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# Potassium Channels in *Chara corallina*[1]

## CONTROL AND INTERACTION WITH THE ELECTROGENIC $H^+$ PUMP

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### ABSTRACT

**Plasmalemma electrical properties were used to investigate $K^+$ transport and its control in internodal cells of *Chara corallina* Klein ex Willd., em R.D.W. Cell exposure to solutions containing 10 mM KCl caused the potential, normally −250 millivolts (average), to depolarize in two steps. The first step was a 21 millivolt depolarization that lasted from 1 to 40 minutes. The second step started with an action potential and left the membrane potential at −91 millivolts, with a 10-fold reduction in resistance. We suggest that the second step was caused by the opening of $K^+$-channels in the membrane. This lowered the resistance and provided a current pathway that partially short-circuited the electrogenic pump. Although largely short-circuited, the electrogenic pump was still operating as indicated by: (a) the depolarized potential of −91 millivolts was more negative than $E_K$ (= −42 millivolts in 10 mM $K^+$); (b) a large net $K^+$ uptake occurred while the cell was depolarized; (c) both the electrogenic pump inhibitor, diethylstilbestrol, and the sulfhydryl-reagent *N*-ethylmaleimide (which increased the passive membrane permeability) further depolarized the potential in 10 mM KCl.**

**A two-phase recovery back to normal cell potentials occurred upon lowering the $K^+$ concentration from 10 to 0.2 mM. The first phase was an apparent Nernst potential response to the change in external $K^+$ concentration. The second phase was a sudden hyperpolarization accompanied by a large increase in membrane resistance. We attribute the second phase to the closing of $K^+$-channels and the removal of the associated short-circuiting effect on the electrogenic pump, thereby allowing the membrane to hyperpolarize. Further experiments indicated that the $K^+$-channel required $Ca^{2+}$ for normal closure, but other ions could substitute, including: $Na^+$, tetraethylammonium, and 2,4,6-triaminopyrimidine. Apparently, $K^+$-channel conductance is determined by competition between $Ca^{2+}$ and $K^+$ for a control (gating ?) binding site.**

Transport processes through the plasmalemma play a key role in the functioning of the cell (1). In the characean cells, these transport processes and their interactions have been extensively studied. The membrane potential of both *Chara* and *Nitella* cells is much more negative than any possible diffusion potential and is probably due to the operation of an ATP-dependent electrogenic $H^+$ efflux system (8, 18, 19). In these plant cells, $K^+$ has the highest passive ionic permeability across the plasmalemma (2); its partial conductance being 0.15 Semen-$m^{-2}$ compared to 0.01 and 0.02 Semen-$m^{-2}$ for $Na^+$ and $Cl^-$, respectively (7). Thus, in the observed hyperpolarized state, there would be net passive movement of $K^+$ into these cells, which would tend to depolarize the potential. If this passive $K^+$ influx were not controlled, not only would the system lose potential energy that would otherwise be available to drive, for example, $Cl^-$ influx (17) but, in addition, cytoplasmic $K^+$ levels may increase beyond physiological limits.

Previous work has shown that the *Chara* cell can exist in two different potential states; termed polarized and depolarized states by Oda (15). A transition between these two states can be caused by changing the external $K^+$ concentration, or by passing current through the membrane (15, 18). The membrane potential in the polarized state was relatively insensitive to changes in $K^+$ concentration, while in the depolarized state it changed by 42 mv for a 10-fold $K^+$ concentration change (15).

The $K^+$ permeability in *Chara* is influenced by the exogenous $Ca^{2+}$/monovalent cation ratio. In the presence of $Ca^{2+}$, the characean membrane electrical properties are essentially independent of $K^+$ concentration, but in the absence of $Ca^{2+}$ the membrane behaves like a $K^+$ electrode (6, 10, 20). If there is a $Ca^{2+}$ binding site for this effect, it is possible that other ions may also bind to it. Recent work suggests that $Mg^{2+}$, $Sr^{2+}$, or $Mn^{2+}$ can substitute for $Ca^{2+}$ under some circumstances (13, 14). It is also possible that other cations may bind to this site, but without having the $Ca^{2+}$ effect. $K^+$ may compete for $Ca^{2+}$ binding sites in nerves (21, 24), and a similar possibility has been suggested for *Chara* (14).

In the present study, we further examined some of the interactions of various ions with the $K^+$-channels in order to advance our understanding of how $K^+$ permeability is controlled. The interaction between the $K^+$-channels and the electrogenic pump was also investigated. The evidence presented is consistent with the hypothesis that in the $K^+$-induced depolarized state, the electrogenic $H^+$ pump remains operational. However, the influence of the $H^+$ pump is masked by the short-circuiting effect of the open $K^+$-channels.

### MATERIALS AND METHODS

**Culture Material.** *Chara corallina* Klein ex Willd., em R.D.W. (= *Chara australis* R.Br.) was grown in a plastic container with 8 to 10 cm of lake mud (Lone Star Lake, Kansas) and 120 liters of solution which initially contained (mM): 3 $Na^+$, 0.2 $K^+$, 0.2 $Ca^{2+}$, 0.2 $SO_4^{2-}$, 1.2 $Cl^-$, and 2 $HCO_3^-$. Fluorescent lights provided 16 h of illumination at an intensity of 17 to 20 w $m^{-2}$ at the solution surface. Internodal cells (about 2–3 cm for electrophysiology and 3–5 cm for $K^+$ determinations) were cut the day before the experiments and incubated overnight in a solution containing (mM): 1.0 NaCl, 0.2 KCl, 0.2 $CaSO_4$, and 1.0 $NaHCO_3$.

**Experimental Solutions.** The basic experimental solution, CPW7[2], was prepared using high purity glass-distilled $H_2O$ and contained (mM): 1.0 NaCl, 0.2 KCl, 0.2 $CaSO_4$, 1.0 Hepes, and

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[2] Abbreviations: CPW, *Chara* pond water; TEA, tetraethylammonium; NEM, *N*-ethylmaleimide; DES, diethylstilbestrol; $E_K$, Nernst potential for potassium; DCCD, dicyclohexylcarbodiimide; AP, 4-aminopyridine; TAP, 2,4,6-triaminopyrimidine; MA, methylamine.

was adjusted to pH 7 using freshly dissolved NaOH. Ca-free CPW7 was as above but without $CaSO_4$. All modifications to this medium are detailed in the text. TEA (Matheson, Coleman, and Bell) was purified by recrystallization from chloroform and vacuum dried in the presence of a liquid $N_2$ cold finger, or used as received (Aldrich). Inorganic chemicals were from Mallinckrodt; all other chemicals were from Sigma. NEM was prepared as a 100 mM stock solution (pH 3.3), stored at 4°C for, at most, 36 h, and diluted into the experimental solution just before use. The DES (40 mM) was dissolved in absolute ethanol and then diluted just before use to 40 μM in the experimental solution. Solutions were flowed through the chamber at a rate of 15 to 25 ml/min.

