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Transient Cl^- and K^+ Currents during the Action Potential in *Chara inflata*

Effects of External Sorbitol, Cations, and Ion Channel Blockers

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In voltage-clamp experiments, a two-pulse procedure was used to investigate the ionic currents underlying the action potential in *Chara inflata*. A prepulse hyperpolarized the membrane from a resting potential of about −100 to −200 mV. The prepulse was followed by a second pulse that changed the potential difference (*p.d.*) to −100 mV and less negative values in steps of 20 mV. This two-pulse procedure induces action potentials that have a reproducible time course, which is essential for any comparative investigation of the action potential. The two-pulse procedure reveals that in the charophyte *C. inflata* the electric current flowing across the cell membranes during positive voltage-clamp steps from the resting *p.d.* consists of a leak current flowing from the start of the pulse, followed by a transient inward-going current, I_i, commencing after a delay, and preceding a delayed transient outward current, I_o. The characteristics of the current components and their response to various ion channel blockers and ionic treatments suggest that: (a) I_i, which is blocked by the external application of 9-anthracenecarboxylic acid, is carried by Cl^- and (b) I_o, which is blocked by the external application of the organic anions tetraethylammonium (TEA^+) and nonyltriethylammonium, is carried mainly by K^+. The magnitude and behavior of these K^+ and Cl^- currents could be modified by changes in the external concentration of $CaCl_2$, LiCl, or NaCl but not sorbitol. Hence, it is concluded that NaCl-enhanced transient inward Cl^- current, I_i, is due to ionic effects of NaCl rather than to its osmotic effects. The modification of the K^+ current, I_o, either by changing external K^+ concentrations or by blocking the current with TEA^+, also alters the Cl^- currents I_i.

In the excitable cells of the charophyte plants *Chara globularis* (Gaffey and Mullins, 1958), *Chara corallina* (Oda, 1976; Kikuyama et al., 1984), *Nitellopsis obtusa* (Kikuyama et al., 1984), *Nitella megacarpa* (Spyropoulos et al., 1961), and *Nitella clavata* (Mullins, 1962), efflux experiments have shown that Cl^- and K^+ flow across the membrane during the action potential. Hope and Findlay (1964) found a significant increase in Cl^- efflux in *C. corallina* but no increase in Ca^{2+} influx, whereas Hayama et al. (1979) found an increase in Ca^{2+} influx in the same species. It has been suggested that an increase in cytoplasmic Ca^{2+} concentration (Williamson and Ashley, 1982; Kikuyama and Tazawa, 1983) causes the cessation of cytoplasmic streaming during the action potential in Characean cells. The cessation of the cytoplasmic streaming in Characean cells is closely related to the occurrence of the action potential (Hayama et al., 1979).

The action potential mechanism appears to be involved in stomatal movements (Blatt, 1991) and salt tolerance (Okazaki and Tazawa, 1990). Data obtained from voltage-clamp experiments have suggested that the transient inward-going current of the action potential in *Nitella* is carried by Cl^- (Kishimoto, 1964) or by Cl^- and Ca^{2+} in *C. corallina* (Beilby and Coster, 1979a, 1979b). A delayed transient outward current was observed in *Nitella* (Findlay, 1962), but this current is not seen in *C. corallina* (Beilby and Coster, 1979a, 1979b; Tester, 1988). Furthermore, patch-clamp experiments on a surgically accessed plasma membrane of *Chara australis* indicate the absence of single-channel activities underlying any outward delayed current (Laver, 1991).

In this paper I describe the results of voltage-clamp experiments on cells of another charaphyte plant, *Chara inflata*. The electric *p.d.* was stepped into the region, less negative than the resting *p.d.*, where the action potential occurs. The electric current flow during such voltage-clamp steps is divided into its separate ionic components and compared with the currents flowing under similar conditions in *C. corallina*.

Previously, the effects of high concentrations of NaCl and sorbitol in the external solution on the electrical properties of the membranes in *C. inflata* were described (Kourie and Findlay, 1990a, 1990b). Those experiments concentrated on the effects on the separate membrane currents when the clamped *p.d.* became more negative than the resting *p.d.* In this paper we describe experiments aimed at (a) developing a voltage-pulse procedure for inducing reproducible action potentials, (b) pharmacologically dissecting the components of the electric current flowing across the cell membranes during depolarizing voltage-clamp steps, and (c) testing the dependency of the magnitude and behavior of these components on changes in the external concentrations of sorbitol, NaCl, LiCl, $CaCl_2$, and KCl.

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Abbreviations: APW, artificial pond water; 9-AC, 9-anthracenecarboxylic acid; I_i, transient inward-going current; I_o, outward transient current; I_L, leak current; $I_{i(max)}$, peak value of I_i; I_m, membrane current; $I_{o(max)}$, peak of I_o; I_{max}, $I_L + I_{o(max)}$; $[NaCl]_o$, external NaCl concentration; *p.d.*, potential difference; TEA^+, tetraethylammonium; V_m, membrane potential.

MATERIALS AND METHODS

Culture Material

Cells of *Chara inflata* were grown in the laboratory in bathing medium of APW, which initially consisted of (concentrations in mM): KNO_3, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0; $Na_2H_2PO_4$, 0.5; $CaCl_2$, 0.1; FeEDTA, 0.002; and trace elements (Coleman and Findlay, 1985). To offset changes in these concentrations as a result of evaporation the levels of the APW in the tanks were kept constant by the addition of distilled water. The temperature was 25°C, and the cells were subjected to a daily cycle of 12 h of light and 12 h of darkness; the light was provided by fluorescent tubes (Philips [Sydney, Australia] BTL 40-W 30RS white and Crompton [Seoul, Korea] F40 TP PG).

Experimental Solutions

The APW bathing *C. inflata* cells contained (in mM): $CaCl_2$, 0.1; KCl, 1.0; $NaHCO_3$, 0.4; Hepes, 4.0; with pH 7.5 adjusted by the addition of about 2.6 mM NaOH. To this basic APW various other solutes were added as required.

Ion Channel Blockers: $LaCl_3$, 9-AC, and TEA^+

In the experiments reported here all *C. inflata* cells were collected from a single culture. A concentration of 250 μM La^{3+} was applied externally for 12 min. This concentration was used because work conducted in the same laboratory indicated that the effect of 100 μM La^{3+} on a hyperpolarization-activated inward current in *C. inflata* was very slow (Tyerman et al., 1986b). 9-AC and TEA^+ were applied for 15 and 5 min, respectively, before voltage clamping the cells.

Intracellular Recording of Electrophysiological Parameters

Unless stated otherwise, the basic methods were as described by Kourie and Findlay (1990a). In all of the experiments described in this paper, the spherical whorl cells of *C. inflata*, of diameter about 1 to 2 mm, were used. Each cell was placed in a small chamber in a Perspex holder and fixed between the end of a glass rod and a flat piece of Perspex. The cell was continuously irrigated with solution at an approximate rate of 50 mL/h. A viewing microscope provided illumination.

