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Control of Membrane Potential and Excitability of *Chara* Cells with ATP and Mg^{2+}

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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. *Plant Cell Physiol.* **16**:611). 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 axillaris* 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.

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, 1970). The membrane potential was found to

be closely related to the intracellular level of ATP[1] in *Neurospora* (Slayman, Long & Lu, 1973). In such studies, the intracellular ATP concentration was controlled by metabolic inhibitors. Therefore, the membrane potential cannot 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 perfuson 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*a, b*). 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 solution 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 in *Chara* cells both ATP and Mg^{2+} in the cytoplasm are necessary for maintaining both the membrane potential and the excitability, and Mg^{2+}-dependent ATPase seems to have a role in electrogenesis.

[1] *Abbreviations:* ATP, adenosine-5′-triphosphoric acid; ADP, adenosine-5′-diphosphoric acid; AMP, adenosine-5′-monophosphoric acid; AMP-PNP, adenylyl imidodiphosphoric acid; cAMP, adenosine 3′,5′-cyclic monophosphoric acid; EGTA, ethyleneglycol-bis-(β-aminoethylether) N,N′-tetra-acetic acid; EDTA, ethylene diamine tetraacetic acid; CyDTA, 1,2-cyclohexane diamine N,N′-tetraacetic acid.

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		
$MgCl_2$	6 mM			6 mM	6 mM
ATP					1 mM
K^+	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

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 KCl, NaCl and $CaCl_2$. 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.

The membrane resistance was measured by applying to the cell either small constant square-shaped current pulses or small constant square-shaped voltage pulses across the plasmalemma.

Excitability of the membrane was judged by the occurrence of the so-called N-shaped voltage-current (*V-I*) 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 medium 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 cock *K* 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 *K*.

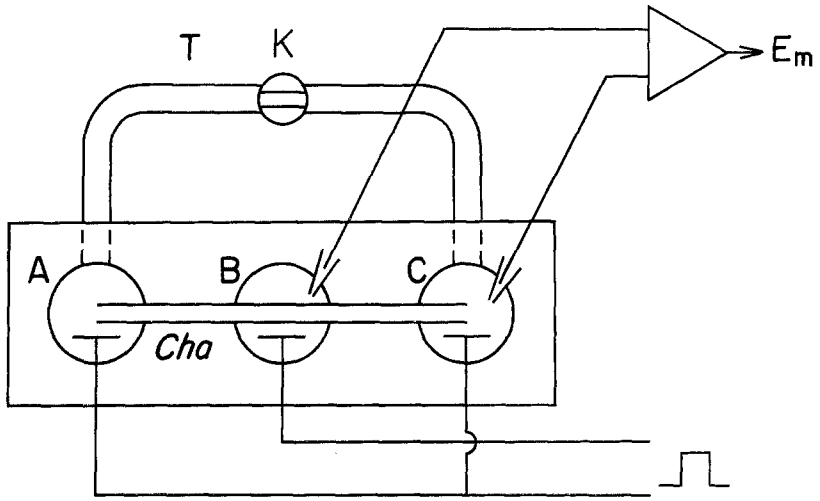


Fig. 1. Polyacrylate vessel used for the open-vacuole method. Chambers *A* and *C* connected with rubber tubing (*T*) were filled with media for intracellular perfusion of the internodal cell (*Cha*). Chamber *B* was filled with iAPW or iAPW containing 5 mM Pb^{2+}. The membrane potential (E_m) of the 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. Internal perfusion occurred when the water level of one chamber was made higher than that of the other chamber after closing cock *K*

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 ±SEM and the number (n) of cells used.

Results

Effects of Depletions 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 open cell ends were closed by ligation with strips of polyester thread. E_m and R_m of the cells in

Table 2. Membrane potential (E_m in mV) and membrane resistance (R_m in $k\Omega cm^2$) of tonoplast-free cells after the first perfusion with EGTA, hexokinase or EDTA medium, and those of cells after second perfusion with Mg-ATP medium which was carried out subsequent to loss of the tonoplast caused by the first perfusion with hexokinase or EDTA medium

	Medium				
1st perfusion	EGTA	Hexokinase	Hexokinase	EDTA	EDTA
2nd perfusion	–	–	Mg-ATP	–	Mg-ATP
E_m	-201 ± 7	-96 ± 4	-168 ± 5	-121 ± 5	-160 ± 13
	(18)	(7)	(6)	(14)	(7)
R_m	68 ± 5	150 ± 27	75 ± 13	89 ± 12	60 ± 8
	(16)	(7)	(6)	(12)	(7)

Numbers of parentheses indicate the number of cells used.

