J. Membrane Biol. 102, 255–264 (1988)

The Journal of
Membrane Biology

Possible Involvement of Protein Phosphorylation/Dephosphorylation in the Modulation of Ca^{2+} Channel in Tonoplast-Free Cells of *Nitellopsis*

T. Shiina,*† R. Wayne,‡ H.Y. Lim Tung,§ and M. Tazawa†

†Department of Biology, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan, ‡Section of Plant Biology, Cornell University, Ithaca, New York 14853-7101 and §Clayton Foundation Biochemical Institute, The University of Texas, Austin, Texas 78712

Summary. The regulation of voltage-dependent Ca^{2+} channels by protein phosphorylation and dephosphorylation was studied using tonoplast-free cells of *Nitellopsis*. Since the Ca^{2+}-channel activation has a dominant role in the membrane excitation of tonoplast-free cells (T. Shiina and M. Tazawa, *J. Membrane Biol.* **96**:263–276, 1987), it seems to be reasonable to assume that any change of the membrane excitability reflects a modulation of the Ca^{2+} channel. When agents that enhance phosphoprotein dephosphorylation (protein kinase inhibitor, phosphoprotein phosphatase-1, -2A) were introduced to the intracellular surface of the plasmalemma (twice-perfused tonoplast-free cells), the membrane potential depolarized and the membrane resistance decreased under current-clamp experiments. By contrast, when cells were challenged with agents that enhance protein phosphorylation (phosphoprotein phosphatase inhibitor-1, α-naphthylphosphate), the membrane potential hyperpolarized, and the membrane resistance increased. When phosphoprotein phosphatase-1 or -2A was perfused, the current-voltage (*I-V*) curve which was obtained under ramp voltage-clamp condition exhibited the so-called N-shaped characteristic, indicating an acceleration of the Ca^{2+}-channel activation. This effect was suppressed by the addition of phosphoprotein phosphatase inhibitors. ATP-γ-S, which is assumed to stimulate protein phosphorylation, decreased the inward current in the *I-V* curve. The dependence of the Ca^{2+}-channel activation on intracellular ATP was different between the once-perfused and twice-perfused cells. In once-perfused cells, the membrane excitability was reduced by low intracellular ATP concentration. By contrast, in twice-perfused cells, excitability was enhanced by ATP.

Key Words Ca^{2+} channel · *Characeae* · membrane excitation · *Nitellopsis* · phosphoprotein phosphatase · protein phosphorylation · tonoplast-free cell

Introduction

Ca^{2+} channels can be modulated by various neurotransmitters or drugs in animal cells (Reuter, 1983). A change in the Ca^{2+} influx would alter various cellular functions such as signal transmission, cell motility or cell development. The activities of ion channels are thought to be modulated by protein phosphorylation and dephosphorylation (Levitan, 1985). For example, various voltage-dependent Ca^{2+} channels in animal cells are positively modulated by cAMP-dependent protein phosphorylation (Osterreider et al., 1982; Doroshenko et al., 1984; Armstrong & Eckert, 1987) and the Ca^{2+}/diacylglycerol-dependent protein kinase (protein kinase C) (DeRiemer et al., 1985; Strong et al., 1986). Protein kinase C also negatively modulates Ca^{2+} channels (Rane & Dunlop, 1986; Hammond et al., 1987).

In plant cells, membrane transport also seems to be modulated by protein phosphorylation. Zocchi, Rogers and Hanson (1983) and Zocchi (1985) showed that an increase in membrane phosphorylation is correlated with an inhibition of the H^+-ATPase. Moreover, Clint and MacRobbie (1987) demonstrated that ATP is required for Na^+ extrusion and discussed the possible involvement of protein phosphorylation in the regulation of Na^+ extrusion in perfused *Chara* cells.

The excitability of tonoplast-free *Chara* cells is lost following the depletion of intracellular ATP (Shimmen & Tazawa, 1977; Lühring & Tazawa, 1985). Moreover membrane excitation of tonoplast-free cells in *Characeae* is caused by the activation of only the voltage-dependent Ca^{2+} channel without any contribution of the Cl^- channel (Kikuyama et al., 1984; Shiina & Tazawa, 1987*a*). Thus we assume that the activity of the Ca^{2+} channel in *Characeae* may be modulated by the degree of protein phosphorylation which is regulated by the intracellular concentration of ATP. In this paper, we tested the effects of highly purified phosphoprotein phosphatases from rabbit skeletal muscle and various

* *Present Address:* Faculty of Integrated Arts and Sciences, Hiroshima University, 1-1-89, Higashisenda-machi, Naka-ku, Hiroshima, 730, Japan.

Table 1. Compositions of perfusion media (in mM)[a]

	High-K medium	Low-K medium	ATP-regenerating medium	HK-medium
PIPES	20	5	5	5
EGTA	5	5	5	5
$MgCl_2$	6	6	6	6
Ficoll 70 (wt/vol)	5	5	5	5
ATP	0–1	0–1	0–1	0
PK	0	0	1	0
PEP	0	0	1	0
HK (mg/ml)	0	0	0	1
Glucose	0	0	0	5
pH	7.0	7.0	7.0	7.0

[a] Osmotic pressures of perfusion media were adjusted to 330 to 350 mOsm with sorbitol and glycerol.

inhibitors of protein kinase and phosphatase on Ca^{2+}-channel activation using tonoplast-free *Nitellopsis* cells. The effects of intracellular ATP concentration on Ca^{2+}-channel activation is also described. A part of the results was presented elsewhere (Shiina & Tazawa, 1986).

Abbreviations

AMP-PNP, adenylyl imidodiphosphate; ATP-γ-S, adenosine-5′-O-(3-thiotriphosphate); α-NP, α-naphthylphosphate; EGTA, ethyleneglycol-bis-(β-aminoethylether)N,N′-tetraacetic acid; HEPES, N-2 - hydroxyethylpiperazine - N′ - 2 - ethanesulfonic acid; HK, hexokinase; PEP, phospho(enol)pyruvate; PIPES, piperazine-N,N′-bis-(2-ethanesulfonic acid); PK, pyruvate kinase; PKI, protein kinase inhibitor.

Materials and Methods

Culture and Preparation

Internodal cells of *Nitellopsis obtusa* were mainly used. The alga was cultured in the laboratory as described in our previous paper (Shiina & Tazawa, 1987*a*). Internodal cells were isolated from neighboring cells and kept in artificial pond water (APW) containing 0.1 mM each of KCl, NaCl and $CaCl_2$. All experiments were carried out at room temperature (20 to 25°C).

Intracellular Perfusion Media and External Medium

The perfusion media used are listed in Table 1. Ficoll 70 was dialyzed against distilled water before use. The high-K medium was used for current-clamp experiments, and low-K medium for voltage-clamp experiments (Shiina & Tazawa, 1987*a*). To maintain a constant ATP level, an ATP-regenerating medium was used. HK-medium was used to deplete the intracellular ATP concentration.