Microelectrodes were inserted with Huxley-type micromanipulators. The measuring electrode, a glass micropipette filled with 3 M KCl, was inserted into the vacuole. The tonoplast and the plasmalemma are in series and thus the *p.d.* between the electrode in the vacuole and the reference electrode is the sum of the *p.d.*s across both membranes. Current was injected into the cell with a Pt Ir alloy wire, electropolished to a fine point and insulated to 100 μm of the tip with glass, inserted into the vacuole. The current was measured with a virtual ground amplifier between the bath and ground.

The Membrane *p.d.*

Nearly all microelectrodes inserted into the cytoplasm very soon sealed or broke through into the vacuole, and long-term measurements of the *p.d.* across the plasmalemma alone were not feasible. In all of the experimental work described in this paper, V_m, which is referred to as the membrane *p.d.*, is the *p.d.* between the vacuole of the cell and the outside solution and thus is the *p.d.* across the plasmalemma and tonoplast in series. V_m has not been corrected for the tonoplast *p.d.* (about 10 mV). This means that V_m is within 10 mV of the true plasmalemma *p.d.* Although the voltage-clamp current maintains the *p.d.* across the plasmalemma and tonoplast constant, the *p.d.* across the separate membranes is, in principle, not constant. However, provided the conductance of the tonoplast is appreciably larger than that of the plasmalemma, the *p.d.* across the plasmalemma will probably remain reasonably constant. It is possible that this condition will not hold at the time of the peak of the inward Cl^- current (Findlay et al., 1990). The effect will be to underestimate the magnitude of this current. It is for this reason that no detailed kinetics analysis was attempted for the Cl^- current that flows during positive voltage-clamp steps.

Experimental Procedure

The experimental procedures have been described in detail by Kourie and Findlay (1990a). The cells of *C. inflata* were voltage clamped in the dark, with their proton efflux not operating (Coleman and Findlay, 1985) and the cell membranes permeable predominantly to K^+. Consequently, the resting *p.d.* was close to the Nernst potential for K^+.

In the voltage-clamp experiments, a two-pulse procedure was used. A prepulse hyperpolarized the membrane from about −100 to −200 mV. Initially, 12-s prepulses were used to ensure the inactivation of the inward K^+ current. However, it was found that the hyperpolarization-activated inward K^+ current decayed to the background current level within 8 s (Kourie and Findlay, 1990a, 1990b). Furthermore, the *p.d.* of the prepulse was not sufficiently negative (e.g. less negative than −225 mV) to activate I_{Cl} (Kourie and Findlay, 1990a). The prepulse was followed by a pulse that took the *p.d.* to −100 mV and less negative values in steps of 20 mV.

In addition to the standard two-pulse voltage protocol, which was usually set for a duration of 20 s, fast bipolar scans could be added on the main depolarizing pulse. The bipolar scans consisted of up to 20 sequential pulses, 50 ms long with variable amplitudes determined by software commands (Tyerman et al., 1986a). Subsequently, these bipolar scans were given by a computer through a Cromemco D+7A 8 bit D/A convertor.

RESULTS

Effects of the Negative Prepulse on the Clamp Current

Figure 1 shows membrane currents flowing in *C. inflata* during three consecutive sets of positive voltage-clamp steps to a range of values of *p.d.*, each lasting 12 s. In Figure 1, A and C, the holding *p.d.* was −100 mV, near the resting level. In Figure 1B a prepulse to −200 mV, lasting 8 s, preceded the positive-going steps. At the end of the main pulse the V_m potential was returned to −100 mV. The prepulse rapidly inactivates the prevailing K^+ conductance (Kourie and Findlay, 1990a)—the cell in the dark has a high K^+

conductance (Coleman and Findlay, 1985)—such that before the depolarizing pulse commences the membrane is in a "K^+-inactivated state" (Kourie and Findlay, 1990a). The data in Figure 1B show that the prepulse had the following effects on the components of the current flowing during the subsequent depolarizing clamp step: (a) it reduced the time to the peak of I_i, (b) it shortened the time to the peak of I_o, (c) it increased the amplitude of I_i, and (d) it tended to make the time courses of the clamp currents less variable for successive steps to the same *p.d.* (data not shown).

The membrane current was analyzed in terms of the following components (Fig. 2): (a) I_L measured at 30 ms after the end of the prepulse and before activation of I_i, (b) I_i component, the $I_{i(max)}$, of which decreases as V_m is clamped to more depolarized levels, and (c) I_o, which reaches $I_{o(max)}$ in 4 to 10 s and then decays to a steady level, usually close to that of the I_L. I_{max} is defined as $I_L + I_{o(max)}$.

The values of the cord conductance for $I_{i(max)}$ and I_{max}, obtained from scans (see "Materials and Methods") in V_m superimposed on the main clamping pulse at locations close to the peak of these currents, were 5.51 and 3.52 siemens m^{-2}, respectively.

Figure 3A shows the effect of the magnitude of the negative prepulse on the clamp current. When the membrane *p.d.* was clamped to −80 mV, the I_i was initiated only when the prepulse was more negative than −150 mV, a membrane *p.d.* that is more negative than the resting potential V_m of *C. inflata* cells in the dark and in APW of pH 7.5. The magnitudes of I_i and I_o, later to be identified as Cl^- and K^+ currents, increased as the prepulse became more negative but tended to maximum values at V_m of about −200 mV.

The Boltzmann equation, which is a description of the gating charge movement in the voltage sensor of voltage-dependent channels (Hille, 1992), was used to examine the

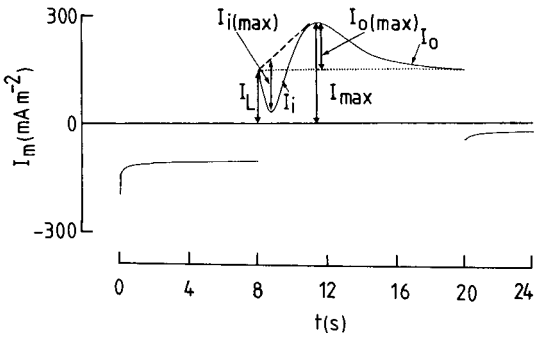

Figure 2. The components of the clamp currents during positive voltage-clamp steps following a negative prepulse. I_L, estimated from the initial current 30 ms after the start of the main pulse, is shown as a dotted line. I_i with $I_{i(max)}$ and I_o with $I_{o(max)}$ are also shown. The transient activation and then inactivation of I_o form the hump, which has an amplitude equivalent to $I_{o(max)}$.