**Measurements of Membrane Potential and Resistance.** The basic details of the electrical measurement methods have been published previously (8). For these experiments, WPI model 750 amplifiers with a differential input (WP Instruments, New Haven, CT) were used. Electrodes were pulled from glass fiber-filled capillary tubing (WPI or Frederick Haer) on a Kopf puller (David Kopf Instruments, Tujunga, CA) to a taper length of 0.5 to 0.8 cm (2–5 Mohm). Contact with the 3M KCl (pH 2) filling solution was made using holders containing AgCl plugs (WPI). Cell resistances were determined by the method of Hogg *et al.* (5) using a Pulsar 4i pulse generator (Frederick Haer and Co., Brunswick, ME) connected to the current electrode through a $10^8$ ohm resistor (0.4 μamp current injected into the cells).

Light was from a Leitz Pradovit projector (quartz iodide lamp; 150 w, 12 v), with the beam attenuated to 20 w $m^{-2}$ using a neutral density filter and 10 cm of water.

**Potassium Concentration.** Potassium concentration in the vacuole was measured by atomic emission spectrophotometry using a Varian model 1200 Atomic Absorption Spectrophotometer. Vacuolar sap (10 μl) was extracted using the technique of Fujii *et al.* (3) and diluted with 10 ml of $H_2O$. $K^+$ standards contained only KCl.

## RESULTS

**Internal Potassium Concentrations.** Table I presents the measured $K^+$ concentrations of extracted vacuolar sap for three different pretreatment conditions. These values, along with other known parameters, can be substituted into the Goldman equation to obtain a value for the diffusion potential. However, in *Chara* the dominant term in this equation is that of $K^+$, resulting in the value of the Goldman equation being only slightly more positive than the value of $E_K$. Thus, $E_K$ provides a negative limit on the diffusion potential and this has been tabulated in Table I for two different external $K^+$ concentrations.

**Membrane Potential and Resistance during and following Exposure to 10 mM KCl.** *Chara* cells in CPW7 ($K^+$ = 0.2 mM) had membrane potentials (mean, −250 mv; range, −220 to −290 mv), that were only slightly altered by changes in external $K^+$. On exposure to 10 mM $K^+$, the potential initially depolarized by 10 to 30 mv (Fig. 1) and then, following an action potential, the potential restabilized at −91 mv (range, −60 to −118 mv). This transition to the depolarized state occurred 1 to 40 min (mean, 16 ± 3 [$n$ = 19] min) after exposure to 10 mM KCl. After this transition, the membrane resistance, originally 10 to 20 kohm-$cm^2$, was 1/10 to 1/20 of the original value, and the potential responded to changes in external $K^+$ as if the membrane were a $K^+$ electrode (99% of theoretical response). The potential stayed in this depolarized state as long as the cell was in the 10 mM KCl solution. When the cell was transferred back into control solution (0.2 mM $K^+$), the membrane potential hyperpolarized (Fig. 1). This recovery exhibited two phases, with a definable break point (Figs. 1 and 2).

Table I. *Vacuolar $K^+$ Level and Nernst Potential for Potassium, $E_K$, in Cells of Chara corallina after Various Pretreatments*

| Pretreatment | Vacuolar Potassium | $E_K$[a] | |
|---|---|---|---|
| | | 0.2 mM External $K^+$ | 10 mM External $K^+$ |
| | *mM* | *mv* | |
| Freshly cut cells | 49 ± 3 ($n$ = 16) | −141 | −41 |
| 16–18 h in CPW + 1.0 mM $NaHCO_3$ | 51 ± 2 ($n$ = 29) | −143 | −42 |
| 16–18 h in CWP7 + 10 mM KCl | 77 ± 3 ($n$ = 18) | −153 | −53 |

[a] $E_K$ was calculated for two different external potassium concentrations.

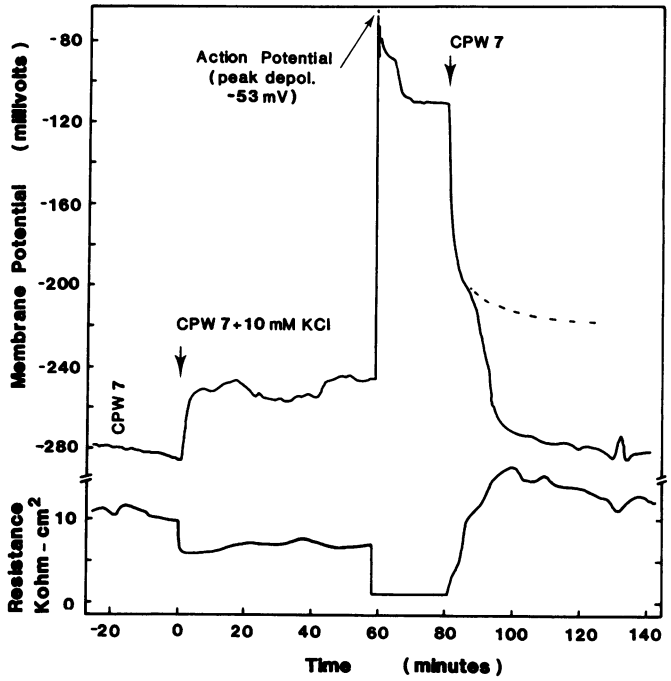


FIG. 1. Membrane potential and resistance changes accompanying exposure of the cell to CPW7 + 10 mM KCl and the later recovery in CPW7. Resistance values were measured once per min; the line on this graph is a fit through these individual points (Fig. 2 has the individual points represented). On return to CPW7, the potential recovered in two phases, the first phase was extrapolated with a dashed line.

In the first phase, which began with the solution change, the potential rapidly approached the break point (−168 ± 3 [$n$ = 22] mv); only toward the end of this first phase did the resistance begin to increase. The first phase was extrapolated to find its asymptote, a value of −191 ± 3 ($n$ = 22) mv was obtained. The change in potential from the 10 mM KCl solution, $E_m$ = −91 ± 3 ($n$ = 22), to the asymptotic value was 100 mv, the same difference as between the $E_K$ values in these two solutions (Table I, line 2). However, both the potential in 10 mM KCl and the asymptotic value were 48 to 49 mv more negative than $E_K$, in the respective solutions.