The external medium was APW-7.5 with the pH adjusted to 7.5 with 2.0 mM HEPES-Na buffer.

Intracellular Perfusion

Intracellular perfusion was performed according to Tazawa, Kikuyama and Shimmen (1976). After loss of turgor pressure, both cell ends were cut off and the cell sap was replaced with the perfusion medium. After perfusion, both cell ends were ligated with polyester thread. The tonoplast disintegrated within 10 min after the perfusion. We call the tonoplast-free cells thus prepared once-perfused cells. Sometimes we perfused the cells again to remove the endoplasmic sol and to control the internal ATP level precisely. The once-perfused cells were kept in a moisture box without ligation for about 15 min and then reperfused and ligated. We call such tonoplast-free cells twice-perfused cells.

Electrical Measurement

Membrane potential (E_m) was measured using the conventional microelectrode method. Details of the current- and voltage-clamp measurements were described in our previous paper (Shiina & Tazawa, 1987*a*). E_m and the membrane current (I_m) were measured using the voltage-measuring and current-measuring circuits, respectively, and recorded on a pen-writing recorder (National VP6521A). In the current-clamp experiments, the membrane resistance (R_m) was measured by applying small constant-current pulses across the plasmalemma. In the voltage-clamp experiments, the current-voltage (*I-V*) curve was obtained by slowly depolarizing the membrane potential (V_m) in a ramp-shaped manner from a slightly hyperpolarized V_m relative to the resting value (rate approx. 400 mV/min). The amplitude of the peak inward current ($(I_m)_p$) was measured as described in our previous paper (Shiina & Tazawa, 1987*a*). We termed V_m at $(I_m)_p$ as $(V_m)_p$. The mean value of $(I_m)_p$ was calculated from that of cells showing N-shaped *I-V* curve. Small constant voltage pulses were applied for the measurements of chord conductance. The chord membrane conductance at $(I_m)_p$ and slope membrane conductance are termed $(G_m)_p$ and $(G_m)_{slope}$, respectively. When the *I-V* curve was not of the N-shaped type, we regarded the chord membrane conductance at the mean $(V_m)_p$ obtained for the excitable cells as $(G_m)_p$.

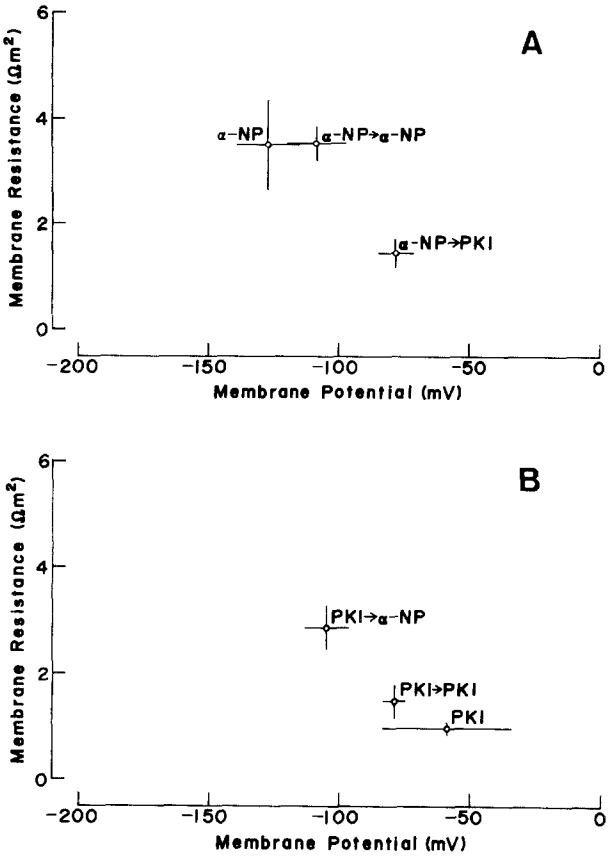

Fig. 1. Effects of α-NP and PKI on the relationship between R_m and E_m in once- or twice-perfused tonoplast-free cells of *Nitellopsis*. High-K perfusion medium containing 1 mM ATP and 50 μM Na_3VO_4 was used. E_m and R_m were measured 30 min after the first perfusion under current-clamp condition. All data are shown as mean ± SEM (n = 3 to 7)

Preparation of Phosphoprotein Phosphatases and Inhibitor-1

Protein phosphatase-1, -2A and the active phosphorylated form of phosphoprotein phosphatase inhibitor-1 were purified to homogeneity from rabbit skeletal muscle after Tung et al. (1984) and Nimmo and Cohen (1978). Activities of enzymes were assayed after Hemmings, Resink and Cohen (1982) and Foulkers and Cohen (1980), and those of the stock solutions were 260 U/ml (phosphatase-1) and 50 U/ml (phosphatase-2A), respectively. The stock solution of the inhibitor-1 contained 56 nmol/ml inhibitor. Inhibitor-1 at 10 to 25 nM inhibited phosphatase-1 at 0.015 U/ml by 100% in vitro (Tung et al., 1984).

Assay of ATP Level

Cells were frozen with liquid nitrogen and stored in a freezer at −20°C. ATP was extracted in boiling buffer containing 25 mM HEPES, 10 mM EDTA and 0.3% H_2O_2 for 5 min. The pH of the buffer was adjusted to 7.4 with KOH. The ATP was measured by the firefly-flash method with an ATP photometer (Chemglow photometer J4-7441; Aminco, Silver Spring, Md.) (Mimura, Shimmen & Tazawa, 1983).

Chemicals

α-NP and PKI (Type II) were purchased from Sigma. ATP-γ-S was purchased from Boehringer Mannheim.

Results

Control of E_m by Protein Phosphorylation and Phosphoprotein Dephosphorylation

In *Nitellopsis* cells, E_m is more negative than the passive diffusion potential as a result of the operation of an electrogenic H^+ pump (Mimura et al., 1983; Takeshige, Shimmen & Tazawa, 1986). Inhibition of the electrogenic H^+ pump either by intracellular ATP depletion (Shimmen & Tazawa, 1977; Mimura et al., 1983) or by intracellular perfusion of vanadate (Shimmen & Tazawa, 1982) causes a depolarization of E_m and an increase in R_m. When the electrogenic H^+ pump activity in twice-perfused cells is reduced by lowering the intracellular ATP concentration, the plasmalemma sometimes falls into the excited state which is characterized by a largely depolarized E_m and a low R_m (Mimura et al., 1983). To compare the membrane excitability under current-clamp condition, we depolarized the E_m near the threshold of the membrane excitation by inhibiting the electrogenic H^+ pump. To this end, we perfused the cells with a high-K media containing 50 μM Na_3VO_4, which is known to inhibit the electrogenic H^+ pump but not influence the membrane excitability in *Nitellopsis* (Shimmen & Tazawa, 1982). We then measured E_m and R_m 30 min after the perfusion.