voltage dependence of the ionic currents underlying the action potential. The voltage-dependent inactivation of $I_{i(max)}$ and activation of I_{max} were studied by applying 8-s conditioning pulses to various potentials between −100 and −225 mV, followed by activating voltage steps to −80 mV. The normalized $I_{i(max)}$, derived from Figure 3A, was plotted as a function of the conditioning voltage and fitted with the Boltzmann equation: $I_{i(max)}/I_{i(max)200} = 1/[1 + \exp(V - V_{½})K]$, where $I_{i(max)200}$ was the current activated from a holding potential of −200 (see Fig. 3A), $V_{½}$ is the *p.d.* at which half of the current is inactivated, and K is a parameter determining the rate of current inactivation. Inactivation was a steep function of voltage, with $V_{½}$ equal to −150 mV and a slope factor of −5 per *e*-fold change (Fig. 3B, solid line). The

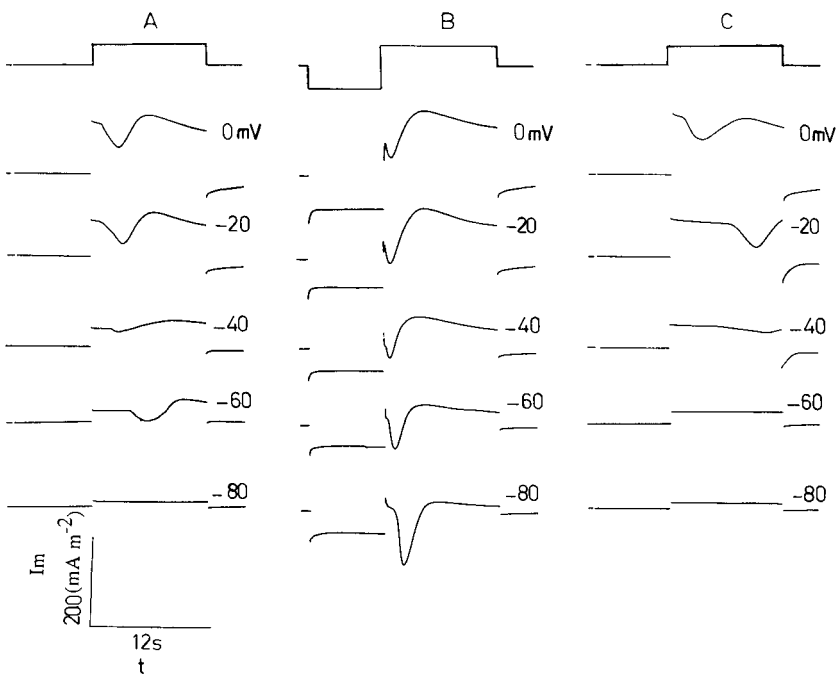

Figure 1. Membrane currents in *C. inflata* during three consecutive sets, A to C, of positive voltage-clamp steps to a range of values of *p.d.*, each lasting 12 s. The level of each step is shown to the right of each current trace. In A and C the holding *p.d.* was −100 mV, near to the resting level. In B a prepulse to −200 mV, lasting 8 s, preceded the positive-going steps. At the end of the main pulse the V_m was returned to −100 mV. The cells were in APW with pH 7.5. Outward current is positive.

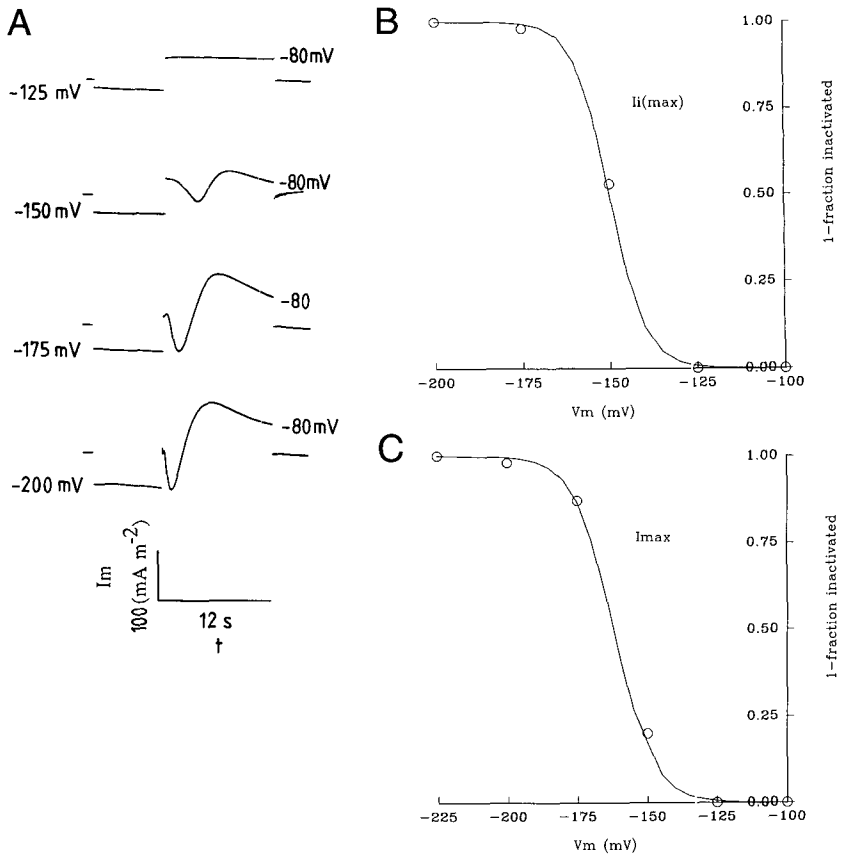

Figure 3. A, Membrane currents during a series of two-step voltage-clamps showing the effect of the level of the 8-s negative prepulse, indicated on the left, on the I_o that flows during the subsequent 12-s step in V_m to −80 mV. B, The voltage dependence of inactivation of $I_{i(max)}$; peak current amplitudes, derived from Figure 3A, normalized to the maximum current activated from −200 mV were plotted against conditioning voltages. The data were fitted to a Boltzmann function (solid line) of the form $I_{i(max)}/I_{i(max)200} = 1/[1 + \exp(V - V_{½})/K]$, where $V_{½}$ = −150 mV and K = −5 mV. C, I_{max}; peak current amplitudes normalized to the maximum current activated from −225 mV were plotted against conditioning voltages. The data were fitted to a Boltzmann function (solid line) of the form $I_{max}/I_{max225} = 1/[1 + \exp(V - V_{½})/K]$, where $V_{½} = -162$ mV and $K = -7$ mV.

normalized I_{max} was also plotted as a function of the conditioning voltage and fitted with the Boltzmann equation: $I_{max}/I_{max225} = 1/[1 + \exp(V - V_{½})K]$, where I_{max225} was the current activated from a holding potential of −225. The values for $V_{½}$ (−162 mV) and K (−7 mV) (Fig. 3C, solid line) are similar to those obtained for $I_{i(max)}$.

