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Maki Katsuhara and Masashi Tazawa

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Calcium-regulated channels and their bearing on physiological activities in Characean cells

MAKI KATSUHARA[1] AND MASASHI TAZAWA[2]

[1]*Research Institute for Bioresources, Okayama University, Kurashiki, 710 Japan*
[2]*Department of Applied Physical Chemistry, Fukui Institute of Technology, Gakuen-cho, Fukui, 910 Japan*

SUMMARY

Calcium is involved in the regulation of cytoplasmic streaming, membrane excitation, turgor regulation and salt tolerance in the giant internodal cells of the Characeae. To analyse the mechanism of Ca^{2+} action, model systems were used, namely, tonoplast-free and plasma membrane-permeabilized cells. In the former, the plasma membrane remained intact and its activity could be investigated by manipulating the cytoplasmic and external media, whereas in the latter, the tonoplast remained intact and its activity could be studied by altering the bathing solution. Studies using these model systems have established the presence of voltage-dependent Ca^{2+}-channels in the plasma membrane and Ca^{2+}-dependent ion channels in both the plasma membrane and the tonoplast. To further analyse Ca^{2+} action on the basis of single channel activities, patch-clamp techniques were applied to plasmolysed protoplasts and isolated cytoplasmic drops. Channel activities were measured using both cell-attached and excised membrane patch modes.

A fresh-water member of the Characeae, *Nitellopsis*, becomes salt-tolerant if millimolar amounts of Ca^{2+} are present in the external medium. Under these conditions, excised patches of the plasma membrane exhibit K^+-channel activity with unitary conductances of 25–50 pS and a permeability ratio (P_{Na}/P_K) of 0.28. These K^+ channels were closed by external Ca^{2+} when ATP was present on the cytoplasmic side of the membrane. ATP could be replaced with AMP, which suggests that ATP acted neither as an energy source nor as a substrate for protein phosphorylation, but rather as an effector. In the tonoplast, K^+ channels having a unitary conductance of 75 pS and a P_{Na}/P_K ratio of 0.2 were not activated by Ca^{2+} when it was present on the cytoplasmic side of the excised patches, but these channels were activated by Ca^{2+} injected into the cytoplasmic drop, which suggested the involvement of an unknown cytoplasmic factor(s) that mediates the Ca^{2+} signal.

The brackish water Characeae, *Lamprothamnium*, can regulate elevated turgor induced by hypotonic treatment only when millimolar amounts of Ca^{2+} are present in the external medium. In this situation, elevated turgor may first activate Ca^{2+} channels and the increased level of Ca^{2+} in the cytoplasm may then activate K^+ and Cl^- channels in both the plasma membrane and the tonoplast. In the cytoplasmic-drop-attached mode, single K^+ channel current–voltage measurements established that the K^+ channel exhibited a unitary conductance of 50 pS for negative shifts of the voltage, while under positive shifts in the voltage 100 pS channel conductance was observed. The channel with a P_{Na}/P_K of 0.02 is highly selective for K^+ against Na^+ and this channel is directly activated by Ca^{2+} added to the cytoplasmic side of the excised patch.

These results suggest that, in *Nitellopsis* and *Lamprothamnium*, Ca^{2+} regulation of channels in both the plasma membrane and the tonoplast may form the molecular basis for Ca^{2+}-regulated physiological functions such as salt tolerance and turgor regulation in characean cells. The mode of Ca^{2+} regulation is discussed in light of current findings.

1. INTRODUCTION

Giant algal cells have been widely used for the study of ionic and water relations, and cytoplasmic streaming in plant cells (Tazawa *et al.* 1987). Among them, internodal cells of the Characeae are special, in that membrane excitation is coupled with the cessation of cytoplasmic streaming, and both phenomena involve Ca^{2+}. For 30 years, there were two hypotheses for the ion-carrying currents during membrane excitation. One was the Cl^--spike hypothesis which associates a large efflux of Cl^- and K^+ with the action potential (Gaffey & Mullins 1958). The other was the Ca^{2+}-spike hypothesis supported by a clear dependence of the peak of the action potential and the maximum inward current, measured under the voltage-clamp condition, on the external [Ca^{2+}] (Hope 1961; Findlay 1961; Hope & Walker 1975). The complex situation of ion species involved in action potentials was thoroughly discussed by Beilby (1984).

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The controversy between the two hypotheses on the nature of characean action potential was finally resolved by evidence for the existence of the plasma membrane Ca^{2+} channel (Shiina & Tazawa 1987*a*,*b*) and the demonstration of a causal relationship between cytoplasmic Ca^{2+} concentration and Cl^--efflux (Lunevsky *et al.* 1983; Shiina & Tazawa 1988). As for the relationship between the action potential and the cessation of cytoplasmic streaming, Ca^{2+} was assumed to be a coupling factor from the effects of external divalent cations on the action potential-induced cessation of cytoplasmic streaming (Barry 1968) and by direct measurement of Ca^{2+} influx induced by the action potential (Hayama *et al.* 1979). The Ca^{2+} hypothesis for the streaming cessation has since been verified by several lines of evidence (Tazawa & Shimmen 1987).

Calcium in characean cells has other implications for the control over membrane properties. The membrane potential in media lacking Ca^{2+} obeys the Goldman diffusion potential for K^+ and Na^+, while in media involving Ca^{2+} there is little dependence on external $[K^+]$ over the lower concentration range (Hope & Walker 1975). Exogenous Ca^{2+} also protects the plasma membrane against depolarization induced by other monovalent cations (Kishimoto 1959) and the import of HCO_3^- is reduced significantly in its absence (Lucas 1976). Furthermore, the hydraulic conductivity of characean cells is reduced by Ca^{2+}, while it is stimulated by K^+ (Tazawa & Kamiya 1965). In *Lamprothamnium*, a brackish-water member of the Characeae, the turgor of the internodal cell, which is increased by treatment of the cell with a hypotonic solution can be recovered by releasing K^+ and Cl^-, and this regulation of turgor is inhibited by removal of Ca^{2+} (Okazaki & Tazawa 1990). Finally, fresh water characean cells are protected against salt stress if Ca^{2+} is present in the external medium (Katsuhara & Tazawa 1986). Thus, the presence of Ca^{2+} in the external medium affects membrane properties of characean cells in a variety of ways.