When cells were perfused with the medium containing 1 mM α-NP, a synthetic phosphoprotein phosphatase inhibitor (Li, 1984; Pondaven & Meijer, 1986), the plasmalemma remained in the resting state with a comparatively hyperpolarized E_m and a high R_m value (Fig. 1*A*). The plasmalemma of the cells twice perfused with α-NP showed the same tendency. However, when 250 μg/ml PKI was introduced in the second perfusion, E_m drastically depolarized and R_m decreased, indicating that the plasmalemma entered the excited state. PKI (Type II) is known to inhibit protein phosphorylation catalyzed by several types of protein kinases (Szmigielski, Guidotti & Costa, 1977). The plasmalemma of cells that were perfused with PKI were in the excited state no matter whether the perfusion was single or double (Fig. 1*B*). However, when cells were perfused first with PKI and next with α-NP,

Table 2. Effects of protein kinase inhibitor (PKI), AMP-PNP and α-NP on E_m and R_m in ATP-depleted twice-perfused cells[a]

		E_m (mV)	R_m (Ωm^2)
PKI	0	−82.9 ± 9.8 (4)	7.73 ± 1.70 (4)
(μg/ml)	3.5	−61.3 ± 11.8 (4)	4.67 ± 1.83 (4)
	35.0	−57.6 ± 3.5 (4)	1.90 ± 0.71 (4)
AMP-PNP	0	−89.9 ± 10.9 (5)	8.66 ± 2.22 (5)
(mM)	0.1	−51.0 ± 7.9 (4)	3.76 ± 1.51 (4)
	1.0	−61.6 ± 4.4 (4)	2.99 ± 0.95 (4)
	1.0 + 1.0 α-NP	−88.7 ± 4.8 (4)	9.25 ± 2.87 (4)

[a] All data are shown as mean ± SEM (number of cells).

Table 3. Effects of phosphoprotein phosphatase-1 (PP-1), phosphoprotein phosphatase-2A (PP-2A) and phosphoprotein phosphatase inhibitor-1 (PPInh-1) on E_m and R_m in ATP-depleted twice-perfused cells[a]

		E_m (mV)	R_m (Ωm^2)
PP-1	0	−92.4 ± 6.8 (4)	3.27 ± 0.59 (4)
(unit/ml)	0.1	−88.8 ± 7.7 (4)	2.33 ± 0.23 (4)
	0.5	−77.8 ± 8.8 (4)	2.25 ± 0.84 (4)
	1.0	−72.7 ± 12.2 (4)	1.48 ± 0.27 (4)
PP-2A	0	−92.4 ± 6.8 (4)	3.27 ± 0.59 (4)
(unit/ml)	0.1	−81.5 ± 2.8 (3)	2.40 ± 0.41 (3)
	1.0	−77.1 ± 11.0 (4)	2.04 ± 0.82 (4)
1.0 unit/ml	0	−72.7 ± 12.2 (4)	1.48 ± 0.27 (4)
PP-1+	0.00224	−84.3 ± 9.0 (3)	2.46 ± 0.69 (3)
PPInh-1 (μM)	0.0112	−80.7 ± 8.7 (4)	4.45 ± 1.62 (4)
	0.112	−106.0 ± 11.0 (4)	3.01 ± 1.30 (4)
	1.12	−108.5 ± 22.2 (3)	3.74 ± 0.94 (3)

[a] All data are shown as mean ± SEM (number of cells).

the plasmalemma returned to the resting state. Although PKI (Type II) from Sigma was an impure preparation, its effects could be completely reversed by the phosphoprotein phosphatase inhibitor, α-NP, indicating that the kinase inhibitor may be an active agent in the preparation.

As shown in Table 2, PKI clearly depolarized E_m and decreased R_m even when the electrogenic H^+ pump was inhibited in ATP-depleted twice-perfused cells. Since the K_m value of the electrogenic H^+ pump for ATP is around 100 μM (Mimura et al., 1983; Takeshige et al., 1986), the electrogenic H^+ pump should be inhibited by intracellular ATP depletion (μM order). However, the K_m of higher plant protein kinases for ATP is assumed to be around 10 μM (*cf.* Davies & Polya, 1983). Thus protein kinases in ATP-depleted twice-perfused cells are assumed to be operating although the electrogenic H^+ is inhibited. AMP-PNP, which cannot serve as a substrate of either ATPase or protein kinase, had the same effect as PKI on E_m and R_m. The addition of 1 mM α-NP reversed the effect of AMP-PNP.

Table 4. Effects of phosphoprotein phosphatases (PP-1, PP-2A) and phosphoprotein phosphatase inhibitors (PPIhn-1, α-NP) on number of cells showing an N-shaped *I-V* curve, $(G_m)_{slope}$ and $(I_m)_p$ in twice-perfused *Nitellopsis* cells[a]

	Number of cells showing N-shaped *I-V* curve	$(G_m)_{slope}$ (S/m^2)	$(I_m)_p$ (mA/m^2)
control	1/4	2.41 ± 0.90 (4)	131.0 (1)
PP-1	5/5	0.69 ± 0.23 (5)	111.8 ± 31.0 (5)
PP-1 + PPInh-1	2/5	2.46 ± 0.77 (5)	56.8 ± 16.0 (3)
control	0/6	3.82 ± 0.85 (6)	—[b]
PP-2A	4/5	1.83 ± 0.62 (5)	164.6 ± 31.6 (4)
PP-2A + -NP	0/4	2.39 ± 1.35 (4)	—[b]

[a] All data are shown as mean ± SEM (number of cells).
[b] $(I_m)_p$ could not be measured in this treatment since there were not any cells that exhibited an N-shaped *I-V* curve.

The effects of phosphoprotein phosphatases and phosphoprotein phosphatase inhibitor on both E_m and R_m of twice-perfused cells are summarized in Table 3. The electrogenic H^+ pump was inhibited by twice perfusing the cells with ATP-free high-K medium. Protein phosphatase-1 and -2A added to the second perfusion medium depolarized E_m and decreased R_m in a concentration-dependent manner, indicating that the tendency of the plasmalemma to enter the excited state was enhanced by dephosphorylation. Moreover, phosphoprotein phosphatase inhibitor-1, which was applied with 1.0 U/ml phosphoprotein phosphatase-1, reversed the effect of phosphatase-1 and restored the plasmalemma from the excited state to the resting state by hyperpolarizing the depolarized E_m and increasing the lowered R_m.