Ionic Components of the Clamp Current

Figure 4 shows the membrane currents in *C. inflata* during depolarizing voltage-clamp steps. Figure 4A shows the pulse protocol. For most cells in APW, pH 7.5, and in the dark, the outward current contains several components (Fig. 4B). However, in some cells (less than 5%) the membrane current lacked some of the components (Fig. 4C). The current-voltage curves show that I_i has a reversal *p.d.* (the *p.d.* where the current is zero) different from those of I_{max} and I_L (see below). For comparison, the membrane current in *C. corallina* during depolarizing voltage-clamp steps was also measured. The outward current lacked $I_{o(max)}$ (i.e. the "hump" that is defined as the transient activation and then inactivation of the outward current, I_o; shown in Fig. 2) and was more or less constant during the clamp step (J.I. Kourie, unpublished data), resembling those currents of *C. inflata* treated with TEA^+ (Fig. 5).

There are difficulties in attempting to dissect the clamp currents into various components and in identifying the components. The complexity is because (a) inactivation of I_i is overlapping with the activation of the delayed outward current and (b) the voltage-clamping pulses are across the plasmalemma and the tonoplast. Somewhat arbitrarily it was assumed that I_o follows the dashed line shown in Figure 2. In fact, with the membrane *p.d.* clamped at +20 mV (see Fig. 4), I_i seems to be small, and there appears to be a delay of up to 1 s before I_o commences. However, the error due to the assumption that I_o follows the dotted line will not be large, since I_i may reverse at a *p.d.* more positive than +40 mV (see below). Under the conditions of the experiments reported here the only ion having a reversal potential at this positive value is Cl^-.

The effects of the K^+ channel blockers TEA^+ (Coleman and Findlay, 1985) on I_o were tested. The hump in I_o was totally blocked by TEA^+ (Fig. 5B). At a clamping potential of +40 mV, I_{max} decreased 39% (Fig. 5C). In contrast, Beilby and Coster (1979a) reported that TEA^+ did not have an effect on the outward current in *C. corallina*. Figure 5 also shows that blocking I_o by TEA^+ makes the I_i more apparent. The current-voltage relation for a voltage-clamped *C. inflata* reveals that the TEA^+-enhanced amplitude of $I_{i(max)}$ is voltage dependent (Fig. 5C). At a clamping potential of −60 mV, the component $I_{i(max)}$ increased 50%, whereas at 0 mV $I_{i(max)}$ decreased 26%.

A second procedure to examine the nature of I_o in *C. inflata* was used. At the time that I_o had increased to its maximum value and with V_m at 0 mV, the current-voltage, I_{max}-V_m, curve was determined by stepping V_m to other levels ranging from −20 mV to −80 mV (Fig. 6A). The reversal *p.d.* of I_{max}

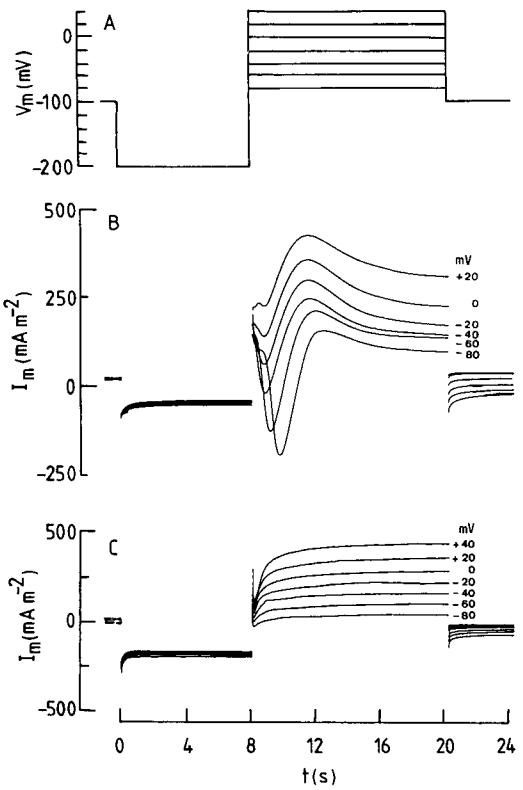

Figure 4. A, Membrane currents during a series of two-pulse voltage-clamp steps. B, The results usually observed. C, Current observed in less than 5% of cells. The cells were bathed in APW with pH 7.5.

is close to the Nernst *p.d.* for K^+ (Fig. 6B), suggesting that this current was carried by K^+.

9-AC, a Cl^- channel blocker (Bryant and Morales-Aguilera, 1971; Tyerman et al., 1986b), when applied to the outside of *C. inflata* at a concentration of 250 μM reduced I_i (Fig. 7). At a clamping potential of −20 mV, the component $I_{i(max)}$ declined from −90 mA m^{-2} in APW to −45 mA m^{-2} in the presence of 200 μM 9-AC. 9-AC also reduced I_i in *C. corallina* but did not eliminate I_i (J.I. Kourie, unpublished data). In both types of cells the time course of I_i had not fully recovered even 60 min after the removal of 9-AC.

The addition of La^{3+} to the external solution also reduced I_i in *C. inflata* (Fig. 8) but did not prevent the cessation of the cytoplasmic streaming. At a clamping potential of −80 mV, the component $I_{i(max)}$ declined from −399 mA m^{-2} in APW to −123 mA m^{-2} in the presence of 200 μM La^{3+}. The blocking effect of La^{3+} was not reversible even 30 min after its removal. Similar results were reported for *C. corallina* by Keifer and Spanswick (1979), Beilby (1984), and Tsutsui et al. (1987). In addition to the reduction in $I_{i(max)}$, La^{3+} also caused a relatively smaller reduction in I_{max} in *C. inflata* (Fig. 9).

