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The Hydration-Induced Ca^{2+} Release Is a General Phenomenon in Characeae

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When an internodal cell of *Nitella axilliformis* that had been treated with 10 mM KCl and 1 mM EGTA was examined for transcellular osmosis, an increase in the cytosolic concentration of Ca^{2+} on the endoosmotic side was observed, as was the case in *Nitella flexilis* even though the increase was much smaller in *Nitella axilliformis*. The hydration-induced calcium release (HICR) was also observed in other species of Characeae, namely, *Chara corallina* and *Nitellopsis obtusa*. HICR was also demonstrated in cytoplasm isolated from *Nitella axilliformis* and *Chara corallina* as from *Nitella flexilis*. Thus, it appears that HICR is not specific to *Nitella flexilis* but may be a general phenomenon in Characeae.

Key words: Aequorin — Ca^{2+} — Characeae — Hydration-induced Ca^{2+} release (HICR) — Osmosis.

When internodal cells of *Nitella flexilis* had been bathed in APW, transcellular osmosis induces considerable membrane depolarization and the inhibition of cytoplasmic streaming on the endoosmotic side of the cell (Hayama and Tazawa 1978), irrespective of whether the cell has been bathed in APW or in 10 mM KCl, which renders the cell inexcitable. This endoosmosis-induced membrane depolarization has been observed only in *Nitella flexilis* and not in other species of Characeae, such as *Nitella axilliformis, Chara corallina* and *Chara australis* (Hayama et al. 1979).

It has been well established that both the membrane potential and cytoplasmic streaming are closely related to the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_c$) (Tazawa and Shimmen 1987). Recently, Tazawa et al. (1994) studied internodal cells of *Nitella flexilis* into which a Ca^{2+}-sensitive photoprotein, aequorin, had been injected, and they demonstrated a significant increase in $[Ca^{2+}]_c$ in the endoosmotic region of the cell. Since light emission from aequorin was also observed in the presence of 5 mM EGTA, they postulated that mobilization of Ca^{2+} from internal stores had occurred. They suggested that hydration of the cytoplasm, which occurs in the endoosmotic region of the cell during transcellular osmosis, might trigger the release of Ca^{2+} from internal stores. This assumption was supported by the emission of light from aequorin upon hypotonic vacuolar perfusion (Tazawa et al. 1995).

The hydration-induced increase in $[Ca^{2+}]_c$ was also observed upon induction of osmotic swelling of the cell, which was brought about by changing the external medium from 10 mM KCl supplemented with 250 mM sorbitol to 10 mM KCl. Since an increase in $[Ca^{2+}]_c$ was also observed in a medium that contained 5 mM EGTA in addition to 10 mM KCl, involvement of external Ca^{2+} could be excluded (Tazawa et al. 1995). The most direct evidence for the hydration-induced Ca^{2+} release (HICR) from cytosolic stores was provided by the demonstration of light emission from aequorin in vitro that was induced by hydration of squeezed-out drops of cytoplasm (Kikuyama et al. 1995).

Experimental results obtained to date suggest that the mechanism of HICR might be specific to *Nitella flexilis*. In the present study we examined whether or not the mechanism for HICR exists also in other characean species, using a very sensitive Ca^{2+}-detecting agent, a recombinant semisynthetic aequorin (Shimomura et al. 1988).

Materials and Methods

Plant materials—Four species of Characeae, namely *Nitella axilliformis, Nitella flexilis, Chara corallina* and *Nitellopsis obtusa* were used. They were cultured in aquaria filled with tap water at about 25°C and all were illuminated with light from two 20 W fluorescent lamps for 16 h a day. Internodal cells of all species except *Nitellopsis obtusa* were isolated from neighboring internodal cells and kept in APW (0.1 mM each KCl, NaCl and $CaCl_2$) for at least one day before microinjection of aequorin. In the case of *Nitellopsis obtusa*, aequorin was applied by microinjection immediately after the isolation of internodal cells, since the isolated cells deteriorated when kept in APW for a long time. After microinjection, cells were kept in 10 mM KCl for several hours to render the plasmalemma inexcitable (cf. Hayama and Tazawa 1978).

Microinjection—Increases in $[Ca^{2+}]_c$ were monitored as increases in the emission of light from aequorin that had been microinjected into the cytoplasm. The injection medium contained 100 mM KCl, 6 mM $MgCl_2$, 0.1 mM EGTA and about 0.5 mg ml^{-1} *h*-aequorin (Shimomura et al. 1988). The pH was adjusted to 7.0 with KOH. The amount of solution injected was several nanoliters which corresponded to a few percent of the cytoplasmic volume.

Abbreviations: APW, artificial pond water; $[Ca^{2+}]_c$, cytosolic concentration of Ca^{2+}; EGTA, ethyleneglycol-bis-(β-aminoethylether)*N,N,N',N'*-tetraacetic acid; HICR, hydration-induced Ca^{2+} release; PM, photomultiplier.