Effects of Phosphoprotein Phosphatases on *I-V* Curve Under Voltage-Clamp Conditions

Figure 2 shows the effects of phosphoprotein phosphatase-1 on the *I-V* curves under voltage-clamp conditions. Cells were first perfused with the low-K medium containing 1 mM ATP. When cells were reperfused with the same low-K medium (control), only one out of four cells showed the typical N-shaped *I-V* curve (Fig. 2(*A*), Table 4). However, all of the cells ($n = 5$), which were reperfused with the low-K medium containing 2 units/ml phosphoprotein phosphatase-1 showed the N-shaped *I-V* curves, indicating that the cells were excitable (Fig. 2*B*). When cells were reperfused with the low-K

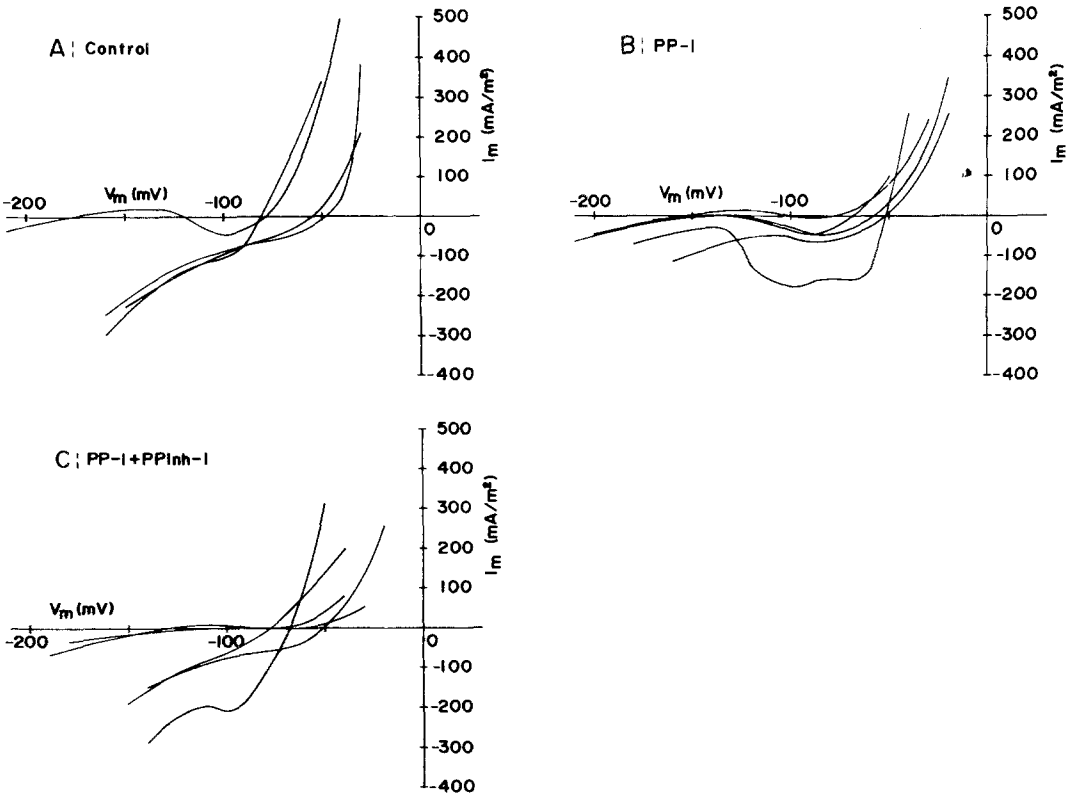

Fig. 2. Effects of phosphoprotein phosphatase 1 (PP-1) and phosphoprotein phosphatase inhibitor-1 (PPInh-1) on *I-V* curves recorded under ramp voltage clamping in twice-perfused tonoplast-free cells of *Nitellopsis*. Low-K perfusion medium containing 1 mM ATP was used. (*A*) control; (*B*) 2 units/ml PP-1; (*C*) 2 units/ml PP-1 and 1 μM PPInh-1

medium containing both 2 units/ml phosphoprotein phosphatase-1 and 1 μM phosphoprotein phosphatase inhibitor-1, the ratio of cells which showed the N-shaped *I-V* curve decreased (Fig. 2*C*) and $(I_m)_p$ was smaller than that of cells perfused with phosphoprotein phosphatase-1 (Table 4). The $(G_m)_{slope}$ at comparatively hyperpolarized region ($V_m < -120$ mV) of cells perfused with phosphoprotein phosphatase-1 was smaller than that of control cells. Protein phosphatase inhibitor-1 reversed the effect of phosphoprotein phosphatase and returned $(G_m)_{slope}$ to the control level.

In another experiment, none of the cells showed an N-shaped *I-V* curve, when they were reperfused with the low-K medium without phosphoprotein phosphatase (control) (Fig. 3(*A*), Table 4). Although $(G_m)_{slope}$ was comparatively high in these cells, they were not in the excited state, because the excited cells had an even higher slope conductance (*cf.* Table 6 OATP). When cells were reperfused with the low-K medium containing 1 unit/ml phosphoprotein phosphatase-2A, the ratio of cells showing an N-shaped *I-V* curve increased (Fig. 3*B*). The *I-V* curve became non-N-shaped by adding phosphoprotein phosphatase inhibitor α-NP to the perfusion medium (Fig. 3*C*). $(G_m)_{slope}$ at the hyperpolarized region also became smaller by the perfusion with phosphatase-2A. This could be partially restored by the phosphatase inhibitor (α-NP).

Effects of ATP-γ-S on $(I_m)_p$

We tested the effect of ATP-γ-S on both $(I_m)_p$ and $(G_m)_p$. ATP-γ-S is a substrate for thiophosphorylation but is not easily dephosphorylated (Gratecos & Fischer, 1974; Morgan, Perry & Ottaway, 1976). Cells were once perfused with the low-K medium lacking ATP. Both $(I_m)_p$ and $(G_m)_p$ of the control cells increased slightly during measurement (Fig. 4). 1 mM ATP-γ-S drastically decreased both $(I_m)_p$ and $(G_m)_p$.

Effects of the Intracellular ATP Concentration on the *I-V* Curve

If the activation of the Ca^{2+} channel in *Nitellopsis* is modulated by protein phosphorylation, depletion of the intracellular ATP would be expected to enhance phosphoprotein dephosphorylation and activate the