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 Ca^{2+} strongly, the concentrations of Ca^{2+} in the cytoplasm are estimated to be very low (2.9×10^{-8} M; Shimmen *et al.*, 1976*a*). This indicates that a very low cytoplasmic Ca^{2+} concentration does not affect the normal functioning of the plasmalemma.

To lower the free Mg^{2+} concentration in the cytoplasm, EDTA was used instead of EGTA in the perfusion medium, since it binds both Ca^{2+} and Mg^{2+}. When the perfusion medium contained hexokinase and glucose, the cytoplasmic ATP concentration decreased after disintegration of the tonoplast, since the enzyme reaction proceeds preferentially to convert ATP and glucose into ADP and glucose-6-phosphate. After loss of the tonoplast, the internal ATP concentration $[ATP]_i$ was 40–50 μM in cells perfused with the EGTA medium and 1 μM 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 $[ATP]_i$ 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 μM $[ATP]_i$. The R_m of cells of low $[ATP]_i$ 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 medium was used, the free Mg^{2+} concentration in the cytoplasm after loss of the tonoplast was calculated to be 3.6×10^{-7} M. Apparent association constants used for calculation were 2.3×10^5 M^{-1} for Mg-EDTA and 2.3×10^7 M^{-1} for Ca-EDTA at pH 7.0. Concentrations adopted for the divalent cations in the cytoplasm were 3.6 mM for total Mg (Williamson, 1975) and 3 mM for total 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

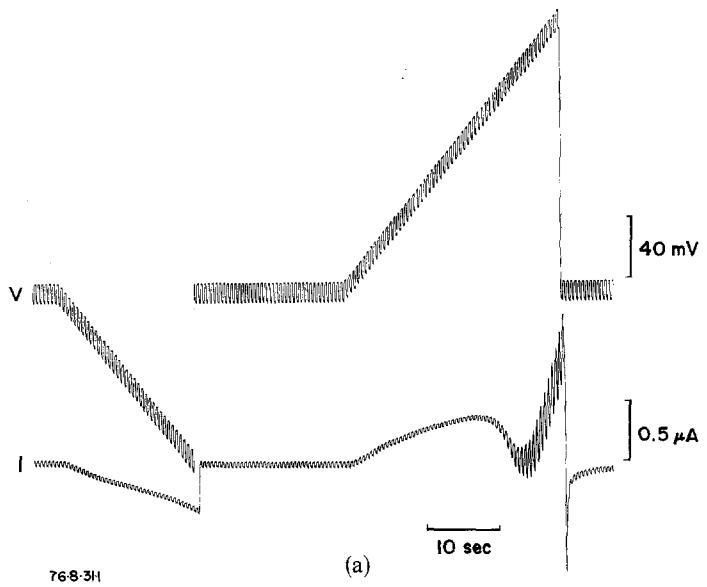
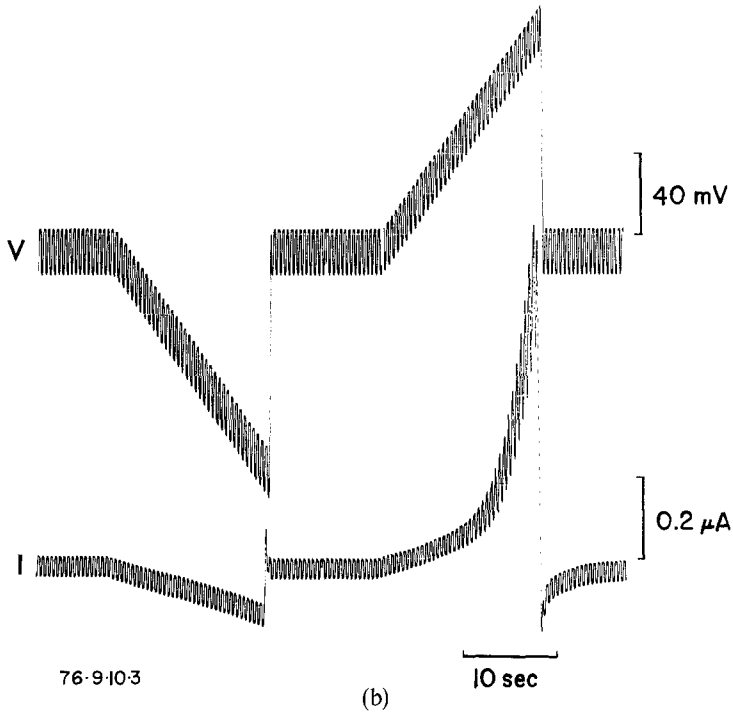
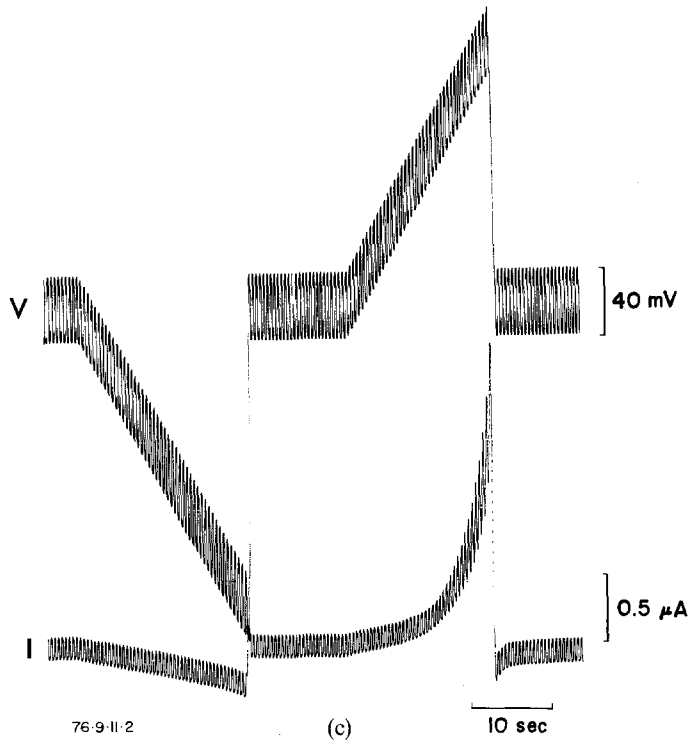