The second phase of recovery involved a further hyperpolarization of the membrane that started very rapidly and was simultaneous with a sudden increase in membrane resistance (Fig. 2). The resistance increase seen towards the end of the first phase may indicate the imminent onset of this second phase. During the second phase, the membrane hyperpolarized to a value similar to, but often slightly more negative than, the initial control potential. Both the response of the cell to changes in the external $K^+$ while in the state induced by 10 mM KCl, and the first phase of the recovery, indicate that the $K^+$ permeability of the membrane is much higher in the depolarized state than in the control.

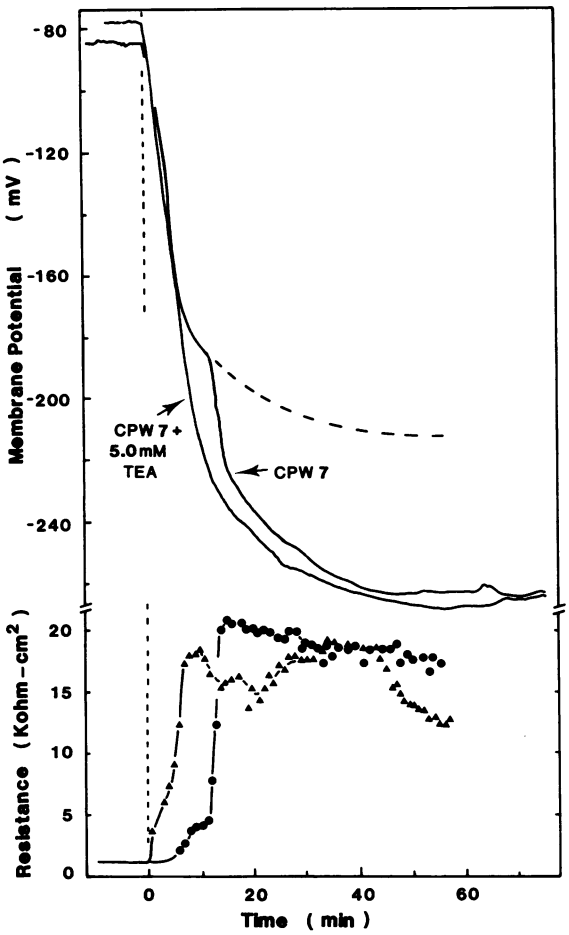


FIG. 2. Recovery time course of potential and resistance following exposure to 10 mM KCl. Prior to the time shown here, cells had been depolarized by exposure to CPW7 + 10 mM KCl (see Fig. 1). At time zero, the solution was changed to either CPW7 or to CPW7 + 5.0 mM TEA. The resistance values were measured once per min in CPW7 (●) or CPW7 + 5.0 mM TEA (▲).

The resistance increase on recovery from the 10 mM KCl solutions occurred with a shorter lag time if 5 mM TEA was present in the solution (Fig. 2). This caused the two phases of recovery to merge into one.

**Experiments under Ca-free Conditions.** Hope and Walker (6) pretreated their *Chara* cells for 2 to 6 h in 5 mM NaCl before inserting microelectrodes, and a similar treatment was used by Spanswick *et al.* (20) on *Nitella*, to produce a membrane that responded to $K^+$ as if the membrane were a $K^+$ electrode. In some experiments, cells were exposed to Ca-free CPW7 + 5 mM NaCl (high NaCl solution) for 6 h following electrode insertion, and the time course of the membrane potential was followed (Fig. 3A). (Increasing the $Na^+$ level enhanced the removal of bound $Ca^{2+}$ from the cell wall and plasmalemma.)

During exposure to high NaCl solutions, the cells did not make a transition, either suddenly or gradually, from the normal hyperpolarized to the depolarized potassium-sensitive state. Upon initial exposure to the high NaCl solution, there was a depolarization of about 20 mv, followed, during the next few h, by a gradual hyperpolarization of about the same magnitude (Fig. 3A). Following an action potential, induced by passing current through the membrane, the potential quickly recovered back to its original, or even more hyperpolarized value when the solution contained 6 mM NaCl (Fig. 3A). Upon changing the solution to Ca-free CPW7, a 20 mv hyperpolarization was seen as the NaCl concentration decreased; the reversal of the depolarization observed on exposure to the high NaCl concentration. The potential would usually stay at this hyperpolarized value (about −280 mv); but, in seven cases (out of 20 experiments), a spontaneous action potential was observed after the cell had been in Ca-free CPW7 for 30 min or more. After an action potential, whether spontaneous or induced by current injection, the potential did not recover back to the extremely hyperpolarized state (−280 mv), but instead, recovered only to −190 to −200 mv. In this new state, the membrane initially exhibited (with one exception out of 20 experiments) a series of spontaneous transient depolarizations to −130 to −150 mv, with a rapid recovery back to −190 to −200 mv. The frequency of these transient depolarizations slowly decreased and finally they ceased completely, leaving the potential steady at −190 to −200 mv.

A transition from the potential level of −190 to −200 back to the extremely hyperpolarized state (−250 mv) occurred when various ions were added to the bathing solution (Fig. 3B): $Na^+$ (5.0 mM addition), $Ca^{2+}$ (0.2–5.0 mM), and TEA (5 mM). Following recovery in either $Na^+$ or TEA, changing back and forth between these two solutions (Fig. 3B) had little effect on the value of the membrane potential (≤5 mv). After any of these treatments ($Na^+$, TEA, or $Ca^{2+}$), switching to Ca-free CPW7 caused the membrane to hyperpolarize a further 20 mv where it remained. In this latter state, an induced action potential given within 2 to 3 h of the previous depolarization in Ca-free CPW7 usually did not cause a transition to the −190 to −200 mv state (data not shown). However, if the cell was left in Ca-free CPW7 until the next morning (8–9 h), then the cell readily made this transition.

When cells were in the hyperpolarized state (−250 mv) in Ca-free CPW7, irrespective of whether or not they had previously made the transition to −200 mv, exposure to Ca-free CPW7 + 10 mM KCl had the same results as found for exposure of normal cells to CPW7 + 10 mM KCl: a depolarization of 20 to 30 mv, followed, after a variable lag time (but shorter than that observed in $Ca^{2+}$-containing solutions), by an action potential that marked the transition to a depolarized state of −90 to −110 mv. Now, in the absence of $Ca^{2+}$, on return to normal levels of $K^+$ (0.2 mM), there was only a one phase recovery to approximately −180 mv (Fig. 4) with only a small increase in resistance. The potential could by hyperpolarized further (−250 mv) by exposing the cell to 0.2 mM $CaCl_2$ or 5 mM TEA.