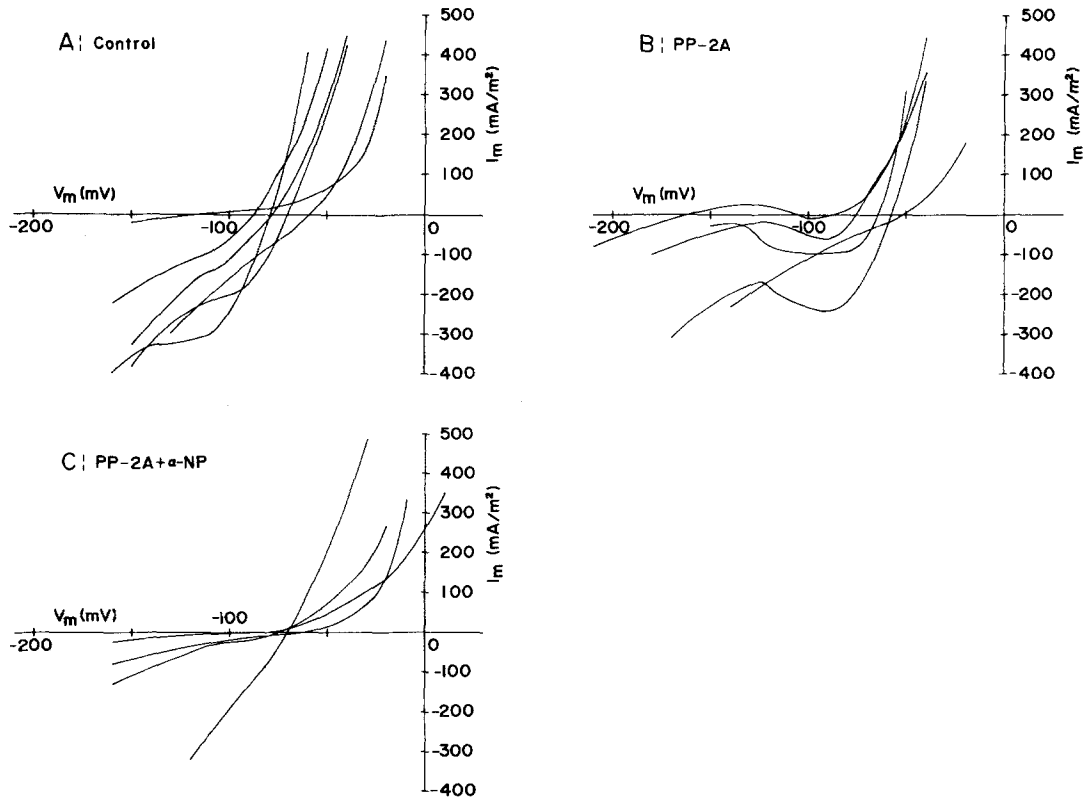

Fig. 3. Effects of phosphoprotein phosphatase-2A (PP-2A) and α-NP on *I-V* curves recorded under ramp voltage clamping in twice-perfused tonoplast-free cells of *Nitellopsis*. Low-K perfusion medium containing 1 mM ATP was used. (*A*) control; (*B*) 1 unit/ml PP-2A; (*C*) PP-2A and 1 mM α-NP

Table 5. Dependence of $(I_m)_p$ and $(G_m)_p$ on the intracellular ATP concentration in once-perfused tonoplast-free cells of *Nitellopsis*[a]

ATP concentration (μM)	$(I_m)_p$ (mA/m^2)	$(G_m)_p$ (S/m^2)	Cell number
0	0	0.74 ± 0.29	5
<200 (96.0 ± 15.7)	52.4 ± 14.9	2.42 ± 0.49	7
>200 (683.0 ± 48.3)	107.4 ± 15.7	3.82 ± 0.40	5

[a] All data are shown as mean ± SEM (number of cells).

Ca^{2+} channel. However, Shimmen and Tazawa (1977) reported that the excitability of the plasmalemma was lost when the intracellular ATP was completely depleted by perfusion with a medium containing hexokinase and glucose. This is contrary to our finding that phosphoprotein dephosphorylation positively modulated the membrane excitability (Shiina & Tazawa, 1986). In order to resolve this discrepancy, we first examined the effect of ATP depletion on the inward current without using the hexokinase system. Cells were once perfused with low-K media containing various concentrations of ATP or HK-medium. The ATP concentration in the tonoplast-free cells varied from cell to cell depending on the ATP concentrations in both the perfusion medium and the cytoplasm. The mean values of $(I_m)_p$ and $(G_m)_p$ relative to 3 ATP levels (0, <200, >200 μM) are shown in Table 5. We used the hexokinase system to deplete the ATP concentration completely in once-perfused cells. The electrophysiological measurements were performed about 40 min after the perfusion. Cells were frozen with liquid nitrogen immediately after the measurement. Both $(I_m)_p$ and $(G_m)_p$ increased as the intracellular ATP concentration increased. This result is in good agreement with those of Shimmen and Tazawa (1977) and Lühring and Tazawa (1985).

However, the above results were almost reversed when cells were perfused twice. We perfused the cells first with the ATP-regenerating medium without ATP, and then with media containing either 0, 100 or 1000 μM ATP. When cells were perfused with 1000-ATP medium, $(G_m)_{slope}$ of the *I-V* curve below −120 mV was comparatively low and half of the curves were N-shaped (Fig. 5(*A*),

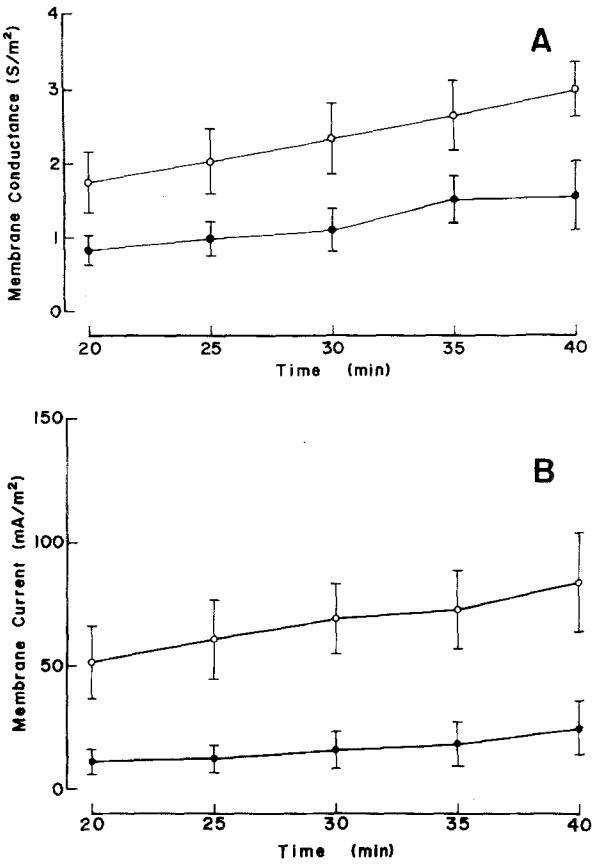

Fig. 4. Effects of ATP-γ-S on $(I_m)_p$ (*A*) and $(G_m)_p$ (*B*) in once-perfused tonoplast-free cells of *Nitellopsis*. ○: control, ●: ATP-γ-S. Low-K perfusion medium containing no ATP was used. The concentration of ATP-γ-S was 1 mM. Horizontal line means the time after perfusion. All data are shown as mean ± SEM (n = 4 to 5)

Table 6). By contrast, the *I-V* curve of cells perfused with 0-ATP medium had very high $(G_m)_{slope}$ (Fig. 5*C*). Since this value is close to $(G_m)_{slope}$ of 1000-ATP cells between −40 to −50 mV (12.44 ± 0.78 S/m² (n = 4)) where the plasmalemma is assumed to be in the excited state, we considered that the membranes of cells treated with 0-ATP were in the excited state and could not return to the resting state even under the voltage-clamp condition. The *I-V* curve of cells perfused with 100-ATP medium was a mixed type. Among five cells, two cells had very high $(G_m)_{slope}$ (12.15 ± 3.97 S/m² (n = 2)), and three cells had comparatively low $(G_m)_{slope}$ (1.48 ± 0.79 S/m² (n = 3)) and showed typical N-shaped *I-V* curves (Fig. 5*B*).