Effects of External NaCl and Sorbitol

When the external osmotic pressure is increased by the addition of 300 mM sorbitol to the bathing medium, the resting V_m depolarizes slowly and an action potential is observed only when the cell is plasmolyzed (Kourie et al., 1990). On the other hand, in the presence of NaCl, the V_m depolarizes rapidly and action potentials are observed even at concentrations as low as 25 mM (Kourie et al., 1990). To separate the osmotic and the ionic effects of NaCl and investigate the nature of NaCl-induced depolarization and

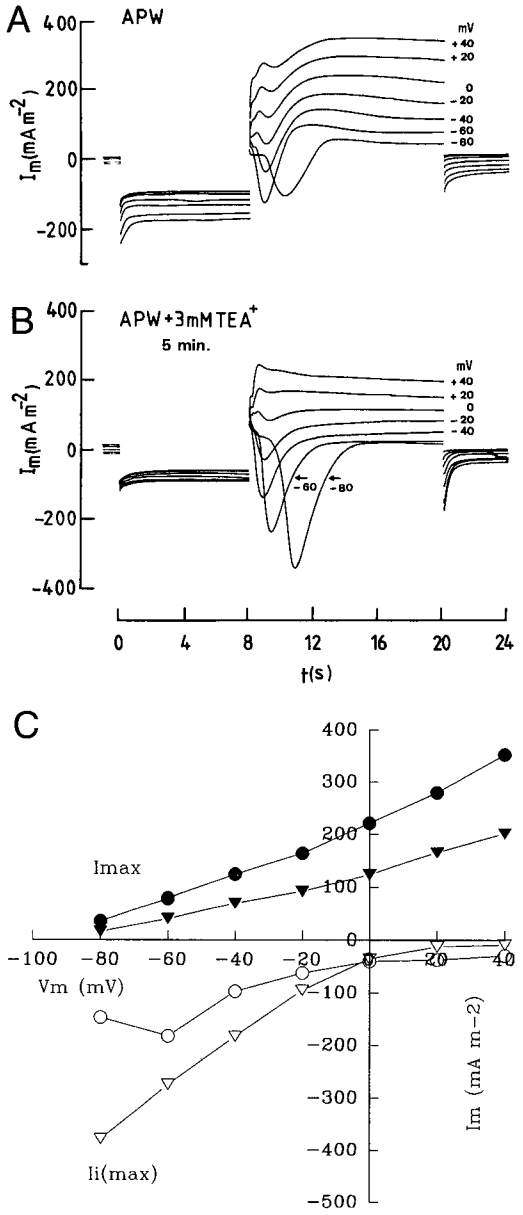

Figure 5. Effect of TEA^+, added externally, on the membrane currents in a voltage-clamped *C. inflata* cell. The V_m is held at −100 mV and then clamped, after the standard negative prepulse of −200 mV, to various potentials shown on the right. A, Membrane currents for a cell in APW. B, Same cell during 5-min exposure to 3 mM TEA^+. C, Current-voltage curves for the components $I_{i(max)}$ (open symbols) and I_{max} (closed symbols) of the clamp current of the same cell shown in A and B, in APW (circles) and for the same cell after 5 min in APW + 3 mM TEA^+ (triangles).

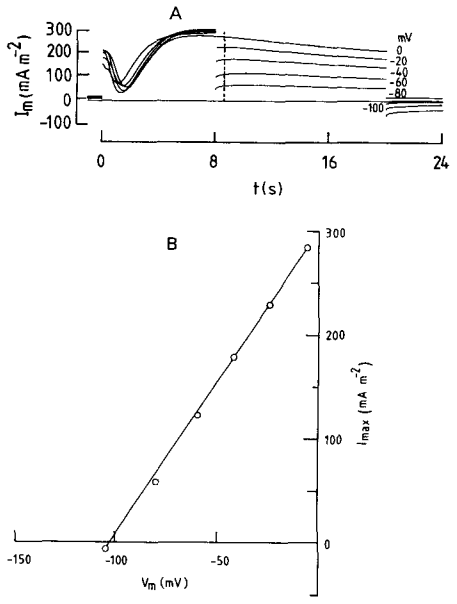

Figure 6. A, Membrane currents during a series of two-step voltage clamps. In each voltage clamp the *p.d.* was stepped initially from a holding *p.d.* of −100 to 0 mV for 8 s, at which point I_o had reached its peak value. The *p.d.* was then stepped back in a sequence incremented by 20 mV. At 20 s after the start of the initial clamp step the *p.d.* was returned to the holding *p.d.* B, The current-voltage curve for I_{max} from A.

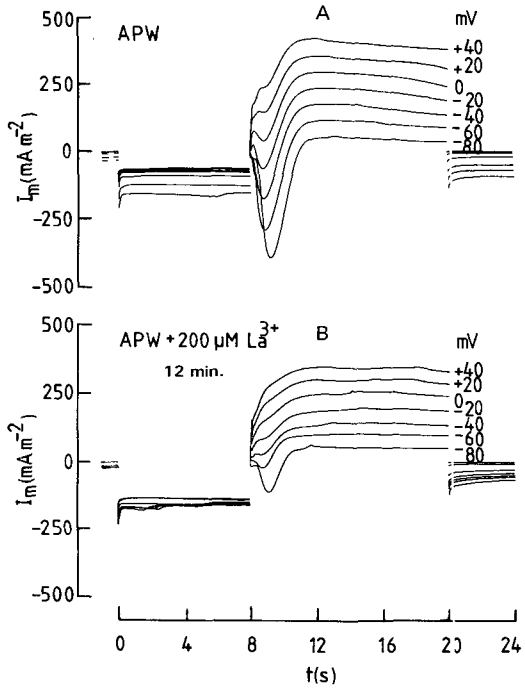

Figure 8. Voltage-clamp currents following step depolarization to various potential levels (shown on the right) from a clamping value of −100 mV for a cell in APW and in the darkness (A) and for the same cell in 200 μm La^{3+} + APW (B).

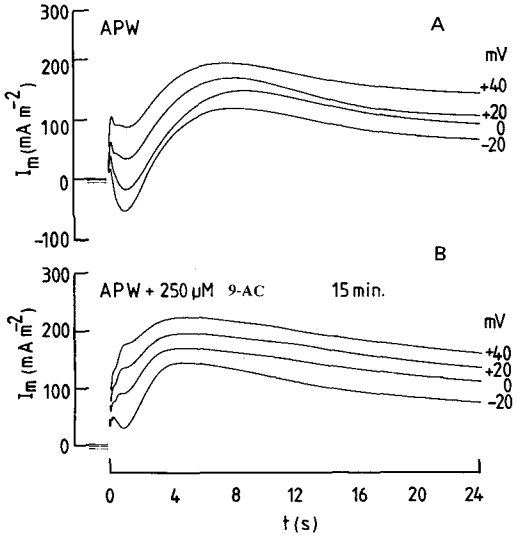

Figure 7. Effect of 9-AC on the membrane currents in a voltage-clamped *C. inflata* cell. The V_m is held at −100 mV and clamped to various potentials shown on the right. A, Membrane currents for a cell in APW. B, Same cell during a 15-min exposure to 250 μM 9-AC.

action potential in the free-running membrane *p.d.*, the effects of NaCl and high concentrations of sorbitol in the external solution on the various clamp current components were examined. This work complements previous studies of NaCl and sorbitol effects on the membrane currents I_K, I_{Cl}, and I_L that flow when the membrane *p.d.* is stepped from the resting level to more negative values (Kourie and Findlay, 1990a, 1990b). Only rather low values of $[NaCl]_o$ were used, because the I_L became large when $[NaCl]_o$ was high. An increase in $[NaCl]_o$ to 10 mM increased the inward-going conductance,