The effects of Ca^{2+} in the external medium raise the question of whether or not Ca^{2+} affects the membrane activities or other cell activities directly, or indirectly, from the outside, via some binding sites, or from the inside after it has crossed the plasma membrane. Support for the former case comes from the role of Ca^{2+} in the salt tolerance of fresh water Characeae, while the latter case gains support from such examples as streaming cessation induced by an action potential and hypotonic turgor regulation. In the present paper, we provide further support for the role of Ca^{2+} in the regulation of ion channels involved in turgor regulation and salt tolerance in characean cells.

2. MATERIALS AND METHODS

(*a*) *Materials*

Two species of freshwater Characeae, *Nitellopsis obtusa* and *Chara corallina*, and one species of brackish water Characeae, *Lamprothamnium succinctum*, were mainly used. Internodal cells (0.4–0.9 mm in diameter and 3–6 cm in length) were separated from the thallus prior to use in experiments. Internodal cells of *N. obtusa* and *C. corallina* were stored in artificial pond water containing KCl, NaCl, and $CaCl_2$ (each at 0.1 mM), whereas cells of *L. succinctum* were stored in one-third diluted artificial seawater in which the alga was cultured (Okazaki *et al.* 1984).

(*b*) *Intracellular perfusion and preparation of tonoplast-free cells*

Tonoplast-free cells were prepared by perfusing the vacuole with media containing a Ca-chelating agent, EGTA (figure 1; Tazawa *et al.* 1976). An internodal cell was placed on a plexiglass bench and, after loss of the turgor, both ends were amputated. Perfusion medium was then placed over the cut ends and a small pressure gradient was established by raising one end of the plexiglass bench. After the perfusion medium had entirely replaced the cell sap, the cell openings were closed by ligating the cell with strips of polyester thread at two loci. In some experiments, cell ends were left open for successive intracellular perfusion in order to vary the cytoplasmic Ca^{2+} concentration (Shiina & Tazawa 1988). Disintegration of the tonoplast, which was caused by reducing Ca^{2+} to very low levels with EGTA, was observed 5–10 min after perfusion, as indicated by the presence of small cytoplasmic fragments (CF, figure 1) floating in the former vacuolar space. CFs had no boundary membrane. The concentration of free Ca^{2+} in tonoplast-free cells containing 5 mM EGTA was estimated to be lower than 10^{-7} M (Katsuhara & Tazawa 1987).

To remove the intracellular ATP, perfusion medium containing hexokinase and glucose was used (Katsuhara & Tazawa 1990). Depletion of ATP was checked by observing the cessation of cytoplasmic streaming, which is driven by MgATP (Tazawa *et al.* 1976).

Microelectrode measurements of the plasma membrane in tonoplast-free cells corresponded to whole-cell recordings using the patch-clamp technique.

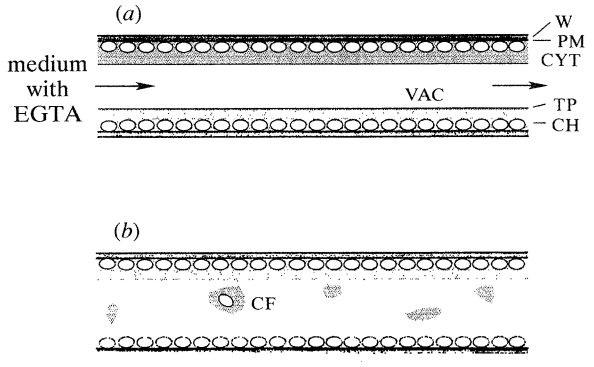

Figure 1. Preparation of tonoplast-free characean internodal cells. (*a*) Intact internodal cell during vacuolar perfusion with a medium containing EGTA. (*b*) Tonoplast-free cell after perfusion. W, cell wall; PM, plasma membrane; CYT, cytoplasm; VAC, vacuole; TP, tonoplast; CH, chloroplast; CF, cytoplasmic fragment.

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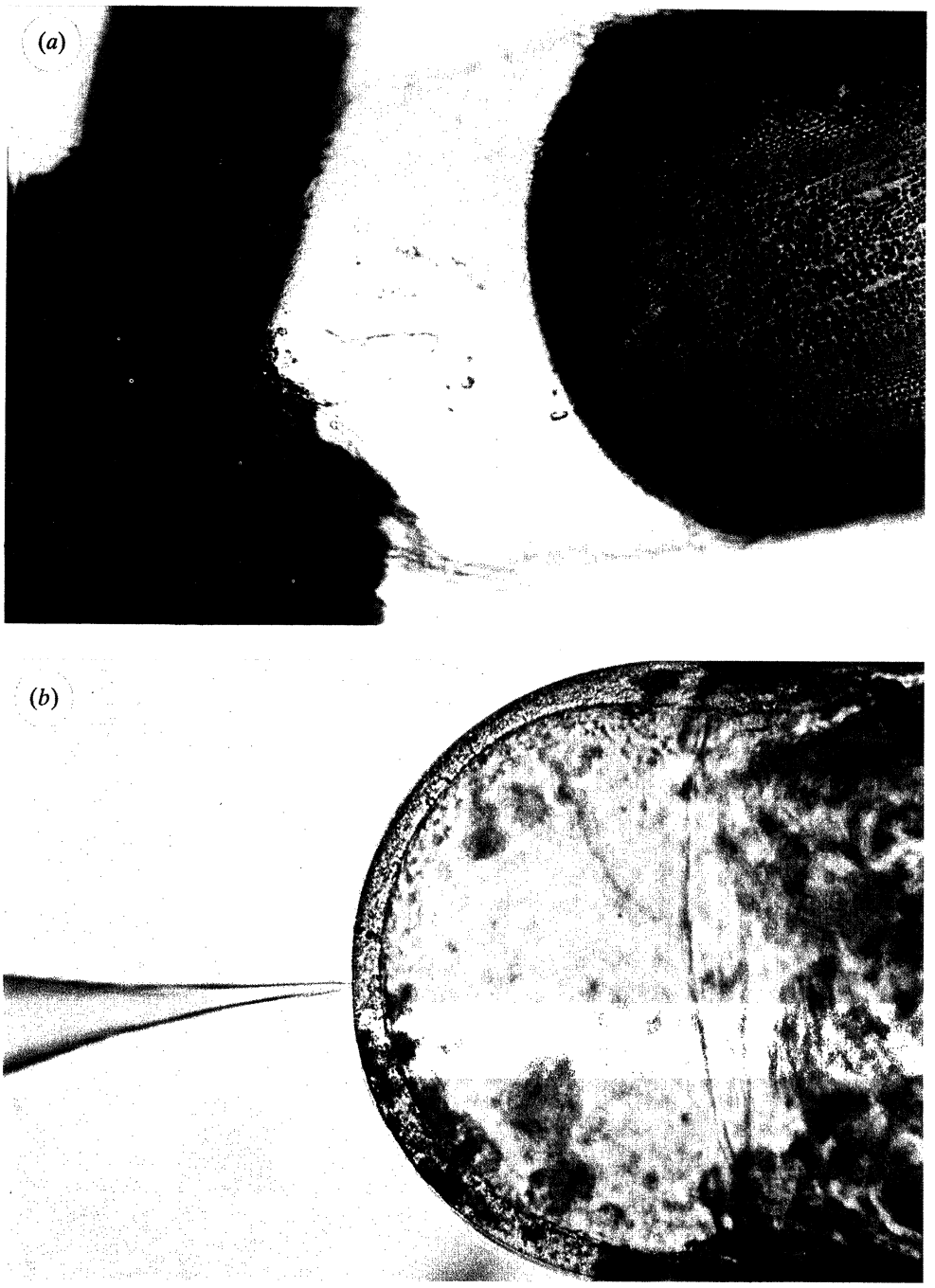