Discussion

We recently demonstrated that only the Ca^{2+} channel is activated during the plasmalemma excitation

Table 6. Effects of intracellular ATP concentration on number of cells showing an N-shaped *I-V* curve, $(G_m)_{slope}$ and $(I_m)_p$ in twice-perfused *Nitellopsis* cells[a]

ATP (μM)	Number of cells showing N-shaped *I-V* curve	$(G_m)_{slope}$ (S/m²)	$(I_m)_p$ (mA/m²)
0	0/4	16.91 ± 3.27 (4)	—[b]
100	3/5	5.75 ± 2.93 (5)	125.0 ± 10.9 (3)
1000	2/4	2.65 ± 0.87 (4)	102.2 ± 31.3 (2)

[a] All data are shown as mean ± SEM (number of cells).
[b] $(I_m)_p$ could not be measured in this treatment since there were not any cells that exhibited an N-shaped *I-V* curve.

in tonoplast-free cells (Shiina & Tazawa, 1987*a*). The Ca^{2+}-activated Cl^- channel which functions in normal cells (Lunevsky et al., 1983; Shiina & Tazawa, 1987*b*; Tsutsui et al., 1987*a*,*b*) is inoperative in tonoplast-free cells. Thus it is reasonable to assume that any modification of the plasmalemma excitability in tonoplast-free cells reflects a change in the activity of the plasmalemma Ca^{2+} channel.

E_m and R_m of the twice-perfused tonoplast-free cells whose electrogenic H^+ pump was inhibited either by Na_3VO_4 or by ATP depletion could be controlled by agents which are known to influence protein phosphorylation or phosphoprotein dephosphorylation. α-NP or phosphoprotein phosphatase inhibitor-1, which are expected to stimulate protein phosphorylation, maintained a hyperpolarized E_m and a large R_m value, indicating that cells were in the resting state. On the other hand, phosphoprotein phosphatase-1 or -2A or protein kinase inhibitor, all of which are assumed to enhance phosphoprotein dephosphorylation, depolarized E_m and decreased R_m. The cells perfused with the latter agents are assumed to be in the excited state. These results are in good agreement with our previous finding that α-NP decreased the inward current, while PKI reversed the α-NP effect (Shiina & Tazawa, 1986).

The number of cells which showed an N-shaped *I-V* curve increased when we perfused the cell with phosphoprotein phosphatase-1 or -2A, and decreased when we perfused the cells with phosphoprotein phosphatase inhibitors (Figs. 2, 3, Table 4). ATP-γ-S which would increase the degree of protein phosphorylation greatly decreased $(I_m)_p$ and $(G_m)_p$ (Fig. 4). Since the commercially available ATP-γ-S is contaminated with a considerable amount of ADP, the intracellular ATP concentration in the cells perfused with ATP-γ-S may be higher than that in the control cells as a consequence of the action of an endogenous adenylate kinase. However, the increase in the ATP concen-

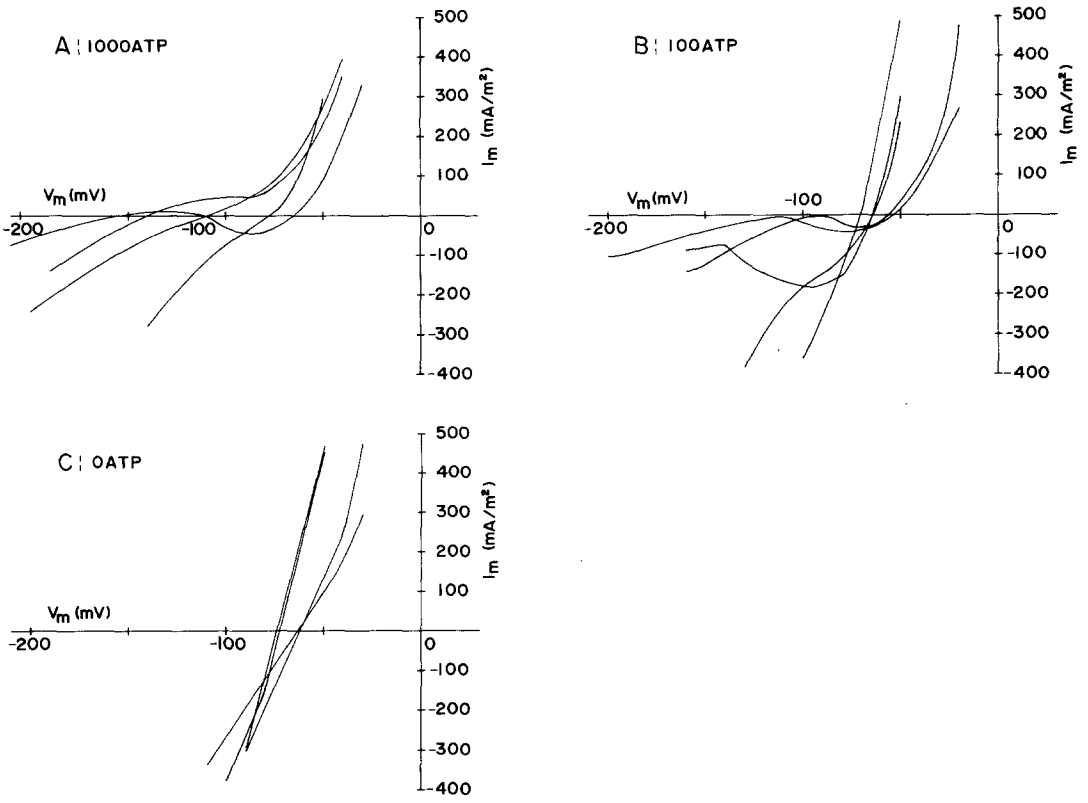

Fig. 5. Effect of intracellular ATP concentration on *I-V* curves recorded under ramp voltage clamping in twice-perfused tonoplast-free cells of *Nitellopsis*. As perfusion media, ATP regenerating media were used. (*A*) 1000 μM ATP; (*B*) 100 μM ATP; (*C*) 0 μM ATP

tration in a once-perfused cell would be expected to increase $(I_m)_p$ (Table 5). Thus we believe that the inhibition of the inward current is not caused by the ATP concentration increase. From these results, it seems to be reasonable that inhibition of the voltage-dependent Ca^{2+} channel in the plasmalemma of tonoplast-free cells is closely related to protein phosphorylation and enhancement of the channel is related to phosphoprotein dephosphorylation. However, we could not exclude the possibility that other ion channels including K^+ channel were also modulated by protein phosphorylation, since the *I-V* curve was very complicated. In addition, protein phosphorylation may influence the leakage properties of the plasmalemma, since $(G_m)_{slope}$ of the *I-V* curve in the hyperpolarized region seems to become smaller when phosphoprotein phosphatases were perfused.