**Effects of NEM and DES.** When cells in CPW7 + 10 mM KCl and with $E_m$ of −91 mv (Fig. 1) were exposed to one to four 30-s pulses of CPW7 + 10 mM KCl + 0.1 mM NEM solution, the potential depolarized by 15 to 30 mv, leaving the cells with a potential of −65 to −80 mv, a value much closer to the calculated diffusion potential of −42 mv, (Table I) than before (Fig. 5A, inset). Exposure of other cells to CPW7 + 10 mM KCl + 40 μM DES caused the potential to depolarize by 10 to 30 mv, often with a transient hyperpolarization following the initial depolarization (Fig. 5A). This treatment significantly increased the resistance over that observed in CPW7 + 10 mM KCl. When 10 mM KCl + DES-inhibited cells were exposed to CPW7 + 40 μM DES (reduction in $K^+$ to 0.2 mM), the potential hyperpolarized to about −160 mv and the resistance increased dramatically.

Although the application of DES depolarized the membrane, it was still more negative than $E_K$. Since it was thought that the high KCl concentration could have been interfering with the operation of DES, three alternate experiments were performed. (a) The $K^+$ concentration was lowered from 10 to 2 mM before DES was applied. This did not increase the DES inhibition. (b) Another inhibitor, DCCD, was used. DCCD (50 μM) depolarized the membrane (20 to 40 mv) somewhat more than did DES. (c) DES was applied first, then cells were exposed to 10 mM KCl (Fig. 5B). This caused a depolarization to about −60 mv, less negative than when DES was applied last, and often the depolarization was in two steps as seen in Figure 5B. This last combination came the

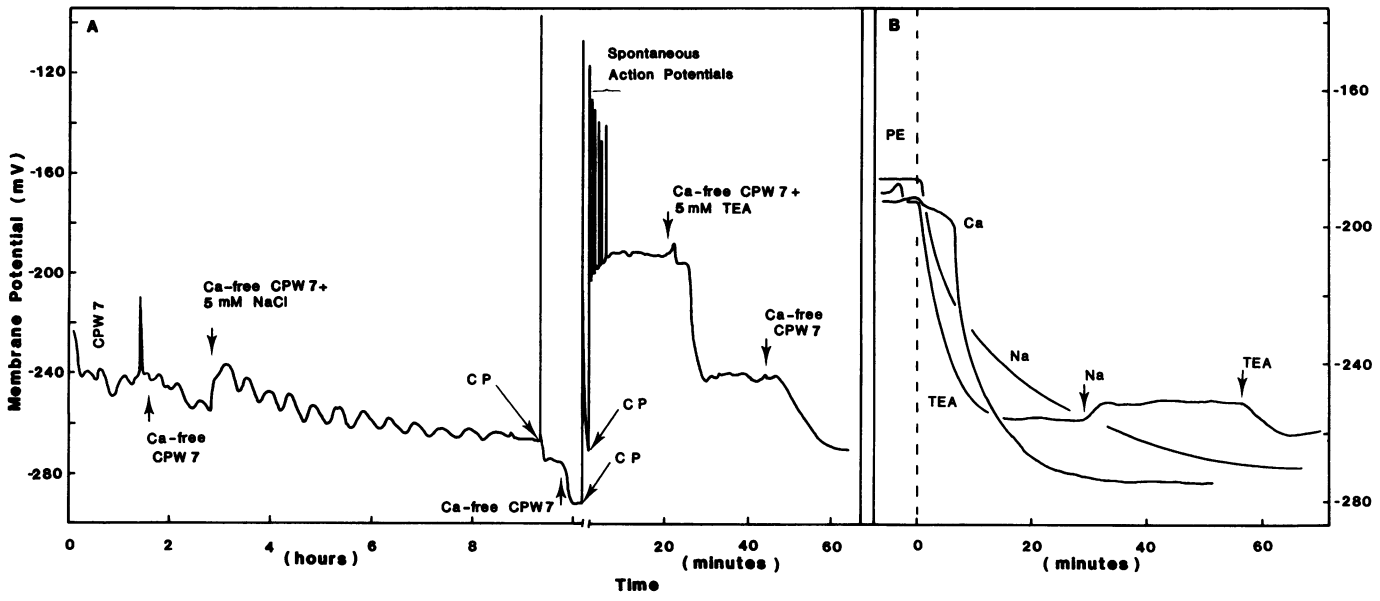


FIG. 3. Time course of the membrane potential in a Ca-free experiment. A, Cell pretreated by a long exposure to Ca-free conditions to remove $Ca^{2+}$ from the wall; to speed this process, the ionic strength of the solution was increased with NaCl (Ca-free CPW7 + 5 mM NaCl). On a few occasions (as shown here) the potential exhibited a slow oscillation. In both the high NaCl solution and in the Ca-free CPW7, current was passed through the membrane (CP) to stimulate an action potential. In Ca-free CPW7, this stimulated action potential (CP) caused the transition to the depolarized state during which a number of experiments were done, one of which appears in A, and three others in B.

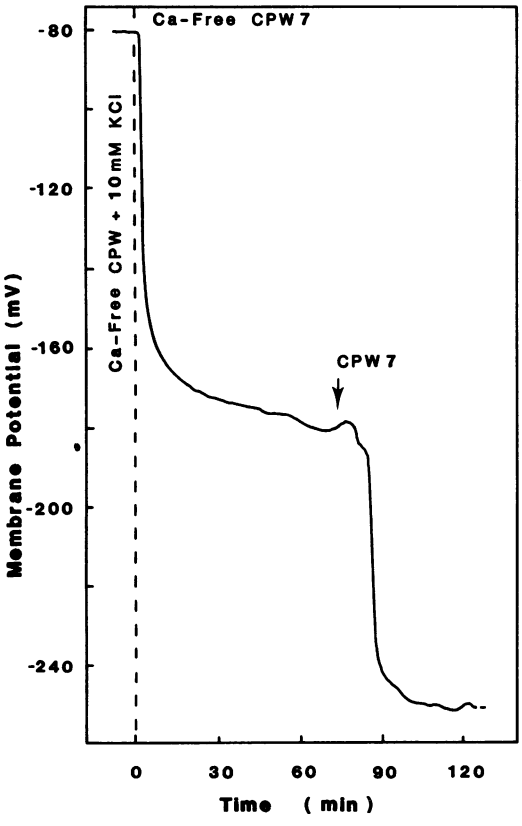


FIG. 4. Membrane potential recovery from a depolarized state induced by Ca-free + 10 mM KCl conditions. Cell pretreatment was similar to Figure 3; following long exposure to Ca-free conditions, the cell was depolarized with Ca-free CPW7 + 10 mM KCl. Reducing the $K^+$ concentration from 10 to 0.2 mM (Ca-free CPW) resulted in a Nernst response of 100 mv. Addition of $Ca^{2+}$ (CPW7) allowed the re-establishment of a control potential.

closest to depolarizing the cell to $E_K$ (= −42 mv).