Figure 2. Light micrographs of a plasmolysed *Nitellopsis* cell (*a*) and protoplast extruded from the cut cell end (*b*). A patch-pipette is attached to the surface of the protoplast. Both the plasma membrane and the tonoplast are visible in (*b*). The diameter of the cylindrical internodal cell is about 0.5 mm.

(*c*) *Preparation of protoplasts for patch-clamping the plasma membrane*

To prepare protoplasts for patch-clamp experiments, internodal cells were plasmolysed (Coleman 1986; Katsuhara *et al.* 1990; Okihara *et al.* 1991). *Nitellopsis* cells were treated with a hypertonic solution of 300 mOsm †(100 mM $Ca(NO_3)_2$, 50 mM sorbitol, 5 mM MES-Tris, pH 6.0) and subsequently with 400 mOsm solution (140 mM $Ca(NO_3)_2$, 70 mM sorbitol, 5 mM MES-Tris, pH 6.0). One to two hours after plasmolysis of the cell, one end was cut off with microscissors. The bathing solution was then replaced with a slightly hypotonic solution. Owing to osmotic swelling, part of the protoplast came out from the cell wall tube (figure 2*a*). After the formation of a tight seal between the patch-pipette and the plasma membrane (figure 2*b*), the bathing solution was exchanged for a low-Ca^{2+} solution (below 10^{-8} M free Ca^{2+}) containing EGTA in order to obtain an excised inside-out patch (Hamill *et al.* 1981). To investigate the effect of ATP or AMP on channel activities, the bathing solution was exchanged for low-Ca^{2+} solutions supplemented with either 1 mM ATP or AMP.

† One osmole contains one mole of osmotically active particles.

(*d*) *Preparation of cytoplasmic drops for patch clamping the tonoplast*

Patch-clamp studies of the channels in the tonoplast were performed on cytoplasmic drops prepared from internodal cells. An internodal cell was exposed to the air until its turgor was lost and then one end of the cell was excised. The cell contents were then gently squeezed out into a bathing solution that was approximately isotonic to the vacuolar sap. Quasi-spherical drops with a diameter of about 150 μm or less were formed. Although it is possible that some plasma membrane and ER membrane coexist, most of the droplet surface was covered with tonoplast (Sakano & Tazawa 1986; Lühring 1986) and could be used to easily form a stable high-resistance seal between the patch pipette and the membrane.

(*e*) *Single-channel recordings*

Single-channel currents were recorded and analysed as described previously (Katsuhara *et al.* 1989, 1991). Single-channel currents were recorded on a VTR after low-pass filtering (1 kHz) with a patch-clamp amplifier (Nihon Kohden, CEZ-2200).

(*f*) *Microinjection*

For microinjection of Ca^{2+} into the cytoplasmic drops of *Nitellopsis*, a glass micropipette containing $CaCl_2$ and sorbitol with an intervening layer of silicon oil was inserted into the cytoplasmic drop during the single-channel recording (Katsuhara *et al.* 1991). For microinjection of aequorin into the cytoplasm of *Lamprothamnium*, a micropipette was set in parallel with the longitudinal axis (figure 3) and inserted into the cytoplasm through the cell end which has a thinner cell wall due to cell ligation (Okazaki *et al.* 1987).

3. Ca^{2+}-REGULATED K^+ CHANNELS IN THE PLASMA MEMBRANE

(*a*) *Evidence for Ca^{2+}-regulated K^+ channels*

Before single-channel studies using patch-clamp techniques, a considerable body of electrophysiological data had accumulated consistent with the existence of K^+ channels in the plasma membrane of characean cells. Potassium channels dominate the diffusion component of the membrane potential in the resting state, and play a major role in the repolarization and hyperpolarization of the potential during the action potential (Shimmen & Tazawa 1983*b*; Tester 1990). Based on measurements of the conductance of the plasma membrane of *Chara corallina*, for different external $[K^+]$, Ohkawa *et al.* (1986) estimated the density of the K^+ channels as 6.5 per 100 μm^2 with a unitary conductance of 80–120 pS for external $[K^+]$ of 0.2–5.0 mM. Azimov & Berestovsky (1988) obtained similar results with *Nitellopsis*. Using calcium channel agonists and antagonists, Tester & MacRobbie (1990) suggested that an increase in cytoplasmic Ca^{2+} accelerates the time and voltage-dependent closing of the TEA-sensitive K^+ current. Under illumination, various forms of K^+ channels seem to be activated by cytoplasmic Ca^{2+} (Stein & Hansen 1988; Vanselow & Hansen 1989).

High concentrations of external Ca^{2+} have been demonstrated to decrease the characean plasma membrane conductances to K^+ (e.g. Kitasato 1976) and Na^+ (Abe & Takeda 1986; Katsuhara *et al.* 1990). It appears that K^+ and Na^+ pass through the same channels with a certain selectivity (Sokolik & Yurin 1986; Katsuhara & Tazawa 1990) or they seem to interfere with each other at the same channels (Tester 1988). The effect of external Ca^{2+} on the Na^+ permeability in characean cells agrees with that in higher plants, where external Ca^{2+} reduces the Na^+ influx and the salt injury caused by high concentrations of external Na-salts.

