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Electrophysiological studies on Chara membrane  
by means of internal perfusion

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of plasmalemma of Chara without tonoplast

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## Chapter I

Demonstration of two stable potential states of plasmalemma  
of Chara without tonoplast 1)

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### Summary

The tonoplast of cells of Chara australis was removed by replacement of the cell sap with a medium containing 5 mM EGTA (ethyleneglycol-bis-( $\beta$ -aminoethyl ether) N, N'-tetraacetic acid). Such cells without tonoplast could generate an action potential of rectangular shape. In the present paper characteristics of the action potential were studied under various external ionic conditions.

Action potentials could be elicited without refractory period and the peak of the action potential was constant among action potentials.

Duration of the action potential decreased under repeated excitations but recovered after pause. Increase in concentrations of alkali metal cations,  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{Rb}^+$  and  $\text{Cs}^+$ , resulted in prolongation of the action potential.

At proper concentrations of monovalent cations the membrane potential could stay either at the resting level or at the depolarized level and could be shifted reversibly from the former level to the latter one or vice versa by applying outward or inward current. Further increase in concentrations of monovalent cations resulted in arrest of the membrane potential at the depolarized level. The critical concentrations of the monovalent cations to hold the membrane potential at the depolarized level were about 10 mM irrespective of the cation species.

Divalent cations,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Mn}^{2+}$ ,

added to bathing medium suppressed the effect of monovalent cations to prolong the action potential.

$\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  added to the bathing medium caused repolarization of the plasmalemma which had been depolarized by application of high concentrations of  $\text{K}^+$  to the bathing medium. The antagonism between monovalent and divalent cations on the state of the plasmalemma of Chara cells was discussed based on the two stable states hypothesis proposed by Tasaki (1968).

### I-1. Introduction

One of the most useful way to study characteristics of a biological membrane is to control its surrounding environments. The external environment can be modified by changing the composition of the bathing medium of the cell. The internal environment can be modified by intracellular perfusion with media of known compositions. For example, the internal space of a squid giant axon can be perfused with artificial salt solutions, thus enabling us to control the internal environment which the plasmalemma faces (Tasaki, Teorell & Spyropoulos, 1961; Baker, Hodgkin & Shaw, 1961). The large central vacuole of an internodal cell of Characeae can be perfused with artificial solutions, thus enabling us to control composition of the fluid occupying the innermost space of the plant cell, the vacuole (Tazawa, 1964). Characeae cells have two membranes,

the plasmalemma and the tonoplast. The vacuolar perfusion, therefore, can only modify the internal environment which the tonoplast faces. Responses of the tonoplast to the modification of the ionic composition of the vacuolar medium of Nitella pulchella were studied in detail by Kikuyama and Tazawa (1976; 1977). More detailed information on properties of Characeae membranes will be obtained, if chemical compositions of the media on both sides of each membrane are controlled. This can be realized, if the cytoplasm is replaced with artificial media of known compositions as is the case in squid giant axons.

To study the electric characteristics of the plasmalemma alone and also to modify the cytoplasmic environment just inside the plasmalemma, a method was developed to prepare the internodal cell without tonoplast (Tazawa, Kikuyama & Shimmen, 1976). Such cells showed not only active cytoplasmic streaming but also membrane excitability. The cell without the tonoplast generates an action potential of rectangular shape (Tazawa et al., 1976). It seems that the membrane of such cell has two stable levels of the membrane potential the presence of which were clearly demonstrated in the squid giant axon (Tasaki, Takenaka & Yamagishi, 1968). The present work was undertaken to demonstrate definitely the presence of the two stable membrane potentials in Chara australis and find factors controlling transitions between the two levels.

## I-2. Materials and Methods

Chara australis, diaecious species of Chara corallina, used throughout this work was cultured outdoors in large pots. Internodal cells were isolated from neighboring cells. Cells without tonoplast were prepared by replacing the cell sap with an artificial solution containing EGTA (Tazawa et al., 1976). Replacement of the cell sap with an artificial medium was carried out by the vacuolar perfusion (Tazawa, 1964) where both cell ends were cut and the artificial medium was perfused through the vacuole by establishing a slight difference in the water level between the cell ends. The perfusion medium contained 5 mM EGTA, 5 mM Tris-maleate, 17 mM KOH, 6 mM  $MgCl_2$  and 290 mM sorbitol (pH 7.0). For convenience, this perfusion medium will be called EGTA medium. Both cell openings were closed by ligating the cell ends with strips of polyester thread. The ligated cell (Cha) was placed on the polyacrylate vessel composed of double chambers as illustrated in Figure 1. Chamber A was filled with artificial pond water (APW; 0.1 mM each of KC1, NaCl and  $CaCl_2$ ) supplemented with 200 mM sorbitol to adjust the osmotic value to that of 110 mM KC1 solution in chamber B. Modification of the bathing medium was done by adding salts to APW and the osmotic value was also adjusted to 200 mM with sorbitol.

The potential difference across the plasmalemma of the cell half in APW ( $E_m$ ) is obtained by the following

equation,

$$E_m = E_{AB} + (E_m)_B. \quad (1)$$

$E_{AB}$  is the potential of the chamber A against chamber B and can be measured with two electrodes made of polyethylene tubing filled with 100 mM KCl - 2 % agar which was connected to Ag-AgCl wire through 3 M KCl. Since the plasmalemma potential in 110 mM KCl which was measured with the micro-electrode method [ $(E_m)_B$  in Eqn. (1)] was found to be very small (cf. Results),  $E_{AB}$  is approximately equal to  $E_m$ .

Electric current pulses for measuring the resistance between the two chambers were applied through Ag-AgCl wire. For the measurement of the membrane resistance small inward current pulses lasting about 1 sec were applied throughout the electric measurements. The electric resistance of the membrane ( $R_m$ ) of the cell half bathed in A is given by the following equation;

$$R_m = R_{AB} - R_B - R_s \quad (2)$$

where  $R_{AB}$  shows the electric resistance between the two chambers,  $R_B$  the membrane resistance of the cell half bathed in 110 mM KCl and  $R_s$  the sum of electric resistances of the external media, the cell wall and the cell sap. Since in the cell bathed in 110 mM KCl the membrane resistance measured by the microelectrode method was found to be negligibly small (about  $0.1 \text{ k}\Omega\text{cm}^2$ ),  $R_B$  in Eqn. (2) can be neglected. Then

$$R_m = R_{AB} - R_s \quad (3)$$

$R_s$  was measured by filling both chambers with 110 mM KC1 solution. For convenience, we call the method described above "K-anesthesia method".

In some cases the potential difference between the cell interior and the exterior was measured by inserting a glass microelectrode into the cell. The ligated cell whose cell sap had been replaced with EGTA medium was partitioned into three parts (Fig. 2). Three chambers were filled with the same solution. The glass microelectrode filled with 3 M KC1 was introduced into the cell segment in B, and the potential difference across the plasmalemma of the cell was measured between the microelectrode and the reference electrode placed in the chamber B. Electric current was applied to the cell between chamber B and chambers A and C through Ag-AgCl wire. The electric potential and the electric current applied were recorded simultaneously with a pen-writing recorder.

Since the K-anesthesia method is simpler than the microelectrode method and is sufficient for the purpose of the present study to demonstrate the presence of the two stable membrane potentials, most of the records represented in this paper were obtained by the former method except that shown in Figure 6.

Experiments were done at room temperature (20 - 25°C) and under dim light. The light intensity was kept at about 100 lux which was too low to induce membrane potential change due to light.

Average values of the electric potentials, the electric

resistances and the concentrations of monovalent cations to arrest the membrane potential at the depolarized level are shown with  $\pm$  SEM and with the number (n) of cells used.

### I-3. Results

#### I-3-1. Electric properties of the plasmalemma at the Resting state

The value of  $E_{AB}$  of normal cells of Chara australis was  $-180 \pm 6$  mV (n = 17) and the membrane resistance was  $40 \pm 3$   $\text{k}\Omega \text{ cm}^2$  (n = 6). Figure 3 shows changes in  $E_{AB}$  and  $R_{AB}$  of the cell whose cell sap was replaced with EGTA medium. The cell was mounted in the chamber (Fig. 1) immediately after the replacement of the cell sap with EGTA medium.  $E_{AB}$  which is roughly equal to  $E_m$  of the cell half in A was low for the initial 10 min and then increased up to  $-170$  mV. Simultaneously with the changes in  $E_{AB}$ ,  $R_{AB}$  increased conspicuously. These characteristic changes in electric potential and resistance are symptoms of disintegration of the tonoplast (Tazawa et al., 1976). As already reported in the previous paper (Tazawa et al., 1976), increases in  $E_{AB}$  and  $R_{AB}$  were observed within 30 min after the perfusion. The average value of  $E_{AB}$  after the loss of the tonoplast was  $-173 \pm 4$  mV (n = 48). This value is nearly equal to  $E_{AB}$  of the normal cell. The membrane potential of the cell measured by the microelectrode method was  $8 \pm 2$  mV (n = 10),

when the cell had been bathed in 110 mM KC1 for 30 min during which the tonoplast was disintegrated. Since this value should be equal to  $(E_m)_B$  in Eqn (1),  $E_m$  can be calculated by adding 8 mV to -173 mV. This value (-165 mV) is nearly equal to the membrane potential of the normal cell. The average value of  $R_m$  of the cells lacking tonoplast is calculated to be  $116 \text{ k}\Omega \text{ cm}^2$  (cf. Eqn. (2)) which is about three times as high as that of normal cells.

### I-3-2. Excitability of the plasmalemma

After the tonoplast was removed, action potentials of rectangular shape were generated by electric stimuli. In Figures 4 and 5 the outward current pulses were applied to the cell after the appearance of the electric symptoms of disintegration of the tonoplast. The current was withdrawn immediately after the action potential was induced. The first action potential lasted for 2 min in Figure 4 and 7 min in Figure 5. Both in Figures 4 and 5 the deflections of the membrane potential due to constant current pulses reflect  $R_{AB}$  of Eqn. (3). It decreased significantly during the depolarized states of the action potential. At the depolarized state  $R_{AB}$  was found to be nearly equal to  $R_s$ , indicating that  $R_m$  is very small (Eqn. (3)). When  $E_m$  was measured with the microelectrode method (Fig. 2), current pulses of the same intensity brought about very small deflections of the potential at the state of depolarization (Fig. 6).

Besides a very long duration of the action potential there are, in addition, three characteristics of the action potential in the tonoplast-free Chara cell. 1) When the cell was repeatedly stimulated, it could generate action potentials without refractory period. Even when the stimulus was given in the falling phase of the action potential, the potential shifted back promptly to the excited depolarized level (Fig. 7). 2) The peak of the action potential was constant on repeated excitations (Figs. 4 and 5). 3) The duration of the action potential decreased with the number of stimuli applied consecutively with short intervals (Figs. 4 and 5). The duration of the action potential which had decreased much on repeated excitations increased again after a long pause. In Figure 4 the action potential lasted for 135 sec in the first excitation and for only 24 sec in the fourth excitation. Keeping the cell at the resting state for 25 min, the duration of the action potential increased to 120 sec and was decreased by repeated excitations.

#### I-3-3. Prolongation of the action potential

There are at least two ways to prolong the duration of the action potential which has been decreased by repeated excitations. One way is to apply an outward current to the cell. In Figure 8 an action potential was induced by the outward ramp current applied during the interval between a and b. The action potential lasted for 37 sec after the current had been withdrawn at b. When the

same cell was stimulated again with a ramp current (d - e) which was superposed on a subthreshold constant outward current (c - f), the duration (80 sec) of the action potential was markedly prolonged.

The other way to prolong the action potential is to add a few mM of alkali metal ions to the bathing medium. Prolongation of the action potential by addition of alkali metal ions was observed in all cells tested, although extent of prolongation varied from cell to cell.

Figure 9 shows one typical example. The duration of the action potential was decreased to 7 sec by repeated excitation of the cell in APW. When the concentration of  $K^+$  in the bathing medium was increased from 0.1 mM to 1.1 mM by adding 0.5 mM  $K_2SO_4$  to APW, the duration of the action potential was prolonged to 22 sec. A further prolongation was observed by increasing the concentration of  $K_2SO_4$  to 1 mM. Other alkali metal ions,  $Li^+$ ,  $Na^+$ ,  $Rb^+$ , and  $Cs^+$ , could also prolong the action potential. Comparisons among abilities of alkali metal ions to prolong the action potential were not done.

#### I-3-4. Transitions of the membrane potential between two levels.

When the concentration of  $K^+$  in the bathing medium was further increased, the effect of  $K^+$  to prolong the action potential was strengthened. For instance, in Figure 5 the duration of the action potential decreased from 7 min to 10 sec due to successive stimulations with short intervals.

After the end of the seventh action potential 2 mM  $K_2SO_4$  was added to the bathing medium(APW), and the cell was stimulated electrically to cause an action potential. When the current was withdrawn, however, the plasmalemma potential did not come back spontaneously to the original resting level but stayed at the depolarized level for a long time. When the inward current was applied to the cell at this prolonged excitited state, the membrane potential showed a hyperpolarizing response accompanying an increase in  $R_m$ . The membrane potential shifted to a value which was more negative than that expected by ohmic change due to the inward current, indicating an increase in the electromotive force of the membrane (cf. Ohkawa & Kishimoto, 1974). Even when the current was removed the membrane potential did not come back to the depolarized level but stayed at the resting level. Such transitions between the two levels triggered by electric stimuli could be repeated many times. The critical concentration of external  $K^+$  at which the membrane potential exhibits reversible transitions between the two stable levels by electric stimili was varialbe between 3 - 10 mM. When the concentration of  $K^+$  was further increased, the plasmalemma showed a hyperpolarizing response to the inward current and the membrane potential returned to the depolarized level on removal of the current. A similar behavior of the membrane potential in response to the increase in ionic concentrations was also observed in other alkali metal ions. The critical concentrations of alkali metal

ions to arrest the membrane potential at the depolarized level were obtained by increasing the concentrations at steps of 2 mM. The critical concentrations of  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Rb}^+$  and  $\text{Cs}^+$  were  $14 \pm 2$  mM ( $n = 5$ ),  $13 \pm 2$  mM ( $n = 5$ ),  $9 \pm 0.4$  mM ( $n = 12$ ),  $10 \pm 2$  mM ( $n = 4$ ) and  $10 \pm 3$  mM ( $n = 4$ ), respectively.

Next, responses of the plasmalemma at each state to the outward and inward electric current were studied. In Figure 10, the cell was bathed in APW containing 2 mM  $\text{K}_2\text{SO}_4$  and the membrane potential was at first at the resting level. When the inward ramp current was applied to the cell at a, the membrane potential showed only an ohmic change. It returned to the original resting level on withdrawal of the current. When the ramp outward current was applied to the cell at b, a characteristic change in the potential showing excitation was observed and the membrane resistance decreased. The membrane potential stayed at the depolarized level after withdrawal of the current. Applying the outward ramp current to the cell at this state (c), only an ohmic change of the membrane potential was observed. To the inward current, however, the cell showed a hyperpolarizing response with an increase in the membrane resistance (d). The membrane potential stayed at the resting level after withdrawal of the current. Thus, the plasmalemma responds differently to the direction of the current according to whether the membrane is at the resting state or at the excited state.

I-3-5. Effect of divalent cations in the bathing medium

When the divalent cations such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Mn}^{2+}$  were added to the bathing medium, they suppressed the effect of monovalent cations to prolong the action potential. One example is shown in Figure 11. At the start of the record, the cell was bathed in APW with 2 mM  $\text{K}_2\text{SO}_4$ . As described in the foregoing section, the membrane potential stayed for a long time either at the resting level, or at the depolarized level. Transitions of the membrane potential between the two stable levels could be controlled by the electric current. Next, the bathing medium was replaced with APW containing 2 mM  $\text{K}_2\text{SO}_4$  and 1 mM  $\text{CaSO}_4$ . Though the outward current caused excitation of the membrane as before, the membrane potential did not stay at the depolarized level but returned to the original resting level soon after withdrawal of the current. The effective concentration of external  $\text{Ca}^{2+}$  to bring spontaneously the depolarized membrane potential back to the resting level changed to a higher value with time during experiment and varied from cell to cell. Essentially the same result was obtained in the experiment using  $\text{Mg}^{2+}$  instead of  $\text{Ca}^{2+}$  (Fig. 12). The other divalent cations,  $\text{Sr}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Mn}^{2+}$ , were also effective in suppressing the effect of monovalent cations to prolong the action potential. When the bathing medium contained 2 mM  $\text{K}_2\text{SO}_4$ , the lowest effective concentrations of divalent cations to reduce the duration of the action potential to less than 1 min were 0.5 mM irrespective of ion species ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Ni}^{2+}$  and  $\text{Mn}^{2+}$ ).

### I-3-6. Transitions of membrane potential by chemical stimuli

Results obtained so far indicate that the tendency of the membrane potential to stay at the depolarized level is strengthened by increasing concentrations of the monovalent cations in the bathing medium and is weakened by adding the divalent cations to the bathing medium. Therefore, it is assumed that the monovalent cations are preferable to bring the membrane from the resting state to the excited state and that the divalent cations from the excited state to the resting state. The assumption was verified by experiments shown in Figure 13, where the bathing medium of the cell was first APW containing 15 mM  $K_2SO_4$  which was later changed for APW containing 15 mM  $CaSO_4$ . Transferring the cell from APW to the  $K_2SO_4$  medium, the membrane potential shifted spontaneously to the depolarized level and stayed there, and the membrane resistance decreased. When the  $K_2SO_4$  medium was replaced with the  $CaSO_4$  medium, the depolarized membrane potential shifted after a short lag abruptly to the polarized resting level and the membrane resistance increased. Addition of  $Mg^{2+}$  instead of  $Ca^{2+}$  to APW also brought back the depolarized membrane potential to the resting level (Fig. 14). When the cell at this state was stimulated electrically, the action potential of rectangular shape was not observed in  $Mg^{2+}$ -enriched APW (Fig. 14) but in  $Ca^{2+}$ -enriched APW (Figs. 13 and 14). It is to be noted that the transitions of the membrane potential between the two

levels without electric stimuli could be repeated many times, indicating that monovalent and divalent cations are essential factors responsible for determining the state of the membrane.

#### I-4. Discussion

##### I-4-1. Intactness of the plasmalemma of tonoplast-free cell

The action potential of the tonoplast-free cell showed peculiar phenomena which were not observed in the normal cell: a very long duration, absence of the refractory period, decrease in duration on repetitive firings with short intervals and recovery of shortened duration under a long pause. These abnormal behaviors suggest that the membrane is modified to some extent by changes in cytoplasmic environments due to loss of tonoplast. However, the modification may be a moderate one which can be reversed by restoring normal cytoplasmic conditions. This presumption was supported by the fact that the duration of the action potential of the tonoplast-free cell became as short as that of the normal Chara cell, when the concentration of  $K^+$  in EGTA medium was raised to that of the normal cytoplasm (Shimmen, Kikuyama & Tazawa, 1976). Furthermore, following facts indicate that the plasmalemma remain almost intact even after loss of the tonoplast. First, the hydraulic

conductivity of the tonoplast-free cell was practically the same as that of the normal cell (Kiyosawa & Tazawa, 1975). Second, light-induced changes in the membrane potential of the tonoplast-free cell were very similar to those of the normal cell (Kikuyama, Fujii & Tazawa, 1976).

I-4-2. Electric potential and resistance before and after tonoplast disintegration

When the cell sap was replaced with EGTA medium, the electric potential ( $E_m$ ) between the interior and the exterior of the cell was first low and increased to that of the normal cell when the tonoplast was removed (Fig. 3). Before removal of the tonoplast  $E_m$  represents the sum of the plasmalemma potential ( $E_{co}$ ) and the tonoplast potential ( $E_{vc}$ ),

$$E_m = E_{vc} + E_{co} \quad (4)$$

In Nitella pulchella decreases in concentration of  $K^+$  and  $H^+$  in the vacuole made  $E_m$  less negative by shifting  $E_{vc}$  to the positive direction (Kikuyama & Tazawa, 1977). Replacement of the normal cell sap of C. australis with EGTA medium decreased the vacuolar concentration of  $K^+$  from 112 mM (Tazawa, Kishimoto & Kikuyama, 1974) to 24 mM and that of  $H^+$  from  $10^{-5.5}$  M (Hirakawa & Yoshimura, 1964) to  $10^{-7}$  M. Therefore, the low value of  $E_m$  before removal of the tonoplast may be due to low concentrations of  $K^+$  and  $H^+$  in EGTA medium.

After removal of the tonoplast  $E_m$  should be equal to  $E_{co}$ . Since  $E_{AB}$  (cf. Eqn. (1)) of the normal cell (-180 mV) was

nearly equal to that of the tonoplast-free cell (-173 mV).  $E_{co}$  seems insensitive to the change in  $K^+$ -concentration of the cytoplasm which was brought about by the loss of the tonoplast. The concentration of  $K^+$  in the cell after loss of the tonoplast is estimated to be 24 mM, since the contribution of the cytoplasmic  $K^+$  to it is about 7 mM (Tazawa et al., 1976). Since the equilibrium potential for  $K^+$  across the plasmalemma in this case is calculated to be -138 mV which is 27 mV more positive than observed  $E_m$  (-165 mV), the observed  $E_m$  can not be explained simply as the diffusion potential of  $K^+$  across the plasmalemma. For further discussions systematic study of the relationship between the plasmalemma potential and the internal concentrations of ions is necessary.

To explain the increase in the membrane resistance observed after replacement of the cell sap with EGTA medium, it was postulated in the previous paper (Tazawa et al., 1976) that the plasmalemma of tonoplast-free cells becomes less permeable to  $H^+$  or  $K^+$ , or to both ions.

#### I-4-3. Two stable potential levels

The most significant result to be stressed in the present study is that the duration of the action potential can be controlled by changing concentrations of monovalent cations and divalent cations in the external medium. Addition of monovalent cations to the external medium prolonged the duration of the action potential. A prolonged action potential was also reported in the normal cell of Chara braunii treated with 20 - 30 mM of  $K^+$  for 30 min or

more (Oda, 1961). At proper concentrations (a few mM) of monovalent cations, the membrane potential of the tonoplast-free cells stayed at the excited level for several tens of minutes or more. Under this condition the membrane potential could be easily shifted by the electric current pulse between the resting and excited levels and could stay at each potential level for a long time.

The fact that there are two stable membrane potentials under the constant internal and external ionic conditions suggests that the plasmalemma has two stable states: one characterized with less negative membrane potential and high membrane conductance, and the other characterized with more negative membrane potential and low membrane conductance. The former state corresponds to the excited state of the membrane and the other to its resting state.

When the concentration of the monovalent cations was increased further, the membrane potential seemed to settle indefinitely at the depolarized level. Such a membrane showed a hyperpolarizing response by applying an inward current. At removal of the current, the membrane potential returned instantly to the depolarized level, as was previously reported in Characeae cells (Ohkawa & Kishimoto, 1974) and squid giant axons (Tasaki, 1959). The critical concentrations of monovalent cations to arrest the membrane potential at the depolarized level were not significantly different among cation species. They were about 10 mM. The fact that in Chara membrane no differences were observed among monovalent cations in the ability to arrest the

membrane potentail at the depolarized level makes striking contrast with the fact that the Characeae membrane shows different permeabilities to these ions (Nakagawa, Kataoka & Tazawa, 1974). The lack of selectivity may be explained by assuming first that the cations act directly on the negatively charged sites of the membrane which are exposed to the external medium and second that the affinities of the sites of the membrane with the cations do not differ much among the cations.