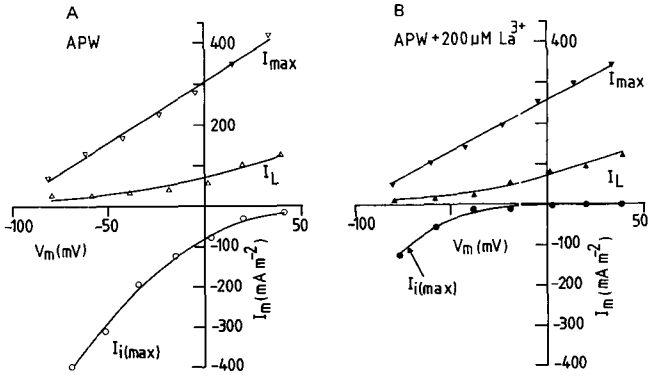

Figure 9. A, Current-voltage curves for the components (see Fig. 3) of the clamp currents of the cell shown in Figure 8. Cell in APW, pH 7.5. B, The effect of the addition of 200 μM La^{3+} to the external APW on the current-voltage curves for the components of the clamp current.

(dI_i/dV_m) in *C. inflata* and in *C. corallina* (J.I. Kourie, unpublished data) and the outward-going conductance in *C. inflata*. In *C. inflata*, at a clamping potential of −80 mV, the component $I_{i(max)}$ increased from −401 mA m^{-2} in APW to −498 mA m^{-2} in the presence of 10 mM NaCl, and the reversal potential of I_{max} changed from about −100 to −75 mV (Fig. 10). LiCl in the external solution at a concentration of 5 mM had similar effects (data not shown).

On the other hand, the addition of 200 mM sorbitol to the external solution had virtually no effect on the clamp current (data not shown).

Effects of Increasing K^+ and Zero External Ca^{2+}

The effects of reducing the external Ca^{2+} on the components of the outward current were examined. The I_i and I_o declined significantly after the cells had been in Ca^{2+}-free APW for 30 min, leaving a steady I_L (Fig. 11). At a clamping potential of −40 mV, the component $I_{i(max)}$ decreased from −43 mA m^{-2} in APW to −7 mA m^{-2} after 12 min of exposure to Ca^{2+}-free APW. In the same cell, the component $I_{o(max)}$ decreased from 37 mA m^{-2} in APW to 23 mA m^{-2}. Figure 11B reveals that after 5 min of cell exposure to Ca^{2+}-free APW $I_{i(max)}$ was reduced to 25% and $I_{o(max)}$ was reduced to 87% of their values in APW. The cytoplasmic streaming did not stop during depolarizing voltage-clamp steps. The effects of increasing the external $[K^+]$ on the components of the outward current were also examined. Increasing the external $[K^+]$ from 0.1 to 0.8 mM reduced, but did not eliminate, I_i or I_{max} (Fig. 12). At a clamping potential of −60 mV, the component $I_{i(max)}$ decreased from −126 mA m^{-2} in APW containing 0.1 mM K^+ to −27 mA m^{-2} after 2 min of exposure to APW containing 0.8 mM K^+. In the same cell and for the same K^+ treatment, the component $I_{o(max)}$ decreased from 28 to 9 mA m^{-2} (Fig. 12B). In addition, these components decreased as the time of $[K^+]_o$ treatments progressed.

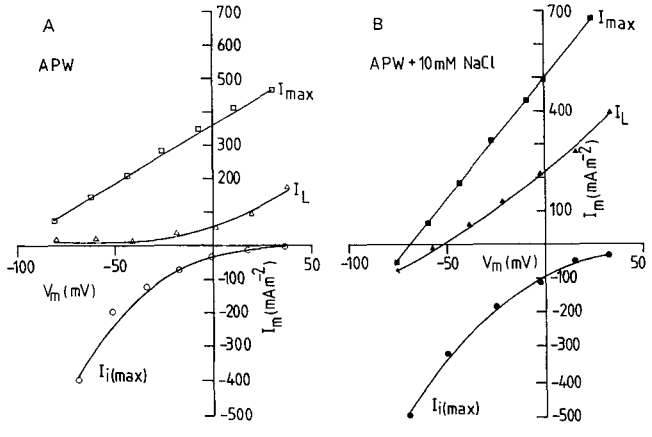

Figure 10. A, Current-voltage curves for the components of the clamp current. B, The effect of the addition of 10 mM NaCl to the external APW on the current-voltage curves for the components of the clamp current.

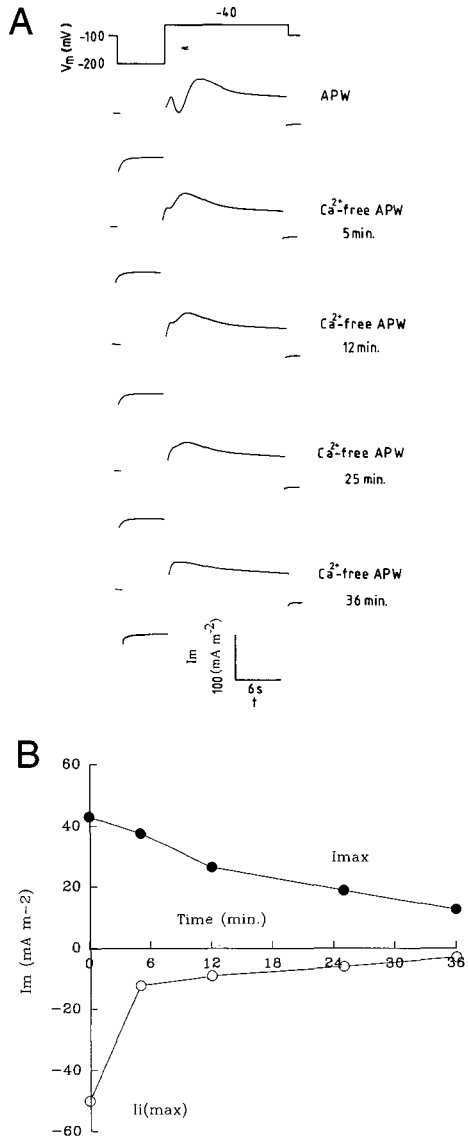

Figure 11. A, Voltage-clamp records illustrating the effect of Ca^{2+}-free APW on clamp currents obtained when the V_m was clamped from a holding potential of −100 mV to a test potential of −40 mV, following a negative prepulse of −200 mV. Both the peak of the I_i and the hump of I_o faded with time. B, The dependence of the current amplitude of the transient inward component $I_{i(max)}$ and the outward current $I_{o(max)}$ on the duration in Ca^{2+}-free APW.