The Ca^{2+} effect of relieving salt injury is specific and can not be replaced by other divalent cations (Katsuhara & Tazawa 1986), although, in some cases, the effect of external Ca^{2+} is mimicked by Mg^{2+} (Bisson 1984), or by Sr^{2+} (Shiina & Tazawa 1987*a*).

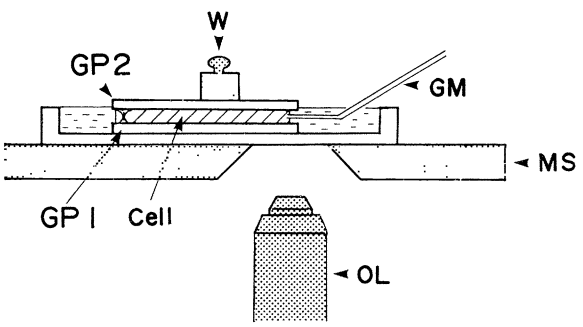

Figure 3. Scheme of the experimental set for microinjection of aequorin into the cytoplasm of *Lamprothamnium* internodal cell. GP 1 and GP 2, glass plates; MS, microscope stage; OL, objective lens; GM, glass micropipette; W, weight. (After Okazaki *et al.* 1987.)

(*b*) *Involvement of external Ca^{2+}-regulated and internal ATP-sensitive K^+ channels in salt tolerance in* Nitellopsis

When the fresh water Characeae *Nitellopsis obtusa* was subjected to salt stress of 100 mM NaCl, the membrane potential depolarized and membrane resistance decreased. A large Na^+ influx and a large K^+ efflux occurred concurrently. Such fluxes were eliminated when 10 mM $CaCl_2$ was added to 100 mM NaCl (Katsuhara & Tazawa 1986). Experiments with tonoplast-free cells (see § 2) suggested that Ca^{2+} acts from the outer side of the plasma membrane since, in such cells, the intracellular free Ca^{2+} concentration was buffered below 10^{-7} M with EGTA (Katsuhara & Tazawa 1987). In tonoplast-free cells, the protective effect of Ca^{2+} was evident only when the intracellular ATP concentration exceeded 0.1 mM (Katsuhara & Tazawa 1990). AMP and adenylyl-imidodiphosphate, a non-hydrolysable ATP analogue, could replace ATP, suggesting that ATP does not act as an energy source nor as a substrate for protein phosphorylation. ATP seems to exert its effect as a co-effector with

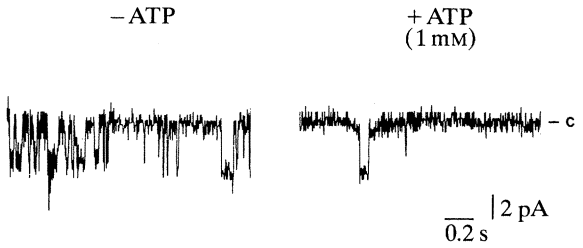

Figure 4. Single-channel recordings from an excised *Nitellopsis* plasma membrane patch before (left) and after (right) applying 1 mM ATP to the cytoplasmic side. Membrane potential (the cytoplasmic side referred to the external side) was clamped at −90 mV. The patch pipette (external side of the membrane) contained 50 mM NaCl, 50 mM Na-gluconate (total $Na^+ = 100$ mM), 0.1 mM K-gluconate, 10 mM Ca-gluconate, 125 mM sorbitol, and 10 mM HEPES-Tris (pH 7.4). The bathing solution (cytoplasmic side of the membrane) contained 50 mM KCl, 1 mM EGTA, 125 mM sorbitol, and 100 mM HEPES-Tris (pH 7.5). Currents from the external to the cytoplasmic side are displayed downward. −C: The level of channel closing. (After Katsuhara *et al.* 1990.)

Ca^{2+} in regulating the Na^+ permeability of the plasma membrane.

To test this hypothesis, inside-out patches were prepared in which we could control the cytoplasmic ATP concentration. The patch-pipette was loaded with a solution containing 10 mM Ca^{2+} and 100 mM Na^+, facing the outer side of the patched plasma membrane. In the absence of ATP, high channel activity was observed with the total open time amounting to 8–34% of the total recording time (figure 4). However, upon addition of 1 mM ATP to the cytoplasmic side, the frequency of channel opening decreased, with the total open time falling to less than 2% (figure 4), while the unitary conductance remained the same. AMP applied to the cytoplasmic side of the excised patch also closed the channels in a similar manner, indicating that suppression of the channels by ATP does not require hydrolysis of ATP. This conclusion was supported by the finding that ATP is still effective in the absence of Mg^{2+}. The same is also true for K^+ channels from the sarcolemma of frog skeletal muscle which are inhibited by micromolar ATP in the absence of Mg^{2+} (Vivaudou *et al.* 1991). Using the Goldman equation, the permeability ratio between Na^+ and K^+ (P_{Na}/P_K) can be calculated to be 0.28. This means that the observed channel activity represents a K^+ channel through which Na^+ can also pass with a permeability one-quarter of that for K^+. The conductance of the observed K^+ channel was 25–50 pS (50 mM K^+ in the inside and 100 mM Na^+ in the outside), which is similar to the conductances of most K^+ channels found in animal plasma membranes. This value is, however, about half the conductance of K^+ channels in the *Chara* plasma membrane estimated using the conventional microelectrode method (Ohkawa *et al.* 1986).

ATP-sensitive K^+ channels, first reported in cardiac muscle (Noma 1983), are widespread in animal cells (Aschroft & Aschroft 1990). Our present results confirm the presence of a similar channel in plant cells. In both animal and *Nitellopsis* cells, ATP hydrolysis is not involved in the suppression of channels by ATP. In animal cells, co-regulation with Ca^{2+} and ATP of single channels has been demonstrated to occur (Findlay 1988), but Ca^{2+} and ATP act on the same side, i.e. the internal side of the membrane. For *Nitellopsis*, direct evidence for the regulation of K^+ channels by external Ca^{2+} at the single-channel level is still lacking.