The depletion of intracellular ATP causes a loss of membrane excitability in *Chara* tonoplast-free cells (Shimmen & Tazawa, 1977; Lühring & Tazawa, 1985). The dependence of the amplitude of the inward current on the intracellular ATP concentration which was obtained using once-perfused cells in *Nitellopsis* supports the previous results (Table 5). When the intracellular ATP concentration decreases, the equilibrium of the protein phosphorylation would shift toward dephosphorylation. If the Ca^{2+} channel in *Nitellopsis* is positively modulated by phosphoprotein dephosphorylation, the measured inward current should increase following ATP depletion. However, the once-perfused cells behaved against the expectation and the inward current became smaller when the intracellular ATP was decreased. On the other hand, in twice-perfused cells the membrane excitability increased as the intracellular ATP concentration decreased (Fig. 5). Therefore the [ATP] does not directly regulate the Ca^{2+} channel. The indirect regulation may depend on the biochemical constituents of the tonoplast-free cell.

Protein phosphorylation usually regulates physiological events through an amplification cascade where many phosphatases and kinases are involved and interrelated (Cohen, 1982). It is likely that some proteins which are concerned with protein phosphorylation may be washed out by the second perfusion. Indeed, rapid perfusion of *Chara* seems to wash out a putative Ca^{2+}-dependent protein kinase whereas it is retained in slowly perfused cells (Tominaga et al., 1987). The degree of protein phosphorylation in a cell should be determined by the

relative activities of both protein kinases and the phosphoprotein phosphatases. The activities of the phosphatases and kinases themselves are regulated through phosphorylation. For example, the Mg-ATP-dependent phosphoprotein phosphatase (the catalytic subunit of which is phosphatase-1) is activated when the regulatory subunit (inhibitor-2) is phosphorylated. The complexity increases when we realize that inhibitor-1 can only inhibit phosphoprotein phosphatase-1 when inhibitor-1 is in its phosphorylated form. Therefore the ability of ATP to regulate the state of phosphorylation will depend on which kinases and phosphatases are present, what substrates these kinases can phosphorylate, and then relative K_ms for ATP and the various substrates.

We could not observe enhancement of the inward current by phosphoprotein phosphatases in once-perfused cells (*data not shown*), although they positively modulated the membrane excitability in twice-perfused cells (Table 3, Figs. 2, 3). If the activity of the endogenous phosphoprotein phosphatase in the endoplasmic sol would be so high that the proteins are in the dephosphorylated state, the exogenous phosphoprotein phosphatase would hardly affect the protein phosphorylation. On the other hand, when phosphoprotein phosphatases in the sol are washed out by the twice perfusion, exogenous phosphatases would be effective.

The Ca^{2+} channel in *Characeae* cells is assumed to play a primary role in excitation-cessation coupling (Hayama, Shimmen & Tazawa, 1979), in turgor regulation (Okazaki & Tazawa, 1986; Okazaki et al., 1987) and in Cl^--channel activation (Findlay & Hope, 1964; Beilby & Coster, 1979; Lunevsky et al., 1983; Tsutsui et al., 1987*a*,*b*; Shiina & Tazawa, 1987*b*). If the activation of the Ca^{2+} channel is related to protein phosphorylation, the control of protein phosphorylation and phosphoprotein dephosphorylation may be of primary importance in the regulation of cytoplasmic streaming (Tominaga et al., 1987), turgor regulation and membrane excitation.

Presently we have no information regarding whether the Ca^{2+} channel is modulated directly through phosphorylation of channel proteins or indirectly through the phosphorylation of the regulatory molecules. Studies on the Ca^{2+} channel using cell-free membrane patches lacking cytoplasmic regulatory mechanisms may elucidate this point.

The authors wish to thank Dr. T. Shimmen (Department of Biology, University of Tokyo) for critically reading this manuscript. This work was supported by a Grant-in-Aid for Special Project Research (No. 60223011) from the Coordination Funds for the Promotion of Science and Technology from the Science and Technology Agency of Japan, and by a Grant-in-Aid for Special Project Research (No. 61215007) from the Ministry of Education, Science and Culture.

References

Armstrong, D., Eckert, R. 1987. Voltage-activated calcium channels that must be phosphorylated to respond to membrane depolarization. *Proc. Natl. Acad. Sci. USA* **84:**2518–2522

Beilby, M.J., Coster, H.G.L. 1979. The action potential in *Chara corallina*. II. Two activation-inactivation transients in voltage clamps of the plasmalemma. *Aust. J. Plant. Physiol.* **6:**323–335

Clint, G.M., MacRobbie, E.A.C. 1987. Sodium efflux from perfused giant algal cells. *Planta* **171:**247–253

Cohen, P. 1982. The role of protein phosphorylation in neutral and hormonal control of cellular activity. *Nature (London)* **296:**613–620

Davies, J.R., Polya, G.M. 1983. Purification and properties of a high specific activity protein kinase from wheat germ. *Plant. Physiol.* **71:**489–495

DeRiemer, S.A., Strong, J.A., Albert, K.A., Greengard, P., Kaczmarek, L.K. 1985. Enhancement of calcium current in *Aplysia* neurons by phorbol ester and protein kinase C. *Nature (London)* **313:**313–316

Doroshenko, P.A., Kostyuk, P.G., Martynyuk, A.E., Kursky, M.D., Vorobetz, Z.D. 1984. Intracellular protein kinase and calcium inward currents in perfused neurons of the snail helix pomatia. *Neuroscience* **11:**263–267

Findlay, G.P., Hope, A.B. 1964. Ionic relations of cells of *Chara australis*. IX. Analysis of transient membrane currents. *Austr. J. Biol. Sci.* **17:**400–411

Foulkers, J.G., Cohen, P. 1980. The regulation of glycogen metabolism. Purification and properties of protein phosphatase inhibitor-2 from rabbit skeletal muscle. *Eur. J. Biochem.* **105:**195–203

Gratecos, D., Fischer, E.H. 1974. Adenosine 5'-O-(3-thiotriphosphate) in the control of phosphorylase activity. *Biochem. Biophys. Res. Commun.* **58:**960–967

Hammond, C., Paupardin-Tritsch, D., Nairn, A.C., Greengard, P., Gerschenfeld, M. 1987. Cholecystokinin induces a decrease in Ca^{2+} current in snail neurons that appears to be mediated by protein kinase C. *Nature (London)* **325:**809–811