Inhibitor experiments were also performed on cells that had been depolarized to −180 to −200 mv by an induced action potential following several h in Ca-free solutions (Fig. 3A). For these cells, one 30-s exposure to 0.1 mM NEM caused the potential to begin an immediate depolarization; in about 30 min, a stable value of about −140 mv was reached (Fig. 5C). After this NEM exposure, increasing NaCl by 5 mM, depolarized the membrane reversibly by about 38 mv (compared to the maximal Nernst response of 46 mv). (In this state, a 10-fold increase in $K^+$ concentration depolarized the membrane by 28–30 mv.) During these treatments, NEM decreased the resistance by a factor of 2 to 3. Both the high $Na^+$ and $K^+$ treatments also decreased the resistance.

A DES treatment, to cells in Ca-free CPW7 following an action potential (Fig. 3A), initially depolarized the membrane by 20 to 40 mv (Fig. 5D), and at times was followed by a transient hyperpolarization (Fig. 5D).

**Reputed $K^+$-Channel Blocking Agents.** Compounds known to block $K^+$-channels in animal tissue were also used: TEA (22), previously discussed, AP (23), and TAP (16). When cells were exposed to 5 mM AP, the membrane depolarized in about 10 min from its normal state (−250 mv) to −150 mv (Fig. 6). This AP-induced depolarization was accompanied by a 5-fold decrease in membrane resistance. Lower concentrations of 0.1 to 0.5 mM AP caused a hyperpolarization of the membrane, the size of which was a function of the AP concentration; in 0.5 mM AP, this hyperpolarization was about 20 mv (Fig. 6). At higher AP concentrations (1.0–1.5 mM), a threshold was reached where, after an initial hyperpolarization, the membrane rapidly depolarized. The rate of this depolarization was a function of the AP concentration, but the final potential in 0.2 mM KCl was −140 to −150 mv, irrespective of the AP concentration, as long as it was greater than the threshold. In this state, with 1.0 mM AP, the potential was sensitive to $K^+$ concentration; increasing $K^+$ concentration to 2.0 mM, a 10-fold increase, depolarized the cell by 40 to 45 mv (data not shown), compared to the theoretical Nernst response of 59 mv. However, a 10-fold reduction of the $K^+$ concentration, to 0.02 mM, hyperpolarized the membrane by only 7 to 10 mv. The resistance

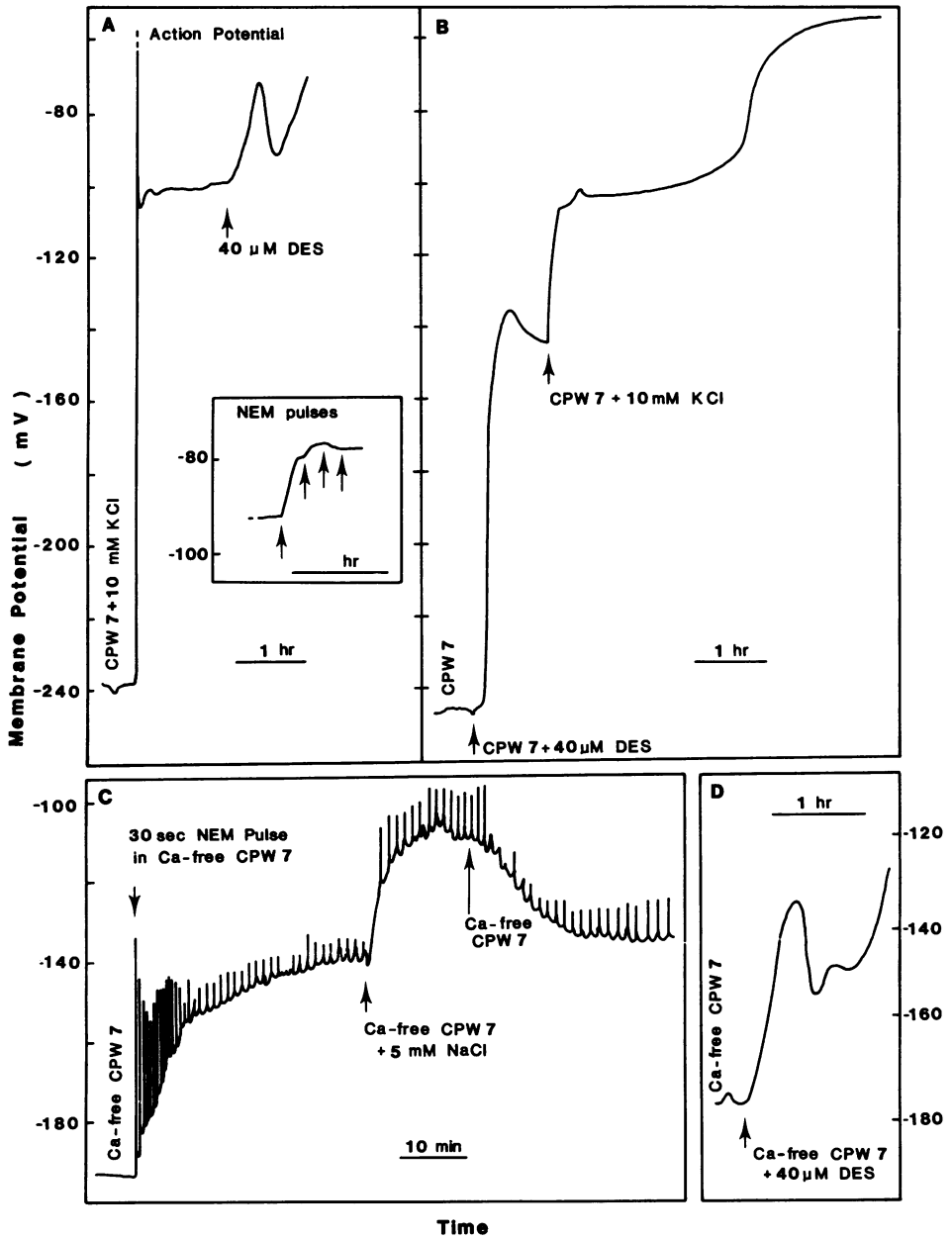


FIG. 5. Effect of NEM and DES on the cell potential. After the cell had been depolarized by exposure to CPW7 + 10 mM KCl, the potential could be further depolarized by treatment with CPW7 + 10 mM KCl + 40 μM DES or with several 30-s exposures to CPW7 + 10 mM KCl + 0.1 mM NEM (at arrows in insert) (A). We reversed the order of treatment, exposing the cell to CPW7 + 40 M DES to inhibit the electrogenic pump(s) before changing to CPW7 + 10 mM KCl (B). Following depolarization with a stimulated action potential in Ca-free CPW7 (see Fig. 3), the potential was further depolarized by a 30-s exposure to Ca-free CPW7 + 0.1 mM NEM (C) or Ca-free CPW7 + 40 μM DES (D). NEM treatment in Ca-free media leaves the cell very sensitive to changes in $Na^+$ concentration (C).

observed in these AP solutions was larger the lower the KCl concentration.