Fig. 2. Voltage-current (*V-I*) relationship for tonoplast-free cells with cell saps replaced with EGTA medium (*a*), EDTA medium (*b*) or hexokinase medium (*c*). The resting potential was −202 mV in (*a*), −120 mV in (*b*) and −100 mV in (*c*). The upward deflections of the *V*- and *I*-curves correspond to changes in E_m in the depolarizing direction and the membrane current in the outward direction, respectively



(b)



(c)

medium. The cell containing EGTA medium showed the typical N-shaped *V-I* relationship when ramp depolarization was applied to the membrane (Fig. 2*a*). Cells containing EDTA or hexokinase medium did not show any sign of the N-shaped *V-I* relationship characteristic of the excitable membrane but did show a significant rectification (Fig. 2*b* and *c*). These results suggest that both 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.

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-ATP medium containing 6 mM Mg^{2+} and 1 mM ATP (Table 1) and both open cell ends were closed as before. E_m and R_m were measured by the microelectrode method and the excitability was tested by checking the N-shaped *V-I* relationship. As shown in Table 2, 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 *V-I* relationship became observable again when Mg-ATP was introduced into Mg^{2+}-depleted cells (Fig. 3*a*) or ATP-depleted cells (Fig. 3*b*).

Time Course of Recovery of Membrane Potential with Mg-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 necessary procedures between perfusion and microelectrode insertion. To observe the time course of recovery of the membrane potential by Mg^{2+} or