Hayama, T., Shimmen, T., Tazawa, M. 1979. Participation of Ca^{2+} in cessation of cytoplasmic streaming induced by membrane excitation in *Characeae* internodal cells. *Protoplasma* **99:**305–321

Hemmings, B.A., Resink, T.J., Cohen, P. 1982. Reconstitution of a Mg-ATP-dependent protein phosphatase and its activation through a phosphorylation mechanism. *FEBS Lett.* **150:**319–324

Kikuyama, M., Oda, K., Shimmen, T., Hayama, T., Tazawa, M. 1984. Potassium and chloride effluxes during excitation of Characeae cells. *Plant Cell Physiol.* **25:**965–974

Levitan, I.B. 1985. Phosphorylation of ion channels. *J. Membrane Biol.* **87:**177–190

Li, H.C. 1984. Activation of brain calcineurin phosphatase towards nonprotein phosphoesters by Ca^{2+}, calmodulin, and Mg^{2+}. *J. Biol. Chem.* **259:**8801–8807

Lühring, H., Tazawa, M. 1985. Cytoplasmic Ca^{2+} has no effect on the excitability of *Chara* plasmalemma. *Plant Cell Physiol.* **26:**769–774

Lunevsky, V.Z., Zherelova, O.M., Vostrikov, I.Y., Beres-

tovsky, G.N. 1983. Excitation of *Characeae* cell membranes as a result of activation of calcium and chloride channels. *J. Membrane Biol.* **72**:43–58

Mimura, T., Shimmen, T., Tazawa, M. 1983. Dependence of the membrane potential on intracellular ATP concentration in tonoplast-free cells of *Nitellopsis obtusa*. *Planta* **157**:97–104

Morgan, M., Perry, S.V., Ottaway, J. 1976. Myosin light chain phosphatase. *Biochem. J.* **157**:687–697

Nimmo, G.A., Cohen, P. 1978. The regulation of glycogen metabolism. Purification and characterization of protein phosphatase inhibitor-1 from rabbit skeletal muscle. *Eur. J. Biochem.* **87**:341–351

Okazaki, Y., Tazawa, M. 1986. Ca^{2+} antagonist nifedipine inhibits turgor regulation upon hypotonic treatment in internodal cells of *Lamprothamnium*. *Protoplasma* **134**:65–66

Okazaki, Y., Yoshimoto, Y., Hiramoto, Y., Tazawa, M. 1987. Turgor regulation and cytoplasmic free Ca^{2+} in the alga *Lamprothamnium*. *Protoplasma* **140**:67–71

Osterreider, W., Brum, G., Hescheler, J., Trautwein, W. 1982. Injection of subunits of cyclic AMP-dependent protein kinase into cardiac myocytes modulates Ca^{2+} current. *Nature (London)* **298**:576–578

Pondaven, P., Meijer, L. 1986. Protein phosphorylation and oocyte maturation. I. Induction of starfish oocyte maturation by intracellular microinjection of a phosphatase inhibitor, α-naphthylphosphate. *Exp. Cell Res.* **163**:477–488

Rane, S.G., Dunlop, K. 1986. Kinase C activator 1,2-oleoylacetylgycerol attenuates voltage-dependent calcium current in sensory neurons. *Proc. Natl. Acad. Sci. USA* **83**:184–188

Reuter, H. 1983. Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature (London)* **301**:569–574

Shiina, T., Tazawa, M. 1986. Regulation of membrane excitation by protein phosphorylation in *Nitellopsis obtusa*. *Protoplasma* **134**:60–61

Shiina, T., Tazawa, M. 1987*a*. Demonstration and characterization of Ca^{2+} channel in tonoplast-free cells of *Nitellopsis obtusa*. *J. Membrane Biol.* **96**:263–276

Shiina, T., Tazawa, M. 1987*b*. Ca^{2+}-activated Cl^- channel in plasmalemma of *Nitellopsis obtusa*. *J. Membrane Biol.* **99**:137–146

Shimmen, T., Tazawa, M. 1977. Control of membrane potential and excitability of *Chara* cells with ATP and Mg^{2+}. *J. Membrane Biol.* **37**:167–192

Shimmen, T., Tazawa, M. 1982. Effects of intracellular vanadate on electrogenesis, excitability and cytoplasmic streaming in *Nitellopsis obtusa*. *Plant Cell Physiol.* **23**:669–677

Strong, J.A., Fox, A.P., Tsien, R.W., Kaczmarek, L.K. 1986. Phorbol ester promotes a large conductance Ca channel in *Aplysia* bag cell neurons. *Biophys. J.* **49**:430a

Szmigielski, A., Guidotti, A., Costa, E. 1977. Endogenous protein kinase inhibitors. Purification, characterization, and distribution in different tissues. *J. Biol. Chem.* **252**:3848–3853

Takeshige, K., Shimmen, T., Tazawa, M. 1986. Quantitative analysis of ATP-dependent H^+ efflux and pump driven by an electrogenic pump in *Nitellopsis obtusa*. *Plant Cell Physiol.* **27**:337–348

Tazawa, M., Kikuyama, M., Shimmen, T. 1976. Electric characteristics and cytoplasmic streaming of Characeae cells lacking tonoplast. *Cell Struct. Func.* **1**:165–176

Tominaga, Y., Wayne, R., Tung, H.Y.L., Tazawa, M. 1987. Phosphorylation-dephosphorylation is involved in Ca^{2+}-controlled cytoplasmic streaming of Characean cells. *Protoplasma* **136**:161–169

Tsutsui, I., Ohkawa, T., Nagai, R., Kishimoto, U. 1987*a*. Role of calcium ion in the excitability and electrogenic pump activity of the *Chara corallina* membrane: I. Effects of La^{3+}, verapamil, EGTA, W-7, and TFP on the action potential. *J. Membrane Biol.* **96**:65–74

Tsutsui, I., Ohkawa, T., Nagai, R., Kishimoto, U. 1987*b*. Role of calcium ion in the excitability and electrogenic pump activity of the *Chara corallina* membrane: II. Effects of La^{3+}, EGTA, and calmodulin antagonists on the current-voltage relation. *J. Membrane Biol.* **96**:75–84

Tung, H.Y.L., Resink, T.J., Hemmings, B.A., Shenolikar, S., Cohen, P. 1984. The catalytic subunits of protein phosphatase-1 and protein phosphatase-2A are distinct gene products. *Eur. J. Biochem.* **138**:635–641

Zocchi, G. 1985. Phosphorylation-dephosphorylation of membrane proteins controls the microsomal H^+-ATPase activity of corn roots. *Plant Sci.* **40**:153–159

Zocchi, G., Rogers, S.A., Hanson, J.B. 1983. Inhibition of protein pumping in corn roots is associated with increased phosphorylation of membrane proteins. *Plant Sci. Lett.* **31**:215–221

Received 21 October 1987; revised 21 January 1988