DISCUSSION

Threshold in Membrane *p.d.* for Initiation of the Action Potential

Previous papers (Kourie and Findlay, 1990a, 1990b) in which aspects of the electrophysiology of the membranes of *C. inflata* were described have been concerned mainly with an examination of the current flow when the membrane *p.d.* was clamped from the resting level to more negative values (Coleman and Findlay, 1985; Tyerman et al., 1986a, 1986b). However, as in many other species of charophyte plants, the

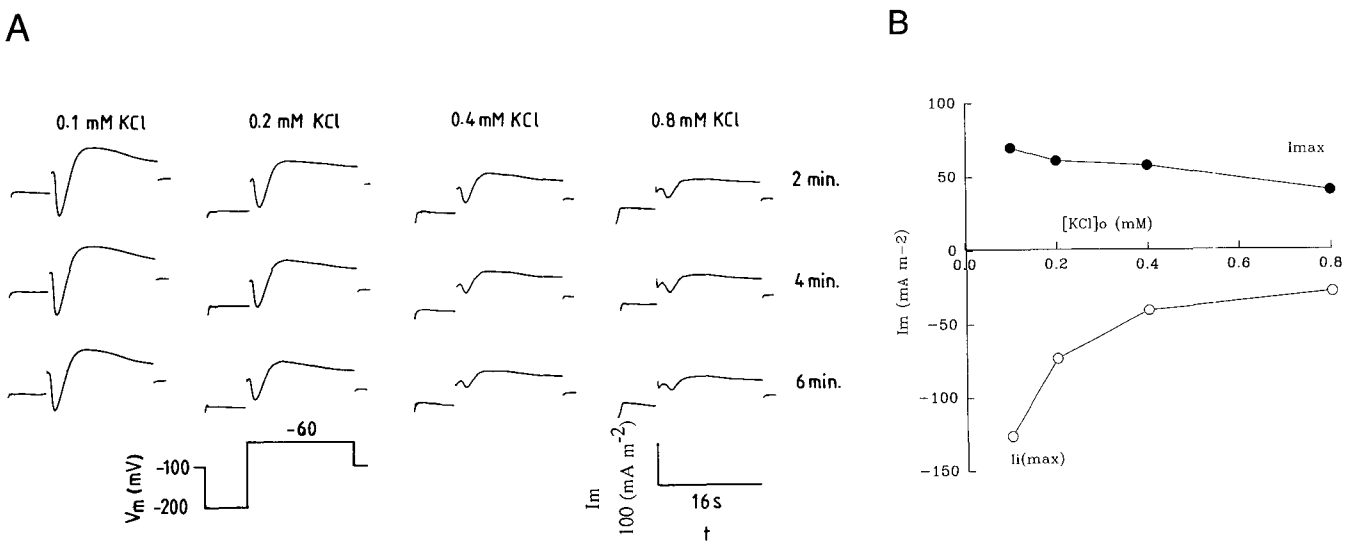

Figure 12. A, Voltage-clamp records illustrating the effect of $[K^+]_o$ and duration of treatment on the components of the clamp currents obtained when the V_m was clamped from a holding potential of −100 mV to a test potential of −60 mV, following a negative prepulse of −200 mV. B, The dependence of the current amplitude of the transient inward component $I_{i(max)}$ and the outward current I_{max} on $[K^+]_o$.

cells of *C. inflata* are excitable, and action potentials can be initiated when the membrane *p.d.* is depolarized beyond a threshold level. Coleman and Findlay (1985) gave a value of −50 mV as the threshold *p.d.* for cells in the light. In this study, the cells were mainly in the dark, and in these circumstances the threshold for initiation of the transient currents associated with the action potential depends on the prior state of the cell, particularly the *p.d.* In fact, it was found that cells in the dark, when the membrane *p.d.* is usually close to the Nernst *p.d.* for K^+, about −100 mV, are often not excitable. They only become so when the *p.d.* is made appreciably more negative (Figs. 1 and 3) and then depolarized beyond −100 mV.

It is thought that *C. inflata*, like other Characean cells, generates action potentials at both the plasmalemma and the tonoplast. The action potential at the plasmalemma precedes the tonoplast action potential (Findlay, 1970; Kikuyama and Tazawa, 1976). It has been suggested that the action potentials at the plasmalemma and the tonoplast are coupled (Findlay, 1970; Kikuyama and Tazawa, 1976). In *Chara*, the increase in the cytoplasmic Ca^{2+} during the action potential at the plasmalemma is the coupling factor that activates Cl^- channels at the tonoplast (Kikuyama, 1986). Thus, the cytoplasmic free Ca^{2+} appears to be more important than the vacuolar Ca^{2+}. The Nernst potential for cytoplasmic Ca^{2+} between 10^{-7} and 10^{-6} M is +58 and +87 mV, respectively. These values support the suggestion that part of I_i could be due to Ca^{2+} influx during the action potential.

Components of the Membrane Current: I_i

The two main components of the membrane current during the action potential were examined in some detail. The I_i is most likely carried by Cl^- as in other Characean species. The evidence for this identification comes from Figures 9 and 10, which show that the reversal *p.d.* for this component must be much more positive than +40 mV. Cl^- is the only ion to have a Nernst *p.d.* in this region, with a vacuolar concentration of about 100 mM (Kourie and Findlay, 1990a) and the external concentration of 1.2 mM, giving a Nernst *p.d.* of +111 mV. Furthermore, I_i is blocked by the external application of Cl^- channel blockers 9-AC and La^{3+}.

The double-pulse technique shows that the peak value of I_i is a function of the holding *p.d.* and the *p.d.* before the depolarizing step. The peak value increases to a maximum at a holding *p.d.* of about −200 mV. There is considerable evidence that the transient nature of I_i results from a mechanism by which the conductance of the plasmalemma to Cl^- is first activated and then deactivated. For the Cl^- mechanism to be fully activated, there is a requirement for the membrane *p.d.* to be at a level substantially more negative than −100 mV, the *p.d.* in the dark, so that Cl^- channels are inactivated, and then for the membrane *p.d.* to be clamped to a level more positive than −100 mV, so that Cl^- channels are now activated. The variability in I_i when the main pulse is not preceded by the negative prepulse probably arises because the mechanism is not activated under this condition. The inward Cl^- current flowing across the membrane during the action potential in *C. inflata* is analogous to the Na^+ inward current in the squid giant axon (Hodgkin and Huxley, 1952).

Tyerman et al. (1986a) have described a Cl^- conductance in the membrane of *C. inflata* that is activated when the membrane *p.d.* is stepped to values more negative than −200 mV. The Cl^- current flowing through this conductance activates rather slowly, over a period of seconds. In cells of *C. inflata* in the light, where the *p.d.* is often as negative as −300 mV, there is good reason to believe that the plasmalemma has a Cl^- conductance of a magnitude comparable to that of the proton extrusion pump.