4. Ca^{2+}-REGULATED K^+ CHANNELS IN THE TONOPLAST AND THEIR BEARING ON TURGOR REGULATION AND SALT TOLERANCE

(*a*) *Evidence for K^+ channels in the tonoplast*

Activities of single K^+ channels in the tonoplast were recorded for *Chara* (Lühring 1986; Laver & Walker 1987; Tyerman & Findlay 1988; Bertl 1989), *Nitellopsis* (Katsuhara *et al*, 1991), and a brackish water charophyte, *Lamprothamnium* (Katsuhara *et al.* 1989). Tonoplast K^+ channels mediate K^+ diffusion across the tonoplast and may be partially responsible for the tonoplast potential. When *Nitellopsis* cells are subjected to salt stress, K^+ in the cytoplasm is rapidly exchanged for external Na^+ (Katsuhara & Tazawa 1986). In such Na^+-loaded cells, cytoplasmic Na^+ is exchanged for vacuolar K^+, causing recovery of the normal ionic condition of the cytoplasm (Katsuhara *et al.* 1991). The exchange between K^+ and Na^+ across the tonoplast may occur through these channels. In *Nitellopsis*, the permeability ratio of the channel between Na^+ and K^+ ($P_{Na}P_K$) is estimated to be 0.2 (Katsuhara *et al.* 1991). In *Chara* (Bisson *et al.* 1989; Bertl 1989) and *Lamprothamnium* (Katsuhara *et al.* 1989), $P_{Na}P_K$ is very low (0.02 or less). This discrepancy may be a reflection of differences in strategy for salt tolerance in these species (Katsuhara *et al.* 1991).

(*b*) *Cytoplasmic Ca^{2+}-regulation of tonoplast K^+ channels in* Lamprothamnium

When internodal cells of the brackish alga *Lamprothamnium* were treated with a hypotonic solution, they readjusted the elevated turgor to the original level by releasing K^+ and Cl^-. If external Ca^{2+} was removed, the turgor regulation was inhibited, indicating the need for Ca^{2+} in the regulation phenomenon (Okazaki & Tazawa 1986*a*). A Ca^{2+}-channel blocker, nifedipine, inhibited turgor regulation even when enough Ca^{2+} was present in the external medium (Okazaki & Tazawa 1986*b*). This suggests that Ca^{2+} becomes effective when it enters the cell through Ca^{2+} channels. Microinjection of the Ca^{2+}-sensitive photoprotein, aequorin, into the cytoplasm demonstrated that an increase in the cytoplasmic Ca^{2+} level preceded turgor regulation (figure 5; Okazaki *et al.* 1987). These results suggest that efflux of K^+ and Cl^- from the vacuole to the extracellular space, during turgor regulation, are triggered by an increase in cytoplasmic Ca^{2+} level. Furthermore, it may be reasonable to

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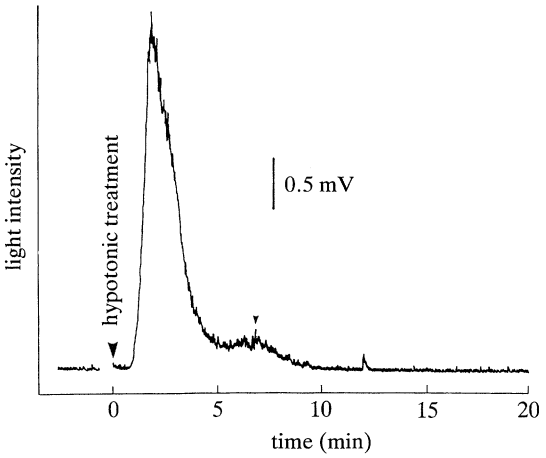

Figure 5. Light emission of aequorin upon hypotonic treatment (transferral from 0.36 Osm solution to 0.21 Osm solution) of an internodal cell of *Lamprothamnium*. External Ca^{2+} of 3.9 mm was constantly present. Time zero represents the start of measurement. (After Okazaki *et al.* 1987.)

assume the existence of Ca^{2+}-sensitive K^+ and/or Cl^- channels in both the plasma membrane and the tonoplast in *Lamprothamnium* cells. To investigate such channels in the tonoplast by the patch-clamp technique, cytoplasmic drops were prepared from internodal cells of *Lamprothamnium* (see § 2).

In patches to which a cytoplasmic-drop has been attached, rectifying channels were recorded (Katsuhara *et al.* 1989). The unitary conductance was 50 pS in the negative voltage region and 100 pS in the positive voltage region. When K^+ in the pipette solution (vacuolar side) was substituted for Na^+, only the current flowing from the cytoplasmic side to the vacuolar side was observed. From the shift of the reversal potential caused by this substitution, the selectivity of this channel to Na^+ relative to K^+ was calculated to be about 0.02, showing that this channel has a high selectivity for K^+ over Na^+. Similar cation channels were also found in the droplet membrane of a salt-tolerant species, *Chara buckellii*, but with a much lower selectivity for K^+ over Na^+ (Bisson *et al.* 1989).

To investigate the direct effects of cytoplasmic Ca^{2+} on the channels, cytoplasmic-side-out patches were prepared (see § 2). Figure 6 shows typical single-K^+-channel recordings. When the cytoplasmic concentration of free Ca^{2+} was below 10^{-8} M (pCa 8, figure 6*a*), the frequency of channel opening was very low and the duration of the opening was very short (about 10 ms). By contrast, an increase in cytoplasmic Ca^{2+} to 10^{-5} M (pCa 5, figure 6*b*) markedly increased both the frequency and the duration of the channel opening, but the channel conductance remained the same. The state of channels activated by Ca^{2+} continued without inactivation for at least a second. Channels activated by high Ca^{2+} (pCa 5) could be inactivated by decreasing the Ca^{2+} level, indicating that the Ca^{2+} regulation is reversible (figure 6*c*). The conductance and rectification of the K^+ channels in the excised patch agreed with values obtained using the attached patch mode. The opening of channels in the high positive voltage region showed subconductance levels of the type reported for K^+ channels in the tonoplast of *Chara* (Lühring 1986) and for the Cl^- channel in