When cells were recovering from 10 mM $K^+$ exposure (Fig. 1), the presence of 1.0 mM TAP caused the recovery to occur in one phase, as did 5.0 mM TEA. The initial rates of hyperpolarization were similar to those obtained with TEA. When applied to an undisturbed cell, TAP caused a slight hyperpolarization (20 mv with 0.5 mM TAP) just as AP did at low concentrations. Unlike AP, even at 1.0 mM, TAP never caused the cell to depolarize.

TAP had no observable effect on the potential or resistance when the cell was in a depolarized state in CPW7 + 5 mM KCl (following a depolarization in a prior exposure to 10 mM KCl). This contrasts with 5.0 mM TEA, which could restore cells to their hyperpolarized state about 50% of the time when they were in CPW7 + 5.0 mM KCl.

Exposure of the cell to a solution containing 5 mM TEA + 1.0 mM AP, after an exposure to 1.0 mM AP, caused a depolarization of about 40 mv. In this new state, with both TEA and AP present in the solution, the potential was relatively insensitive to changes in $K^+$ concentration; a change from 0.2 to 1.0 mM KCl caused a potential change of 7 to 10 mv.

**Methylamine.** At pH 7, MA is a cation which could also interact with $K^+$-channels. Cell exposure to 0.05 mM MA caused a rapid depolarization of 20 to 25 mv. At higher concentrations, 0.2 mM MA, the depolarization was 60 to 80 mv (Fig. 7A). This exposure reduced the resistance to one-half to one-third of the control,

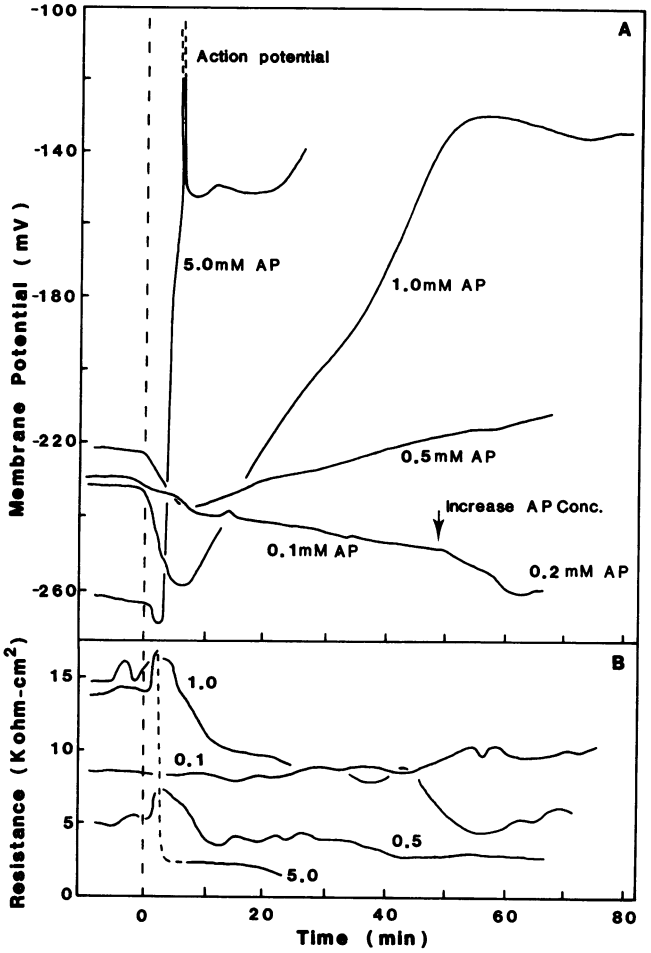


FIG. 6. Membrane potential (A) and resistance (B) changes caused by exposure to different concentrations of AP at time zero.

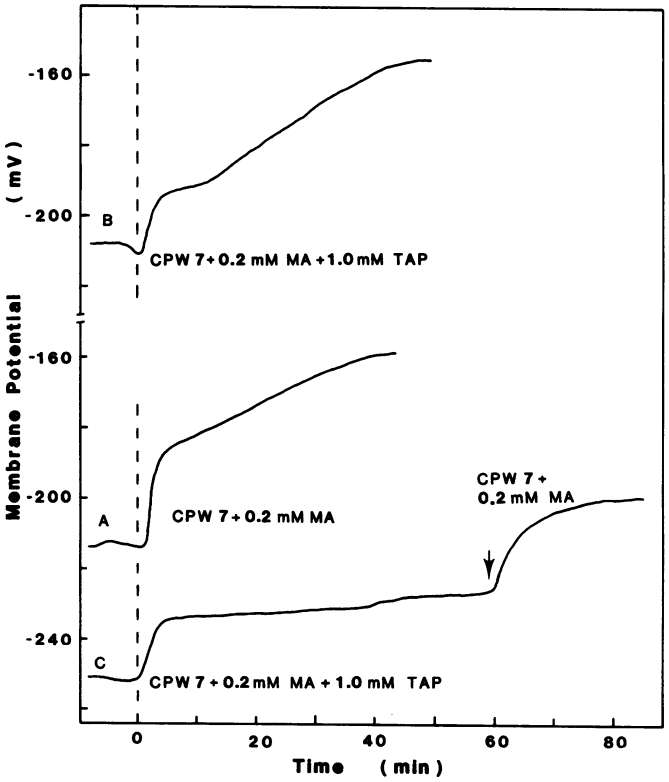


FIG. 7. Effect of MA and TAP on the membrane potential. A, Effect of MA on control (CPW7) cells. B, Cell pretreated with CPW7 + 1.0 mM TAP prior to MA treatment. C, Cell given a complex pretreatment (preexposed to CPW7 + 10 mM KCl to open $K^+$-channels, and then introduction of CPW7 + 10 mM KCl + 1.0 mM TAP, followed by recovery from high KCl, in CPW7 + 1.0 mM TAP) before introducing MA. TAP removal caused a further depolarization such that the total depolarization was similar to the control (A).

consistent with charge movement through the membrane.

If the cell was exposed to TAP (1.0 mM) prior to the MA (0.2 mM) exposure, this depolarization (Fig. 7B) was reduced about 20% compared to the control (Fig. 7A). However, if the prior exposure to TAP began while cells were depolarized in 10 mM KCl, then the depolarization on exposure to MA (Fig. 7C) was reduced to about 55% of the control depolarization.