An interesting question is whether the populations of Cl^-

channels that constitute these two Cl^- conductances are the same or different. At present there is no clear answer, but given the rather different kinetics of the conductances, together with the observation that high concentrations of sorbitol in the external solution affect only the Cl^- conductance activated by negative steps in *p.d.*, it would be more likely that there are two separate populations of Cl^- channels but that both are blockable by 9-AC.

The effect of La^{3+} in reducing I_i is more likely to be attributable to its blocking effect on an inward Ca^{2+} current during the action potential, which in turn activates the Cl^- current, rather than a direct blocking effect on the Cl^-current. The blocking effect on the Cl^- current during negative steps in membrane *p.d.* (Tyerman et al., 1986b) is also likely to be from the same cause, particularly because it has been shown by Kourie and Findlay (1990b) that this Cl^- current is Ca^{2+} dependent and is reduced when the concentration of Ca^{2+} is reduced. La^{3+} presumably displaces Ca^{2+} from sites associated with the activation of the Cl^- channel.

It is possible that the transient nature of the delayed outward current, $I_{o(max)}$, is a manifestation of a transient increase in the free cytoplasmic Ca^{2+}, which activates K^+ channels. Furthermore, the La^{3+}-induced blocking effect on Ca^{2+} influx caused a reduction in I_{max}. However, patch-clamp experiments are needed to verify the presence in *C. inflata* membranes of La^{3+}-sensitive Ca^{2+} channels and Ca^{2+}-activated K^+ channels underlying $I_{o(max)}$.

Components of the Membrane Current: I_o

The I_o is carried predominantly by K^+. The evidence for this is 2-fold. The current-voltage curves for I_{max} have a reversal *p.d.* close to the Nernst *p.d.* for K^+ (Figs. 6, 9, and 10), and I_o is blocked by TEA^+ (Fig. 5). Furthermore, the response of I_o to TEA^+ indicates that I_o may consist of two K^+ components, the transient hump overlying a steady component, with the transient consistently blocked by TEA^+, and the steady component blocked to a lesser extent by TEA^+. This steady component is apparent in the membrane currents of some voltage-clamped *C. inflata* cells (Fig. 4C).

The transient nature of I_o also seems to be associated with the mechanism of I_i activation during the action potential. First, the activation of $I_{i(max)}$ and I_{max} was voltage dependent and it could be described by the Boltzmann equation (Fig. 3, B and C). The values of $V_{½}$ and K for $I_{i(max)}$ and I_{max} are similar, being −150, −5 mV and −162, −7 mV, respectively. Second, the component $I_{o(max)}$ is not seen without the corresponding $I_{i(max)}$. The data in Figure 4, in which I_i is absent, show that I_o, although showing some time dependence, only increases to a steady level during the voltage-clamp steps. The nature of the dependence of I_o on the occurrence of I_i can only be speculated ipon at present, but it is possible that during the action potential in *C. inflata* there is also an influx of Ca^{2+}, as in *C. australis* (Williamson and Ashley, 1982; Kikuyama and Tazawa, 1983; Tsutsui et al., 1987), and that the transient I_o, like I_i, is a function of the concentration of Ca^{2+} in the cytoplasm and follows an increase and decrease of cytoplasmic $[Ca^{2+}]$. Certainly, removal of Ca^{2+} from the external solution prevents the occurrence of the action potential (Fig. 11).

Effects of NaCl, Sorbitol, and Other Ions in the External Solution

When $[NaCl]_o$ is high, it seems that both I_L and I_o may contain a component carried by Na^+, because the reversal *p.d.* for these currents becomes less negative, being between the Nernst *p.d.* for K^+ at about −100 mV and that for Na^+ at about −50 mV. Of course, a component due to Cl^- cannot be ruled out. The simplest explanation for the similarity between the effects of NaCl and LiCl is that both of these ions go through the same channel pathway. If there were a separate pathway for Li^+, and given that the internal concentration of Li^+ is zero, the Nernst *p.d.* for this ion would be very considerably negative, and its effect would be to make the reversal *p.d.* for I_o more negative, rather than less negative, as observed.

The NaCl-induced increase in the transient inward Cl^- current, I_i, and repetitive action potentials (Kourie et al., 1990) could be expected to cause the depletion of Cl^- in the cytoplasm unless new supplies were to arrive. Two possible mechanisms involved in supplying Cl^- are influx of external Cl^- and efflux of vacuolar Cl^-. The passive influx of external Cl^- is uphill, suggesting the mechanism for Cl^- efflux from the vacuole. The most likely pathways for Cl^- efflux from the vacuole during the action potential would be Cl^- channels in the tonoplast. The evidence for the presence of Cl^- channels in the tonoplast is inferred from the findings that the action potential, which is due to increases in Cl^- and K^+ conductances, occurs at both the plasmalemma and the tonoplast (Hope and Findlay, 1964). Recently, patch-clamp experiments have confirmed the presence of Cl^- and K^+ channels in the tonoplast of Characean cells (Tyerman and Findlay, 1989).

The modification of the behavior of K^+ channels by increasing $[K^+]_o$ altered the behavior of the inactivation of I_i. This could be due to an increase in the number of activated K^+ channels as a result of increasing $[K^+]_o$. It was shown that rapid inactivation of K^+ channels, with a −200 mV prepulse (Kourie and Findlay, 1990a, 1990b), aids the activation of the I_i and the delayed outward current (Fig. 1). The blockade of K^+ channels by the external addition of TEA^+, such as a −200 mV prepulse, alters the behavior and amplitude of the inactivation of $I_{i(max)}$ (Fig. 5). This is partially due to the fact that in the presence of TEA^+ the activation of the K^+ current is absent, and hence $I_{o(max)}$ is not overlapping with the activation of the inward component $I_{i(max)}$. Consequently, the amplitude of $I_{i(max)}$ in the presence of TEA^+ is larger than $I_{i(max)}$ in APW (Fig. 5).

CONCLUSIONS

In conclusion, in whole-cell voltage-clamp experiments, a two-pulse procedure induces reproducible action potentials that have distinct behavior. The electric current during these action potentials consists of I_L, flowing from the start of the pulse, followed by I_i, commencing after a delay, and preceding a delayed I_o. The characteristics of the current components and their response to various ion channel blockers and ionic composition of the bathing solutions suggest that: (a) I_i is carried by Cl^- and (b) I_o is carried mainly by K^+. The mag-

nitude and behavior of these K^+ and Cl^- currents could be modified by changes in the external concentration of $CaCl_2$, LiCl, and NaCl but not by sorbitol. The NaCl enhancement of I_i is due to ionic effects of NaCl rather than to its osmotic effects.

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