In the squid giant axon effective concentrations of monovalent cations to depolarize the membrane differ much among cation species (Tasaki et al., 1968). The negatively charged sites of the axon membrane may have a higher selectivity to monovalent cations than those of the Chara membrane. Alternative explanation is that the negatively charged sites of the membrane are not directly exposed to the external meidum and therefore that accesses of the cations to them are limited by the permeabilities of the membrane to these ions.

Monovalent and divalent cations seem to act on the plasmalemma competitively and determine its state. No detectable differences were observed among divalent cations in their ability to suppress the effect of monovalent cations to prolong the action potential (Figs. 11 and 12). However, there is a significant difference between  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  in the shape of the action potential elicited, when their concentrations are high (Fig. 14).

Transitions of the membrane potential between the two levels by chemical stimuli were reported also in the squid

giant axon (Tasaki et al., 1968) and the Ranvier node of the toad (Spyropoulos, 1961). In the squid giant axon the critical concentrations of monovalent cations in the bathing medium to induce the abrupt depolarization of the membrane increase with increasing the concentration of  $\text{CaCl}_2$  in the bathing medium (Tasaki et al., 1968).

Results obtained in the present study on the effect of monovalent and divalent cations in the external medium on the excitation of the plasmalemma of Chara cells can well be accounted for on the basis of the two stable states hypothesis proposed by Tasaki on squid giant axons (Tasaki, 1968). According to the hypothesis, the membrane takes the resting state when the fixed negative charges at the external layer of the membrane are occupied by divalent cations and does the excited state when the divalent cations are exchanged for monovalent cations. Since cations in the external medium act directly on the external layer of the membrane, the monovalent/divalent cation ratio in the external medium can modify the same cation ratio in the membrane. When the action potential was elicited by the electric stimulus in the presence of a few mM of monovalent cations and 0.1 mM  $\text{Ca}^{2+}$  in the external medium, the membrane stayed at the excited state for a longer period (Fig. 9). On the basis of the two stable states theory this fact implies that the exchange of monovalent cations occupying the negatively charged sites in the membrane for divalent cations from the external medium takes a longer time. The fact that the duration of excitation was shortened by raising the

divalent/monovalent cation ratio (Fig. 11 and 12) is explained by the acceleration of the exchange of monovalent cations for divalent cations.

That the essential process of membrane excitation is a chemical one was clearly demonstrated by the fact that both excitation and repolarization were induced only by changing the external monovalent/divalent cation ratio without applying electric current (Figs. 13 and 14).

The action of the outward electric current in inducing the action potential can be explained as the processes of expelling  $\text{Ca}^{2+}$  from the membrane into the bathing medium and of carriage of  $\text{K}^+$  into the membrane from the cell interior (Tasaki, 1968). Then the negatively charged sites in the membrane are occupied by  $\text{K}^+$  and the membrane is brought into the excited state. When the outward current is put off, exchange of  $\text{K}^+$  for  $\text{Ca}^{2+}$  from the external medium may proceed and the membrane returns to the original resting state. Inhibition of the exchange by a continuous supply of  $\text{K}^+$  into the membrane and/or continuous repelling of  $\text{Ca}^{2+}$  from the membrane by the outward electric current will result in a prolongation of the action potential (Fig. 8).

The presence of the two stable membrane potential states suggests that the action potential is the reversible transitions of the plasmalemma between the two stable states. During the action potential the membrane first shifts from the resting state to the excited state and then from the excited state to the resting state. In the normal cell it is difficult to characterize the excited state of the membrane

since the duration of the peak of the action potential is not long enough to allow the exact measurements of the electric and ionic parameters. In the tonoplast-free cell, however, the excited state extends to several or sometimes to several tens of minutes by raising external  $K^+$  concentration. In the tonoplast-free cell, therefore, it is easy to characterize the excited state of the membrane in comparing with the resting state. There are many reports that both  $K^+$ - and  $Cl^-$  effluxes increase during the action potential of Characeae (Mullins, 1962; Hope & Findlay, 1964; Mailman & Mullins, 1966; Oda, 1976). Our preliminary experiments using tonoplast-free cells showed that  $K^+$ -efflux at the excited state was  $84 \text{ pmoles cm}^{-2} \text{ s}^{-1}$  which is about 30 times as high as the resting efflux ( $3 \text{ pmoles cm}^{-2} \text{ s}^{-1}$ ).

#### I-4-4. Constancy of the peak potential

In tonoplast-free cells the potential at the peak of the action potential remained constant on repeated excitations with short intervals (Figs. 4, 5, 6 and 7), while in normal cells it fluctuates to some extent. The action potential observed in normal cells is the sum of the action potential generated at the tonoplast and that at the plasmalemma (cf. Eqn. (4)). In Nitella pulchella the action potential at the tonoplast always accompanies the action potential at the plasmalemma with a short lag and has a longer refractory period than that of the plasmalemma (Kikuyama & Tazawa, 1976). Difference in refractory periods and variations of the lag between the two action potentials should cause fluctuations of the magnitude and duration of the action potential in

normal cells. After disintegration of the tonoplast the action potential represents only the action potential of the plasmalemma. Absence of both tonoplast action potential and refractory period of the plasmalemma may be responsible for the constancy of the peak levels of the repeated action potentials in tonoplast-free cells.

#### I-4-5. Internal $\text{Ca}^{2+}$ - and $\text{K}^+$ -concentrations

In all experiments shown in this paper intracellular perfusion was conducted only once. Concentrations of the ions in the cell can be estimated from the contents of the ions in the cytoplasm and in the perfusion medium under the assumption that the ions in the cytoplasm are distributed homogeneously in the cell after disappearance of the tonoplast (Tazawa et al., 1976). When the present EGTA medium was used, the internal concentrations of  $\text{K}^+$ ,  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  after the loss of the tonoplast were estimated to be 24 mM, 14 mM and  $2.9 \times 10^{-8}$  M, respectively. Generation of the action potential under the extremely low internal  $\text{Ca}^{2+}$  concentration indicates that the presence of  $\text{Ca}^{2+}$  inside the plasmalemma is not necessary for excitation of the plasmalemma. This coincides with the result obtained on squid giant axons that the presence of  $\text{Ca}^{2+}$  in the cell is unfavorable for excitation (Tasaki, Watanabe & Takenaka, 1962).

In squid giant axons the internal condition necessary for generation of the action potential of longer duration is not lowering of the ionic strength but lowering of concentration of a special cation species,  $\text{K}^+$  (Tasaki, Lerman & Watanabe,

1969). Implications of internal ionic strength and ion species in the duration of action potential of Chara australis will be dealt with in subsequent papers.

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## Legend

Fig. 1. Diagram of apparatus to measure the membrane potential and resistance by "K-anesthesia method". Internodal cell (Cha) was placed on the polyacrylate vessel composed of two chambers. Ramp and rectangular electric current for the stimulation and for the measurement of the membrane resistance was applied between chamber A and chamber B through Ag-AgCl wire. The potential difference between two chambers ( $E_{AB}$ ) was recorded on a pen-writing recorder.

Fig. 2. Diagram of the apparatus to measure the membrane potential and the membrane resistance by "microelectrode method". Internodal cell (Cha) was placed on the polyacrylate vessel composed of three chambers. Rectangular electric current for the stimulation and for the measurement of the membrane resistance was applied to the cell part in the chamber B by passing the current between the chamber B and chambers A and C through Ag-AgCl wire. Membrane potential ( $E_m$ ), represented by the potential difference between reference electrode and microelectrode inserted into the cell, was recorded on the pen-writing recorder.

Fig. 3. Increase in membrane potential and membrane resistance after the cell sap was replaced with EGTA medium. The cell was placed on the chamber (Fig. 2) within a few min after replacement of the cell sap and APW was used as

the bathing medium. Rectangular electric current pulses of 0.02  $\mu$ A were applied during the measurement (lower trace). The value of  $E_{AB}$  is shown in mV.  $R_s$  in Eqn. (3) was 150 k $\Omega$ .

Fig. 4. Decrease in the duration of the action potential by repeated excitations and its recovery after pause. Four action potentials were repeatedly elicited by outward current and four action potentials were elicited again after 25 min of pause in APW. Rectangular electric current pulses of 0.02  $\mu$ A were applied during the measurement.  $R_s$  in Eqn. (3) was 150 k $\Omega$ .

Fig. 5. Decrease in the duration of the action potential in APW and the reversible transition of the membrane potential by electric current in APW containing 2 mM  $K_2SO_4$ . Seven action potentials were elicited in APW and the duration of the action potential decreased. Reversible transitions of the membrane potential between two levels by electric current were observed after the bathing medium was changed for APW containing 2 mM  $K_2SO_4$ . Rectangular electric current pulses of 0.05  $\mu$ A were applied during the measurement.  $R_s$  in Eqn. (3) was 100 k $\Omega$ .

Fig. 6. Action potentials and membrane resistance measured by the microelectrode method. Action potentials were elicited in APW. The duration of the action potential decreased with numbers of the action potential and the

membrane resistance decreased significantly during the plateau of action potentials. Rectangular electric current pulses of 0.05  $\mu$ A were applied during the measurement.

Fig. 7. Generation of action potentials without refractory period. The cell was bathed in APW and stimulated repeatedly. Action potential could be elicited just after the preceding action potential or even in its falling phase.

Fig. 8. Prolongation of the action potential by application of outward electric current to the cell. Two action potentials were elicited by outward ramp current under the absence (a - b) or presence (d - e) of constant outward current in APW. Rectangular electric current pulses of 0.05  $\mu$ A were applied during the measurement.  $R_s$  in Eqn. (3) was 150 k $\Omega$ .

Fig. 9. Prolongation of the action potential by application of  $K_2SO_4$  to the external medium. After the duration of the action potential had been decreased by repeated excitations in APW, prolonged action potentials were elicited under the presence of 0.5 mM or 1 mM  $K_2SO_4$  in the bathing medium. Rectangular electric current pulses of 0.05  $\mu$ A were applied during the measurement.  $R_s$  in Eqn. (3) was 130 k $\Omega$ .

Fig. 10. Responses of the plasmalemma to the outward and inward ramp currents in resting and depolarized states. Outward and inward ramp currents were applied alternately to the cell bathed in APW containing 2 mM  $K_2SO_4$ . The cell

responded to outward current (b) but not to inward current (a and e) in the resting state and did to inward current (d) but not to outward current (c) in the depolarized state. Rectangular electric current pulses of 0.05  $\mu$ A were applied during the measurement.  $R_s$  in Eqn. (3) was 150 k $\Omega$ .

Fig. 11. Suppression of the effect of  $K^+$  to prolong the duration of the action potential by  $Ca^{2+}$ . The prolonged action potential was induced by outward ramp current in APW containing 2 mM  $K_2SO_4$  and the action potential was abolished by inward ramp current. The action potential was elicited again in APW containing 2 mM  $K_2SO_4$  and 1 mM  $CaSO_4$  and the duration was shortened. Rectangular electric current pulses of 0.05  $\mu$ A were applied during the measurement.  $R_s$  in Eqn. (3) was 130 k $\Omega$ .

Fig. 12. Suppression of the effect of  $K^+$  to prolong the duration of the action potential by  $Mg^{2+}$ . The prolonged action potential was induced by outward ramp current in APW containing 2 mM  $K_2SO_4$  and the action potential was abolished by inward ramp current. The action potential was elicited again in APW containing 2 mM  $K_2SO_4$  and 1 mM  $MgSO_4$  and its duration was shortened. Rectangular electric current pulses of 0.024  $\mu$ A were applied during the measurement.  $R_s$  in Eqn. (3) was 240 k $\Omega$ .

Fig. 13. Chemical stimulation of Chara. The bathing medium was first APW containing 15 mM  $K_2SO_4$  and next APW

containing 15 mM  $\text{CaSO}_4$ . The action potential of rectangular shape was elicited by electric outward current, when the plasmalemma repolarized in 15 mM  $\text{CaSO}_4$ . Rectangular electric current pulses of 0.02  $\mu\text{A}$  were applied during the measurement.  $R_s$  in Eqn. (3) was 100  $\text{k}\Omega$ .

Fig. 14. Chemical stimulation of Chara. The bathing medium was first APW containing 15 mM  $\text{K}_2\text{SO}_4$ , then APW containing 5 mM  $\text{MgSO}_4$  and finally APW containing 5 mM  $\text{CaSO}_4$ . Action potentials were induced in both  $\text{Mg}^{2+}$ - and  $\text{Ca}^{2+}$ -media by electric current (downward arrows). Rectangular electric current pulses of 0.02  $\mu\text{A}$  were applied during the measurement.  $R_s$  in Eqn. (3) was 200  $\text{k}\Omega$ .