**Action Potential Initiation.** Exposure of *Chara* to 10 mM $K^+$ caused an action potential to occur, with a lag time of typically 10 to 20 min. At lower $K^+$ concentrations the lag time was longer, until at a threshold concentration of 2 to 3 mM $K^+$, no action potential occurred (12). When this experiment was repeated in the presence of 0.2 mM MA, 2.0 mM $K^+$ routinely initiated action potentials with a lag time of 5 to 30 min. In the presence of 0.2 mM MA, changing from 0.02 to 0.2 mM KCl caused, at most, a 5 to 6 mv change in potential, about one-tenth of the theoretical Nernst response.

## DISCUSSION

**Effect of, and Recovery from, 10 mM KCl Treatment.** Two phases of membrane potential change were observed, both when the cell depolarized on exposure to 10 mM KCl and later when it repolarized after the exposure was terminated. The transition between these two phases was correlated with a large change in membrane resistance and in the sensitivity of the membrane to changes in external $K^+$ concentrations. It is believed that these phenomena indicate the operation of a gated $K^+$-channel across the plasmalemma. On exposure to 10 mM KCl, the membrane initially depolarized slightly, then after a variable lag time, the resistance decreased as these $K^+$-channels opened, providing a large conductance through the membrane. This $K^+$ conductance depolarized the membrane potential by short-circuiting the electrogenic pump in the plasmalemma (8).

The first phase of recovery from the 10 mM KCl exposure is assumed to be the voltage change expected if the membrane were responding as a $K^+$ electrode to the reduction in external $K^+$ from 10 mM ($E_K = -42$ mv; Table I, line 2) to 0.2 mM ($E_K = -143$ mv). The difference between these two calculated Nernst potentials is 101 mv, which compares closely with the potential change of 100 mv measured when the first phase of recovery was extrapolated (Figs. 1 and 2). This first phase of recovery is initially accompanied by only a small resistance change which can be attritubed to the reduction of available charge carriers when the $K^+$ concentration is lowered.

The increase in membrane resistance that occurred at the transition from the first to the second phase of recovery could be accounted for by a closure of $K^+$-channels. This explanation would be consistent with the observed decrease in $K^+$ permeability following recovery from a high $K^+$ treatment. The sudden hyperpolarization that accompanied the resistance increase could also be explained in terms of $K^+$-channel closure. Open $K^+$-channels would short-circuit the electrogenic pump, thereby reducing the maximum observable electrogenic component of the membrane potential. As these $K^+$-channels close (and the resistance increases), this short-circuit would be eliminated and the electrogenic component of the potential would become more apparent, thus the sudden hyperpolarization.

The two phases of potential recovery from 10 mM KCl exposure were completely separated in the $Ca^{2+}$-free experiments (Fig. 4). In the first phase, the membrane is still responsive to $K^+$, due to open $K^+$-channels, and, hence, the potential change is that due to readjustment in the diffusion potential. When the $K^+$-channels were closed by introduction of $Ca^{2+}$ (0.2 mM), the membrane became relatively insensitive to $K^+$; a state reflected by an increase in membrane resistance. Under these conditions, the second phase of membrane potential recovery, back to the control state, was observed (Fig. 4).

**Electrogenic Potentials.** The potential of −91 mv observed in the depolarized state in the presence of 10 mM KCl was much more negative than any diffusion potential ($E_K$ = −42 mv, and $E_K$ is more negative than the Goldman diffusion potential for $K^+$, $Na^+$, and $Cl^-$). This potential in 10 mM KCl probably reflects the operation of an electrogenic pump in the membrane, just as was observed for the normal state of the cell (8). Further evidence for an electrogenic potential was provided by the large increase in internal $K^+$ concentration following an overnight incubation in 10 mM KCl (Table I). During most of the 16 to 18 h uptake period, the cell would be in the depolarized (−91 mv) state since this state is established within 40 min. For this large net accumulation, there must be a substantial driving force on $K^+$; for passive $K^+$ movement, this would be provided by the membrane potential (−91 mv) that is more negative than $E_K$ (= −42 mv). Our present results, which indicate that the electrogenic pump is still operating in the high $K^+$ depolarized state, is counter to the hypothesis of Walker and his collaborators (25, 27). These workers postulated that the electrogenic proton pump was inactivated when the membrane was depolarized by high $K^+$ treatment.

The potential observed in Ca-free solutions (−190 to −200 mv) was more negative than $E_K$ (= −143 mv since external $K^+$ = 0.2 mM); hence, in this state, there must also be an electrogenic component. Further evidence for both this state and that in 10 mM KCl being electrogenic was provided by the ability of DES and NEM to depolarize the membrane. DES inhibits the electrogenic pump of *Chara* (8, 9) either directly or through a reduction of ATP levels. By inhibiting the electrogenic pump, the membrane should depolarize towards a diffusion potential, and it did for both of these cases (Fig. 5). NEM increases the $K^+$ permeability of *Chara* (11) and should further short-circuit the electrogenic pump, depolarizing the membrane to a value nearer to $E_K$, which was observed for both cases (Fig. 5). If, however, the membrane potential were only a diffusion potential prior to NEM exposure, then increasing the $K^+$ permeability would have had little effect, since the $K^+$-permeability would already have been the dominant influence in the diffusion potential. The sensitivity of the membrane potential to $Na^+$ concentration changes in the Ca-free experiments, after NEM exposure, indicates that NEM probably alters both the $Na^+$ and $K^+$ permeability.

**Reputed $K^+$-Channel Blocking Agents.** The presence of 5 mM TEA caused the recovery from the 10 mM KCl exposure to occur with only one phase, and a shorter lag time before the resistance increased. This suggests that TEA causes the $K^+$-channels to close much sooner, accounting for the earlier resistance increase and merging of the two recovery phases. TEA appears to function as a gate on the $K^+$-channel.

The agents, which reputedly block the $K^+$-channels of animal cells, did not all have the same effect in *Chara*. Our results are consistent with both TEA and TAP blocking $K^+$-channels once the channels have been opened. But, AP apparently opens the normally closed $K^+$-channels, depolarizing the membrane to a value near $E_K$. AP could work by competing for binding sites, displacing $Ca^{2+}$, and yet not blocking the channels when bound.

Because of the different effects of TEA and AP, cells were exposed to solutions containing both of these agents, following an initial AP exposure to open $K^+$-channels. The membrane potential was now relatively insensitive to $K^+$ as if TEA had closed the channels. However, the combination of these two agents depolarized the membrane as if the electrogenic pump had been further short-circuited, instead of the hyperpolarization expected if the $K^+$-channels had been closed and the short-circuit on the electrogenic pump removed.