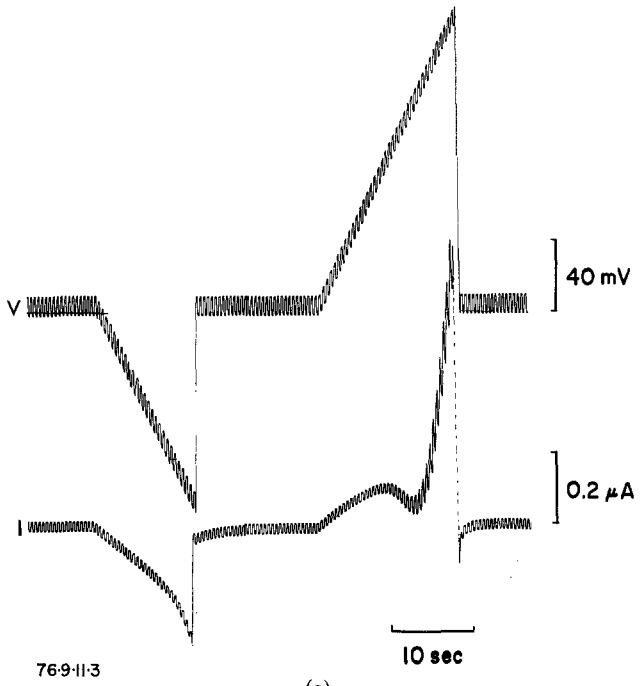


Fig. 3. Recovery of excitability by introducing Mg^{2+} into Mg^{2+}-depleted cell containing EDTA medium (*a*) or ATP into ATP-depleted cell containing hexokinase medium (*b*). The resting potential was -172 mV in (*a*) and -134 mV in (*b*). The upward deflections of the *V*- and *I*-curves correspond to changes in E_m in the depolarizing direction and the membrane current in the outward direction, respectively

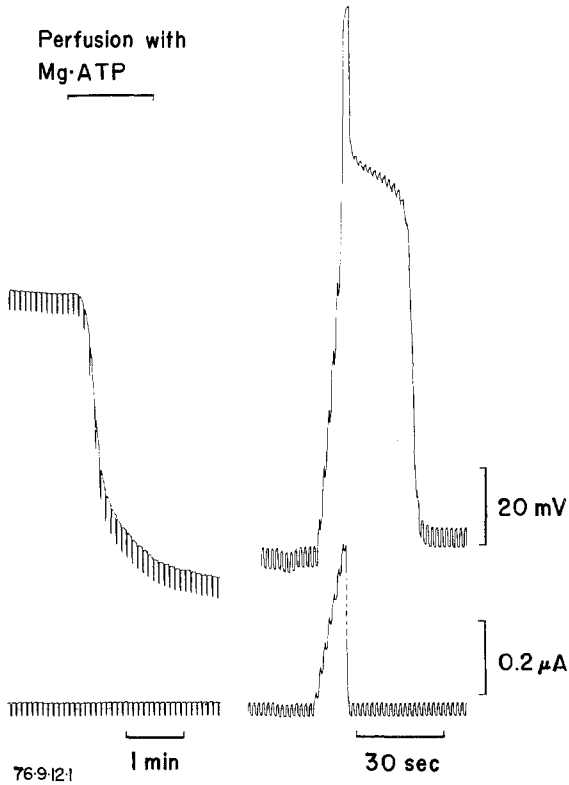


Fig. 4. Membrane repolarization by introducing 1 mM Mg-ATP into Mg^{2+}-depleted cell containing EDTA medium. The action potential was generated by electrical stimulus after repolarization. E_m at the start of the record was -74 mV

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 3.6×10^{-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.

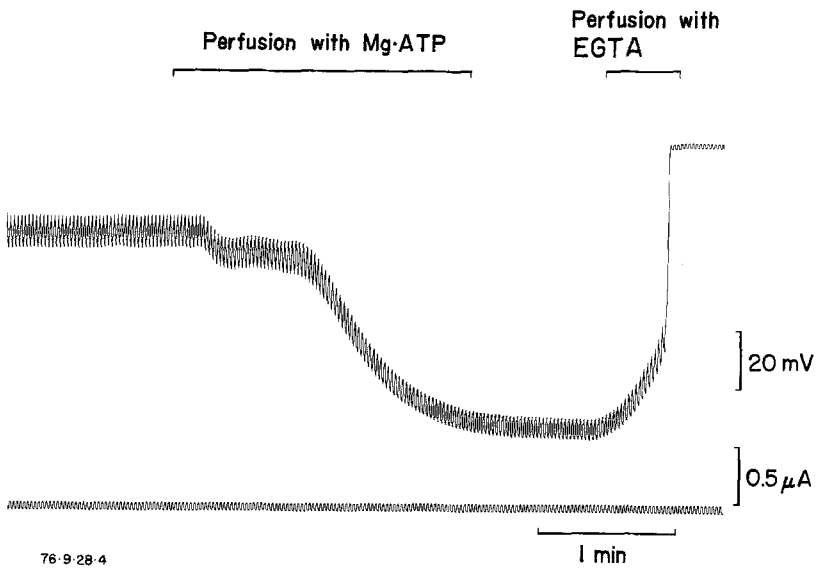


Fig. 5. Repolarization of the once-depolarized membrane potential of the ATP-depleted cell by introducing 1 mM Mg-ATP and depolarization by subsequent intracellular perfusion with EGTA medium. Notice that the cell was excited when E_m reached a threshold level. E_m at the start of the record was -80 mV

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 depo-

larization or transferred E_m in the excited state to the resting state (Shimmen *et al.*, 1976*a*) also could not repolarize the membrane.