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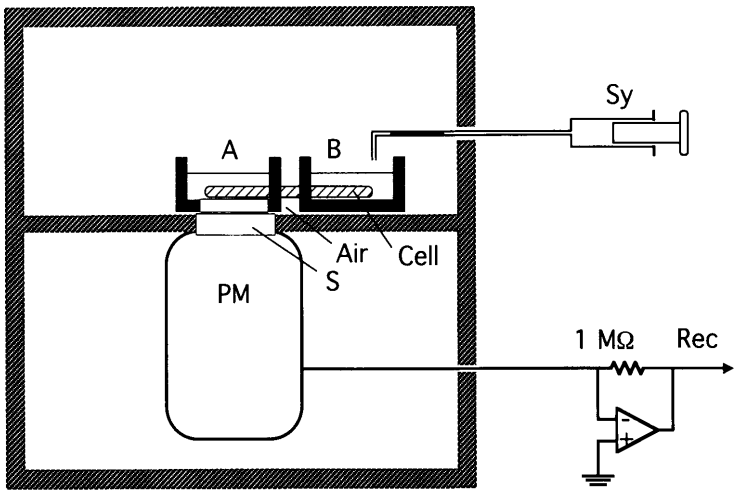

Fig. 1 Apparatus for measuring changes in $[Ca^{2+}]_c$ during transcellular osmosis. An internodal cell (Cell) that had been loaded with aequorin was placed in a vessel. Pool A and B were first filled with 1 mM EGTA. Pool A was located over the photomultiplier tube (PM). Transcellular osmosis was induced by replacing the external solution in pool B with the same solution supplemented with sorbitol. Air, Air gap; S, shutter; Sy, syringe; Rec, recorder.

After the injection, internodal cells were returned to the same 10 mM KCl medium and incubated for more than one hour. Before transcellular osmosis, cells were placed in a medium that contained 1 mM EGTA and 4 mM PIPES (pH 7.0 adjusted with KOH, and referred to below only as 1 mM EGTA) for 5 min to remove extracellular Ca^{2+}.

Measurements of the emission of light from aequorin during transcellular osmosis—An internodal cell loaded with aequorin was placed in a vessel, as shown in Figure 1. The plexiglass vessel for induction of transcellular osmosis had two pools (A and B) with an air gap between them. Each pool and the air gap were connected via a narrow groove in which the cell was placed. The groove was filled with vaseline to prevent possible leakage of the medium from the two side pools. Both pools were first filled with 1 mM EGTA. The vessel was placed in the upper chamber of the measuring box such that pool A was located over the photomultiplier tube (PM). Then the chamber was closed with a lid. After checking that the PM current was stable, we interrupted the measurement to remove the 1 mM EGTA from pool B. Then the measurement was restarted, when transcellular osmosis had been induced by adding 1 mM EGTA supplemented with sorbitol to pool B via the injection syringe (Sy). Only the change in the PM current on the endoosmotic side (A side) was recorded. The PM current was converted to a voltage signal through a 1 MΩ resistor and recorded on a recorder (Memory Hicoder 8840; Hioki, Tokyo). The peak PM current, referred to the base-line value before treatment, was designated ΔL. After the measurement, the medium in each pool was changed to 1 mM $CaCl_2$ and the cell was cut with a razor blade to measure the amount of aequorin that remained in the cytoplasm (Fig. 3b; cf. Kikuyama and Tazawa 1983, Tazawa et al. 1995). The ΔL measured in this way was designated ΔL_{max}.

In some cases, not only changes in the emission of light from aequorin but also changes in the membrane potential (E_m) on the endoosmotic side were measured simultaneously by the conventional microelectrode method. In this case, both measurements were made in APW. A micropipette electrode, filled with a saturated solution of KCl and connected to an Hg-Hg_2Cl_2 half-cell, was inserted into the part of the cell in the air gap (Air in Fig. 1). The reference electrode, which was an APW-agar salt bridge that was also connected to Hg-Hg_2Cl_2 half-cell, was placed in pool A where endoosmosis occurred. The E_m was the potential difference between two electrodes. To measure the membrane conductance (g_m), small electric pulses were applied to the cell through a pair of Ag-AgCl wires placed in each pool. Both E_m and the PM current were also recorded simultaneously by the recorder.

Measurements of changes in $[Ca^{2+}]_c$ of a drop of cytoplasm during hydration—The vacuolar sap of an internodal cell was replaced with perfusion medium (240 mOsm), which contained 5 mM EGTA, 1 mM ATP, 10 mM PIPES, 6 mM $MgCl_2$ and 200 mM sorbitol (pH 7.0, adjusted with KOH) to remove vacuolar Ca^{2+}. The perfusion medium and the cytoplasm, which included many chloroplasts, were squeezed out with forceps as a small drop (for convenience, such drops are referred to below as cytoplasmic drops) in silicone oil in a glass chamber (cf. Kikuyama et al. 1995). A small amount of aequorin was added to the drop. Measurements of the emission of light from aequorin were made in a similar manner to that described above. Hydration of the drop was induced by the addition of an almost equal volume of the hypotonic medium, the osmolality of which had been adjusted to half of that of the perfusion medium by reducing the concentration of sorbitol from 200 mM to 80 mM.

Estimation of changes in the emission of light from aequorin—When the amplitude of the change in the PM current was sufficiently large as shown, for example, in Figure 3a, the increase in light emission (ΔL) was estimated simply as the difference between the peak value of the PM current and the value before the treatment. When the amplitude was small, ΔL was estimated as follows. Since the recorder trace of the PM current was composed of the current that was due to the emission of light from aequorin and the dark current, with random pulses, we assumed that the lower edge of the trace of the PM current corresponded to the light emission from aequorin. In order to isolate the aequorin-generated current from the dark current, we used the following procedure. First, numerical data from the recording were transferred to a computer (Macintosh; