamp currents just prior to excitation to document an inflow of Ca\textsuperscript{2+} into the cytoplasm. Thus some cytoplasmic factor, possibly Ca\textsuperscript{2+} released from internal stores, facilitates the Cl\textsuperscript{−} channel activation. Kikuyama et al. (1993) observed sparklike random spots of high Ca\textsuperscript{2+} appearing at the time of AP in Nitella, suggesting that the AP is a superposition of local Ca\textsuperscript{2+} increases, that in turn activate spikes of Cl\textsuperscript{−} current. It was obvious that the channel, which provides the depolarizing phase of the AP, is gated in a different way than the axon Na\textsuperscript{+} channel. Thiel and coworkers started to design experiments to reveal the mechanism by which the Ca\textsuperscript{2+} was released from internal stores when the membrane PD was depolarized (see Section III.A).

3. Potassium Channels

The increase of both Cl\textsuperscript{−} and K\textsuperscript{+} effluxes at the time of AP has been measured by many researchers (Haapanen and Skoglund, 1967; Hope and Findlay, 1964; Kikuyama, 1986a, 1987a,b; Kikuyama et al., 1984; Oda, 1975, 1976).
Kikuyama et al. (1984) calculated $K^+$ permeability at the time of Chara AP and found no increase compared to that at resting state. They suggested that the $K^+$ efflux arose from the depolarization of the membrane PD.

In *N. axillaris* (Barry, 1968; Kamitsubo, 1980) and *N. axilliformis* (Shimmen and Tazawa, 1983b), the repolarizing PD often overshot the pre-AP resting level. This behavior became known as “after-hyperpolarization.” This effect only occurred if the initial resting PD was more positive than $E_K$ and was inhibited by $K^+$ channel blocker tetra ethyl ammonium (TEA). Thus $K^+$ channel activation was postulated.

Thiel and coworkers (Homann and Thiel, 1994; Thiel, 1995; Thiel et al., 1993, 1997) used Chara cell-attached configuration and found $K^+$ channels with linear conductance of about 40 pS. They showed (by comparing patches in excited cells and cells with depolarized PD) that these channels are activated by depolarization. The channels were identified as the $K^+$ selective outward rectifier, which is not sensitive to TEA. Beilby and Coster (1979a) found that TEA had no effect on the shape of the AP in Chara.

How can we reconcile data from Chara and *N. axillaris* and *N. axilliformis*? Chara plasmalemma contains large conductance $K^+$ channels, which are sensitive to TEA (Beilby, 1985). These channels are activated by depolarization and $K^+$ concentration in the medium above about 1 mM. It is not probable that these channels are activated at the time of the AP, while the cells are in APW (0.1-mM $K^+$). The large conductance $K^+$ channels might be faster to activate in *N. axillaris* or *N. axilliformis*, or the outward rectifier has a slow inactivation and sensitivity to TEA.

4. Proton Pump

Kishimoto et al. (1985) suggested that the pump conductance decreases and the pump current increases at the time of the AP. Both of these effects follow from the I/V profile of the pump and the depolarization of the membrane PD due to the increased conductances of the Cl$^-$ and $K^+$ channels. The I/V profile of the pump was obtained by fitting the HGSS model (Hansen et al., 1981) with stoichiometry of 2H$^+$ per 1 ATP. Similar interpretation applies for a model with the stoichiometric ratio 1H$^+$ per 1 ATP (Beilby, 1984b). Working with cells in $K^+$ state (in which large conductance $K^+$ channels dominate the plasmalemma electrical characteristics), Beilby found that the H$^+$ pump appeared to “switch off” in this state and reactivated slowly as the $K^+$ channels were closed by decreasing $K^+$ in the outside medium (Beilby, 1985). It was also found that prolonged voltage clamping to depolarized PDs for 5–10 min resulted in decline of the pump activity (Beilby, unpublished). Although the transient depolarization due to the AP is shorter (~5 s), there is still a transient effect on the pump conductance as found by Smith and Beilby (1983). The actual mechanism by which the pump is inhibited is not known.
In the case of the AP, the transient rise of Ca$^{2+}$ in the cytoplasm might inhibit the pump. However, in K$^+$ state, the streaming rate is fast (indicating low Ca$^{2+}$ in the cytoplasm), but the pump needs many minutes to recover after the K$^+$ state is terminated by lowering the K$^+$ concentration (Beilby, 1985).

Employing the AP-clamp (see Section II.A.1), Thiel (1995) selectively inhibited the K$^+$ or the Cl$^-$ current. To balance the charge lost, the two currents should be symmetrical as functions of time. However, the Cl$^-$ current was not balanced by the K$^+$ current in the first 2 s of the AP. Later in the AP the K$^+$ current dominates over the Cl$^-$ current. Thiel et al. (1997) suggested the excess of Cl$^-$ current may be balanced by the increase in the pump current.

F. Manipulating Inside and Outside Media

1. Ion Concentrations

Changes of medium Cl$^-$ or Ca$^{2+}$ were used in very early experiments (Findlay, 1962; Findlay and Hope, 1964a,b; Kishimoto, 1964, 1966b) to distinguish which of these ions is responsible for the depolarizing phase of the AP. Hope (1961a) and Findlay (1961, 1962) found that the peak PD of the AP (measured between the vacuole and outside) was a linear function of log [Ca$^{2+}$]o, whereas changes in the [Cl$^-$]o between 0–10 mM had little effect. Kishimoto (1966b), working on N. axillaris, found that the N-shaped part of the I/V curve became smaller in high Cl$^-$ and more pronounced in high Ca$^{2+}$. Beilby and Coster (1979a) observed an increase in the second transient peak of the voltage clamp current near the excitation threshold and a reversal of the prompt sharp peak at very depolarized PDs. None of these results were clear-cut and it was the measurements of the Cl$^-$ outflow that identified Cl$^-$ as the depolarizing ion (see Section II.E.2). Now we interpret the effect of high medium Ca$^{2+}$ as increasing the amount of Ca$^{2+}$ in the internal stores and activating more Cl$^-$ channels upon release (Plieth et al., 1998). Shiina and Tazawa (1987) repeated the changes of medium Cl$^-$ or Ca$^{2+}$ to study excitation in tonoplast-free cells and concluded that Ca$^{2+}$ is the main depolarizing ion in this case (see also Section II.D).

An increase in K$^+$ concentration of the medium above 1 mM opens the large conductance K$^+$ channels (Beilby, 1985; Smith and Walker, 1981). Cells in K$^+$ state can be excitable if the resting PD is not too depolarized (Beilby, 1985).

Different divalent ions invoke a range of responses in various charophytes. In C. corallina replacing the Ca$^{2+}$ in the APW (artificial pond water) by 5-mM Sr$^{2+}$ reversibly decreased the tonoplast AP after 16 h (Kikuyama, 1986b).
The plasmalemma AP exhibited a wide flat peak in Sr$^{2+}$ APW (Findlay and Hope, 1964a). The streaming stopped at the time of excitation. Using 5-mM Ba$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$ reversibly abolished excitation at both membranes. Beilby (1984a) observed that the excitation clamp currents were abolished in Mg$^{2+}$ APW, but not the streaming stoppage. Barry (1968) worked with *N. axillaris* and found that the cells produced APs in 6 mM of CaCl$_2$, SrCl$_2$, MgCl$_2$, and BaCl$_2$. Streaming stoppage was observed in CaCl$_2$ and SrCl$_2$ only. Cells were not excitable in MnCl$_2$ or ZnCl$_2$. The shape of the AP became progressively more rectangular in Sr$^{2+}$, Mg$^{2+}$, and Ba$^{2+}$. Kikuyama (1986b) used *N. axilliformis* and this charophyte exhibited APs in Ba$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$ APW, but the tonoplast AP was reversibly abolished after repeated stimulation. The streaming did not stop. Injection of Ca$^{2+}$ into the cytoplasm induced an AP at both membranes in *Chara* and *N. axilliformis*, whereas injection of Cl$^-$ had no effect. Kikuyama and Tazawa (1976) perfused the vacuole of *N. pulchella* and found that the amplitude and direction of the tonoplast AP was strongly dependent on [Cl$^-$]$_{vac}$ (Fig. 7A).

Beilby (1981) diminished the cytoplasmic Cl$^-$ concentration by Cl$^-$ starvation. The transient voltage clamp currents decreased as a result. Shimmen and Tazawa (1980), using tonoplast-free *Chara*, varied internal K$^+$ and Cl$^-$ concentrations in tonoplast-free cells. They found that the duration of the AP is strongly dependent on intracellular K$^+$ concentration. At 100-mM K$^+$, the AP resembles that of intact cells (Fig. 5B). Na$^+$ could substitute for K$^+$, but the effect was weaker. AP occurred with very low Cl$^-$ inside the cell or with EGTA, which prevented Ca$^{2+}$ concentration inside the cell to rise. The Japanese researchers assumed the under some circumstances, Ca$^{2+}$ inflow alone produced the depolarizing phase of the AP (see Section II.D). At the other extreme, if the internal concentration of Cl$^-$ increased above 29 mM, the cells lost excitability. The result suggests a low concentration of Cl$^-$ in the cytoplasm of intact cells. Coster (1966) measured cytoplasmic Cl$^-$ in *Chara* as 10 mM.

2. pH

The pH of “normal” charophyte medium, such as artificial pond water (APW), is usually between pH 7.0 and 7.5. Beilby (1982) and Beilby and Bisson (1992) studied excitation in *Chara* in a large range of medium pH from 4.5 to 11.5. The pH 4.5 is the lower limit for excitability: the resting PD approached—100 mV and excitation disappeared after about 60 min at this pH. The excitation currents appeared at more positive voltage clamp levels and with longer initial delay than at higher pH. The time scale was longer. The differences were much less pronounced at more depolarized levels (at and above −35 mV). Consistently, the AP form was also stretched with time.
Above pH 10, Chara cells enter the H\(^+\)/OH\(^-\) state (Bisson and Walker, 1980), in which H\(^+\)/OH\(^-\) channels dominate the plasmalemma conductance. The form of the AP was conserved at high pH. Under voltage clamp conditions, some differences in the excitation transients emerged. Although the currents at very depolarized PD levels did not show large effects, the currents near the threshold appeared after longer delays and with slower rise and fall times. The changes developed over many minutes.

Tazawa and Shimmen (1982) explored the variation of the internal pH in tonoplast-free cells. Chara remained excitable between internal pH limits from pH 6.0 to 9.0, whereas Nitellopsis exhibited a narrower range between pH 6.6 and 7.9. The peak PD of the AP was almost independent of the internal pH.

As both internal and external pH deviations from “normal” values caused depolarization of the membrane resting PD, the changes in excitation might stem from this rather than the pH change itself.

3. Inhibitors, Antagonists, and Agonists

Tsutsui et al. (1986, 1987a) tested effects of Ca\(^{2+}\) channel blockers, La\(^{3+}\) and verapamil, Ca\(^{2+}\) chelating agent ethylene glycol bis (beta-aminoethyl ether)-N, N, N\(^{0}\), N\(^{0}\)-tetraacetic acid (EGTA), and calmodulin antagonists W-7 and trifluoperazine (TFP) on the AP and the current-voltage (I/V) profiles of intact cells of Chara corallina. In small concentrations of 10-μM LaCl\(_3\) and 100-μM verapamil, the resting PD depolarized, the amplitude of the AP decreased irreversibly, and the streaming did not stop at the time of excitation. At 100-μM TFP, the resting PD depolarized and the AP lost amplitude (AP peak at more negative PD) with longer time scale. The streaming did not stop. A similar effect was observed with 40-μM W-7. Tsutsui et al. (1987b) employed the ramp voltage clamp command and found that 20-μM LaCl\(_3\) removed the excitable N-shaped part of the I/V profile, 75-μM N, N\(^{0}\)-dicyclohexylcarbodiimide (DCCD) affected the proton pump, without disturbing the excitation channels. 0.5-mM EGTA reversibly depolarized the resting PD and removed excitability. The calmodulin antagonists, TFP (10 μM) and W-7 (20 μM), depolarized membrane PD, reduced excitation, and shifted it to more positive PDs. The effects were partially reversible. Beilby and MacRobbie (1984b) exposed Chara cells to 5 μM of TFP. They also found depolarization and slowing of the excitation transients. However, in their experiments the peak of the AP became positive and cells exhibited spontaneous APs with concurrent slowing of cytoplasmic streaming. Beilby (1984b) found that 100 μM LaCl\(_3\) irreversibly removed excitation and the cells could then be returned to APW for collecting I/V profiles over wide PD window.
The effect of putative Ca\(^{2+}\) blockers was explicable in terms of no Ca\(^{2+}\) inflow, either directly at the time of excitation or to replenish the internal stores. Calmodulin binds Ca\(^{2+}\) and the complex then influences ATPases and protein kinases in animal cells (Cheung, 1980). The antagonists TFP and W-7 interfere with these functions. However, the effects of these substances on excitation are not easily explicable in terms of any model. Perhaps, the most significant correlation is that 40-\(\mu\)M W-7, TFP, or CPZ abolished the patch-clamp currents of the Ca\(^{2+}\)-dependent Cl\(^{-}\) channel (Okihara et al., 1991).

Lunevsky et al. (1983) used Cl\(^{-}\) channel blocker ethacrynic acid to reveal a prompt transient at the beginning of excitation clamp currents across plasmalemma of *Nitellopsis obtusa* (Fig. 7B). They were able to show that this current component responded not only to Ca\(^{2+}\), but also to a range of divalent and monovalent cation concentration changes. They suggested that the underlying channel is a cation channel, activated by depolarization, which passes Ca\(^{2+}\) at the time of excitation.

As mentioned earlier (Section II.E.3), “after-hyperpolarization” was observed after the AP in *N. axillaris* (Barry, 1968; Kamitsubo, 1980), *N. axilliformis* (Shimmen and Tazawa, 1983b), and *N. flexilis* (Belton and Van Netten, 1971). In the first two experimental systems, the effect was abolished by K\(^{+}\) channel blocker TEA. In *N. flexilis* the after-hyperpolarization was abolished by procaine hydrochloride (which reduced K\(^{+}\) and Na\(^{+}\) current activation in animal AP). The AP duration was not affected. The exposure to TEA and BaCl\(_2\), on the other hand, did prolong the AP duration.

Thiel (1995) and Thiel et al. (1997) employed the AP clamp on intact *Chara* cells while blocking the Cl\(^{-}\) current with niflumic acid and/or the K\(^{+}\) current with Ba\(^{2+}\). They were able to resolve the time-dependence of these currents at the time of the excitation.

Shiina and Tazawa (1987) employed ramp voltage clamp command to study excitability of tonoplast-free cells of *Nitellopsis obtusa*. Cl\(^{-}\) channel blocker 4, 4'-di-isothiocyanostil-bene-2, 2'-disulphonate (DIDS) did not suppress the inward excitation current component of the I/V curve, whereas Ca\(^{2+}\) channel blockers La\(^{3+}\) and nifedipine removed it irreversibly. Ca\(^{2+}\) channel agonist BAY K8644 enhanced excitation. The authors concluded that excitability in tonoplast-free cells is based on activation of Ca\(^{2+}\) channels alone.

Shiina et al. (1988) suggested that the Ca\(^{2+}\) channel is inhibited by protein phosphorylation and activated by protein dephosphorylation. Agents enhancing dephosphorylation, protein kinase inhibitor, or phosphoprotein phosphatase-1,2A, were perfused into tonoplast-free cells of *Nitellopsis obtusa*, causing the plasma membrane to depolarize and become more conductive. The reverse effect was obtained by agents enhancing phosphorylation, phosphoprotein phosphatase inhibitor I, or alpha-naphthylphosphate. These results were challenged by Zherelova (1989), who found that Ca\(^{2+}\) channels
were activated by phosphorylation via protein kinase C. Polymyxin B, which inhibits protein kinase C, blocked the Ca\(^{2+}\) current in *N. syncarpa*.

**G. Effect of Temperature**

*Kishimoto (1972)* changed medium temperature from 4.5°C to 30°C, while he applied ramp voltage clamp command to cells of *C. australis*. He found that the resting PD became slightly more positive as temperature decreased. The amplitude of negative current decreased with temperature, while the threshold PD became more positive. Excitation disappeared altogether at about 8°C.

*Blatt (1974)* changed medium temperature between 3°C and 28°C and observed the resting PD and the AP in *N. flexillis*. Both the resting PD and the AP amplitude declined with decreasing temperature. The threshold became more positive and the refractory period was longer at lower temperature. The rise and decay times increased two orders of magnitude with falling temperature, but the form of the AP did not change. Arrhenius plot of the rise and fall times showed a relatively sharp break at 13.5°C. The author suggested that the discontinuity might be related to a “phase change” of the membrane.

*Beilby and Coster (1976)* studied the temperature dependence of excitation of *C. corallina* (Fig. 3). The duration both of the vacuolar AP and the vacuolar excitation clamp currents increased with decreasing temperature from \(~1\) s at 40°C to \(~30\) s at 3.5°C. The peak PD of the AP did not change greatly with temperature. The resting PD depolarized by about 70 mV between 20°C and 5°C. Consequently, the amplitude of the AP decreased with temperature. Similar to Blatt (1974), the authors found that the AP form did not change. The change of duration was quantified by taking the AP or clamp current at 20°C as standard and finding a factor F by which the transients at different temperature have to be shrunk or expanded. The Arrhenius plot of F was curved with no obvious breaks. Activation enthalpies varied from \(~7\) kJ/mol for temperatures above 20°C to \(~350\) kJ/mol for temperature less than 7°C. The authors suggested that as temperature increased, a smaller degree of dehydration was necessary for the ions to permeate through the channels that underlie excitation.

**H. Signal Propagation**

Similarly to the nerve AP, the plant AP propagates along the cell. The conduction velocity in moist air of 0.3–2.3 cm/s was measured in *N. mucronata* (Umbrath, 1933), 1.1–2.1 cm/s in *N. translucens* (Auger, 1933), 4.4 cm/s in
N. flexilis, and 1.4 cm/s in C. braunii (Sibaoka, 1958). Tabata and Sibaoka (1987) found that the speed of AP propagation in C. braunii increased from 0.21 cm/s in moist air to 1.5 cm/s in APW. The AP propagated at almost constant velocity along the cell, but near the end of the cell the velocity in APW increased. The increase was of the right order of magnitude to that predicted by conduction velocity equations (Hodgkin, 1954). The AP in charophytes can be transmitted from cell to cell (Sibaoka and Tabata, 1981; Tabata, 1990).

I. Capacitance at the Time of Excitation

The capacitance of many animal and plant membranes has been estimated as 0.01 F/m^2 (e.g., see Cole, 1970). However, Kishimoto (1972) superimposed small sinusoidal current on the voltage clamp command and measured the phase angle of the resulting clamp current. He concluded that the capacitance increased at the peak of the current transient. Beilby and Beilby (1983) utilized greater computing power to calculate capacitance at the time of voltage clamp to excitable levels, finding transient decrease followed by an increase. The full meaning of these measurements still awaits interpretation.

J. Early Models

1. States of Plasmalemma of Tonoplast-Free Cells

Shimmen et al. (1976) explained the behavior of the tonoplast-free cells using the Tasaki (1968) two-state hypothesis, which proposed that negatively charged sites on the outer membrane surface are occupied by divalent cations in resting state and by monovalent cations in excited state. The Japanese researchers viewed the depolarized level as an “excited” level (see Fig. 8A). The excited level was further split into +ATP and −ATP.

This data may be interpreted another way. In tonoplast-free cells, the APs and the transient-clamp currents inactivated very slowly (Fig. 6A and B). However, the excitation-clamp currents in tonoplast-free cells did diminish with time (Beilby et al., 1993). Consequently, although the AP may be prolonged, the excitation state is never stable in low medium K^+. In high medium K^+ the membrane appeared to have two stable states: depolarized and hyperpolarized. The increase of medium K^+ together with depolarization opens the large conductance K^+ channels (sometime called maxi K^+) at the plasmalemma. These channels become the dominant membrane conductance and the membrane behaves as a K^+ electrode (K^+ state). The maxi K^+ channels can be activated in the tonoplast-free Chara cells (Smith, 1984; Smith and Walker, 1981). These channels are closed by increase in medium Ca^{2+} and hyperpolarization of the membrane PD (Beilby, 1985, 1986a,b;
Smith, 1984). Furthermore, the I/V characteristics of the membrane in K\textsuperscript{+} state display region of negative conductance: N-shaped curve, which is similar to that of excitation (e.g., Fig. 5A). The negative conductance is not an exclusive signature of excitation: it arises with any channel with strong PD dependence if the membrane PD is swept through the activation PD of the channel (Beilby, 1986b). Consequently, the steady “excited state” is probably K\textsuperscript{+} state. Tazawa and Shimmen (1980) found that the excitability is lost after about 10 min of perfusion with HK medium to remove ATP. My interpretation is that after the Ca\textsuperscript{2+} in the internal store becomes depleted, ATP is necessary

**FIG. 8** Models of excitation and membrane states. (A) Four stable states of Chara plasmalemma in tonoplast free cells. R and E refer to resting and excited states, respectively (Tazawa and Shimmen, 1980). (B) Ionic events occurring during a charophyte AP. Solid line arrows show net fluxes of ions, dashed line arrows indicate Ca\textsuperscript{2+} activation of other channels (Shimmen et al., 1994).
to replenish it. Thus no excitation occurs if no ATP is available in the cytoplasm and consequently, there is no \(-\)ATP-excited state.