Fig. 1

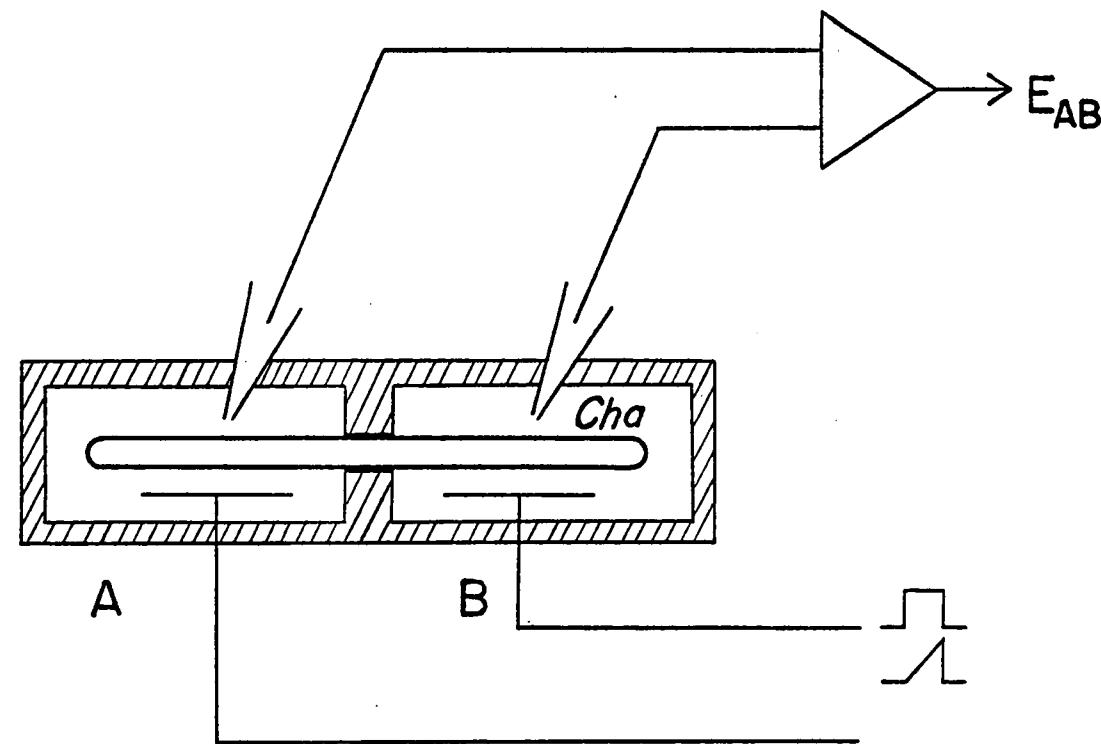


Fig. 2

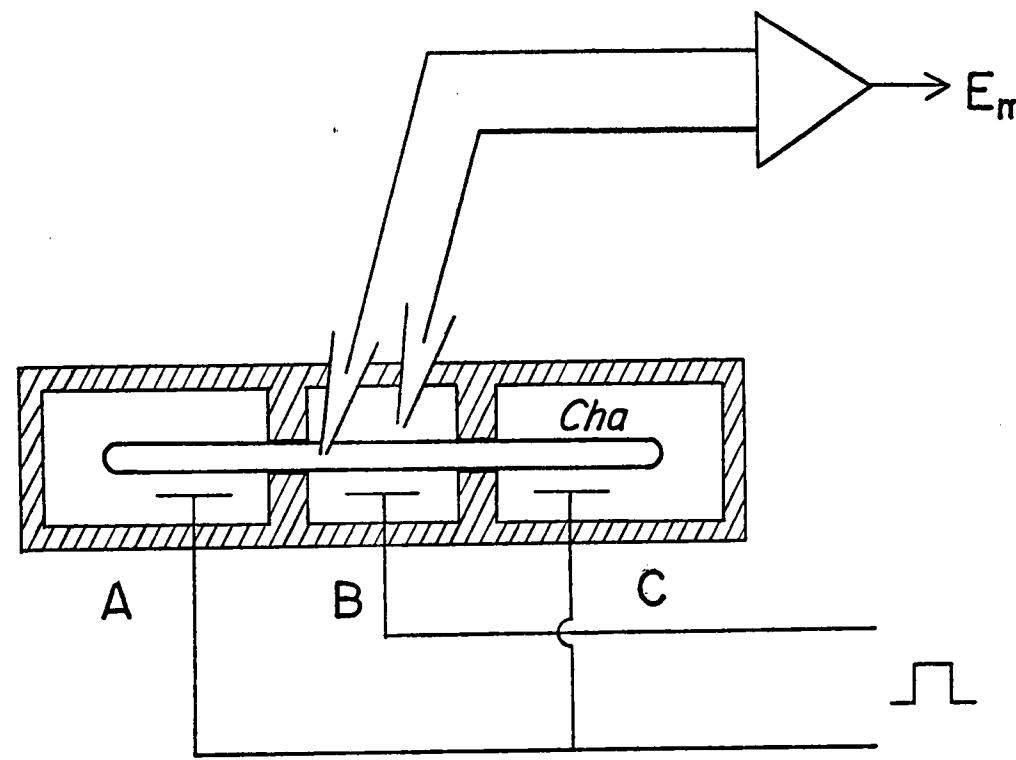


Fig. 3

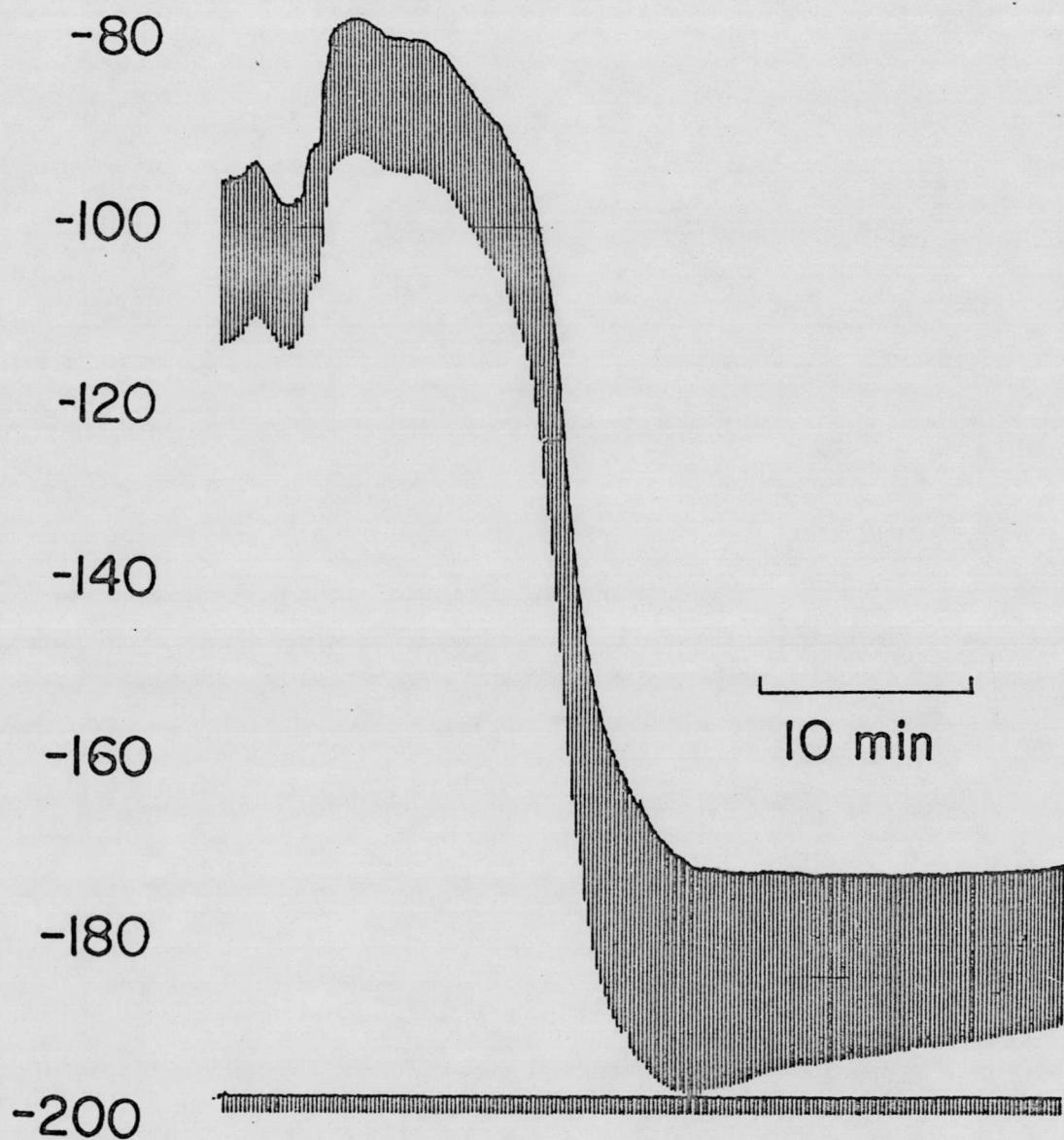


Fig. 4

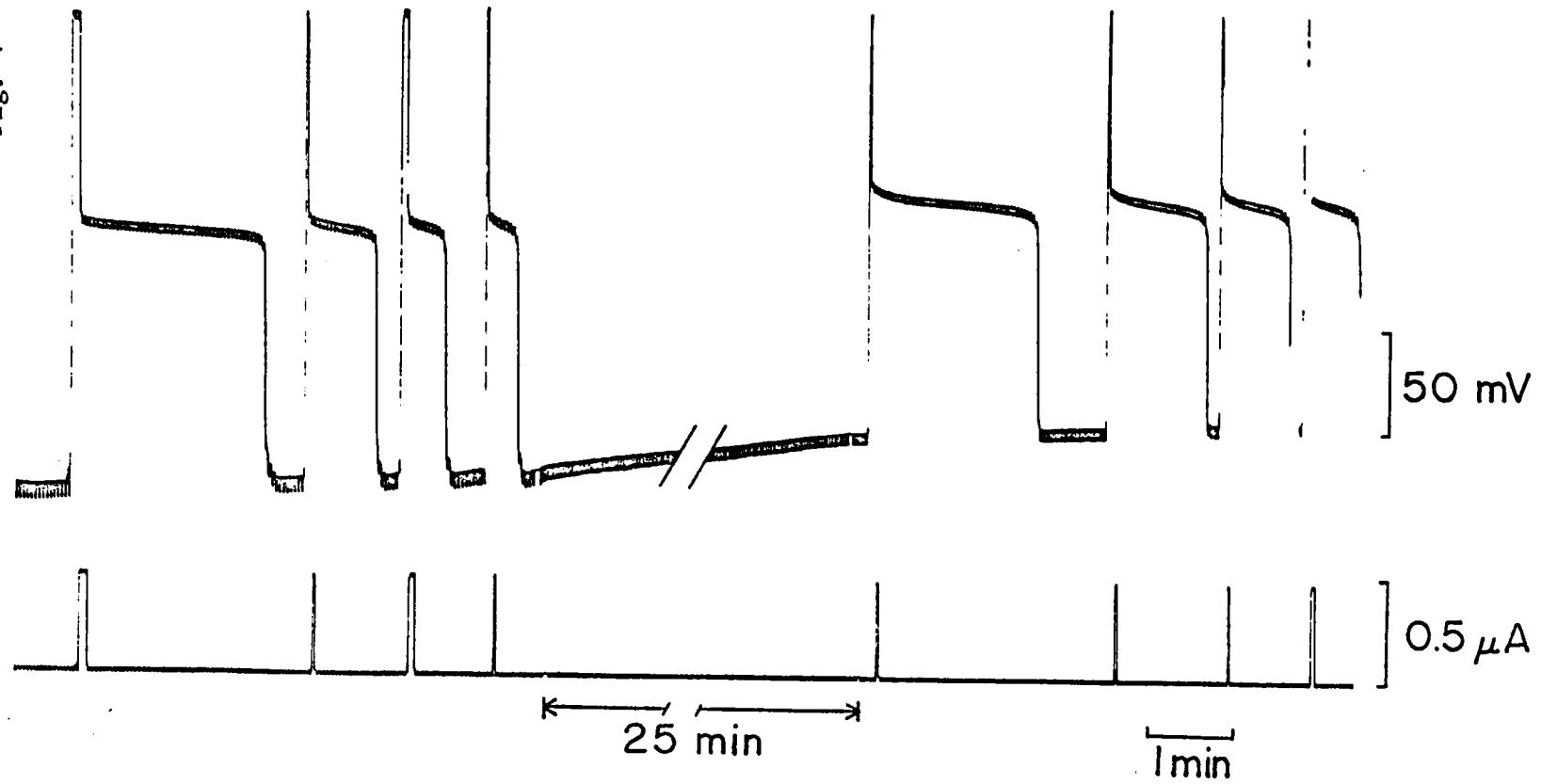


Fig. 5

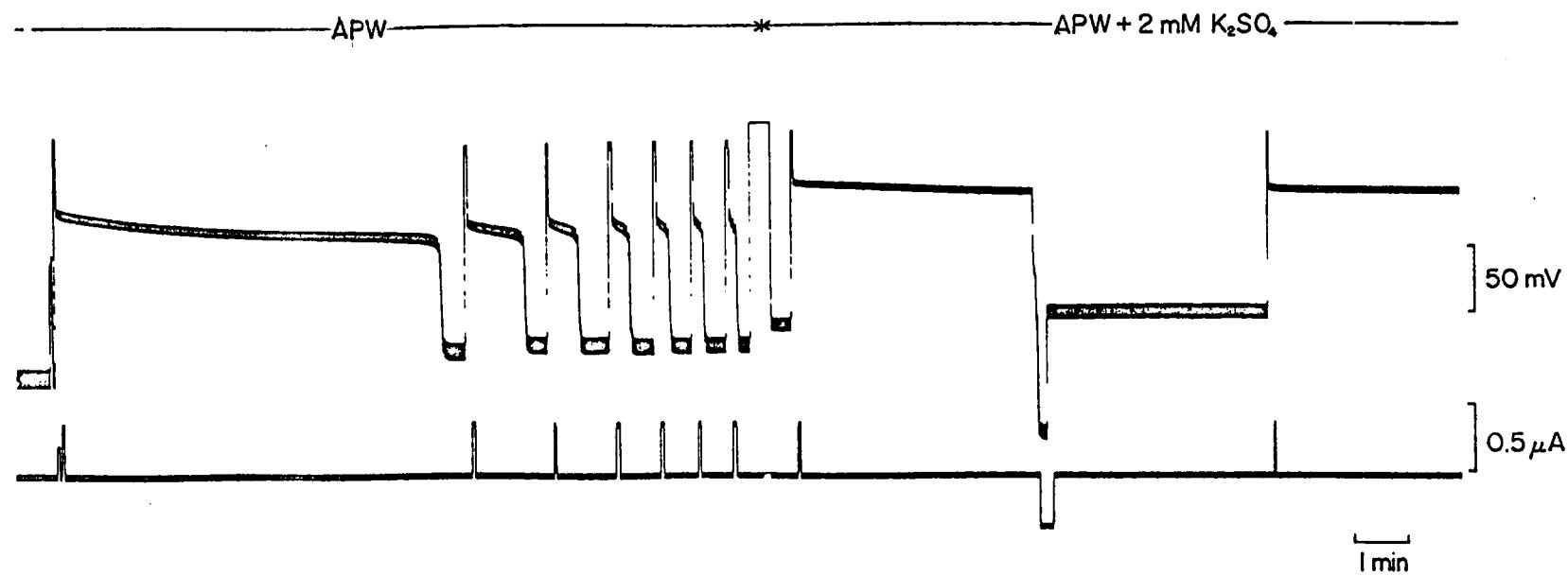


Fig. 6

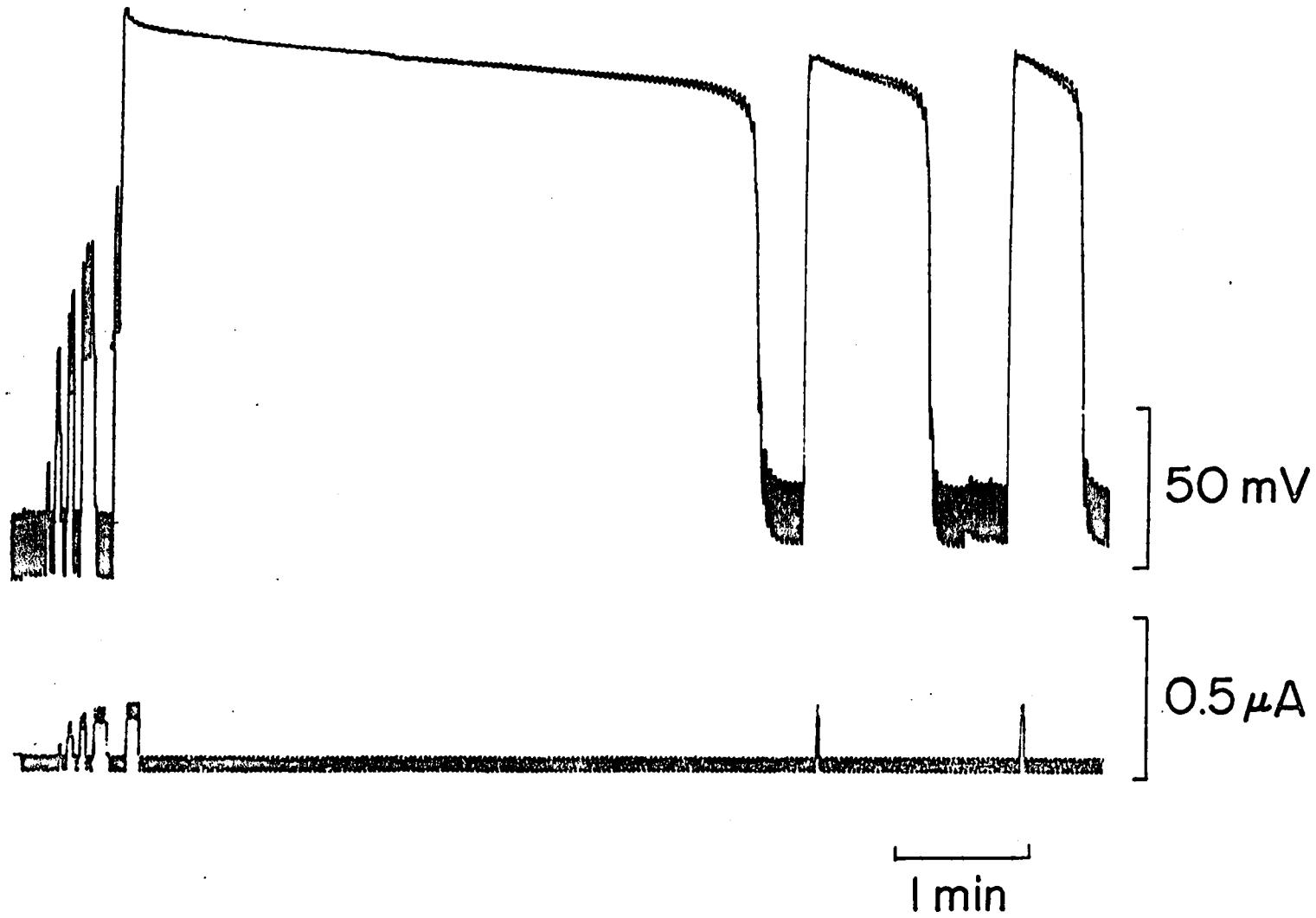


Fig. 7

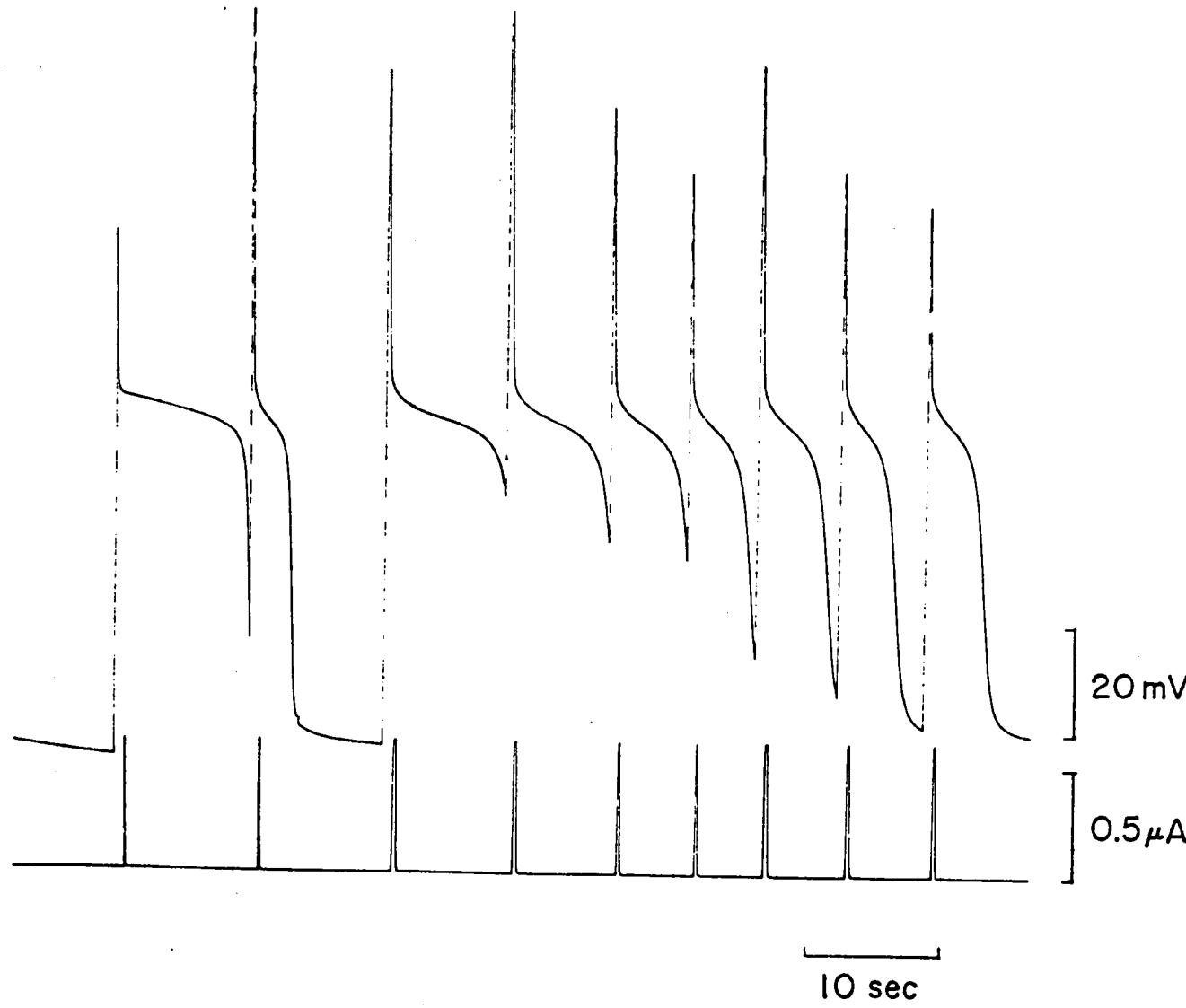


Fig. 8

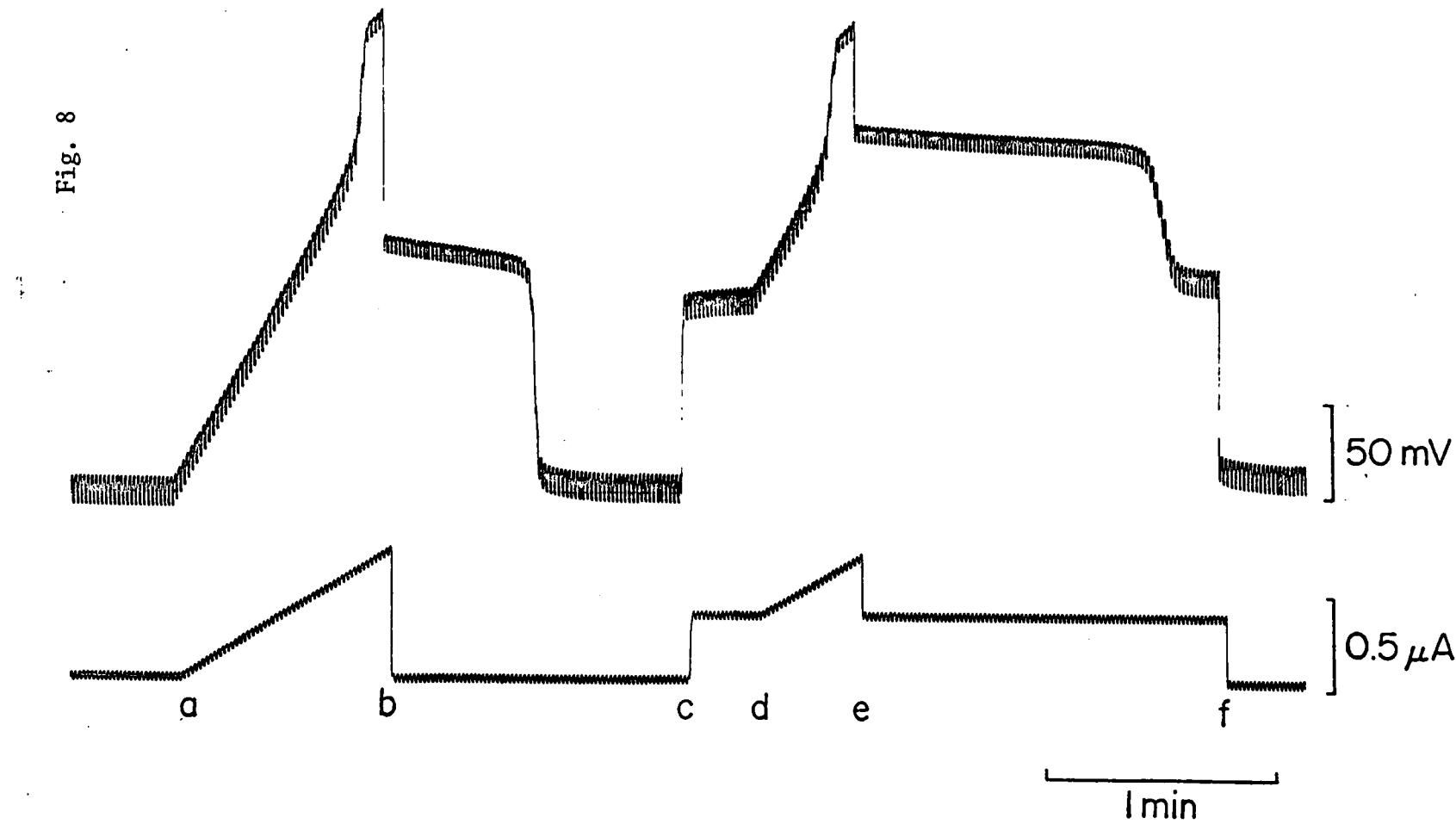


Fig. 9

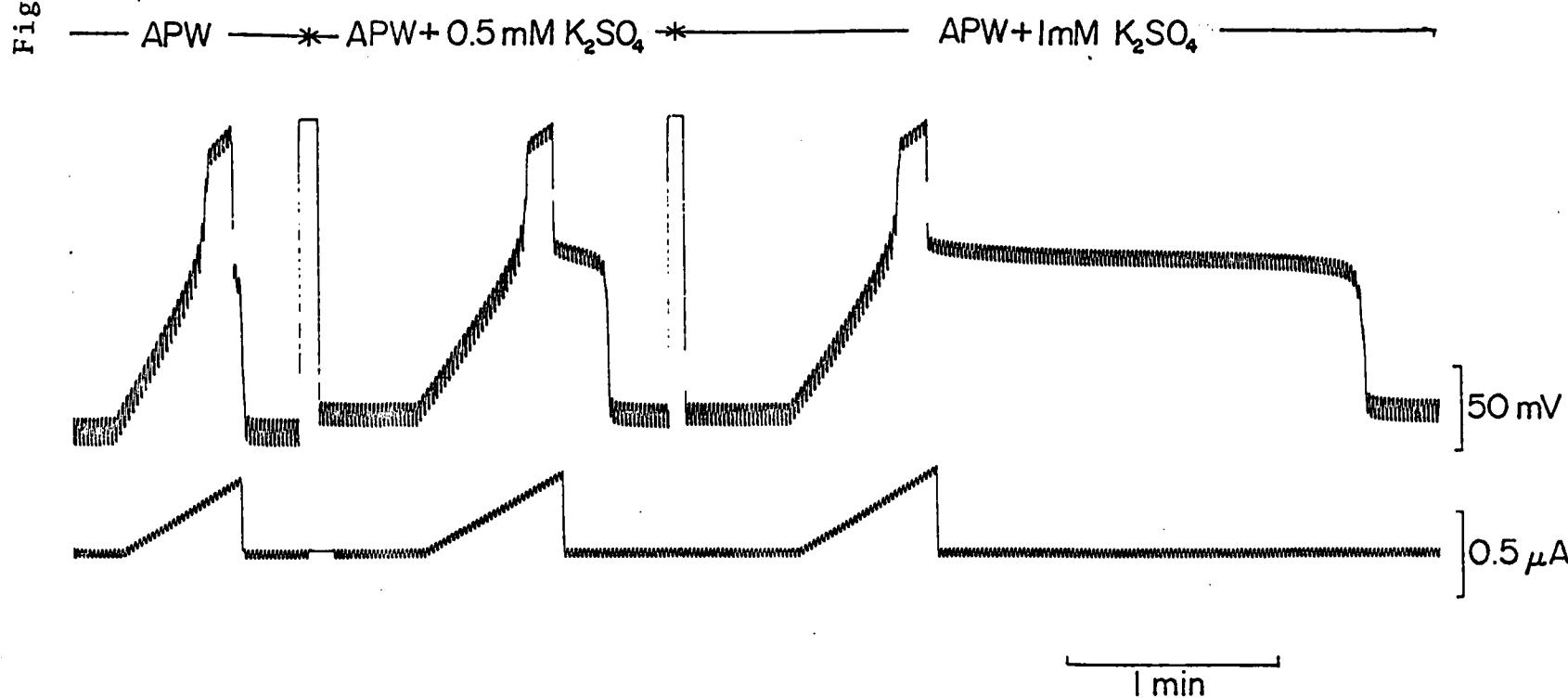
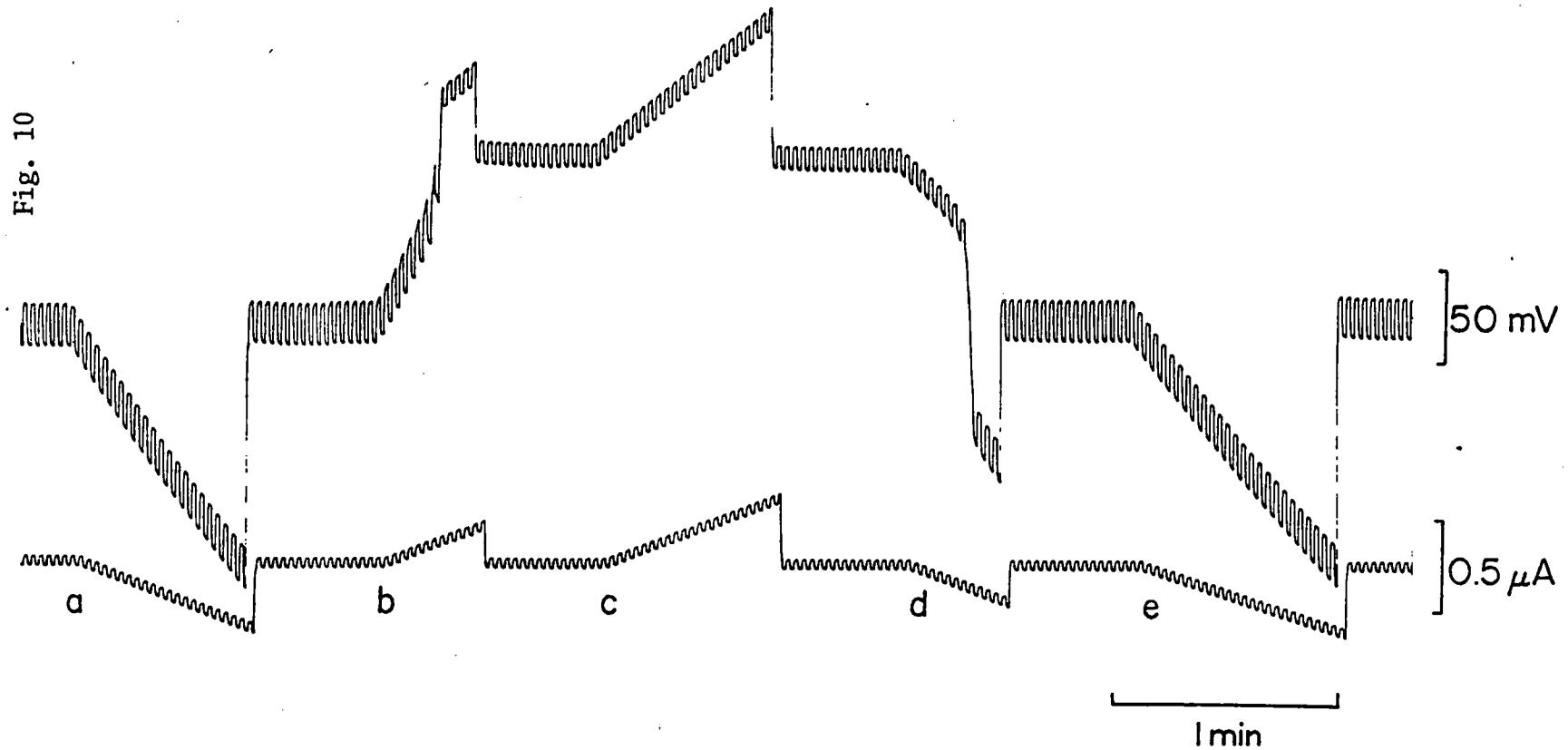


Fig. 10



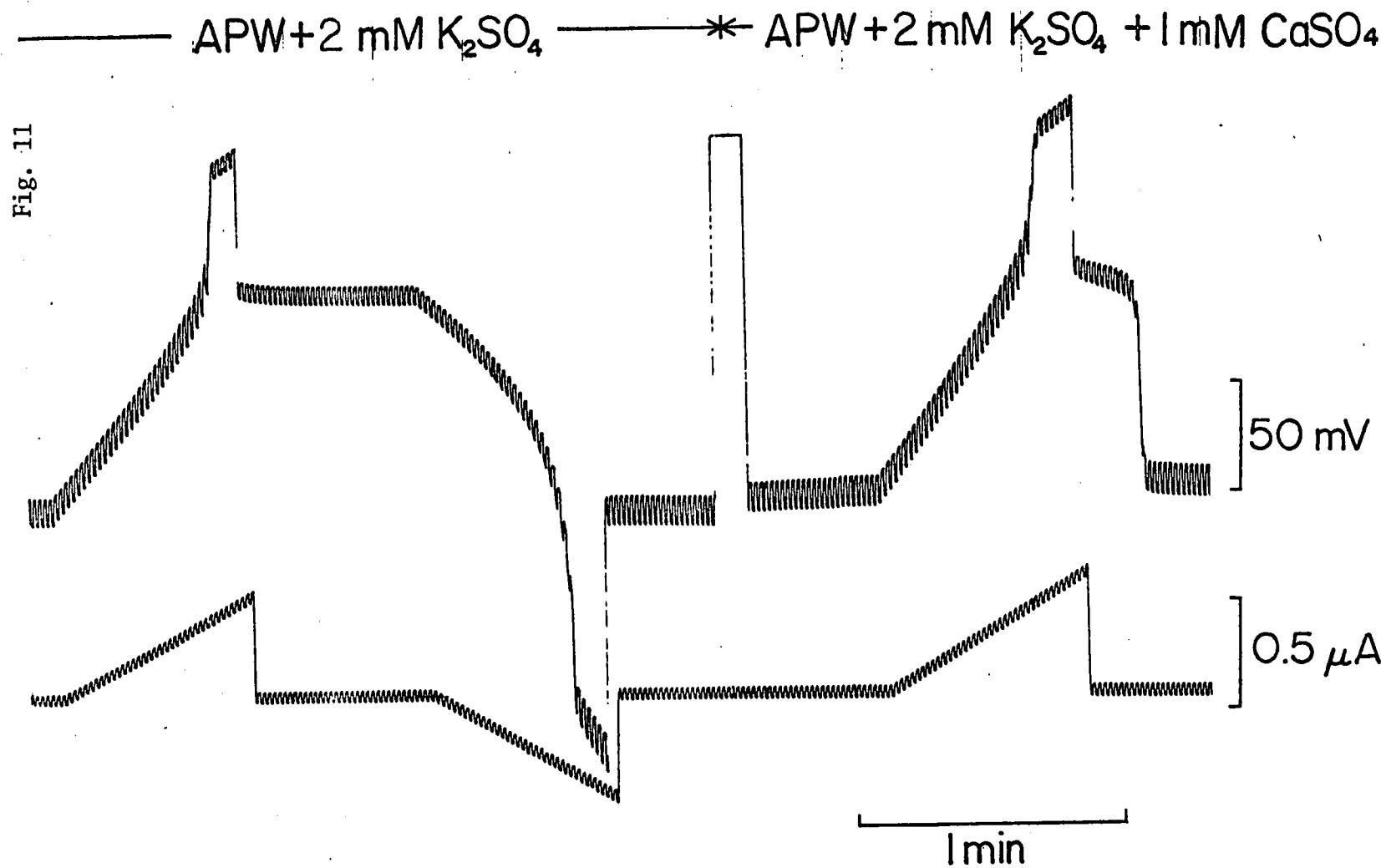


fig. 12

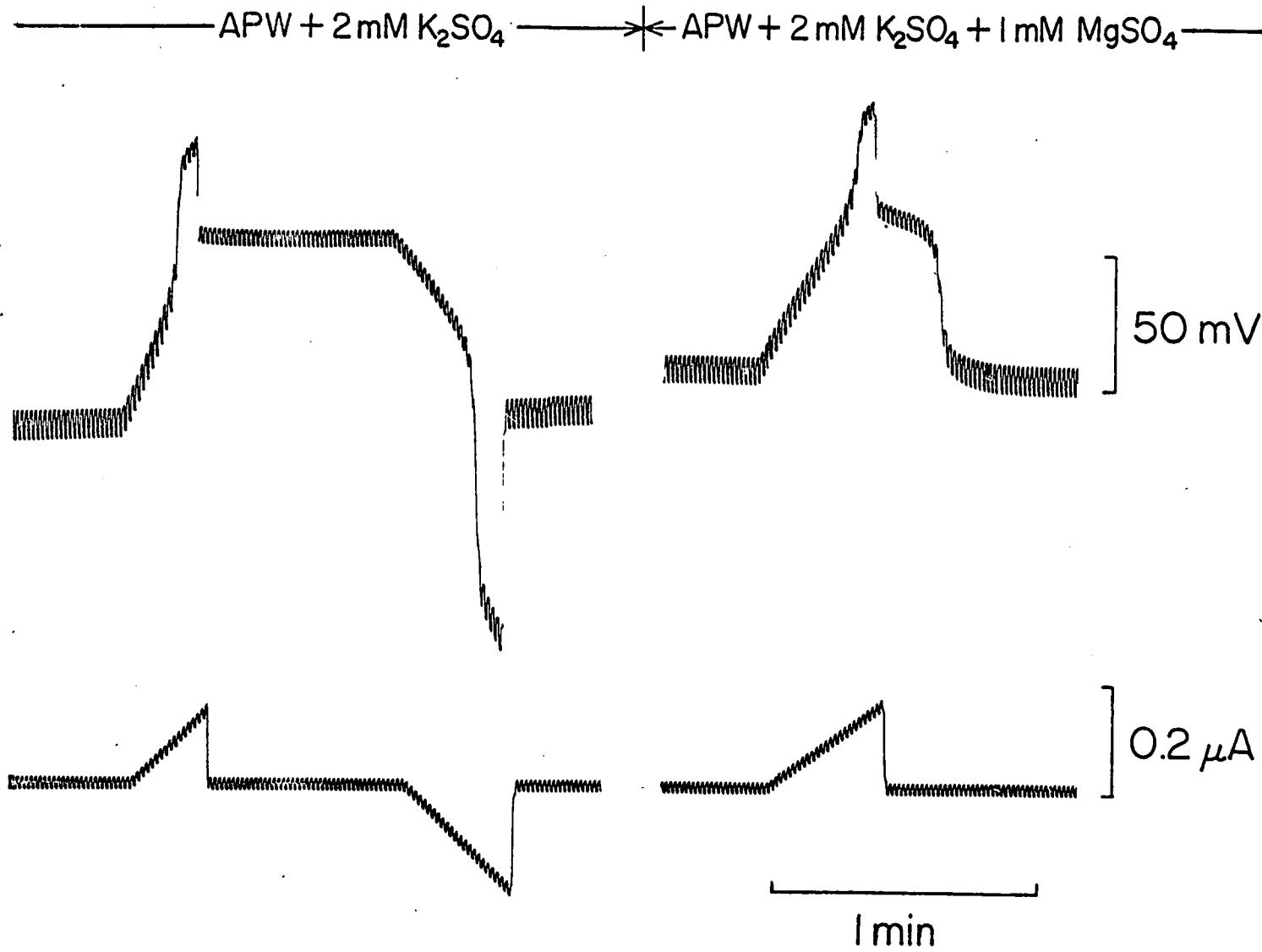


Fig. 13

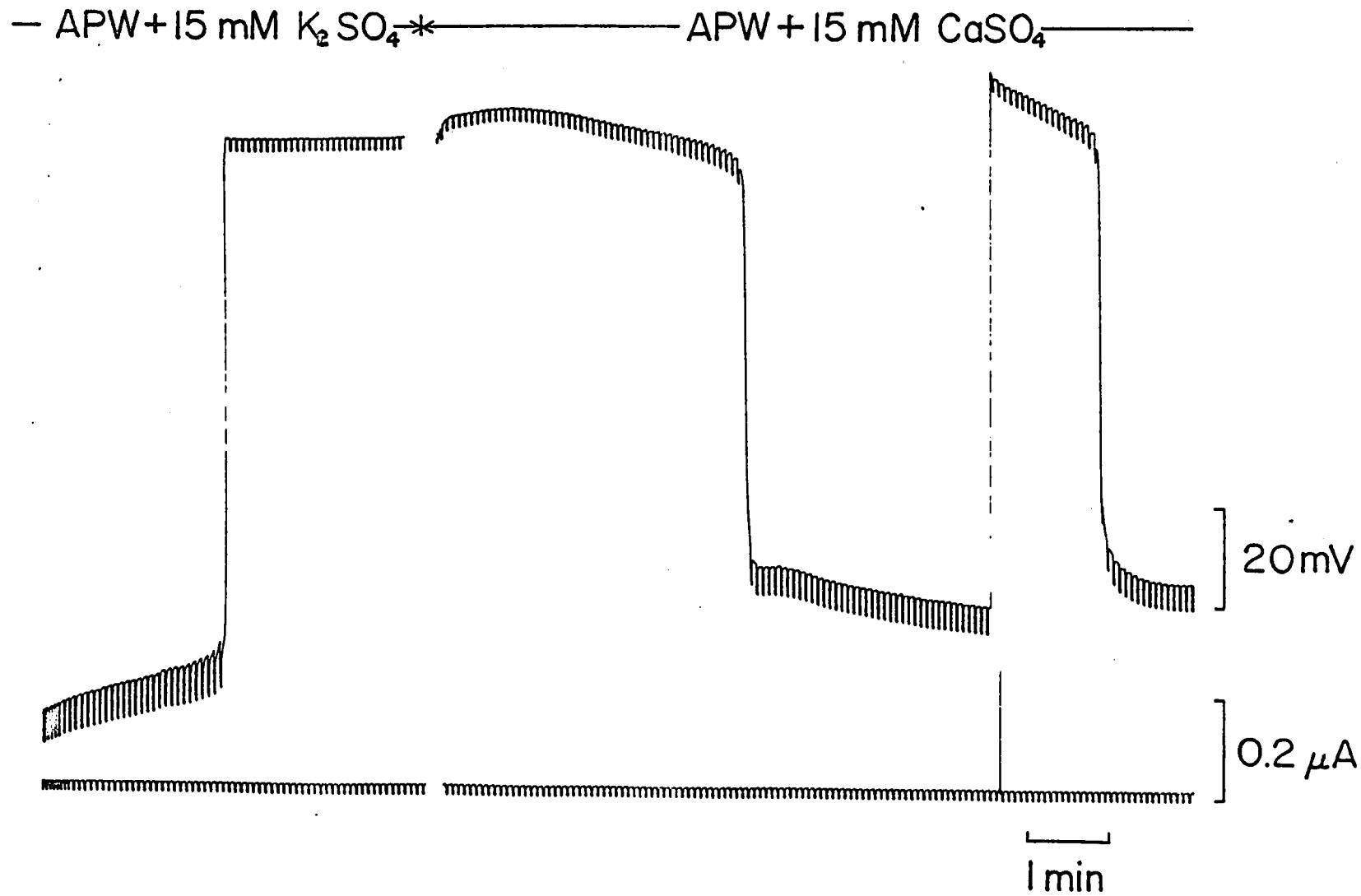
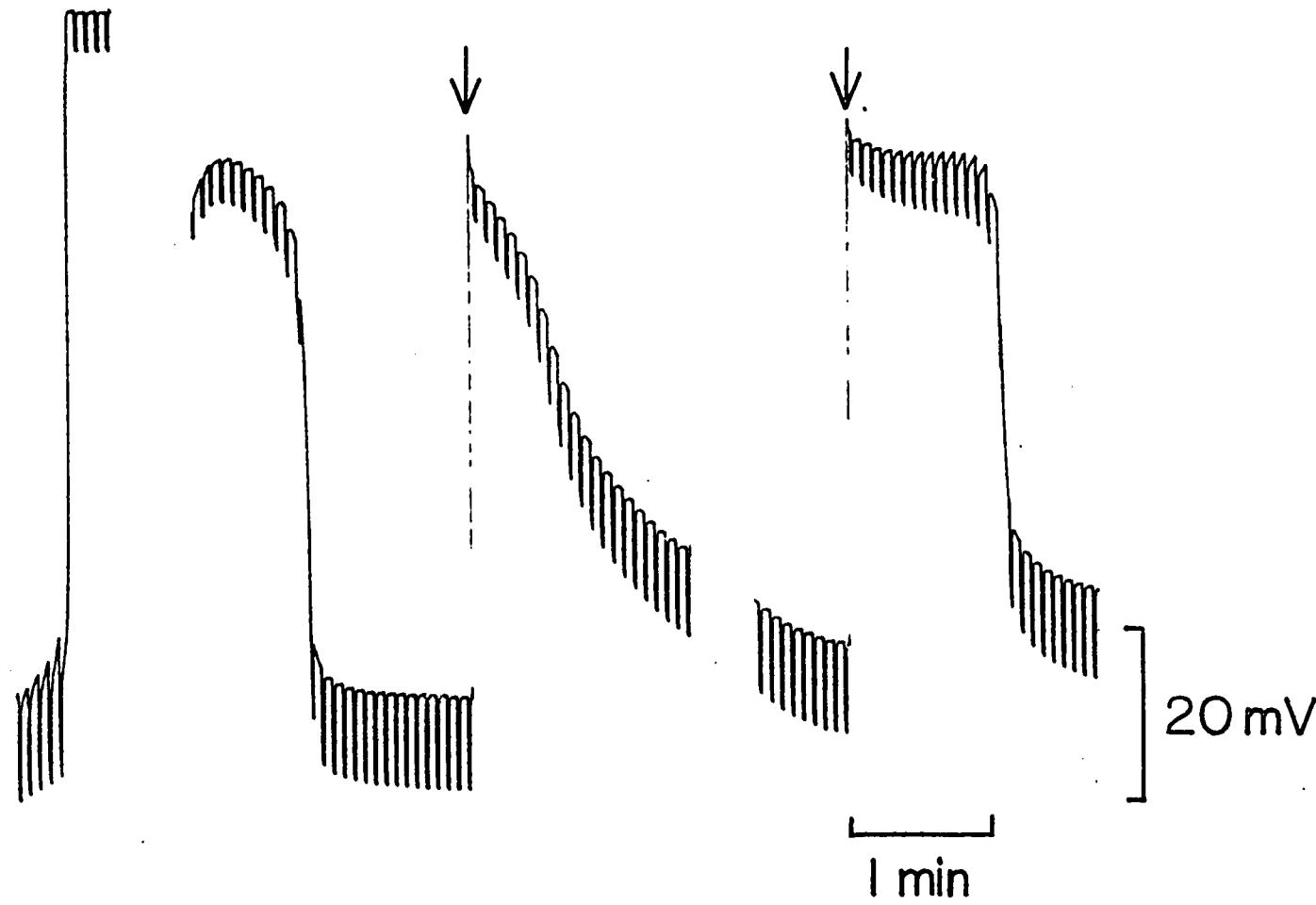


Fig. 14

APW +  
15 mM  $K_2SO_4$   APW + 5 mM  $MgSO_4$   APW + 5 mM  $CaSO_4$



## Chapter II

Control of membrane potential and excitability of Chara  
with ATP and  $Mg^{2+}$

### Abbreviations

ATP, adenosine-5'-triphosphoric acid; ADP, adenosine-5'-diphosphoric acid; AMP, adenosine-5'-monophosphoric acid; c-AMP, adenosine 3', 5'-cyclic monophosphoric acid; EGTA, ethyleneglycol-bis-( $\beta$ -aminoethyl ether) N, N'-tetra-acetic acid; EDTA, ethylene diamine tetraacetic acid; CyDTA, 1, 2-cyclohexane diamine N, N'-tetraacetic acid

## Summary

Electric characteristics of internodal Chara australis cells, from which the tonoplast had been removed by vacuolar perfusion with media containing EGTA, were studied in relation to intracellular concentrations of ATP and  $Mg^{2+}$  using the ordinary microelectrode method and the open-vacuole method developed by Tazawa, Kikuyama and Nakagawa (1975). The concentration of ATP was decreased by introducing hexokinase and glucose into the cell and that of  $Mg^{2+}$  by introducing EDTA or CyDTA. The membrane potential decrease and the membrane resistance increase were both significant when the ATP or  $Mg^{2+}$  concentration was decreased. An ATP-dependent membrane potential was also found in other species of Characeae, Nitella axilliformis and N. pulchella. Excitability of the membrane was also completely lost by reducing the ATP or  $Mg^{2+}$  concentration. Both membrane potential and excitability were recovered by introducing ATP or  $Mg^{2+}$  into ATP- or  $Mg^{2+}$ -depleted cells.

The time course of membrane potential recovery was followed by the open-vacuole method. Recovery began as soon as intracellular perfusion with medium containing ATP and  $Mg^{2+}$  was started. Reversible transition of the membrane potential between polarized and depolarized levels by controlling the intracellular concentration of ATP or  $Mg^{2+}$  could be repeated many times by the open-vacuole method, when the excitability was suppressed by addition of  $Pb^{2+}$ .

to the external medium.

The ineffectiveness of an ATP analog, AMP-PNP and the synergism of ATP and  $Mg^{2+}$  in maintaining the membrane potential and excitability strongly suggest that ATP act via its hydrolysis by  $Mg^{2+}$ -activated ATPase. The passive nature of the membrane, as judged from responses of the membrane potential to changes of the external  $K^+$  concentration, was not altered by lowering the ATP concentration in the cell. The mechanism of membrane potential generation dependent on ATP is discussed on the basis of an electrogenic ion pump. Involvement of the membrane potential generated by the ion pump in the action potential is also discussed.

### II-1. Introduction

Close correlation of the membrane potential with the metabolic activities of cells has been reported for many plant materials and the involvement of an ion pump in generating the membrane potential has been suggested (cf. Slayman, 1975). The membrane potential was found to be closely related to the intracellular level of ATP in Neurospora (Slayman, Long & Lu, 1973). In such studies, the intracellular ATP concentration was controlled by metabolic inhibitors. Therefore, the membrane potential can not be unequivocally said to be directly dependent on ATP. Direct control of ATP concentration without using inhibitors is necessary to answer the question of

whether or not ATP is the direct source of energy for the electrogenic ion pump. The intracellular perfusion technique fulfills this requirement and also provides further advantages for studying the mechanism of membrane potential generation, since ion species, including  $H^+$ , and their concentrations inside the cell can be modified easily.

Vacuolar perfusion of Characeae cells allows free adjustment of the cell sap composition (Tazawa, 1964). This technique has been used to intensively study tonoplast responses to changes in ionic compositions of characean cell sap (Tazawa & Kishimoto, 1964; Kishimoto, 1965; Kikuyama & Tazawa, 1976; 1977). Media containing  $Ca^{2+}$  which stabilize the tonoplast were used as artificial cell saps. Recently, Tazawa, Kikuyama and Shimmen (1976) succeeded in removing the tonoplast by introducing solutions containing EGTA, a  $Ca^{2+}$  chelator, into the vacuole to allow modification of the cytoplasm composition. The effects of modifying the ionic composition of the cytoplasm on excitation of the plasmalemma of Chara cells have been studied in detail (Shimmen, Kikuyama & Tazawa, 1976 b) and the presence of two stable potential states in the plasmalemma has been reported (Shimmen, Kikuyama & Tazawa, 1976 a)

A normal membrane potential (-165 mV) is maintained (Shimmen et al., 1976 a) in tonoplast free cells in which the intracellular ATP concentrations are about 1/10 the normal level (Tazawa et al., 1976). However, extreme lowering of the ATP concentration results in very low membrane potentials and loss of light-induced potential changes

(Kikuyama, Fujii, Hayama & Tazawa, unpublished). These results suggested that the role of the intracellular ATP level in generating the membrane potential should be studied. This study showed that the excitability of the Chara membrane is clearly dependent on the ATP concentration, cytoplasmic  $Mg^{2+}$  is necessary for maintaining both the membrane potential and the excitability, and  $Mg^{2+}$ -dependent ATPase seems to have a role in electogenesis.

## II-2. Materials and Methods

Internodal cells of Chara australis cultured outdoors were isolated from neighboring cells and stored in pond water at least overnight before use.

The external medium was artificial pond water (APW) containing 0.1 mM each of KC1, NaCl and CaCl<sub>2</sub>. Chemicals were dissolved in APW. Standard media for intracellular perfusion are shown in Table 1.

The membrane potential ( $E_m$ ) was measured by the conventional microelectrode method. The cell sap of the internodal cell was replaced with an artificial solution by vacuolar perfusion (Tazawa et al., 1976). The osmolarities of the perfusion media were made equal to or slightly higher than the osmotic values of the cells which were about 0.3 M sorbitol equivalent. After replacement of the cell sap with the artificial solution, both cell ends were ligated with strips of polyester thread. In tonoplast-free cells,

the  $E_m$  values thus measured represents the potential difference across the plasmalemma.

Excitability of the membrane was judged by the occurrence of the so-called N-shaped voltage-current (I-V) relationship on application of ramp-shaped depolarization by the voltage-clamp technique (Ohkawa & Kishimoto, 1977).

$E_m$  during internal perfusion was measured by the open-vacuole method (Tazawa et al., 1975). The cell (Cha) was placed on a polyacrylate vessel having three chambers, then both cell ends were cut (Fig. 1). Chambers A and C were filled with perfusion media and chamber B was filled with isotonic APW (iAPW), whose osmolarity was adjusted with sorbitol. When another chemical was added to iAPW, the osmotic value was adjusted by decreasing the sorbitol concentration.  $E_m$  of the cell part in chamber B was measured by measuring the potential difference between chambers B and C. The electric current for stimulation or measurement of the membrane resistance ( $R_m$ ) was applied between chamber B and chambers A and C through the Ag-AgCl wire. When valve V was open, there was no flow of the perfusion medium through the cell, since the water level between chambers A and C was same. The cell interior was perfused with the solution in chamber A or C by creating a difference in the water level between chambers A and C after closing V.

Loss of the tonoplast was confirmed by the appearance of endoplasmic fragments or masses in the original vacuolar space. Absence of the boundary between the endoplasm and the vacuole also indicated loss of the tonoplast.

The present paper uses the term "internal cell space" for the whole space inside the plasmalemma. This internal cell space after loss of the tonoplast is composed of an outermost cytoplasm with chloroplasts, endoplasm, and a mixture of endoplasm and perfusion medium. Concentrations of diffusible substances in the gel cytoplasm in direct contact with the plasmalemma were assumed to be equal to those of the whole internal cell space.

Electrical potential and current were recorded with a pen-writing recorder.

Experiments were done at room temperature (20-25°C) under dim light (about 100 lux).

Average values of  $E_m$  and  $R_m$  are shown with  $\pm$  SEM and the number (n) of cells used.

### II-3. Results

#### II-3-1. Effects of depletion of cytoplasmic ATP and $Mg^{2+}$ on membrane potential and excitability

The cell sap of Chara internodal cells was replaced with EGTA, EDTA or hexokinase medium (Table 1). Both opened cell ends were closed by ligation with strips of polyester thread.  $E_m$  and  $R_m$  of the cells in APW were measured by the microelectrode method after disintegration of the tonoplast. In previous studies (Tazawa et al., 1976; Shimmen et al., 1976 a), the cell sap of the Chara internode was replaced with EGTA medium and electric characteristics of the

plasmalemma were studied after disintegration of the tonoplast. The cells possessed high resting potential and generated action potential on electric stimulation. The cell also showed a light-induced potential change (Kikuyama et al., unpublished). Thus the cell can maintain important characteristics of the plasmalemma even after loss of the tonoplast. Since EGTA binds  $\text{Ca}^{2+}$  strongly, the concentrations of  $\text{Ca}^{2+}$  in the cytoplasm are estimated to be very low ( $2.9 \times 10^{-8}$  M; Shimmen et al., 1976 a). This indicates that a very low cytoplasmic  $\text{Ca}^{2+}$  concentration does not affect the normal functioning of the plasmalemma.

To lower the free  $\text{Mg}^{2+}$  concentration in the cytoplasm, EDTA was used instead of EGTA in the perfusion medium, since it binds both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . When the perfusion medium contained hexokinase and glucose, the cytoplasmic ATP concentration decrease after disintegration of the tonoplast, reaction since the enzyme  $\wedge$  proceeds preferentially to convert ATP and glucose into ADP and glucose-6-phosphate. After loss of the tonoplast, the internal ATP concentration ( $\text{ATP}_i$ ) was 40-50  $\mu\text{M}$  in cells perfused with the EGTA medium and 1  $\mu\text{M}$  or less in cells perfused with the hexokinase medium (kikuyama et al., unpublished).

Table 2 shows membrane potentials and membrane resistances of cells containing EGTA, EDTA and hexokinase media. The decrease in ( $\text{ATP}_i$ ) of cells containing hexokinase medium resulted in a conspicuous decrease in  $E_m$  and a significant increase in  $R_m$ . The membrane depolarization was so large that  $E_m$  dropped to about half that of cells containing

EGTA medium with about 50  $\mu\text{M}$  (ATP)<sub>i</sub>. The  $R_m$  of cells of low (ATP)<sub>i</sub> increased to twice that of cells containing EGTA medium. The same tendency was observed in cells with EDTA medium, although  $E_m$  decrease and  $R_m$  increase were less conspicuous than in the case of the hexokinase medium. When EDTA meium was used, the free  $\text{Mg}^{2+}$  concentration in the cytoplasm after loss of the tonoplast was calculated to be  $3.6 \times 10^{-7}$  M. Apparent association constants used for calculation were  $2.3 \times 10^5 \text{ M}^{-1}$  for  $\text{Mg}\cdot\text{EDTA}$  and  $2.3 \times 10^7 \text{ M}^{-1}$  for  $\text{Ca}\cdot\text{EDTA}$  at pH 7.0. Concentrations adopted for the divalent cations in the cytoplasm were 3.6 mM for Mg (Williamson, 1975) and 3 mM for Ca (Tazawa et al., 1976). Furthermore, it was assumed that both Mg and Ca were dispersed in the whole internal cell space after loss of the tonoplast and the cytoplasm volume was 1/10 of the total cell volume.

In parallel with changes in the membrane potential and membrane resistance, excitability was lost in cells perfused with EDTA or hexokinase medium. The cell containing EGTA medium showed the typical N-shaped I-V relationship when ramp depolarization was applied to the membrane (Fig. 2a). Cells containing EDTA or hexokinase medium did not show any sign of the N-shaped I-V relationship characteristic of the excitable membrane but did show a significant rectification (Figs. 2b and c). These results suggest that both  $\text{Mg}^{2+}$  and ATP are essential for normal functions of the membrane, such as maintaining a high resting membrane potential and generating the action potential.

### II-3-2. Reversibility of effects of $[Mg^{2+}]_i$ and $[ATP]_i$

Next, reversibility of the effects of  $[Mg]_i$  and  $[ATP]_i$  on  $E_m$  and  $R_m$  was tested. After the cell sap had been replaced with EDTA or hexokinase medium, the cell was closed by ligating both ends with strips of polyester thread and kept in APW for 30-60 min. During this period, the tonoplast disintegrated and ATP and  $Mg^{2+}$  of the cytoplasm were assumed to be dispersed in the whole internal space. The concentration of  $Mg^{2+}$  or ATP was decreased by the action of EDTA or hexokinase. The decrease in  $[Mg^{2+}]_i$  or  $[ATP]_i$  was checked by observing the cessation of cytoplasmic streaming which requires both  $Mg^{2+}$  and ATP (Williamson, 1975; Tazawa et al., 1976). Next, the cell interior was perfused again with  $Mg\cdot ATP$  medium containing 6 mM  $Mg^{2+}$  and 1 mM ATP (Table 1) and both opened cell ends were closed as before.  $E_m$  and  $R_m$  were measured by the micro-electrode method and the excitability was tested by checking the N-shaped I-V relationship. As shown in Table 3, the plasmalemma was repolarized markedly and  $R_m$  decreased significantly when  $Mg^{2+}$  was introduced into  $Mg^{2+}$ -depleted cells and ATP into ATP-depleted cells. In addition, the N-shaped I-V relationship became observable again when  $Mg\cdot ATP$  was introduced into  $Mg^{2+}$ -depleted cells (Fig. 3a) or ATP-depleted cells (Fig. 3b).

### II-3-3. Time course of recovery of membrane potential with $Mg\cdot ATP$

In the above experiments on the recovery of the membrane

potential, at least 5 min lapsed before the  $E_m$  measurement began after the first contact of the membrane with Mg·ATP medium, due to the procedures necessary between perfusion and microelectrode insertion. To observe the time course of recovery of the membrane potential by  $Mg^{2+}$  or ATP, the open-vacuole method was employed, since  $E_m$  could be followed during perfusion.

In the experiment shown in Fig. 4, the cell containing EDTA medium was first incubated in APW. After cytoplasmic streaming had ceased, the cell was placed on the perfusion vessel used for the open-vacuole method. Chambers A and C (Fig. 1) were filled with Mg·ATP medium and chamber B was filled with iAPW. Before internal perfusion with Mg·ATP medium was begun, the internal concentration of  $Mg^{2+}$  was as low as  $3.6 \times 10^{-7} M$  and  $E_m$  was at the depolarized level. As soon as intracellular perfusion was started,  $E_m$  shifted in the more negative direction and attained a steady value within 1.5 min. When the  $E_m$  changes had ceased, perfusion was stopped.  $E_m$  stayed at the polarized level and when the cell was stimulated with the ramp outward current, an action potential of rectangular shape was elicited.

In the next experiment (Fig. 5), the cell containing hexokinase medium was prepared and kept in APW. After cytoplasmic streaming had ceased,  $E_m$  was measured by the open-vacuole method. Chambers A, C and B were filled with Mg·ATP medium, EGTA medium and iAPW, respectively. Soon after perfusion with Mg·ATP medium had started,  $E_m$  began to shift in the more negative direction. Perfusion was stopped

when  $E_m$  had attained a steady value. Next, perfusion was done from C to A, i.e. Mg-ATP medium in the cell was replaced with EGTA medium containing no ATP. In this case,  $E_m$  began to change in the less negative direction. Since the only difference in composition between the Mg-ATP and EGTA media was the presence or absence of ATP, the membrane depolarization observed should be caused by the decrease in ATP concentration in the cell. When  $E_m$  reached the threshold level, the action potential was elicited. The plasmalemma depolarized rapidly and stayed at the depolarized level which is more positive than that before the perfusion with Mg-ATP medium. The membrane resistance decreased significantly (Fig. 5). In such a case,  $E_m$  and  $R_m$  did not recover with subsequent perfusion with Mg-ATP medium (data not shown). The inward current which sometimes effectively abolished the membrane depolarization or transferred  $E_m$  in the excited state to the resting state (Shimmen et al., 1976a) also could not repolarize the membrane.

#### II-3-4. Control of the membrane potential by intracellular ATP or $Mg^{2+}$

During perfusion with ATP- or  $Mg^{2+}$ -depleted medium, action potentials were often elicited by unknown reasons. Since in this case the membrane was irreversibly depolarized, generation of the action potential had to be avoided. The excitability of Nitella can be lowered simply by adding  $Pb^{2+}$  to the external medium (Kamitsubo, 1976). In the open-vacuole method, chamber B was filled with iAPW to which

5 mM Pb<sup>2+</sup> as acetate or nitrate had been added. Chamber A was filled with Mg-ATP medium and chamber C was with EGTA medium. The cell was placed on the open-vacuole vessel and both of its ends were cut. The cell sap was replaced with Mg-ATP medium. When the tonoplast had been removed, the membrane was in the polarized state and  $E_m$  was high, since the cell contained enough Mg<sup>2+</sup> and ATP (Fig. 6). When the cell interior was perfused with EGTA medium,  $E_m$  changed in the less negative direction without eliciting an action potential and stayed at the depolarized level. The depolarization caused by lowering  $[ATP]_i$  is different from that caused by excitation, since  $R_m$  increased in the former but decreased conspicuously in the latter (Fig. 5). When the cell was perfused with Mg-ATP medium,  $E_m$  was recovered to its original level and stayed at the polarized level. This reversible transition could be repeated many times.

In another experiment, chambers A, C and B were filled with Mg-ATP medium, CyDTA medium containing 1 mM ATP and iAPW containing 5 mM Pb-acetate, respectively. CyDTA was used instead of EDTA, because its affinity to Mg<sup>2+</sup> is stronger than that of EDTA. First, the cell sap was replaced with Mg-ATP medium. After loss of the tonoplast, high  $E_m$  values were recorded (Fig. 7). Next, the cell interior was perfused with CyDTA medium containing 1 mM ATP. During the perfusion,  $E_m$  began to change in the positive direction and stayed at the depolarized level after perfusion was stopped.  $E_m$  was recovered with a subsequent perfusion with Mg-ATP medium. These transitions of  $E_m$  also could be repeated many times.

The results indicate that both cytoplasmic  $Mg^{2+}$  and ATP are necessary to keep the membrane polarized.

Next, the question of whether the effects of  $Mg^{2+}$  and ATP depletions were cooperative or not was studied. Chambers A, C and B were filled with Mg·ATP medium, CyDTA medium containing 1 mM ATP and iAPW containing 5 mM Pb-acetate, respectively. When the cell sap was replaced with Mg·ATP medium,  $E_m$  was high after disintegration of the tonoplast. Next, when the same cell was perfused with the CyDTA medium containing 1 mM ATP, the membrane was depolarized instantly, since the intracellular  $Mg^{2+}$  concentration was almost zero (Fig. 8). The decreased  $E_m$  recovered to the former polarized level upon perfusion with Mg·ATP medium. Next, the solution in chamber C was replaced with CyDTA medium without ATP and internal perfusion was done with this medium. As the result, the plasmalemma was depolarized, since both  $Mg^{2+}$  and ATP concentrations in the cytoplasm decreased. The depolarized membrane potential could be repolarized again by intracellular perfusion with Mg·ATP medium. The membrane depolarization caused by introduction of CyDTA medium not being influenced by the presence or absence of ATP suggests that  $Mg^{2+}$  and ATP are synergistic.

#### II-3-5. Effects of other adenine nucleotides and phosphorous compounds

Whether hydrolysis of ATP is necessary for maintaining the membrane potential and excitability, i.e., involvement of

ATPase, was examined. AMP-PNP, an ATP analog which is not hydrolyzed by typical  $\beta$ - $\gamma$ ATPase but binds its active site (Yount, Ojala & Babcock, 1971), was used instead of ATP. Chambers A, C and B in Fig. 1 were filled with Mg-ATP medium, EGTA medium and iAPW containing 5 mM  $\text{Pb}(\text{NO}_3)_2$ , respectively. Since in winter the resting potential was low in APW, the pH of iAPW containing 5 mM  $\text{Pb}(\text{NO}_3)_2$  was increased from 5.6 to 7.5 by adding 5 mM Tris- $\text{H}_2\text{SO}_4$  buffer (Shimmen et al., 1976 b). First, the cell sap was replaced with Mg-ATP medium.  $E_m$  was high after disintegration of the tonoplast (Fig. 9). When the cell was perfused with EGTA medium containing  $\text{Mg}^{2+}$  but no ATP, the membrane was depolarized.  $E_m$  was recovered by intracellular perfusion with Mg-ATP medium. Transition of  $E_m$  between polarized and depolarized levels could be repeated. Next, the solution in chamber C was replaced with AMP-PNP medium of the same composition as Mg-ATP medium, except that 1 mM AMP-PNP was used instead of 1 mM ATP. When the cell containing EGTA medium was perfused with AMP-PNP medium, only a very small repolarization and increase in the membrane resistance were observed. After perfusion with AMP-PNP medium had been stopped, Mg-ATP medium was again introduced into the cell by reversing the direction of the perfusion.  $E_m$  recovered quickly.

In another experiment, the effect of a higher concentration of AMP-PNP was tested by the microelectrode method. First, the cell sap was replaced with EGTA medium, and the cell was kept in APW. After disintegration of the tonoplast, the cell was perfused again with a solution

containing 10 mM AMP-PNP; the composition of this medium was the same as that of the Mg-ATP medium, except that 10 mM AMP-PNP instead of 1 mM ATP and 11 mM  $MgCl_2$  instead of 6 mM  $MgCl_2$  were used. Since AMP-PNP was used as the tetralithium salt, the concentration of  $Li^+$  in the perfusion medium was 40 mM.  $Li^+$  at this concentration probably does not affect this system because the excitability and the membrane potential were maintained even in the presence of 100 mM  $Li^+$  in the cytoplasm (Shimmen et al., 1976 b). After perfusion with the 10 mM AMP-PNP medium, both cell ends were closed by ligation and  $E_m$  was measured by the microelectrode method. With such a high concentration of AMP-PNP,  $E_m$  was low ( $-80 \pm 5$  mV,  $n = 5$ ) and  $R_m$  was high ( $93 \pm 17$   $k\Omega cm^2$ ,  $n = 5$ ). Furthermore, excitability could not be detected. Thus, AMP-PNP clearly can not replace ATP in maintaining high  $E_m$  values and excitability.

Next, the abilities of AMP and ADP to maintain the membrane potential were tested by the open-vacuole method (Fig. 10). First, the cell contained Mg-ATP medium and therefore  $E_m$  was high. The AMP medium composition was the same as that of the Mg-ATP medium, except that 1 mM AMP was used instead of 1 mM ATP. The plasmalemma was depolarized and remained in this state after perfusion had stopped.  $E_m$  was recovered by perfusion with Mg-ATP medium. Next, ADP medium containing 1 mM ADP instead of ATP was introduced into the cell. The plasmalemma was relatively slowly depolarized. After the perfusion had been stopped  $E_m$  changed slowly in the negative direction, but did not attain the original potential level. In some cells  $E_m$  stayed at the depolarized level even after

perfusion with ADP medium had ceased.

In another experiment, the effect of ADP was studied by the microelectrode method. The cell was perfused with ADP medium after disintegration of the tonoplast with EGTA medium. Both cell ends were closed by ligation. Measurements of  $E_m$  and  $R_m$  by the microelectrode method gave  $-166 \pm 17$  mV ( $n = 3$ ) for  $E_m$  and  $40 \pm 13$   $\text{k}\Omega\text{cm}^2$  ( $n = 3$ ) for  $R_m$ . The N-shaped I-V relationship showing the presence of excitability was observed.

The polarized membrane potential level could not be maintained by 1 mM c-AMP, 1 mM phosphate (mixture of  $\text{K}_2\text{HPO}_4$  and  $\text{KH}_2\text{PO}_4$ ) or 1 mM pyrophosphate (data not shown).

II-3-6. Response of the membrane to  $[\text{K}^+]_o$  in the resting, excited and low  $[\text{ATP}]_i$  states

The effect of  $\text{K}^+$  in the external medium on the membrane potential of cells perfused with the EGTA or hexokinase medium was examined. After replacement of the cell sap with the perfusion medium, the cell was ligated and  $E_m$  was measured by the microelectrode method. After disintegration of the tonoplast,  $[\text{K}^+]_o$  was increased by adding  $\text{K}_2\text{SO}_4$  in APW and the membrane potentials were measured at various  $[\text{K}^+]_o$  (Fig. 11). When the cells were perfused with EGTA medium, both the resting and the peak action potentials were measured. Fig. 11 shows that the slope of the curve showing the response of the resting potential to  $[\text{K}^+]_o$  of the cells containing hexokinase medium was similar to that of the cells containing EGTA medium. However,  $E_m$  of cells containing EGTA medium

showed different sensitivities to  $[K^+]_o$  for the excited and resting states.

#### II-3-7. ATP dependency of $E_m$ in other Characeae species

Preliminary experiments to see the effect of ATP on  $E_m$  in other Characeae species revealed that essentially the same ATP-dependent transition phenomenon of  $E_m$  also occurred in Nitella axillaris and N. pulchella, although the magnitude of the ATP-dependent membrane potential was smaller (-40 - -50 mV) than in C. australis (Fig. 6).

### II-4. Discussion

#### II-4-1. Requirement of both ATP and $Mg^{2+}$ for generation of membrane potential

The present experiment in which the cytoplasmic concentrations of ATP and  $Mg^{2+}$  were changed by internal perfusion, clearly demonstrated that both ATP and  $Mg^{2+}$  are essential for maintaining the normal high membrane potential. ATP and  $Mg^{2+}$  show synergism which is so strong that  $E_m$  in the absence of one agent has almost the same value as in the absence of both.  $E_m$  values of cells with vacuoles perfused once with EDTA medium were higher than those of the hexokinase-treated (ATP-depleted) cells (Table 2). This may be explained by assuming that the  $Mg^{2+}$  concentration in EDTA-treated cells, which

was calculated to be  $3.2 \times 10^{-7}$  M, was not low enough to completely inhibit the Mg-ATP-dependent part of the membrane potential.

AMP-PNP, which is known to bind with active sites of the myosin ATPase (Yount et al., 1971) and mitochondrial ATPase (Melnick, Tavares de Dousa, Maguire & Packer, 1975), competes for the sites with ATP, but is not hydrolyzed by the ATPase (Yount et al., 1971). AMP-PNP is as effective as ATP for generating the membrane potential of the chromaffin granule (Pollard, Zinder, Hoffman & Nikodejenic, 1976). This means that hydrolysis of ATP is not necessary in this organell. On the contrary, the fact that in Chara ATP can not be substituted for by AMP-PNP to maintain high  $E_m$  levels (Fig. 9), suggests that ATP in Chara membrane acts through its hydrolysis. ATP can be partially replaced by ADP. This may be explained by assuming the presence of adenylylkinase in the cell. Williamson (1975) also suggested the presence of adenylylkinase in Chara corallina.

Affinity of ATP for this ATPase seems very high compared with that of the membrane ATPase of Neurospora, the presence of which was assumed by Slayman et al. (1973). ATP concentrations in the cytoplasm of normal Chara cells are around 0.5 mM (Hatano & Nakajima, 1963; Kikuyama, et al., unpublished). If all the ATP originally present in the cytoplasm disperses homogeneously in the whole internal cell space after disintegration of the tonoplast, the ATP concentration in the cell become 0.05 mM. As reported in the previous paper (Shimmen

et al., 1976 a), the membrane potential of cells perfused with EGTA medium is almost equal to that of normal cells. This means that the ATP effect of generating the membrane potential is saturated even at a low ATP concentration such as 0.05 mM.

#### II-4-2. ATP in relation to an electrogenic ion pump

Although no evidence was found in the present study to show the mechanism of the generation of ATP-dependent membrane potential, one plausible mechanism is an electrogenic ion pump. Kitasato (1968) assumed the presence of an electrogenic  $H^+$  pump to explain the discrepancy between the membrane potential observed in cells of Nitella clavata and the calculated diffusion potential involving  $K^+$ ,  $Na^+$ , and  $H^+$ . Many data suggesting the existence of an electrogenic  $H^+$  extrusion pump have been accumulated in algal (Spanswick, 1972; Saito & Senda, 1973 a; b; 1974) and higher plant cells (Bentrup, Gratz & Unbehauen, 1973). Hyphae of the fungus Neurospora excretes  $H^+$  actively (Slayman, 1970; Slayman & Slayman, 1968) and the membrane potential is a function of the concentration of ATP in the cytoplasm (Slayman et al., 1973). Slayman et al. deduced that ATP is the direct energy source for the electrogenic ion pump, from an experiment using KCN which blocks respiration and promptly reduces the intracellular ATP level. However, the inhibitors may not only lower the ATP level but also affect either the pump directly or other processes

which control pump activity. In the present experiment using the open-vacuole method, no inhibitor was applied to the cell with the intracellular ATP concentration being changed directly using media with or without ATP.

II-4-3. Dependencies of passive and active components of  $E_m$  on  $[K^+]_0$

Using the equivalent circuit (Fig. 12) proposed by Slayman et al. (1973), we tried to explain the ATP-dependent membrane potential of Chara. This model contains in parallel an ion pump as an ideal current source and ion diffusion regimes composed of an electromotive force,  $(E_m)_0$ , and resistance,  $R_m$ . From the equivalent circuit, the total membrane potential,  $E_m$ , is calculated by the following equation.

$$E_m = (E_m)_0 + E_a \quad (1)$$

$$E_a = i_p \cdot R_m \quad (2)$$

$(E_m)_0$ ,  $E_a$ ,  $i_p$  and  $R_m$  are the passive membrane potential independent of ATP, the membrane potential supported by the electrogenic pump, the current supplied by the pump, and the membrane resistance, respectively.  $E_m$  and  $(E_m)_0$  are assumed to be the membrane potential of the cell perfused with EGTA medium and that of the cell perfused with hexokinase medium, respectively. As shown in Fig. 11 and 12,  $E_a$ , which is obtained by subtracting  $(E_m)_0$  from

$E_m$ , is nearly constant for the range of  $[K^+]_o$  between 0.1 and 10 mM. This means that passive permeability of the plasmalemma to  $K^+$  seems to be unaltered even when the ATP concentration is drastically changed. Similar results were reported for the responses to the change of  $[K^+]_o$  of the membrane potentials of  $NaN_3$ -treated and -untreated hyphae of Neurospora (Slayman, 1965) and dark-treated and illuminated Vallisneria leaf cells (Bentrup, Gatz & Unbehauen, 1973). The difference in the membrane potentials between azide-treated and -untreated cells or dark-treated and illuminated cells was interpreted as being caused mainly by an electrogenic ion pump. In Vallisneria, increase in permeability of the membrane to  $H^+$  due to illumination is also involved.

In Neurospora, the pump acts as an ideal current source and the magnitude of its current is independent of the level of  $E_m$  (Slayman & Slayman, 1975). In the case of Chara,  $i_p$  at various  $[K^+]_o$  can be calculated by the following equation:

$$i_p = \frac{E_m - (E_m)_0}{R_m} \quad (3)$$

Since the difference between  $E_m$  and  $(E_m)_0$  is nearly constant between 0.1 and 10 mM of  $[K^+]_o$  and  $R_m$  of the cell perfused with EGTA medium changes significantly according to  $[K^+]_o$ , the calculated  $i_p$  can not be constant for the change in  $[K^+]_o$  (Fig. 12). This means that  $i_p$  is dependent on either  $E_m$  or  $[K^+]_o$ . To know the dependency of  $i_p$  on  $E_m$ , it is necessary to measure  $i_p$  under various transmembrane voltages using voltage-clamp technique.

II-4-4. Effects of inhibitors and low temperature on  $R_m$  and  $E_m$

When cells of Nitella were treated with DNP,  $E_m$  was decreased and  $R_m$  was increased (Kitasato, 1968; Saito & Senda, 1973 b). As the direct dependence of  $E_m$  and  $R_m$  on ATP was demonstrated in this study, it is understandable that treatment with inhibitors causing a decrease in internal ATP concentration would result in low membrane potentials and high membrane resistances. A decrease in  $E_m$  and increase in  $R_m$  upon treatment of cells with low temperature were reported for Nitella translucens (Hogg, Williams & Johnston, 1968), Chara corallina, Griffithsia (Hope & Aschberber, 1970) and Chara australis (Kishimoto, 1972). In <sup>the</sup> light of the present results indicating strong participation of ATP in generation of  $E_m$ , the changes might be caused by lowering of the ATP level and/or inhibiting the activity of the putative membrane ATPase itself. Since the permeability of  $K^+$  into the Chara membrane does not seem to change with depletion of ATP as discussed <sup>above</sup>, the increase in  $R_m$  must be caused by the decrease in permeabilities of ions other than  $K^+$ . The membrane permeability of  $H^+$  does decrease in Vallisneria, whose electrogenic ion pump is inactive in darkness (Bentrup et al., 1973).

II-4-5. Excitability and ATP

Chara plasmalemma has two stable potential states, like the squid giant axon membrane, and these states

are controlled by the ratio between monovalent and divalent cations in the external medium (Shimmen et al., 1976 a). Dependency of the duration of the action potential on the cytoplasmic  $K^+$  concentration in Chara cells (Shimmen et al., 1976 b) basically agrees with data for squid nerve cells (Tasaki, Lerman & Watanabe, 1969). However, the fact that ATP and  $Mg^{2+}$  in the cytoplasm are necessary for excitation of the Chara plasmalemma strikingly contrasts with data for squid giant axons showing that continuous internal perfusion with media lacking ATP can maintain the excitability of the membrane and that  $Mg^{2+}$  is unfavorable for excitation (Tasaki, Watanabe & Takenaka, 1962).

In the case of Chara, low membrane potential may be responsible for the lack of excitability in ATP- or  $Mg^{2+}$ -depleted cells, since the membrane may already be at the excited state. When Nitella membrane was depolarized by adding high concentrations of  $K^+$  to the external medium, it showed hyperpolarizing response on application of inward current. Furthermore when the membrane which was polarized by applying constant inward current was stimulated by outward current, firing of action potential was observed (Ohkawa & Kishimoto, 1974). Similar experiments were carried to see whether ATP- or  $Mg^{2+}$ -depleted cells are at the excited state or not. Increasing the transmembrane potential by applying a ramp-shaped hyperpolarizing voltage change did not change the electromotive force in ATP- or  $Mg^{2+}$ -depleted cells (Figs. 2b and c). In addition, ATP-depleted cells whose transmembrane potentials

were fixed by the voltage-clamp technique at -200 mV, which is the  $E_m$  level of ATP-rich cells, did not show a change in the electromotive force during the ramp-shaped depolarization (data not shown). That the membrane in the ATP-depleted state differs in nature from the membrane in the excited state was further indicated by the fact that  $R_m$  in the former state was significantly higher than that in the latter state and also that the response of  $E_m$  to  $[K^+]_o$  in the former state was more sensitive than that in the latter state (Fig. 11).

The fact that AMP-PNP can not act as a substitute for sustaining excitability, suggests that the membrane excitability is also maintained by dissipating energy. Thus, hydrolysis of ATP via a  $Mg^{2+}$ -activated ATPase provides energy not only for the putative electrogenic ion pump but also for maintaining the membrane structure responsible for the excitability. ATP is known to be necessary for maintaining the membrane structure in human erythrocytes (Nakao, Nakao, Yamazoe & Yoshikawa, 1960; Quist & Roufagalis, 1976). From the present experiments, it is difficult to determine whether the ion pump itself is involved in the excitability or another ATPase is concerned with the maintenance of membrane excitability.

#### II-4-6. Changes in passive and active components of $E_m$ on excitation

After it became evident that in Chara membrane  $E_m$  in the resting state is composed of the passive  $((E_m)_0)$  and

active components, the question arises how  $(E_m)_0$  and  $E_a$  contribute to  $E_m$  in the excited state. The fact that the response of  $E_m$  to  $[K^+]_0$  in the excited state was different from that in the resting state clearly shows that the passive nature of the membrane changed during excitation. Changes in passive nature of the membrane are also clearly demonstrated by drastic increase in the permeability of the membrane to  $Cl^-$  (Mullins, 1962; Hope & Findlay, 1964; Mailman & Mullins, 1966; Oda, 1976). All these facts indicate that  $(E_m)_0$  which is represented by the well-known Goldmann equation should change during excitation.

In addition to the change in  $(E_m)_0$ ,  $E_a$  is also expected to change on excitation, since  $R_m$  decreases significantly (cf. Eq. 2). When cells of Chara australis containing EGTA medium were excited in APW supplemented with 4 mM  $K^+$ ,  $R_m$  decreased from  $32 \pm 3 \text{ k}\Omega\text{cm}^2$  ( $n = 11$ ) to  $2 \pm 0.4 \text{ k}\Omega\text{cm}^2$  ( $n = 10$ ). Then, even when the pump current ( $i_p$ ) is not affected by the excitation,  $E_a$  in the excited state should decrease to only 1/17 the value in the resting state. In order to decide whether or not the membrane excitation accompanies changes in ion pump activity, it is necessary to know experimentally the contribution of  $E_a$  to  $E_m$  under excitation.

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## Legends

Fig. 1. Polyacrylate vessel used for the open-vacuole method. Chambers A and C connected with rubber tubing (T) were filled with media for intracellular perfusion of the internodal cell (Cha). Chamber B was filled with iAPW or iAPW containing 5 mM  $Pb^{2+}$ . The membrane potential ( $E_m$ ) of the cell part in B was measured by measuring the potential difference between chambers B and C. Electric current for stimulation or measurement of the membrane resistance was applied between chamber B and chambers A and C through Ag-AgCl wire. Intenal perfusion occurred when the water level of one chamber was made higher than that of the other chamber after closing valve V.

Fig. 2. Voltage-current (I-V) relationship for tonoplast-free cells with cell saps replaced with EGTA medium (a), EDTA medium (b) or hexokinase medium (c).

Fig. 3. Recovery of excitability by introducing  $Mg^{2+}$  into  $Mg^{2+}$ -depleted cell containing EDTA medium (a) or ATP into ATP-depleted cell containing hexokinase medium (b).

Fig. 4. Membrane repolarization by introducing 1 mM

$Mg \cdot ATP$  into  $Mg^{2+}$ -depleted cell containing EDTA medium. The action potential was generated by electrical stimulus after repolarization.

Fig. 5. Repolarization of the once-depolarized membrane potential of the ATP-depleted cell by introducing 1 mM  $Mg \cdot ATP$  and depolarization by subsequent intracellular perfusion with EGTA medium. Notice that the cell was excited when  $E_m$  reached a threshold level.

Fig. 6. Reversible transition of  $E_m$  between polarized and depolarized levels controlled by internal ATP concentration.  $E_m$  was measured by the open-vacuole method. The external medium was iAPW containing 5 mM Pb-acetate. First, the cell sap was replaced with  $Mg \cdot ATP$  medium,  $E_m$  was high after disintegration of the tonoplast.  $E_m$  was depolarized by perfusion with EGTA medium and recovered by perfusion with  $Mg \cdot ATP$  medium. This transition could be repeated many times.

Fig. 7. Reversible transition of  $E_m$  between two levels by controlling intracellular  $Mg^{2+}$  concentration.  $E_m$  was measured by the open-vacuole method. The central chamber of the measuring vessel (Fig. 1) was filled with iAPW containing 5 mM Pb-acetate. Internal  $Mg^{2+}$  concentration was controlled by alternating perfusion with  $Mg \cdot ATP$  medium containing 6 mM  $Mg^{2+}$  and CyDTA medium containing 1 mM ATP.

Fig. 8. Comparison of the level of depolarization caused by depletion of both  $Mg^{2+}$  and ATP with that caused by depletion of  $Mg^{2+}$  alone.  $E_m$  was measured by the open-vacuole method. The central chamber of the measuring vessel (Fig. 1) was filled with iAPW containing 5 mM Pb-acetate.  $Mg^{2+}$  in the cell was depleted by perfusion with CyDTA medium containing 1 mM ATP while both  $Mg^{2+}$  and ATP were depleted by perfusion with CyDTA medium without ATP.

Fig. 9. Effect of AMP-PNP on  $E_m$ .  $E_m$  was measured by the open-vacuole method. The central chamber of the measuring vessel (Fig. 1) was filled with iAPW containing 5 mM  $Pb(NO_3)_2$  at pH 7.5. First, the cell sap was replaced with  $Mg\cdot ATP$  medium. Transition of  $E_m$  between higher and lower levels was repeated twice by alternating perfusion of the cell with EGTA medium and  $Mg\cdot ATP$  medium. After  $E_m$  had been depolarized by perfusion with EGTA medium, the cell was perfused with AMP-PNP medium containing 1 mM AMP-PNP and then with  $Mg\cdot ATP$  medium. Notice that the high potential level could not be recovered with addition of AMP-PNP.

Fig. 10. Effects of AMP and ADP on  $E_m$ .  $E_m$  was measured by the open-vacuole method. The central chamber of the measuring vessel (Fig. 1) was filled with iAPW containing 5 mM  $Pb(NO_3)_2$ . Depolarization occurred when the cell containing  $Mg\cdot ATP$  medium was perfused with medium containing 1 mM AMP. The membrane was repolarized by perfusion with  $Mg\cdot ATP$  medium.

Subsequent perfusion with ADP medium containing 1 mM ADP caused depolarization which was followed by a slow repolarization.  $E_m$  was recovered completely by perfusion with the Mg-ATP medium.

Fig. 11. Responses of membrane potential of cells containing EGTA medium at resting (●) and excited (○) states and those containing hexokinase medium (○).

Fig. 12. Equivalent circuit of plasmalemma of Chara.

$(E_m)_o$ : passive electromotive force independent of ATP.

$R_m$ : membrane resistance. P: electrogenic ion pump

$i_p$ : current produced by the pump (P).  $E_m$ : total membrane potential measured.

Fig. 13. The current produced by the pump,  $i_p$  (○), with different external  $K^+$  concentrations,  $[K^+]_o$  was calculated from the active component of the membrane potential,  $E_a$  (○), and the membrane resistance,  $R_m$  (●), according to Eq. 3, in which  $[E_m - (E_m)_o]$  is equal to  $E_a$ . For further explanation, see text.

Fig. 1

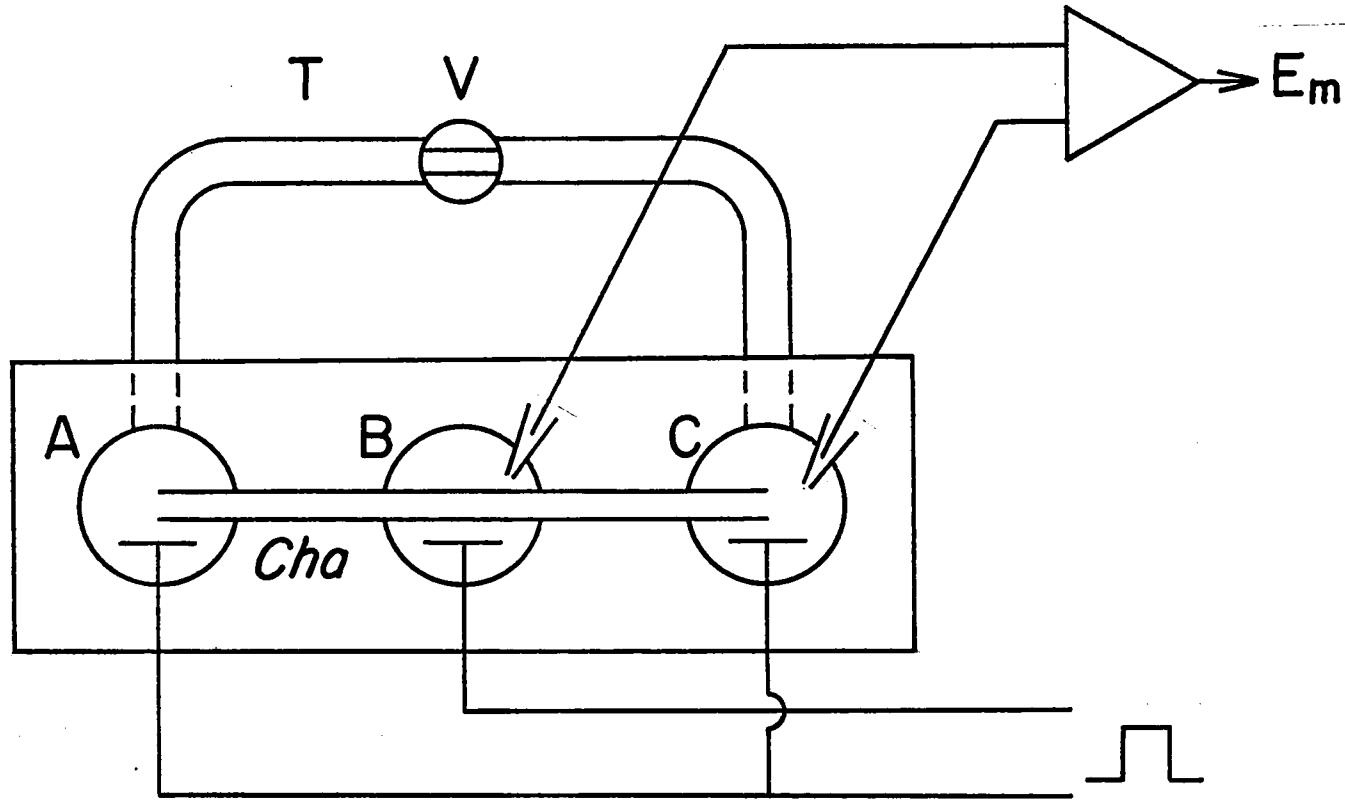
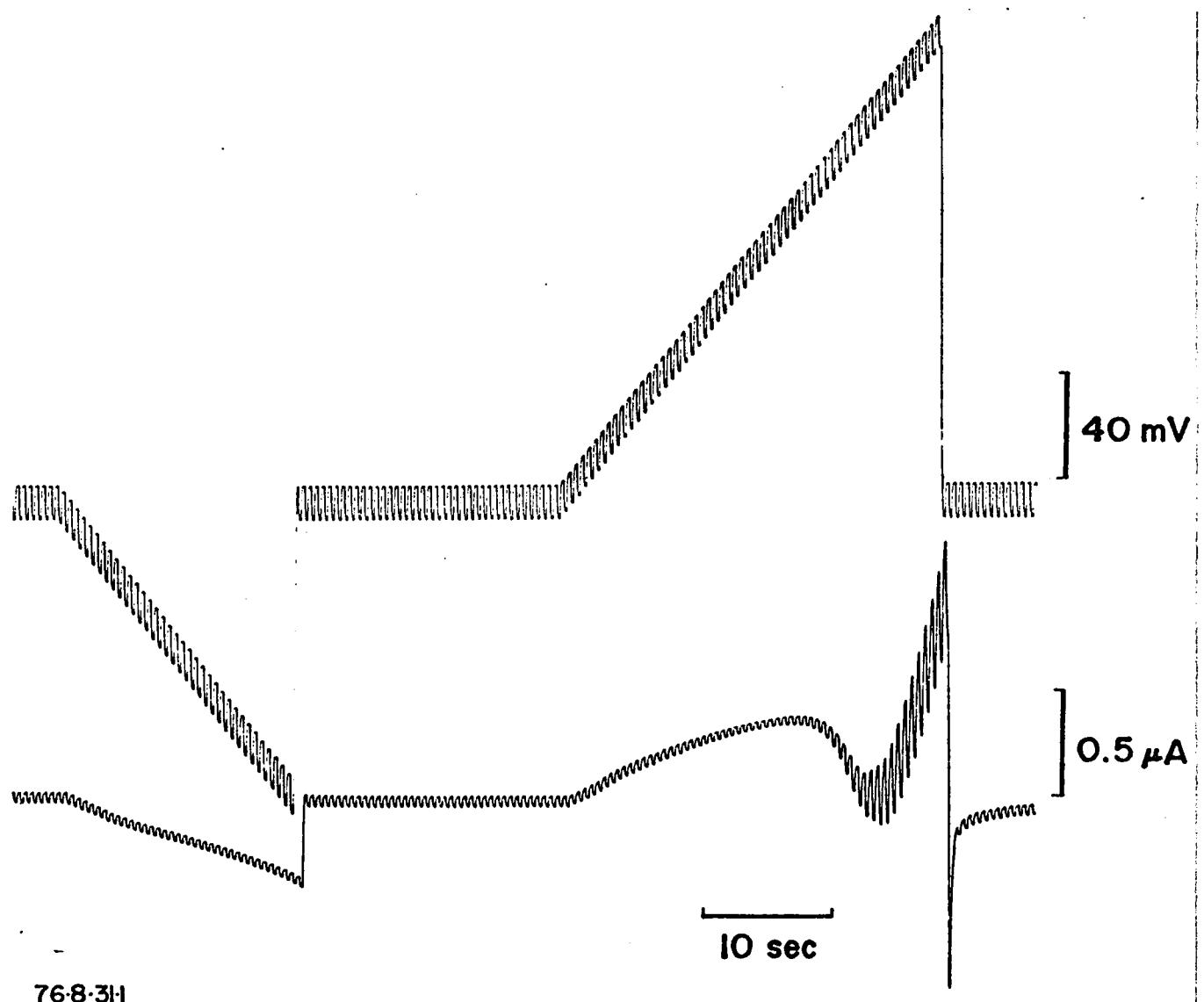
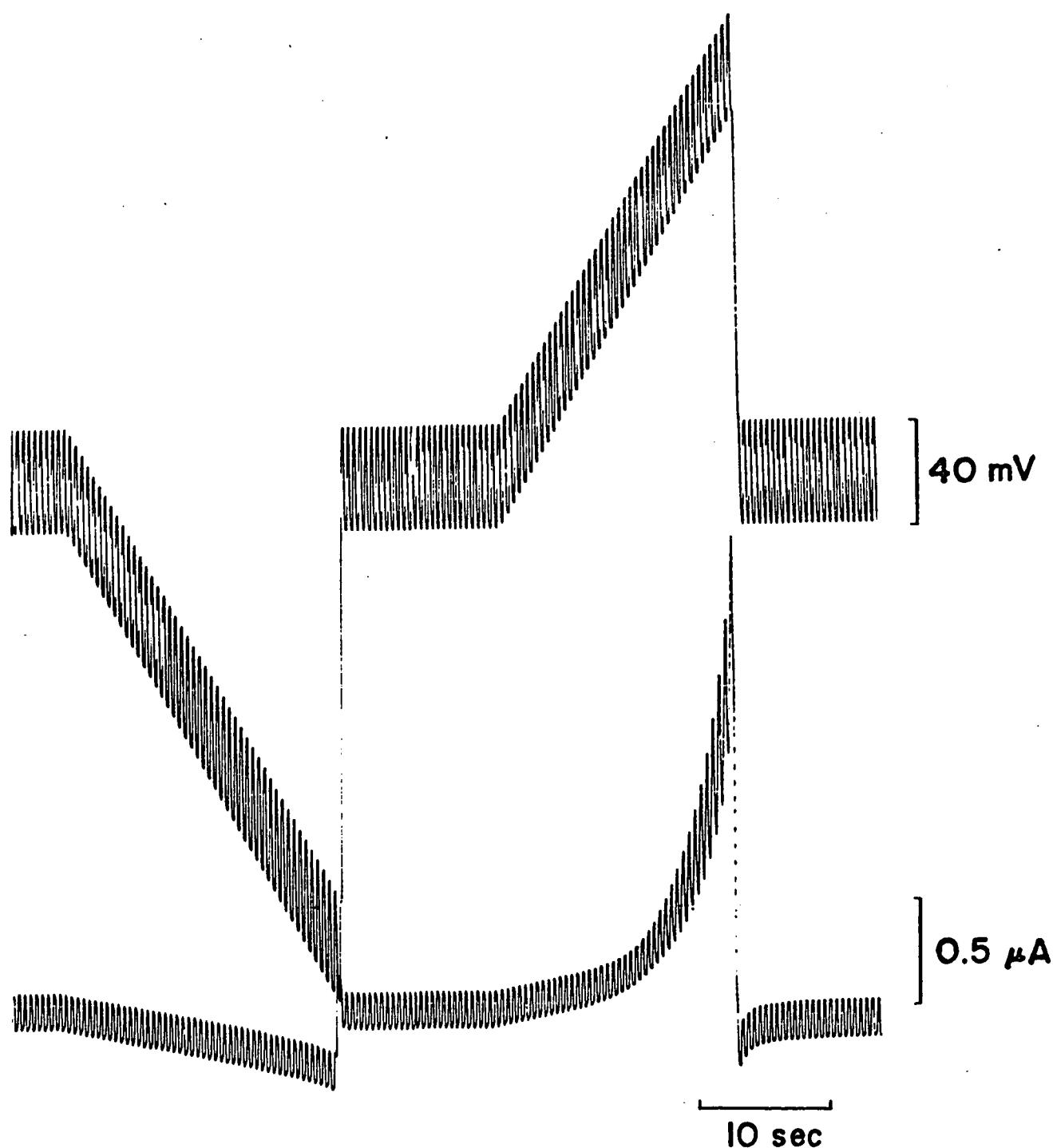