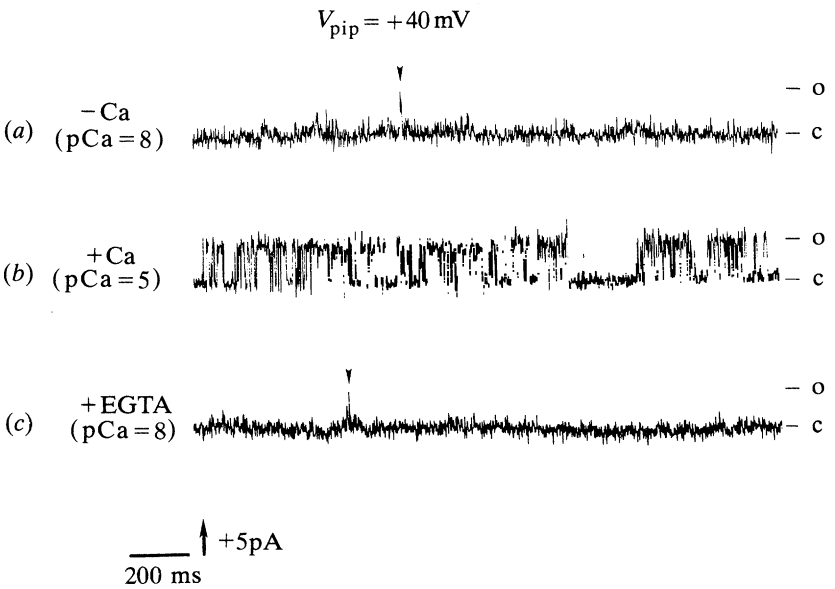

Figure 6. Single-channel recordings from a cytoplasmic-side-out patch of *Lamprothamnium* tonoplast in the bathing solution (cytoplasmic side) containing 1 mM EGTA (*a*), after addition of 1 mM calcium gluconate (*b*), and after subsequent addition of EGTA (10 mM, final concentration) (*c*). Membrane potential (the cytoplasmic side against the vacuolar side) was clamped at +40 mV. The patch pipette (vacuolar side) contained 220 mM KCl, 20 mM $MgCl_2$, 10 mM $CaCl_2$, and 200 mM HEPES-KOH (pH 7.4). The bathing solution (cytoplasmic side) initially contained 220 mM KCl, 20 mM $MgCl_2$, 1 mM EGTA, and 200 mM HEPES-KOH (pH 7.4). Currents from the cytoplasmic side to the vacuolar side are displayed upward. Arrowheads indicate the channel opening at pCa 8. −C, Channel closing; −O, Channel opening. (After Katsuhara *et al.* 1989.)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

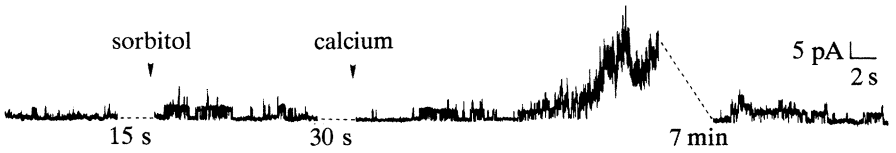

Figure 7. Effects of microinjected sorbital and Ca^{2+} on single-channel currents in a cytoplasmic-drop-attached patch. At the indicated times, 1.3 M sorbital and 0.5 M $CaCl_2$ were microinjected (1 to 5 pl, about 1% of the volume of the cytoplasmic drop). Currents from the cytoplasmic side to the vacuolar side through open channels were recorded. Transient channel activation was observed after Ca^{2+} injection. Membrane potential (the cytoplasmic side against the vacuolar side) was clamped at +40 mV. The patch pipette (vacuolar side) contained 60 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 100 mM HEPES-KOH (pH 7.4). (After Katsuhara *et al.* 1991.)

the plasma membrane of *Chara* (Coleman 1986). We conclude that the Ca^{2+} regulation of the observed K^+ channels in the tonoplast may provide a molecular basis for the turgor regulation in *Lamprothamnium*.

(*c*) *Ca^{2+}-regulation of tonoplast K^+ channels in* Nitellopsis *and* Chara

Ca^{2+}-activation of the tonoplast K^+ channels was also observed in the fresh water charophyte *Nitellopsis* (Katsuhara *et al.* 1991) and *Chara* (Laver & Walker 1991) when the patch-clamp technique was applied to cytoplasmic drops. In cell-free excised patches from *Nitellopsis*, single K^+ channels were identified. The unitary conductance was 75 pS (100 mM K^+ on both sides of the membrane) and the permeability ratio between Na^+ and K^+ was 0.2. No influence of Ca^{2+} on this channel was observed after addition of Ca^{2+} to the cytoplasmic side of the excised patches. However, in the case of attached patches, microinjection of Ca^{2+} into the cytoplasmic drop brought about enormous activation of K^+ channels after a lag of about 10 s (figure 7). Since microinjection of sorbitol had no effect (figure 7), the activation by Ca^{2+} was concluded to be due to neither an osmotic effect nor changes in the membrane tension. Channel activation by Ca^{2+} lasted for a few minutes and then disappeared. These results suggest that some cytoplasmic factor(s) may mediate the Ca^{2+} signal to the tonoplast to activate K^+ channels. A preliminary experiment showed that addition of Ca-calmodulin (spinach) to the cytoplasmic side of excised patches had no effect on channel activity.

In Chara, cytoplasmic Ca^{2+} at concentrations above 0.5 μM activates tonoplast K^+ channels (Laver & Walker 1991). Higher concentrations of Ca^{2+} (0.2 mM on the cytoplasmic side or 20 mM on the vacuolar side; Laver 1990) or other divalent cations (Sr^{2+} and Ba^{2+}; Laver & Walker 1991) block these channels, reducing the unitary conductance and inducing flickering of channels. Activation of K^+ channels by Ca^{2+}, which occurs with a delay after Cl^- channel activation during an action potential, may function to restore the tonoplast potential to the resting level which is close to the K^+ equilibrium potential (Laver & Walker 1991).

5. InsP_3-RELATED CHANNELS

In higher plant cells, as in animal cells, inositol 1,4,5-trisphosphate (InsP_3) is involved in the release of Ca^{2+} from intracellular stores, mainly from vacuoles (e.g. Schumaker & Sze 1987). In the red beet tonoplast, Ca^{2+} channels are activated directly by InsP_3 (Alexandre *et al.* 1990).