2. Ionic Events at the Time of Excitation

By the 1990s it was obvious that the initial response to depolarization past the threshold PD is inflow of Ca\(^{2+}\) from the outside medium through cation channels (which are probably not very selective). The increase of Ca\(^{2+}\) in the cytoplasm induces Ca\(^{2+}\) release from internal stores close to the inner face of the plasmalemma (Fig. 8B). Once the Ca\(^{2+}\) concentration reaches a certain level, the Cl\(^{-}\) channels are activated on both plasmalemma and tonoplast, producing a flow of anions out of the cell and making membrane PD more positive. The depolarization activates the outward rectifier channels, causing K\(^{+}\) outflow. At the same time the Ca\(^{2+}\) level in the cytoplasm starts to drop and the Cl\(^{-}\) channels inactivate. The reason for the Ca\(^{2+}\) level dropping has not been adequately addressed, but emptying of the internal stores was suggested. While the Cl\(^{-}\) channels on both plasmalemma and tonoplast are Ca\(^{2+}\) activated, it is not clear whether the tonoplast K\(^{+}\) channels need cytoplasmic Ca\(^{2+}\) increase. The patch clamp studies on cytoplasmic droplets of Chara suggest Ca\(^{2+}\) involvement (Laver and Walker, 1991), although the analysis of the hypotonic effect in Lanthrothamminium revealed increase in K\(^{+}\) conductance at both membranes in lanthanised cells, where the streaming did not stop.

3. Adaptation of Hodgkin-Huxley Model

The equations describing the Na\(^{+}\) transient in the nerve (Hodgkin and Huxley, 1952a) have been adapted to describe the Cl\(^{-}\) transient and the putative Ca\(^{2+}\) transient (Beilby and Coster, 1979b,c).

\[
I_\text{Cl}^- = g_\text{Cl}^- (E_m - E_{\text{Cl}^-}) \tag{1}
\]

where \(I_\text{Cl}^-\) is chloride current, \(g_\text{Cl}^-\) is the chloride conductance, \(E_m\) is the membrane PD, and \(E_{\text{Cl}^-}\) is the equilibrium potential for Cl\(^{-}\).

\[
g_{\text{Cl}^-} = m^3 h g_{\text{max}} \tag{2}
\]

where \(g_{\text{max}}\) is the maximum conductance, \(m\) and \(h\) are activation and inactivation parameters, respectively.

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

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

where \(t\) is time and \(\delta_{\text{Cl}^-}\) is a delay before excitation begins. Delays were later found in the nerve by Keynes and Rojas (1976). Equations 3 and 4 describe how the \(m\) and \(h\) parameters evolve from their resting values \(m_0\) and \(h_0\), to the long time values \(m_\infty\) and \(h_\infty\) with time constants \(\tau_m\) and \(\tau_h\), respectively.
Similar set of equations was used for the second transient. As the membrane PD depolarizes, the outward rectifier is activated and $K^+$ flows out restoring the resting PD (together with $Cl^-$ inactivation). The PD-dependent activation of the outward rectifier is much faster than the other currents and is taken as “instantaneous.”

The AP can be reconstructed:

$$\frac{dV}{dt} = -\frac{1}{C}[g_{Cl^-}(E_m - E_{Cl^-}) + g_{Ca^{++}}(E_m - E_{Ca^{++}}) + I_{ss}]$$

where $I_{ss}$ is “steady stream current,” which was measured at each PD level.

The AP shape is well approximated by Equation 5. A similar approach was taken by Hirono and Mitsui (1981) in describing excitation in single membrane constructs of $N. axilliformis$. The resting PD restoring current appears to activate more gradually in this charophyte and was fitted with an equation similar to that used in the HH for the nerve.

$$I_K = g_{K}^* n^4 (V - V_K)$$

$$n = n_{\infty} - (n_{\infty} - n_0) \exp[(\delta_K - t)/\tau_n]$$

where $g_{K}^*$ is the maximum $K^+$ conductance, $V_K$ is the equilibrium PD for $K^+$, and $n$ is the activation parameter.

The fit of the HH equations has been very useful to quantify many aspects of excitation in plants. For instance, Beilby and Coster (1979c) analyzed the temperature dependence of the time constants obtaining activation enthalpies of \(\sim 60\, kJ/mol\) for activation process and \(\sim 40\, kJ/mol\) for the inactivation process. They found that the enthalpies are independent of membrane PD and do not involve movement of charged moieties normal to the membrane. The delays in excitation indicate existence of intermediate step(s) before excitation channels can be activated. These ideas are compatible with data on the cytoplasmic second messenger taking part in the plant AP and the new models necessary to describe them.