Fig. 2 a



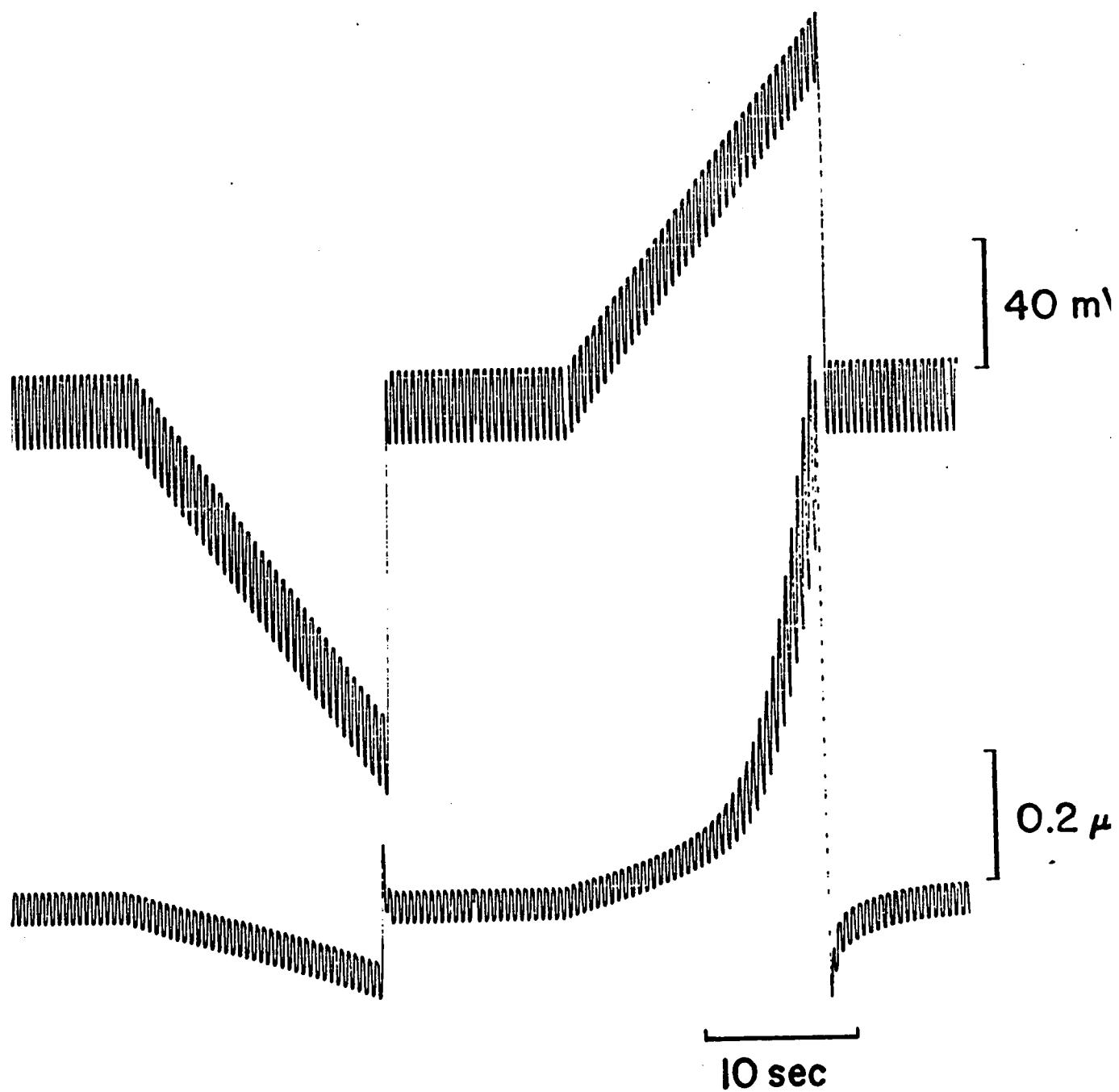
76-8-3H

Fig. 2 b



76·9·II·2

Fig. 2 c



76.9.10.3

Fig. 3 a

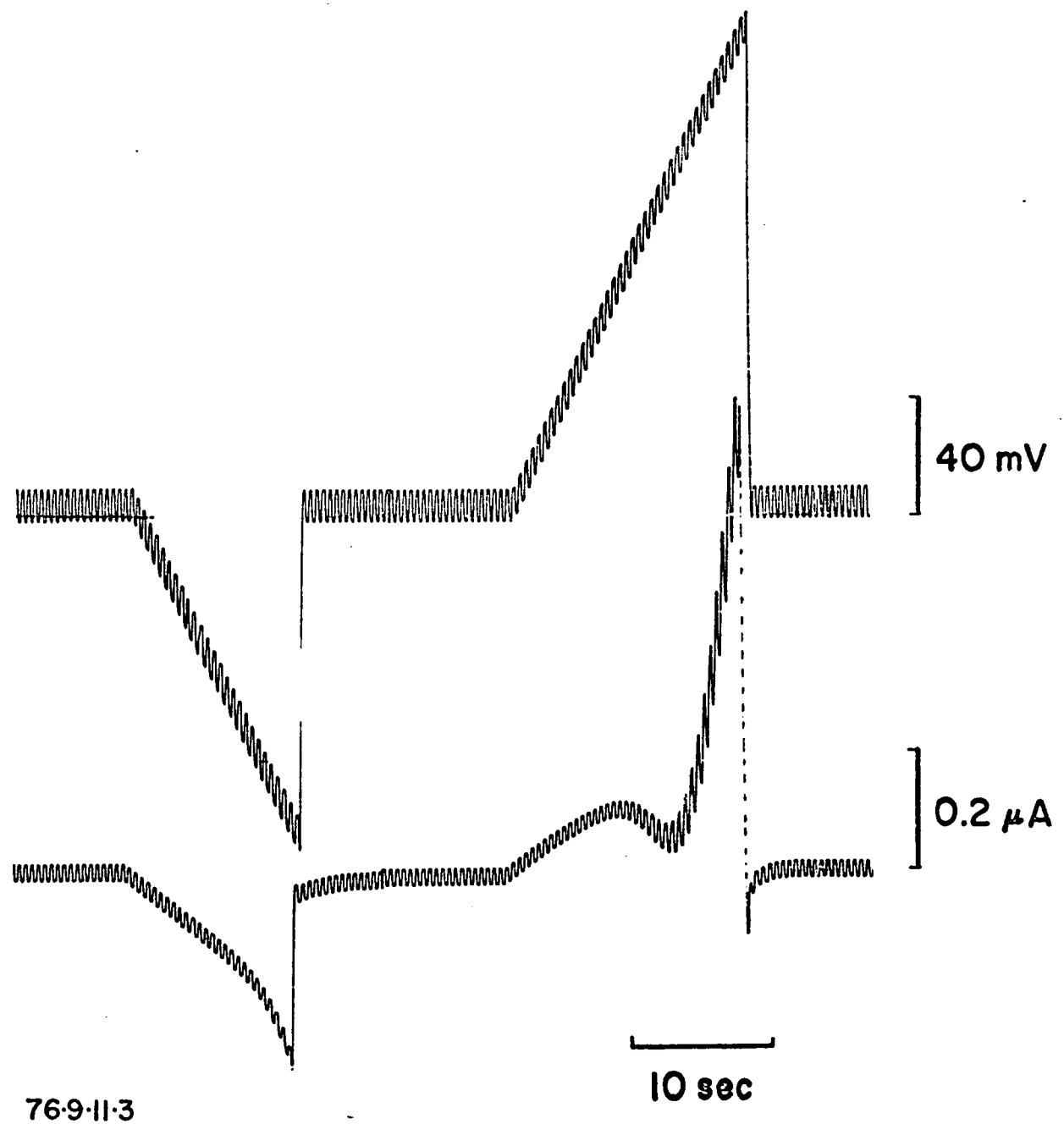


Fig. 3 b

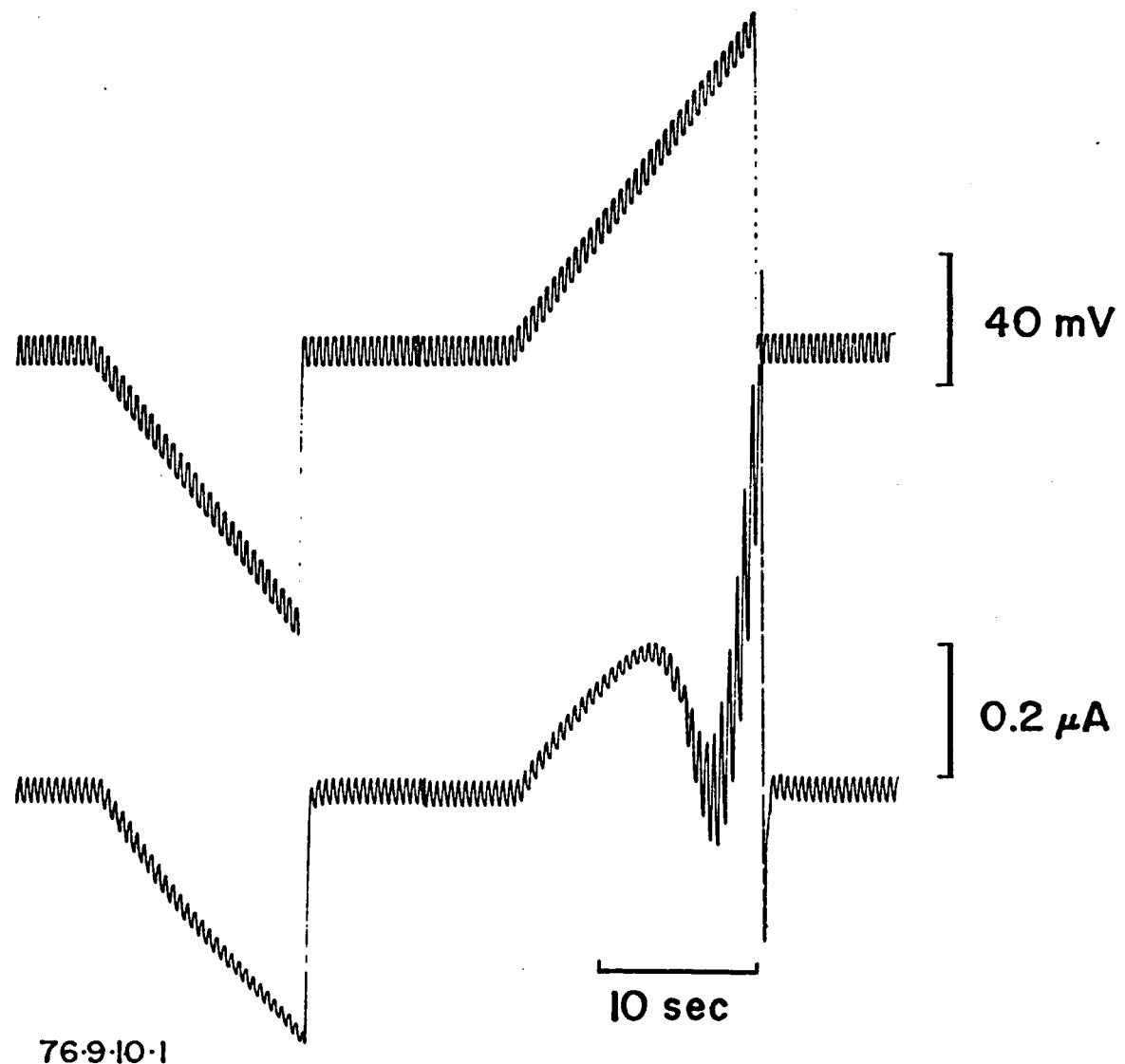


Fig. 4

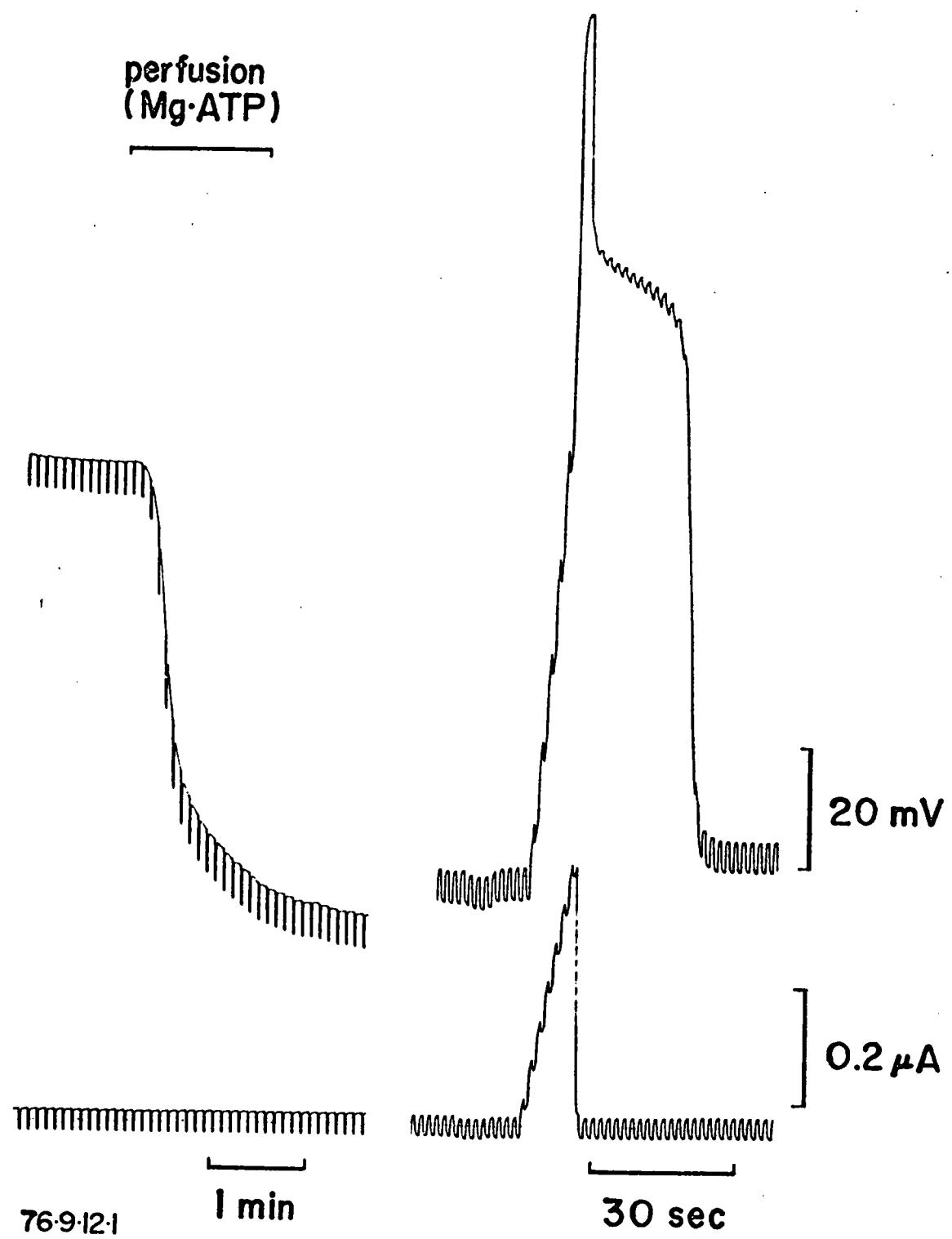


Fig. 5

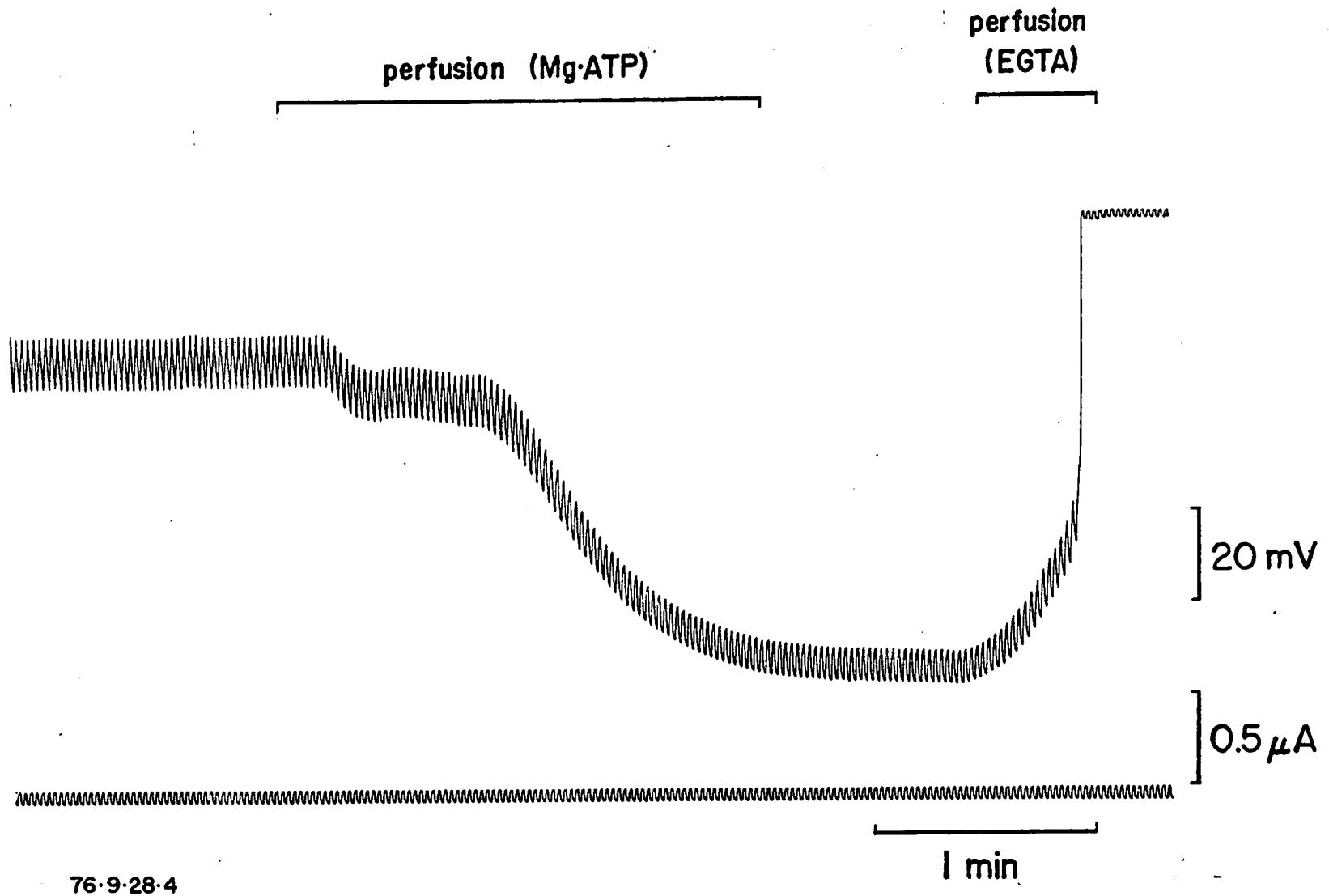


Fig. 6

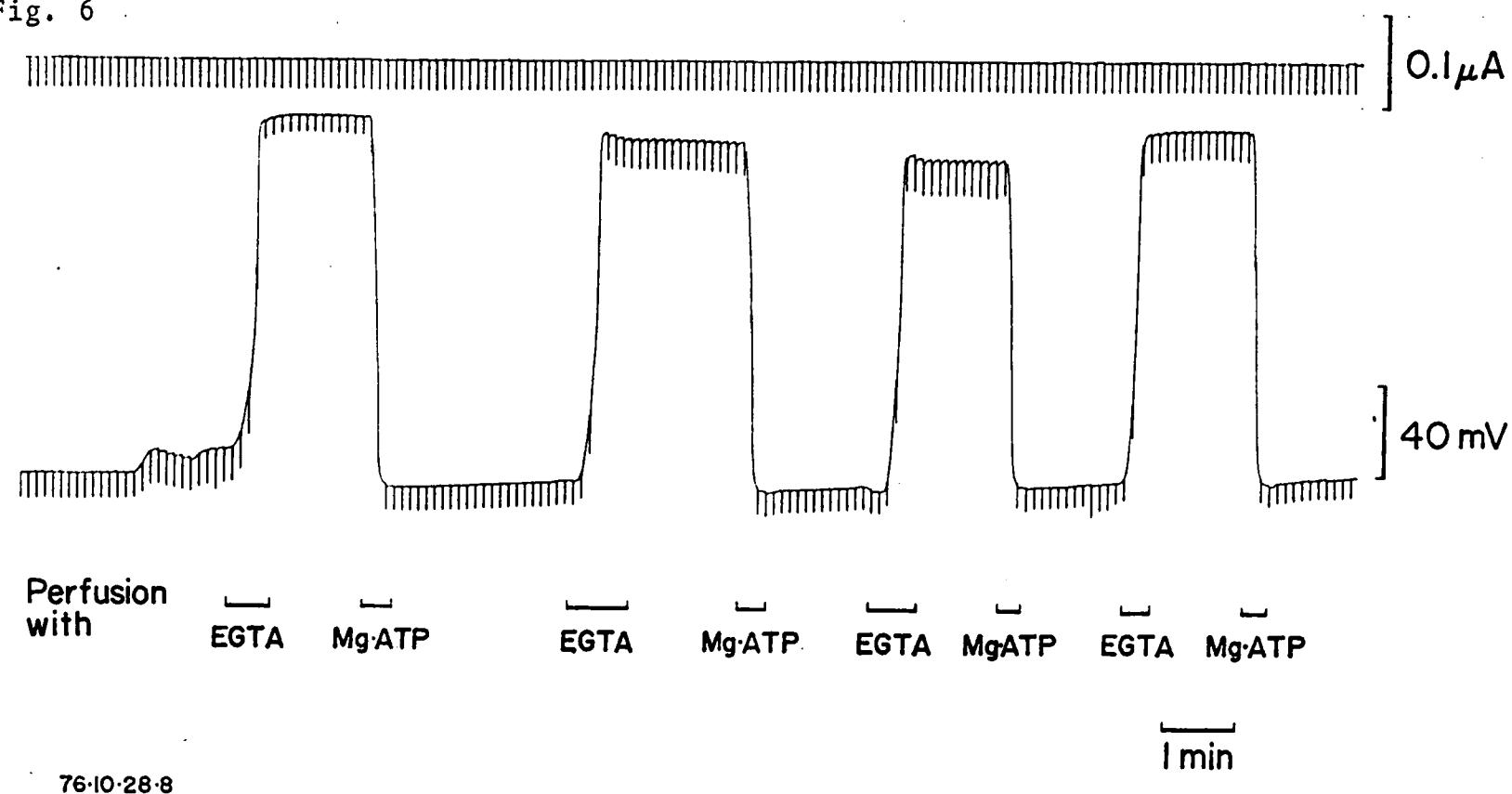


Fig. 7

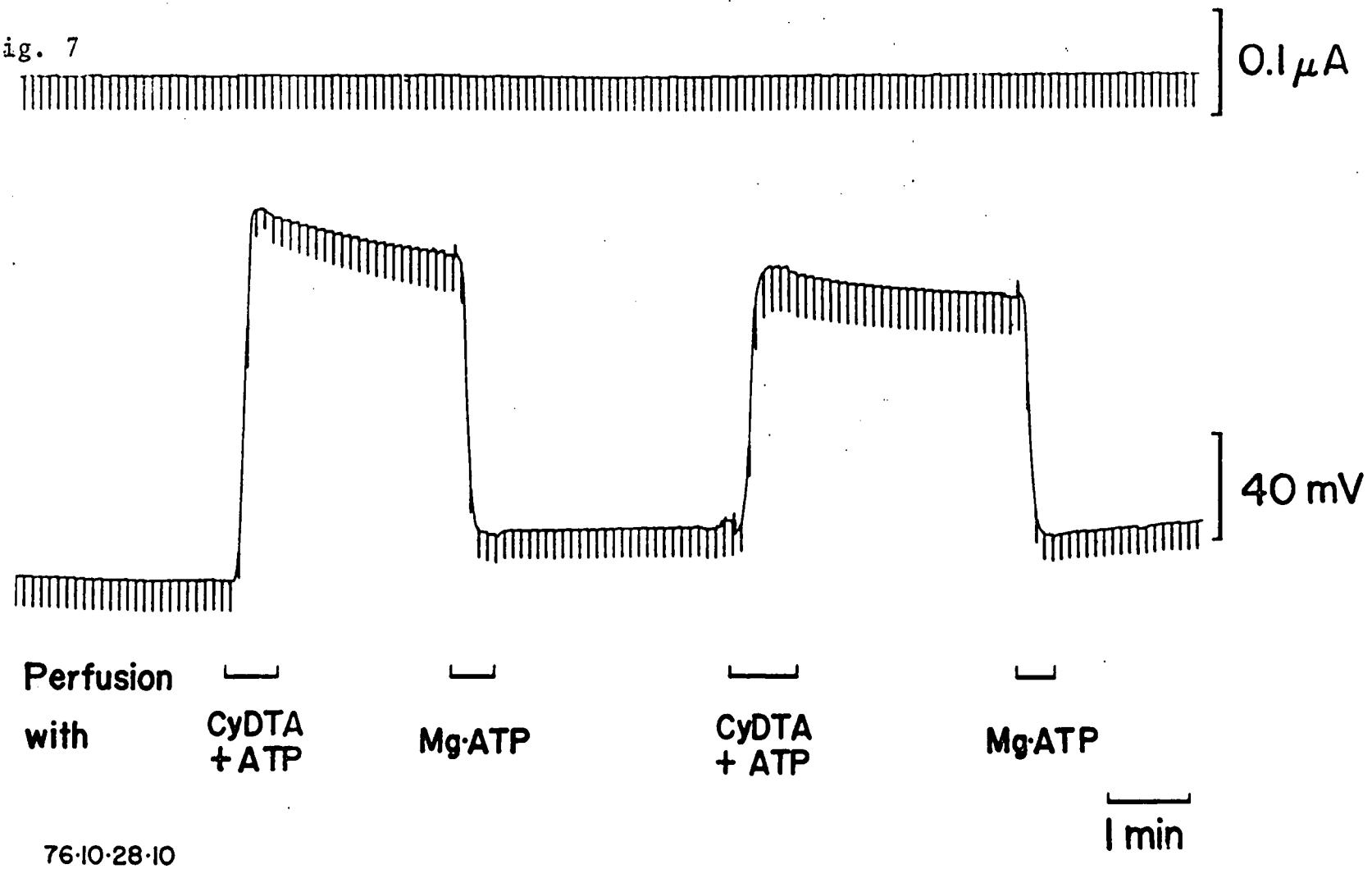


Fig. 8

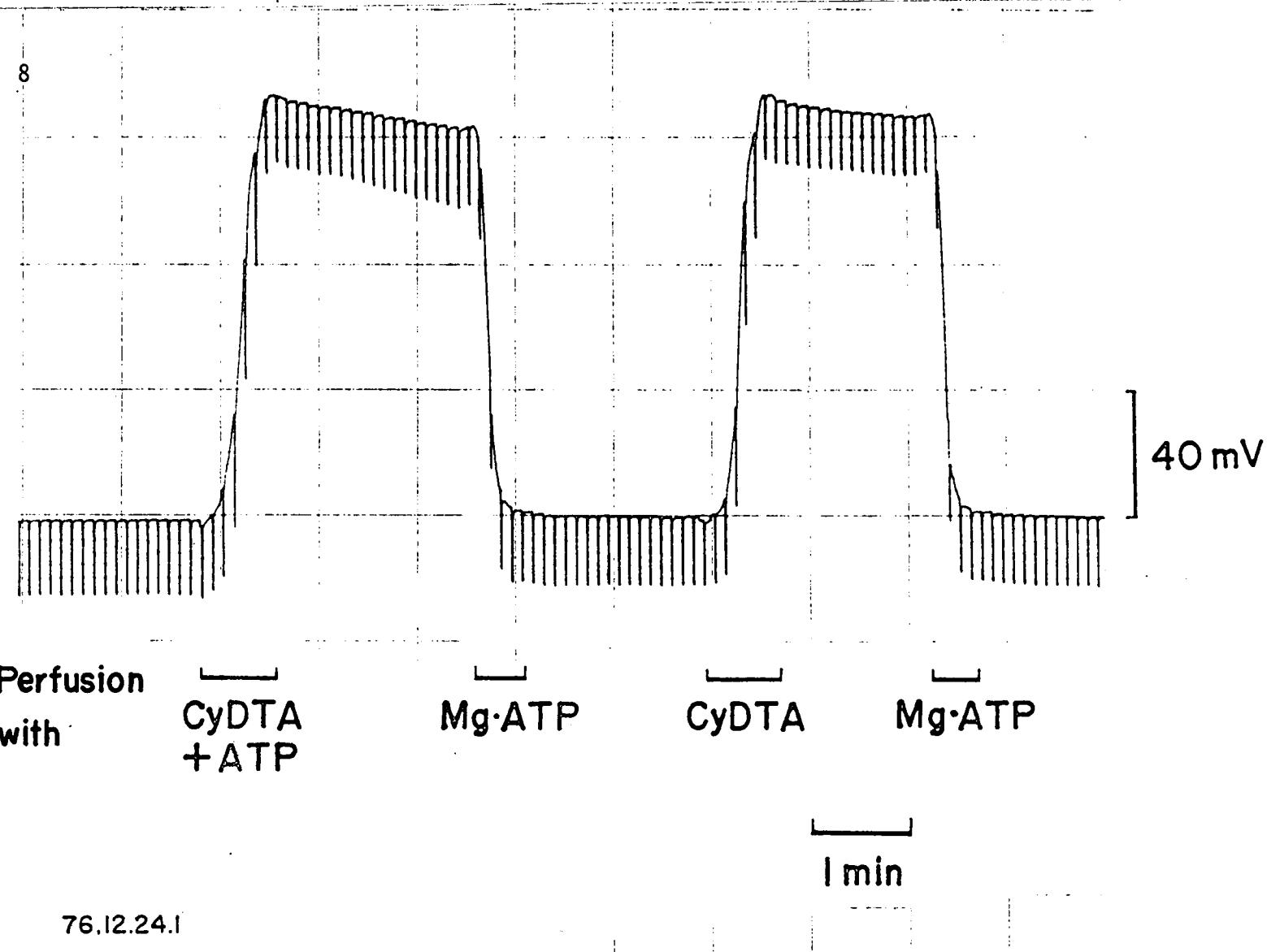
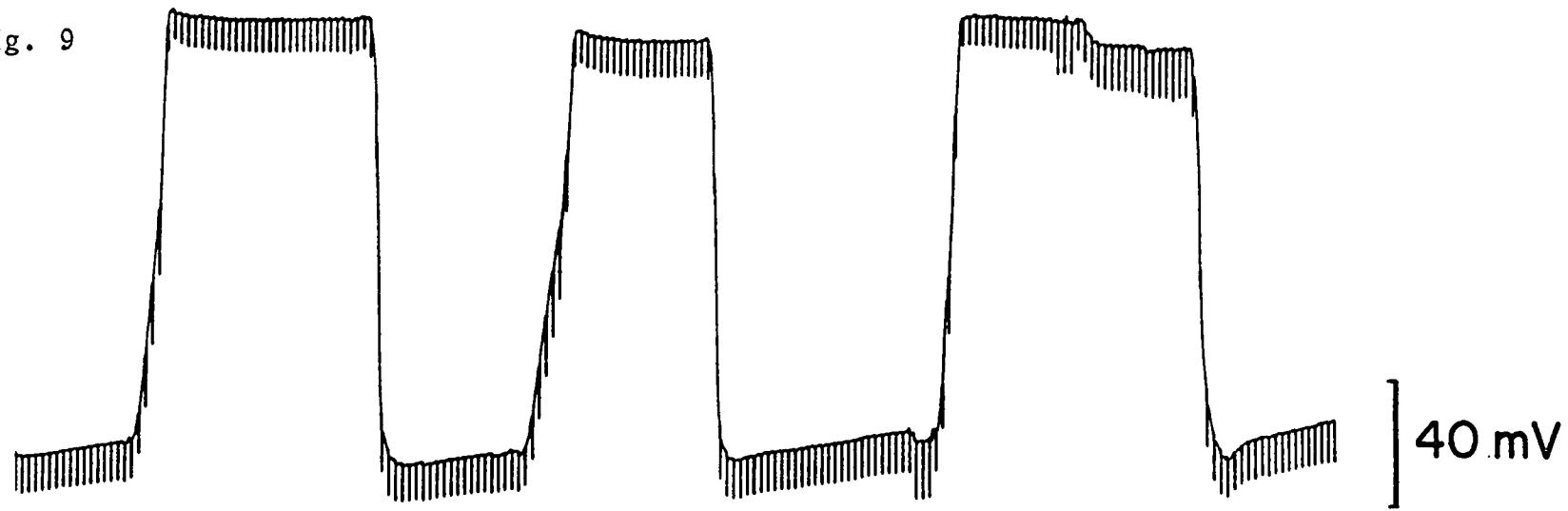


Fig. 9



94

Perfusion —  
with EGTA Mg·ATP EGTA Mg·ATP EGTA —  
AMP-PNP

1 min

76-II-10-3

Fig. 10

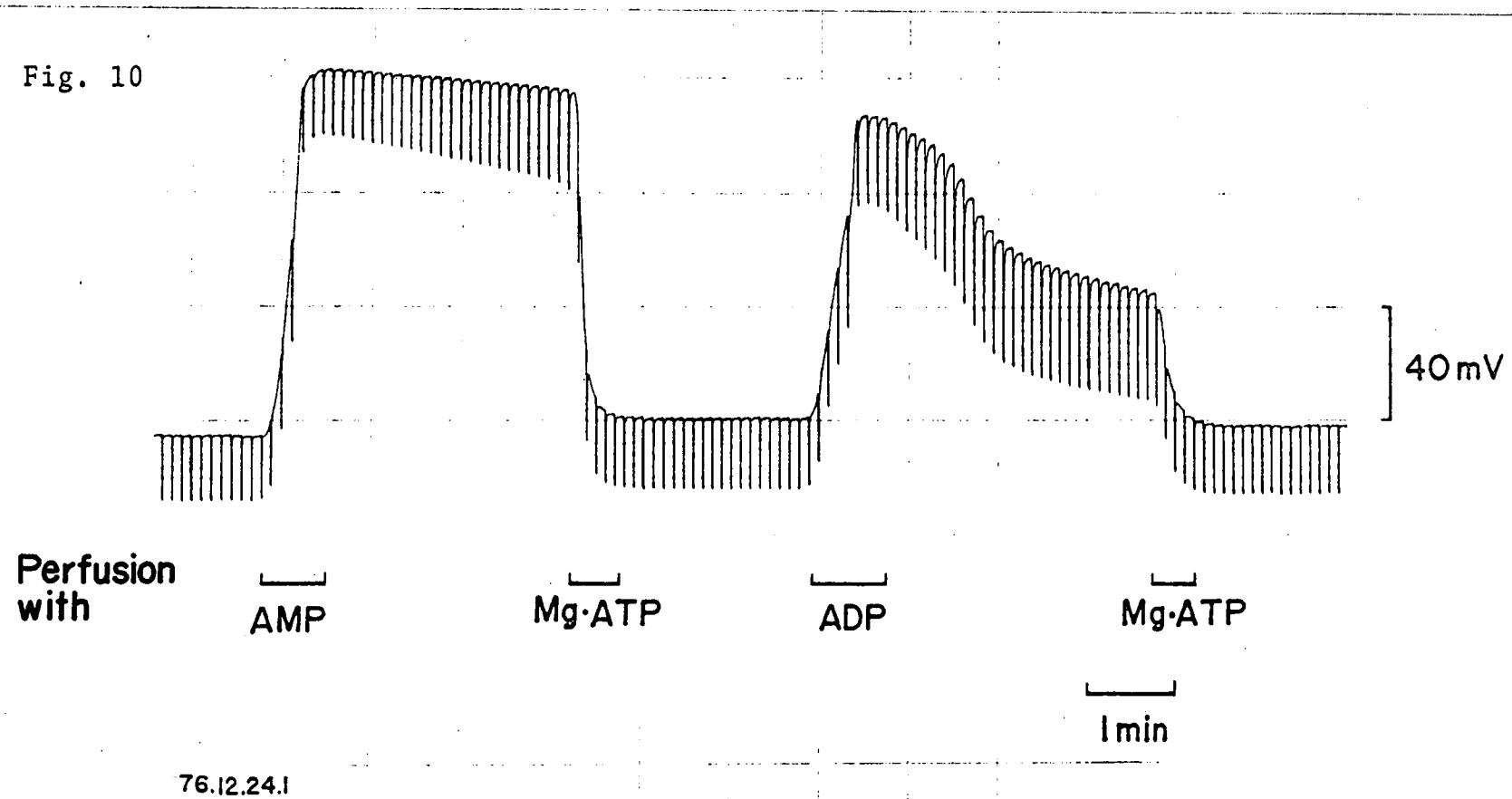


Fig. 11

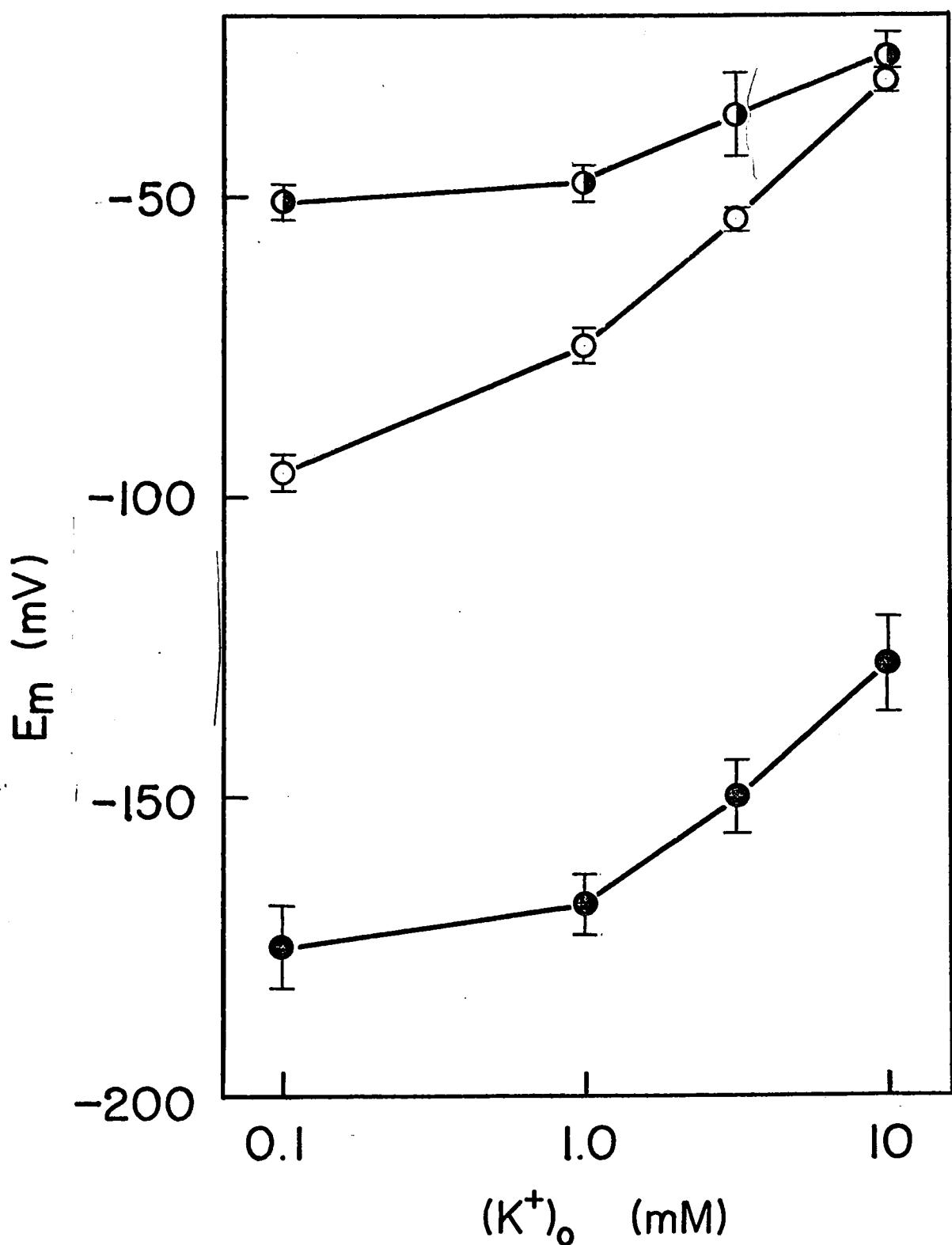


Fig. 12

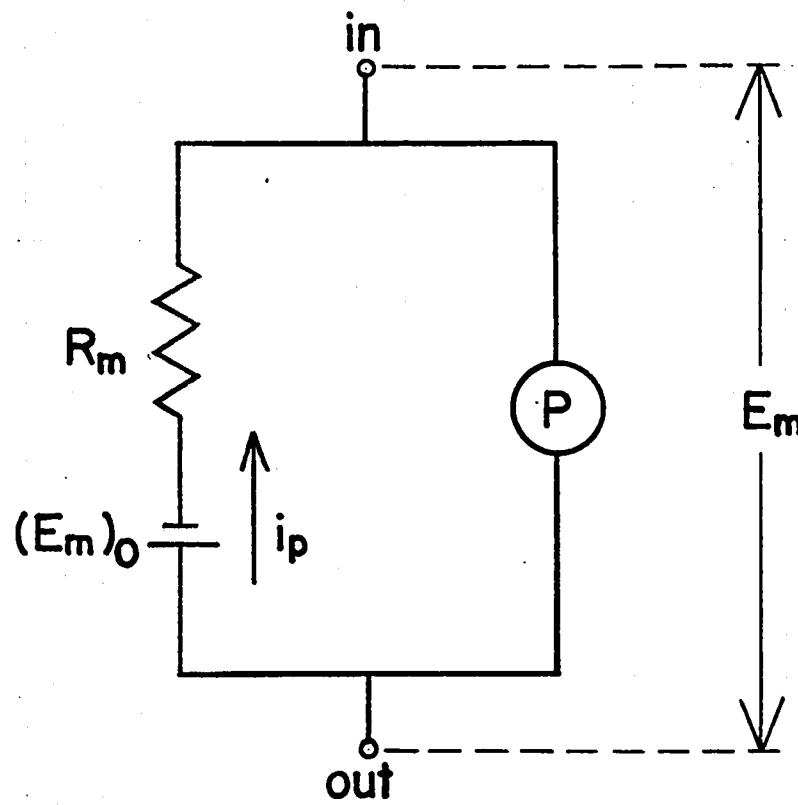


Fig. 13

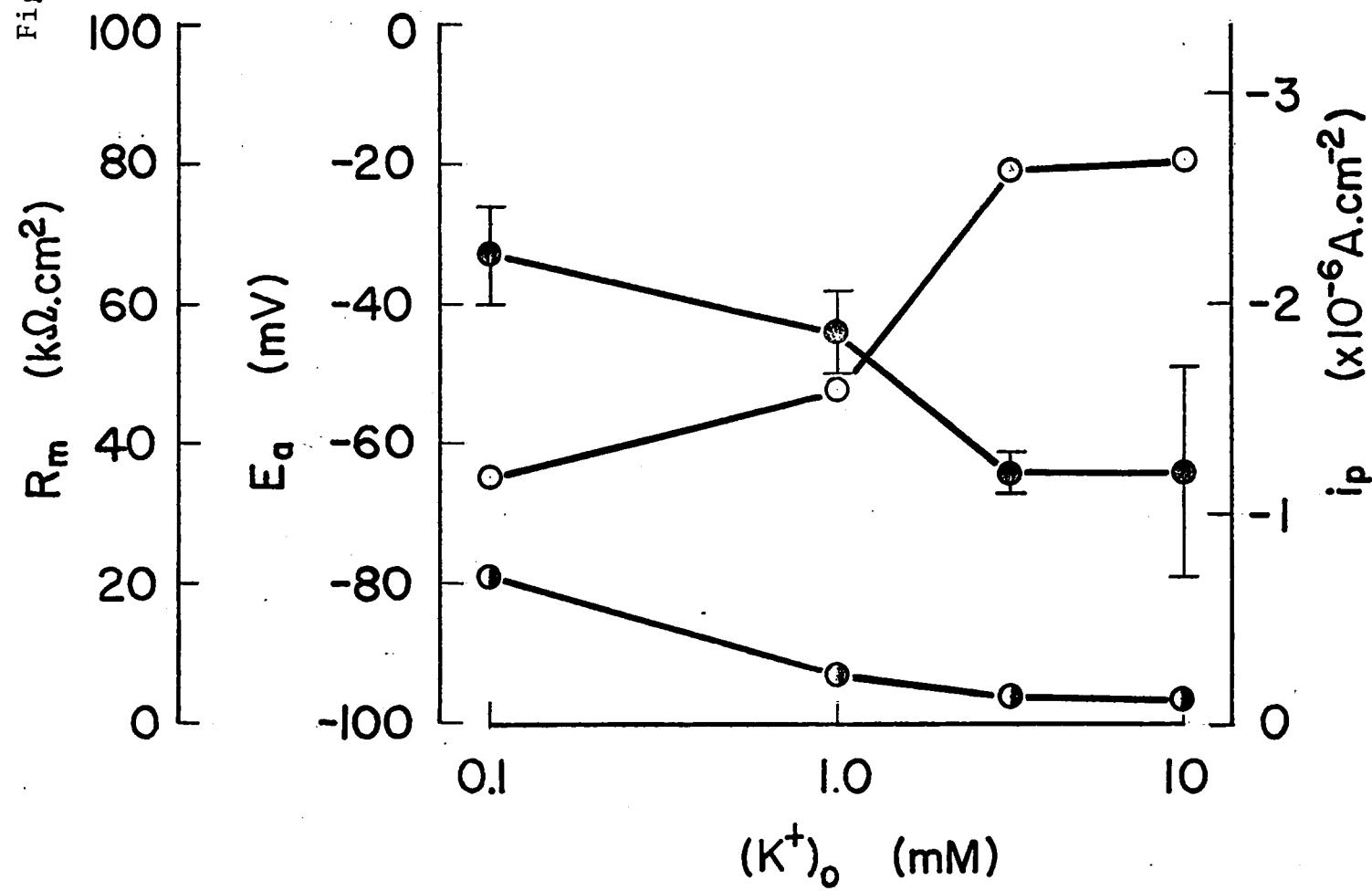


Table 1. Compositions of internal perfusion media

Medium	EGTA	EDTA	CyDTA	hexokinase	Mg:ATP
EGTA	5 mM			5 mM	5 mM