In *Nitella*, the plasma-membrane Cl^- channels were suggested to be activated by external InsP_3 (Zherelova 1989), although permeation of InsP_3 into the cell was not demonstrated. InsP_3 probably acts via an increase in cytoplasmic Ca^{2+} in *Chara* and *Nitella* (Thiel *et al.* 1990). Cytoplasmic InsP_3 may first activate Ca^{2+} channels in the plasma membrane or the tonoplast (Ranjeva *et al.* 1988), and then the plasma membrane Cl^- channels become activated by the increased cytoplasmic Ca^{2+}. Simultaneously, K^+ channels in the plasma membrane may be activated in a voltage-dependent manner. Although the presence of a signal transduction system involving InsP_3 has been demonstrated in charophytes, the existence of external effectors (first signals) which increase cytoplasmic InsP_3 are not yet known. To reveal the physiological role of InsP_3 in characean cells, further studies are needed (Irvine 1990).

6. CYTOPLASMIC Ca^{2+}-ACTIVATED Cl^- CHANNELS IN THE PLASMA MEMBRANE

During the plasma membrane excitation of characean cells, a large transient inward current under voltage-clamp and a large Cl^- efflux are observed. This suggests that plasma membrane Cl^- channels are involved in membrane excitation, or the generation of an action potential. Actually, a Cl^- channel blocker, A-9-C (9-anthracenecarboxylic acid), reduces both the Cl^- efflux and the membrane depolarization during membrane excitation (Shiina & Tazawa 1987*b*). On the other hand, reduction of external Ca^{2+} inhibits the inward current (Findlay & Hope 1964). Calcium channel blockers also inhibit the membrane excitation (Tsutsui *et al.* 1986), suggesting that Ca^{2+} channels are also involved in the plasma membrane excitation. These results may be explained by the Ca^{2+}-activated Cl^--channel hypothesis. Upon excitation, a Ca^{2+} influx occurs first through the plasma membrane Ca^{2+} channels, and then an increased cytoplasmic level of Ca^{2+} activates the Cl^- channels. The existence of such Ca^{2+}-activated Cl^- channels is supported by the following experimental findings.

In *Nitellopsis* cells, when Ca^{2+} channels were

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

blocked by La^{3+}, Ca^{2+} influx was inhibited and Cl^- efflux was reduced (Shiina & Tazawa 1987*b*). Depletion of the external Ca^{2+}, which reduces the increase in the cytoplasmic Ca^{2+} during membrane excitation, also resulted in a decrease in the Cl^- efflux.

Using tonoplast-free cells, when the intracellular (cytoplasmic, in this case) Ca^{2+} was increased artificially by intracellular perfusion, the inward current under voltage clamping of the plasma membrane in *Nitellopsis* cells was increased greatly (Kataev *et al.* 1984). In this study the inward current was assumed to be carried by Cl^- ions. More direct evidence for the Ca^{2+}-induced Cl^- efflux was presented by Shiina & Tazawa (1988). They intracellularly perfused tonoplast-free *Nitellopsis* with media containing various concentrations of Ca^{2+} and found that the Cl^- efflux increased with an increase in Ca^{2+} concentration. As for ion specificity, Sr^{2+} activates the Cl^- channels only partially (Kataev *et al.* 1984; Shiina & Tazawa 1987*b*), although it can pass through the plasma membrane Ca^{2+} channel as easily as Ca^{2+} (Shiina & Tazawa 1987*a*). Neither Mg^{2+} nor Ba^{2+} can activate the Cl^- channels (Kataev *et al.* 1984).

Single-channel currents through the plasma membrane Cl^- channel were first recorded by Coleman (1986) using *Chara*, which actually represents the first single-channel recording of Cl^- channels in a plant plasma membrane. Okihara *et al.* (1991) established that Ca^{2+} regulates the Cl^- channel in excised patches of the *Chara* plasma membrane. In cytoplasmic Ca^{2+} of 1 μM, the strongest activation of Cl^- channels was noted. Ths channel also showed voltage-dependency (strongest activation at -80 to -100 mV). These results suggest that the observed channels are the excitable Ca^{2+}-activated Cl^- channels in the plasma membrane. Because antagonists of calmodulin (W-7, chlorpromazine, and trifluoperazine at 40 μM of each) reduced the channel activity, the involvement of calmodulin in the Cl^- channel activation was proposed. However, in tonoplast-free *Nitellopsis* cells, the Ca^{2+}-dependent Cl^- efflux was not affected by 100 μM W-7 (Shiina & Tazawa 1988).

7. CYTOPLASMIC Ca^{2+}-REGULATED Cl^- CHANNELS IN THE TONOPLAST

As with Cl^- channels in the plasma membrane, so Cl^- channels in the tonoplast are activated by an increase in cytoplasmic Ca^{2+}. Tonoplast Cl^- channels seem to be responsible for the tonoplast action potential, as its amplitude changes with the concentration of vacuolar Cl^-, as expected from the Goldman equation (Kikuyama & Tazawa 1976). Findlay (1970) and Kikuyama & Tazawa (1976) demonstrated that the tonoplast action potential followed the plasma membrane action potential. The factor coupling both action potentials was suggested to be Ca^{2+} in *Nitella axilliformis* (Kikuyama 1986) and in *Nitella flexilis* (Shimmen & Nishikawa 1988). Using cells with a permeabilized plasma membrane, in which the ionic composition on the cytoplasmic side of the intact tonoplast could be controlled (Shimmen & Tazawa 1983*a*), the critical concentration of cytoplasmic Ca^{2+} needed to activate tonoplast Cl^- channels was determined to be 1 μM (Kikuyama 1989).

Single-Cl^--channel currents in the tonoplast have been recorded in *Chara* (Tyerman & Findlay 1988), *Nitellopsis* (Katsuhara *et al.* 1991) and *Lamprothamnium* (Katsuhara *et al.* 1989). In *Chara*, the conductance of this channel was estimated to be about 30 pS. Interestingly, these anionic channels were shown to be twice as permeable to NO_3^- as to Cl^- (Tyerman & Findlay 1988). It remains unclear whether the observed channels are those responsible for the tonoplast action potential, or whether they are activated by cytoplasmic Ca^{2+}, as their activity was easily lost, especially in excised patches. This situation is the same in *Lamprothamnium*. Although we could register activi-

Table 1. *Ca^{2+}-regulated channels likely to exist in freshwater and brackish water Characeae, Nitellopsis obtusa and Lamprothamnium succinctum*