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^{2+} as acetate had been added. Chamber *A* was filled with Mg-ATP medium and chamber *C* was filled 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^{2+} 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 changed little in the former (Fig. 6) but decreased conspicuously in the latter (Fig. 5). When the

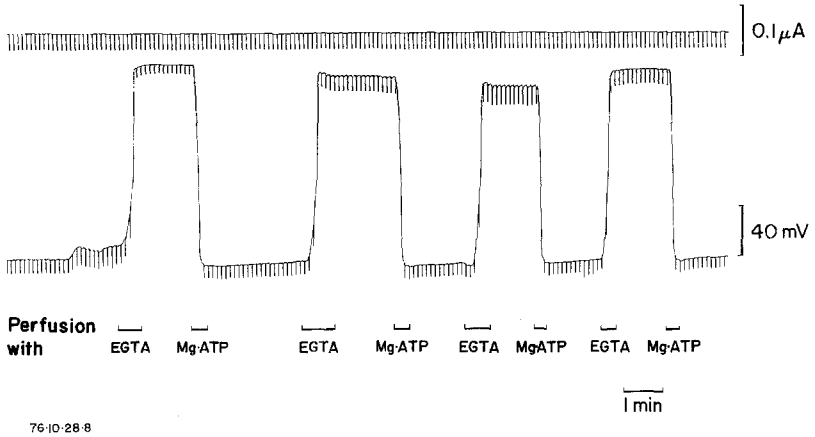


Fig. 6. Reversible transition of E_m between polarized and depolarized levels controlled by internal ATP concentration. E_m was measured by the open-vacuole method. The external medium was iAPW containing 5 mM Pb-acetate. First, the cell sap was replaced with Mg-ATP medium, E_m was high after disintegration of the tonoplast. E_m was depolarized by perfusion with EGTA medium and recovered by perfusion with Mg-ATP medium. This transition could be repeated many times. E_m at the start of the record was −202 mV

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 Fig. 6, where R_m was measured just after replacement of Mg-ATP medium with ATP-depleted medium, R_m showed a decrease in the first depolarization and a slight increase in the following depolarizations. Thus, no concurrent change in R_m with E_m was observed in the measurements using OV-methods. On the other hand it was emphasized in Table 2 that depletion of internal ATP or Mg^{2+} caused significant increase in R_m in parallel with decrease in E_m. The discrepancy between the results shown in Table 2 and Fig. 6 may be explained by the fact that in the former R_m was measured 20–30 min after replacement of the cell sap with hexokinase medium. The delayed effect of ATP depletion on R_m will be referred to in the Discussion.

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^{2+} 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 rapidly 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 in-

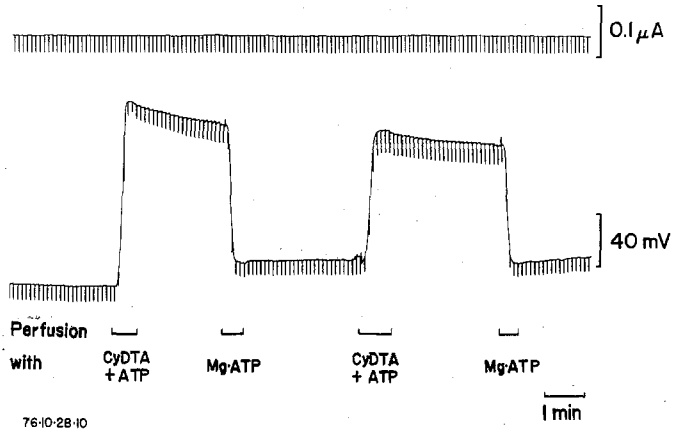


Fig. 7. Reversible transition of E_m between two levels by controlling intracellular Mg^{2+} concentration. E_m was measured by the open-vacuole method. The central chamber of the measuring vessel (Fig. 1) was filled with iAPW containing 5 mM Pb-acetate. Internal Mg^{2+} concentration was controlled by alternating perfusion with Mg-ATP medium containing 6 mM Mg^{2+} and CyDTA medium containing 1 mM ATP. E_m at the start of the record was -176 mV