EDTA		5 mM			
CyDTA			5 mM		
MgCl <sub>2</sub>	6 mM			6 mM	6 mM
ATP					1 mM
K <sup>+</sup>	18 mM	20 mM	21 mM	18 mM	22 mM
hexokinase				1 mg/ml	
glucose				5 mM	
Tris-maleate	5 mM	5 mM	5 mM	5 mM	5 mM
sorbitol	290 mM	310 mM	310 mM	285 mM	290 mM
pH	7	7	7	7	7

Table 2. Membrane potentials and membrane resistances of tonoplast-free cells containing EGTA, hexokinase or EDTA medium

Medium	EGTA	Hexokinase	EDTA
$E_m$ (mV)	201 $\pm$ 7 (n = 18)	96 $\pm$ 4 (n = 7)	121 $\pm$ 5 (n = 14)
$R_m$ ( $k\Omega \cdot cm^2$ )	68 $\pm$ 5 (n = 16)	150 $\pm$ 27 (n = 7)	89 $\pm$ 12 (n = 12)

Table 3. Recovery of membrane potentials and membrane resistances by introduction of  $Mg^{2+}$  or ATP into  $Mg^{2+}$ - or ATP-depleted cells

First perfusion medium	Hexokinase	EDTA
$E_m$ (mV)	$168 \pm 5$ (n = 6)	$160 \pm 13$ (n = 7)
$R_m$ (k $\Omega$ cm)	$75 \pm 13$ (n = 6)	$60 \pm 8$ (n = 7)

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