		Nitellopsis		*Lamprothamnium*	
channel		plasma membrane	tonoplast	plasma membrane	tonoplast
K^+					
unitary conductance		25–50 pS[b]	75 pS[c]		50 pS[a]
condition	out.	100 mM Na^+ 10 mM Ca^{2+}			
	cyt.	50 m MK^+	100 mM K^+		300 mM K^+
	vac.		100 mM K^+		300 mM K^+
P_{Na}/P_K		0.28	0.2		0.02
Ca^{2+} outside					
+ATP in cyt.		close[b]			
−ATP in cyt.		open[b]			
mode of activation by Ca^{2+} in cyt.		unknown	indirectly[c]	indirectly[d,c]	directly[a]
role of Ca^{2+} regulation		salt tolerance	salt tolerance	turgor regulation	turgor regulation
Cl^-					
mode of activation by Ca^{2+} in cyt.		directly[f,g]	unknown	indirectly[e]	unknown
role of Ca^{2+} regulation		action potential		turgor regulation	

References: [a] Katsuhara *et al.* (1989); [b] Katsuhara *et al.* (1990); [c] Katsuhara *et al.* (1991); [d] Okazaki & Tazawa (1987); [e] Okazaki *et al.* (1987); [f] Shiina & Tazawa (1987*b*); and [g] Shiina & Tazawa (1988).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

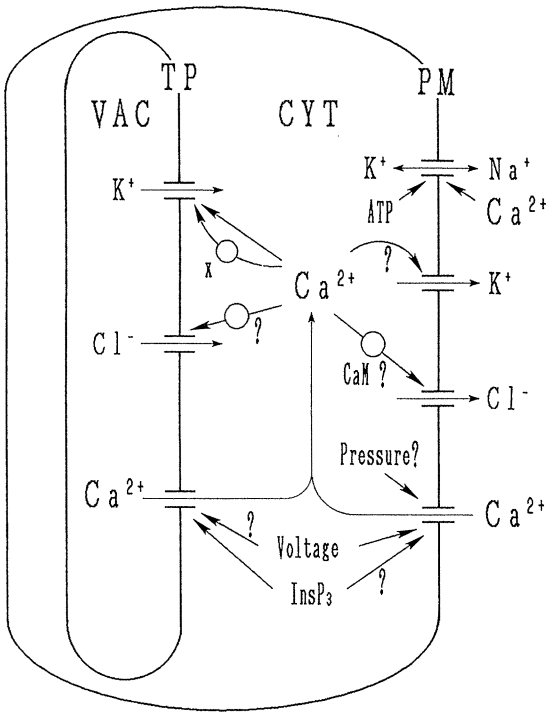

Figure 8. Putative Ca^{2+}-related channels in the plasma membrane and the tonoplast of characean cells. Circles represent putative cytoplasmic factors mediating Ca^{2+} signals. PM, plasma membrane; CYT, cytoplasm; TP, tonoplast; VAC, vacuole; CaM, calmodulin; InsP_3, inositol 1,4,5-trisphosphate; x, unknown cytoplasmic factor(s).

ties of the Cl^- channels, observations are of such low frequency that we have not yet examined the effect of Ca^{2+} on Cl^--channel activity. However, activation of tonoplast Cl^- channels by cytoplasmic Ca^{2+} is assumed to occur in *Lamprothamnium* cells during turgor regulation (Okazaki & Tazawa 1990).

8. CONCLUDING REMARKS

Evidence has accumulated for the presence of Ca^{2+}-regulated ion channels in both the plasma membrane and the tonoplast of characean cells. Table 1 summarizes the characteristics of Ca^{2+}-regulated K^+ and Cl^- channels in fresh and brackish water Characeae which we mainly studied. What needs to be done next is to find whether Ca^{2+} regulates the channels directly or indirectly. A possible mode of Ca^{2+}-regulation of plasma membrane and tonoplast ion channels is displayed in figure 8. In tonoplast K^+ channels of *Nitellopsis*, the Ca^{2+} signal may be mediated by putative cytoplasmic factor(s). In contrast, Ca^{2+} seems to act directly on the tonoplast K^+ channels of *Lamprothamnium*, as it activates single K^+-channel currents in isolated membrane patches (Katsuhara *et al.* 1989). However, there is the possibility that some factors which bind strongly to the excised membrane mediate the activation of K^+ channels by Ca^{2+} in the *Lamprothamnium* tonoplast. The results obtained by Okihara *et al.* (1991) suggest that Cl^- channels and a calmodulin-like factor may co-exist even in the excised *Chara* plasma membrane. In tonoplast-free *Nitellopsis* cells. Shiina & Tazawa (1988) demonstrated that introduction of Ca^{2+} into the cytoplasmic side activated Cl^- channels in the plasma membrane but that a calmodulin antagonist (W-7) had no effect on the Ca^{2+} activation. Clearly, the mode of Ca^{2+} activation may differ from species to species as was observed in the Ca^{2+}-regulation of tonoplast K^+ channels between *Nitellopsis* and *Lamprothamnium*. Furthermore, in the same species, *Nitellopsis obtusa*, Mimura & Tazawa (1983) could not detect the Ca^{2+}-induced Cl^- efflux from the tonoplast-free cells. Under certain conditions, some cytoplasmic factor(s) mediating the Ca^{2+}-signal may be lost.

Studies on *Lamprothamnium* offer preliminary evidence suggesting the involvement of a cytoplasmic factor in the signal transduction occurring during turgor regulation. Normal cells of *Lamprothamnium* lose K^+ and Cl^- when the turgor is elevated by hypotonic treatment. A steep rise of Ca^{2+} concentration in the cytoplasm triggers the efflux of ions across the plasma membrane and the tonoplast (Okazaki & Tazawa 1990). In tonoplast-free cells, however, hypotonic treatment fails to stimulate this efflux even when the intracellular Ca^{2+} concentration is increased up to 10^{-5} M (Okazaki & Tazawa 1987). This implies the loss of some cytoplasmic factor which mediates the Ca^{2+} signal to ion channels in normal cells.

The mode of Ca^{2+} activation of ion channels in characean cells is rather complex. The transduction system connecting Ca^{2+} to ion channels may be channel-specific. It may be modified according to the experimental and physiological conditions of the cells. Further studies are necessary to substantiate the involvement of cytoplasmic factors in the signal transducing system related to Ca^{2+}-regulated ion channels in characean cells.

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gure 2. Light micrographs of a plasmolysed *Nitellopsis* cell (*a*) and protoplast extruded from the cut cell end (*b*). A tch-pipette is attached to the surface of the protoplast. Both the plasma membrane and the tonoplast are visible (*b*). The diameter of the cylindrical internodal cell is about 0.5 mm.