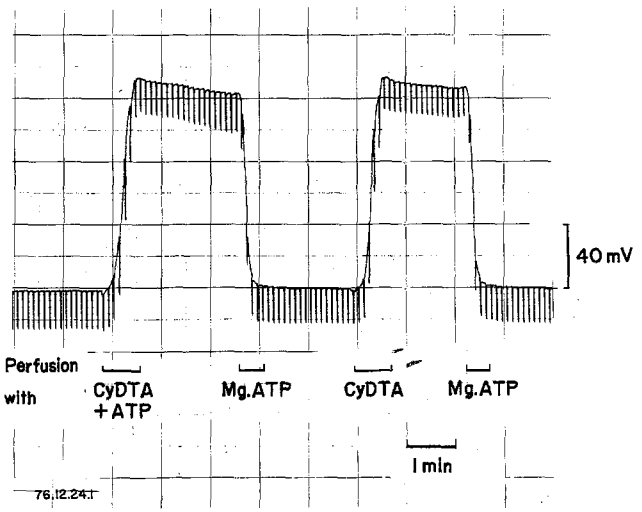


Fig. 8. Comparison of the level of depolarization caused by depletions of both Mg^{2+} and ATP with that caused by depletion of Mg^{2+} alone. E_m was measured by the open-vacuole method. The central chamber of the measuring vessel (Fig. 1) was filled with iAPW containing 5 mM Pb-acetate. Mg^{2+} in 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. E_m at the start of the record was -120 mV. During the recording current pulses of 0.03 μA were applied to the cell

tracellular 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.

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 β-γATPase 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 $Pb(NO_3)_2$, respectively. Since in winter, when the experiment shown in Fig. 9 was conducted, the resting potential was low in APW, the pH of iAPW containing 5 mM $Pb(NO_3)_2$ was increased from 5.6 to 7.5 by adding 5 mM Tris-H_2SO_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 Mg^{2+} but no ATP, the membrane was depolarized. E_m was recovered by intracellular perfusion with Mg-ATP me-

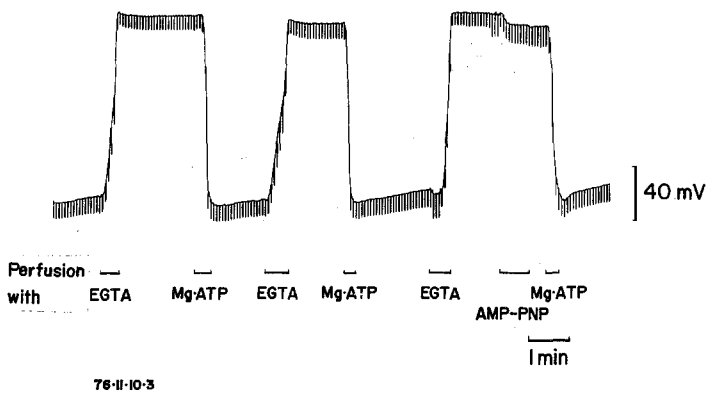


Fig. 9. Effect of AMP-PNP on E_m. E_m was measured by the open-vacuole method. The central chamber of the measuring vessel (Fig. 1) was filled with iAPW containing 5 mM $Pb(NO_3)_2$ at pH 7.5. First, the cell sap was replaced with Mg-ATP medium. Transition of E_m between higher and lower levels was repeated twice by alternating perfusion of the cell with EGTA medium and Mg-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-ATP medium. Notice that the high potential level could not be recovered with addition of AMP-PNP. E_m at the start of the record was −184 mV. During the recording current pulses of 0.02 μA were applied to the cell

dium. 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 ± 5 mV; n=5) and R_m was high (93 ± 17 kΩcm^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.