**Calcium.** We interpreted the results of the Ca-free experiments as showing that $Ca^{2+}$ is the normal substrate required to close the $K^+$-channels. Long exposures to Ca-free conditions do not by themselves open these channels, probably because (a) the $Ca^{2+}$ that closes the channel is bound too tightly to be removed by this treatment alone or (b) the $Ca^{2+}$ does leave the channel binding sites, but the channels remain closed until they are stimulated to open by either an action potential or exposure to high levels of KCl (10 mM) (which stimulates an action potential as well). In cells in which the $K^+$-channels are open, the potential developed by the electrogenic pump would be at least partially short-circuited; thus, the potential would be depolarized by these open channels to a value that is intermediate between the normal hyperpolarized electrogenic potential and the diffusion potential. Under normal conditions, the passive diffusion potential is given by the Goldman equation and is slightly more positive than $E_K$. In cells in which the $K^+$-channels are open, the $K^+$ permeability is much larger so the importance of $K^+$ is increased and the diffusion potential would be essentially equal to $E_K$. In a solution containing 0.2 mM $K^+$, $E_K$ in the cells would be near −143 mv. In the Ca-free experiments described above, where either an action potential or a brief exposure to high KCl placed the cell in a depolarized state, the potential in 0.2 mM $K^+$ is always more negative than −143 mv, suggesting that the electrogenic pump is operating and able to hyperpolarize the membrane to values of −180 to −200 mv, even with the load of open $K^+$-channels. When the $K^+$-channels are closed by $Na^+$, $Ca^{2+}$, or TEA, the short-circuit load on the pump is removed, and the membrane rapidly hyperpolarizes to values of −250 to −290 mv (Fig. 3B). A $K^+$-sensitive membrane potential, observed in either *Chara* or *Nitella* (6, 20) under $Ca^{2+}$-free conditions, probably represents a state in which the $K^+$-channels have been opened.

**Methylamine.** It has been suggested that MA enters *Chara* as does $NH_4^+$ and $K^+$ (4, 26). The inhibition of MA entry by TAP is the predicted result of TAP blocking $K^+$-channels if MA enters through the $K^+$-channels. The TAP inhibition was larger (40–50%) if TAP was applied when the $K^+$-channels had been opened by 10 mM KCl than if TAP was applied to a normal cell (20% inhibition). This is consistent with the ability of TAP to block $K^+$ movement; TAP is relatively ineffective at reaching the blocking sites unless the channels have been opened. Thus, this TAP inhibition provides further evidence for MA movement through $K^+$-channels. Blockage by TAP may involve first removing $Ca^{2+}$ from the binding sites with 10 mM KCl, and then binding TAP to block the channel. Thus, TAP protection against the depolarization by MA is effective only when TAP is applied while the $K^+$-channels are open.

The interaction between MA and $K^+$ could be explained in two ways: (a) MA acts directly by opening $K^+$-channels, thus potentiating the ability of $K^+$ to initiate action potentials; or (b) MA acts indirectly through its known depolarization of the membrane, and this depolarization then alters the action of $K^+$ on the $K^+$-channels. The first explanation is supported by the observation that MA reduces the membrane resistance to one-half to one-third of the control. The second explanation is supported by voltage clamp experiments (D. W. Keifer and W. J. Lucas, unpublished observations) in which the voltage for action potential initiation was found to be dependent upon the exogenous $K^+$ level. At higher concentrations of $K^+$, the membrane has to be depolarized less to initiate an action potential; the action potential occurs at more negative values of the membrane potential. The first expla-

nation would require that in the presence of MA the membrane potential be more responsive to changes in the external $K^+$ concentration; but, in fact, it was found to respond by only about one-tenth of the theoretical Nernst response. We feel, therefore, that it is more likely that MA interacts with $K^+$ only indirectly through the depolarization of the membrane by MA.

**Speculative Model.** The $K^+$-channel in the plasmalemma of *Chara* has a cation binding site associated with it, presumably at the exterior entrance to the channel. When this site is occupied by $Ca^{2+}$, the channel is closed, and when the site is occupied by $K^+$, the channel is open. The membrane, which presumably contains a large population of channels, has a non-zero $K^+$ permeability even when substantial amounts of $Ca^{2+}$ are available. At least three explanations are possible: (a) there are two populations of channels, only one of which is blocked by $Ca^{2+}$; (b) a given channel is only partially blocked by $Ca^{2+}$, and at any given time some amount of $K^+$ can pass through the channels; (c) $Ca^{2+}$ totally blocks the channel, but $Ca^{2+}$ bound to the channel is in equilibrium with free-$Ca^{2+}$, so that in a population of channels some will not have a $Ca^{2+}$ bound and will thus be open.

This binding site may have its highest affinity for $Ca^{2+}$, but it is not entirely specific for $Ca^{2+}$. Other ions will compete for this site and displace $Ca^{2+}$, including: $K^+$, $Na^+$, TEA, TAP, and AP. The concentration of the various exogenous ions that it takes to displace $Ca^{2+}$ varies widely; some of these differences in concentrations could be due to the cation exchange properties of the cell wall which would concentrate divalent cations more than it would monovalent cations. Concentrations in the following discussion will refer to those in the external solution. $Ca^{2+}$ (0.2 mM) can be displaced by 10 mM $K^+$ (with a probable threshold of 2–3 mM $K^+$), leaving the channels open. In zero $Ca^{2+}$, 6 mM $Na^+$ can fill the sites displacing 0.2 mM $K^+$ and close the channels. If the $K^+$ is reduced to 5 mM, 5 mM TEA can sometimes displace the $K^+$ to close the channels, but it is a slow process. $K^+$ can always be displaced by 2.0 mM $Ca^{2+}$, but again it is slow. While all the possibilities and combinations have not been investigated, our data suggest the following series: $Ca^{2+} \gg TAP > K^+ \simeq TEA > Na^+$, for the binding affinity. All of these ions close the channel except $K^+$, which allows $K^+$ passage through the channel. Of these ions, $K^+$ is also the one with the smallest hydrated radius, so it may be able to bind to the site at the channel mouth and not obstruct $K^+$ access to the channel itself.

The state of the $K^+$-channel, whether open or closed, plays a substantial role in determining the membrane potential. Probably, the best example is during the Ca-free experiments where the $K^+$-channels opened without any change in $K^+$ concentration. The membrane potential changes from typically −280 mv when the $K^+$-channels are closed, to −190 mv when they are open. In the state with the open $K^+$-channels, the electrogenic pump is still operating, keeping the potential substantially more negative than $E_K$ (= −143 mv). Further evidence for this is (a) the depolarization of this potential by inhibiting the electrogenic pump with DES or by short-circuiting the pump by further increasing $K^+$ permeability with NEM and (b) the large uptake of potassium driven by this potential.

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