## III. New Approaches in the New Century

### A. IP$_3$ (Inositol 1,4,5,-triphosphate) and Ca$^{2+}$ from Internal Stores

The experiments with Mn$^{2+}$ as a quencher of fura-2 fluorescence (Plieth et al., 1998) confirmed the importance of internal stores as the main source of Ca$^{2+}$ at the time of the AP (see Section II.E.1). Further, the elevation of inositol-1,4,5,-triphosphate (IP$_3$) in the cytoplasm was able to elicit APs
The enzyme phospholipase C (PLC) is responsible for mobilizing IP₃ from its membrane-bound precursor phosphatidyl inositol 4,5-biphosphate (PIP₂). Inhibitors of PLC, Neomycin, and U73122 inhibited excitation in Chara (Biskup et al., 1999).

The Ca²⁺ concentration in the cytoplasm was monitored, while the depolarizing pulses of varying height and width were applied. A sharp threshold existed in the Ca²⁺ concentration rise with respect to the strength/duration of the stimulus (Wacke and Thiel, 2001). The response in the Ca²⁺ rise was all or none. If two subthreshold pulses were applied in a certain regime, the full response was also obtained. The experimental results supported the hypothesis that a second messenger, such as IP₃, is produced by the stimulating voltage step and has to reach a critical concentration to liberate Ca²⁺ from internal stores. The double pulse experiments provided information about the dynamics of IP₃ formation and decay into IP₂, as well as refilling of the PIP₂ pool. The double subthreshold pulse produced a response only if the interval between pulses was shorter than ~3 s. This time was within the lifetime of IP₃ in the cytoplasmic compartment and the IP₃ produced by the first pulse was added to the IP₃ produced by the second pulse, reaching the threshold concentration. If the pulses come too close together (less than ~0.3 s), the PIP₂ pool has not been replenished and no further IP₃ was produced.

Wacke et al. (2003) borrowed another animal model (Othmer, 1997) to set up a quantitative description of Chara AP mediated by IP₃ activated Ca²⁺ channels and Ca²⁺ pumps on the endomembranes of internal stores, such as the ER. Othmer (1997) described the IP₃-sensitive Ca²⁺ channels as having four states: (1) unbound, (2) bound to IP₃, (3) bound to IP₃ and activating Ca²⁺ molecule, and (4) bound to IP₃ and to a second inactivating Ca²⁺ molecule. The channel is only conductive in state 3 and as Ca²⁺ concentration in the cytoplasm rises, the channels undergo transition into inactivated state 4. For the channels to be activated by IP₃ again, the transition into state 1 is necessary. Using sophisticated periodic stimulation technique, Wacke et al. (2003) established that the long lifetime of state 4 was responsible for the refractory period. In the simulation using many parameter values from Othmer’s animal model, the refractory period was about 30 s. The model also confirmed the previous assumptions (Wacke and Thiel, 2001), that the all-or-none response and the steep dependence of Ca²⁺ response on strength/duration of the stimulatory pulse arises from the dynamics of the model variables.

Although the Wacke et al. (2003) modeling of the AP looks very encouraging, Tazawa and Kikuyama (2003) failed to duplicate some of the preceding experiments in Chara. Their injection of IP₃ into cytoplasm did not produce Ca²⁺ release; and inhibitors of PLC, U73122, and neomycin, did not affect generation of the AP. An inhibitor of the IP₃ receptor, 2APB, did make
cells inexicitable, but the same effect was observed in tonoplast-free cells, in which the AP transient is thought to be carried by inflow of Ca\(^{2+}\) from the outside. The authors suggested, that the inhibitor affects the plasma membrane rather than Ca\(^{2+}\) channels on the endomembranes. More experiments will be necessary to understand why the different results were obtained by the German and Japanese groups.

B. Involvement of AP in Wound Signaling

Wounding induces a range of responses in plants. One of the earliest reactions is localized depolarization of membrane PD in cells close to the site of damage. There are also APs and variation potentials that propagate away from the injury site. The experiments with complex tissues of higher plants are too difficult to analyze. Consequently, Shimmen (2001, 2002) employed a much simpler two Chara cell system, in which one cell (victim cell) was cut off and the reaction of the other cell (receptor cell) was measured. The receptor cell generated four kinds of depolarization: (1) rapid component, (2) slow and long-lasting component, (3) action potential, and (4) small spikes. The first two responses were observed in most experiments and their origin is assumed to be at the nodal end. The APs and the small spikes were not always present. The AP was generated on the flank of the receptor cell near the cut end and propagated to the far end.