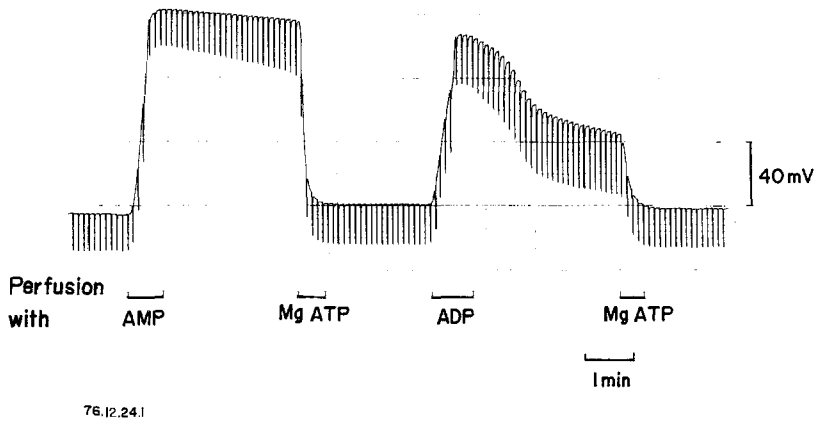


Fig. 10. Effects of AMP and ADP on E_m. E_m was measured by the open-vacuole method. The central chamber of the measuring vessel (Fig. 1) was filled with iAPW containing 5 mM $Pb(NO_3)_2$. Depolarization occurred when the cell containing Mg-ATP medium was perfused with medium containing 1 mM AMP. The membrane was repolarized by perfusion with Mg-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. E_m at the start of the record was −122 mV. During the recording current pulses of 0.03 μA were applied to the cell

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 ± 17 mV (n=3) for E_m and 40 ± 13 kΩcm^2 (n=3) for R_m. The N-shaped *V-I* relationship showing the presence of excitability was observed.

The polarized membrane potential level could not be maintained by 1 mM cAMP, 1 mM phosphate (mixture of K_2HPO_4 and KH_2PO_4) or 1 mM pyrophosphate (data not shown).

Response of the Membrane to $[K^+]_o$ in the resting, excited and low $[ATP]_i$ states

The effect of 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, $[K^+]_o$ was increased by adding K_2SO_4 in APW and the membrane potentials were measured at various $[K^+]_o$

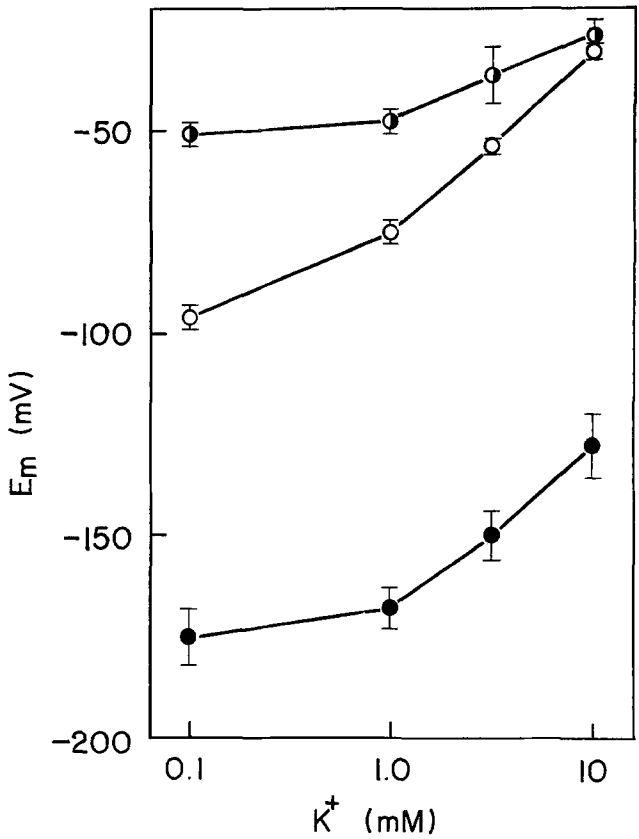


Fig. 11. Responses of membrane potential of cells containing EGTA medium at resting (●) and excited (○) states and those containing hexokinase medium (○). The membrane potential at the excited state represents the potential at the peak of action potential

(Fig. 11). When the cells were perfused with EGTA medium, both the resting potential and the potential at the peaks of action potentials were measured. Fig. 11 shows that the slope of the curve showing the response of the resting potential to $[K^+]_o$ 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.

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. pul-*

chella, although the magnitude of the ATP-dependent membrane potential (-40 to -50 mV) was smaller than in *C. australis* (Fig. 6).

Discussion

Requirements 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 3.2×10^{-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 controlling the membrane potential of adrenalin secreting chromaffin granule (Pollard, Zinder, Hoffman & Nikodejenic, 1976). This means that hydrolysis of ATP is not necessary in this organelle. On the contrary, the fact that in *Chara* ATP cannot 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 adenylkinase in the cell. Williamson (1975) also suggested the presence of adenylkinase 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 becomes 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.

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* excrete 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.