C. AP and Turgor Regulation

Beilby and Shepherd (2006) measured the I/V characteristics of the Ca\(^{2+}\)-activated Cl\(^{-}\) channels at the time of hypotonic regulation in Lamprothamnium succinctum. They suggest that the same Cl\(^{-}\) channels participate in the hypotonic effect and in the excitation. However, the Ca\(^{2+}\) increase at the time of hypotonic effect lasts for more than 10 min (Okazaki et al., 2002). This is far too long for the dynamics of IP\(_3\)-activated Ca\(^{2+}\) channels, which close within 15 s (Thiel et al., 1997). Experiments of Kikuyama and Tazawa (2001) suggest that the Ca\(^{2+}\)-containing cytoplasmic organelles become mechanically distorted by the hypotonic stress and that there are stretch-activated channels that allow Ca\(^{2+}\) to flow into the cytoplasm. This might be an alternative mode of cytoplasmic Ca\(^{2+}\) increase, independent of the IP\(_3\) signal cascade.

Similarly to the AP, the fast hypotonic regulation involves outflow of Cl\(^{-}\) and K\(^{+}\). In Lamprothamnium the Ca\(^{2+}\)-activated Cl\(^{-}\) channels open first, followed by the K\(^{+}\) channels. In this case the large conductance K\(^{+}\) channels, rather than the outward rectifier, are involved (Beilby and Shepherd, 1996).
In *C. longifolia*, the sequence of channel activation is reversed (Stento *et al.*, 2000). As the loss of KCl is the prime objective of the hypotonic regulation, the sequence of channel activation is not important.

Hoffmann and Bisson (1990) observed APs in *C. longifolia* upon exposure to hypertonic medium. This was not observed at the time of hypertonic regulation of *L. succinctum* (Beilby and Shepherd, 2001). Although *C. longifolia* is able to regulate its turgor and survive in a range of salinities, there is a similarity to salt-sensitive charophytes, which respond with APs when faced with increase in medium salinity (Kishimoto, 1966b; Shepherd *et al.*, in preparation).

**IV. Summary**

My scientific career started by investigating the charophyte AP (Beilby and Coster, 1976, 1979a, b, c). I have moved on to other transporters in charophyte and other giant-celled organisms, but I kept up my interest in the AP research. So, it was a great pleasure for me to refresh in my memory all the elegant experiments investigating excitation over many years. I hope that the readers will get the same enjoyment, following the intricate story of the charophyte AP. I have described the research up to the mid-1990s in greater detail, as the papers are not available on the World Wide Web and are likely to be less accessible to young researchers.

The late-1990s saw a paradigm shift for the charophyte AP: from PD-activated plasmalemma channel mechanisms, described so well by HH equations, to a combination of several chemical reactions and second messenger-activated channel, liberating Ca$^{2+}$ from internal stores (Biskup *et al.*, 1999; Wacke and Thiel, 2001; Wacke *et al.*, 2003). IP$_3$ can diffuse freely in the cytoplasm, whereas Ca$^{2+}$ is not mobile (Trewavas, 1999). It is thought that the involvement of IP$_3$ produces Ca$^{2+}$ waves in many types of cells and enables the AP to travel along the cell. The dynamics of IP$_3$ production and decay can account for the shape of the AP (Fig. 9), delays between the stimulus and excitation onset, the multiple peaks in the clamp current and for the refractory period. New questions that we have to ask include how is IP$_3$ release triggered by a threshold PD change across plasmalemma? What can we learn about the Ca$^{2+}$ pumps that return the cytoplasmic Ca$^{2+}$ to a low level after excitation? How is accommodation produced by the IP$_3$ model? Why do salt-sensitive charophytes, exposed to concentrated saline media, exhibit repetitive firing? The removal of the tonoplast greatly changes the AP form, removes refractory period, but the threshold behavior is retained. The excitation currents are much slower to turn off. Is IP$_3$ still involved? Can Ca$^{2+}$ diffuse in tonoplast free cells? What happens to ER in these cells? Clearly, the charophyte cells are an
excellent system for this research. Cell perfusion combined with inhibitors of IP$_3$ production and decay, fluorescence microscopy, and electrophysiology will lead us to the understanding of the synergy of structural elements, biochemical reactions, and ion flows that produce the plant AP.

FIG. 9 Simulated transient Ca$^{2+}$ concentration rise as a result of IP$_3$-activated endomembrane Ca$^{2+}$ channels. (A) Effect of pulse duration. (B) Effect of pulse strength (Wacke et al., 2003).
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References