Dependencies of Passive and Active Components of E_m on $[K^+]_o$

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.

$$E_m = (E_m)_0 + E_a \tag{1}$$

$$E_a = i_p \cdot R_m. \tag{2}$$

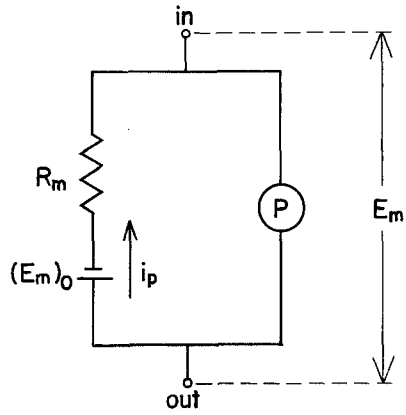


Fig. 12. Equivalent circuit of plasmalemma of *Chara*. $(E_m)_o$: passive electromotive force independent of ATP. R_m: membrane resistance. P: electrogenic ion pump i_p: current produced by the pump (P). E_m: total membrane potential measured

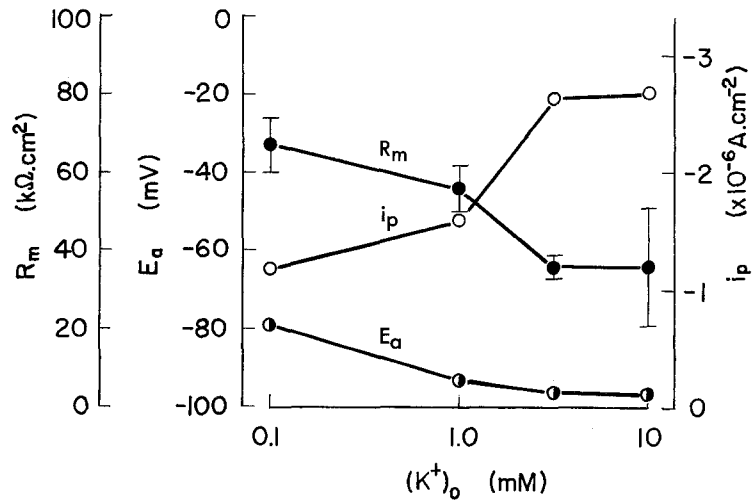


Fig. 13. The current produced by the pump, i_p (○), with different external K^+ concentrations, $[K^+]_o$ was calculated from the active component of the membrane potential, E_a (○), and the membrane resistance, R_m (●), according to Eq. (3), in which $[E_m-(E_m)_o]$ is equal to E_a. For further explanation,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

$(E_m)_0$, 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 Figs. 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 *et al.*, 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 & Gradmann, 1975). In the case of *Chara*, i_p at various $[K^+]_o$ can be calculated by the following equation:

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

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.

Dependence of Membrane Resistance (R_m) on $[ATP]_i$ and $[Mg^{2+}]_i$

The Mg-ATP-dependent potential change is probably not directly related to changes in passive ionic conductances, since R_m did not show concurrent changes with changes in E_m which were caused by modifying intracellular ATP-level (*cf.* Figs. 6–9). Lack of simultaneous changes in E_m and R_m due to decrease in $[ATP]_i$ was also demonstrated in *Neurospora*. When *Neurospora* hyphae are treated with azide and N_2, R_m begins to increase after the membrane depolarization has ceased (Slayman, 1965). The retarded increase in R_m observed in *Chara* (Table 2) as well as in *Neurospora* (Fig. 8 in Slayman, 1965) suggests that the membrane

structure responsible for higher ionic permeabilities is probably dependent on functioning of the ion pump driven by ATP. In this connection it is to be mentioned that E_m of *Vallisneria* leaf cells becomes sensitive to external H^+ in the light which hyperpolarizes the membrane via activating the putative H^+ pump, while it is insensitive to H^+ in darkness (Bentrup *et al.*, 1973). This fact suggests that during functioning of the ion pump the membrane is permeable to H^+.

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 out to see whether ATP- or Mg^{2+}-depleted cells are in 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 (Fig. 2*b* 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 mem-

brane 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 cannot 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 sturcture responsible for the excitability. ATP is known to be necessary for maintaining the membrane structure in human erythrocytes (Nakao, Nakao, Yamazoe & Yoshizawa, 1961; Quist & Roufogalis, 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.

Changes in Passive and Active Components of E_m on Excitation

After it became evident that in *Chara* 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 $[K^+]_o$ 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 addtion 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 ± 3 kΩcm^2 (n=11) to 2 ± 0.4 kΩcm^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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During preparation of this manuscript we received information from Mr. P. Smith (University of Sydney) that he, in collaboration with Dr. N.A. Walker, also found the presence of ATP-dependent membrane potential in *Chara corallina*.

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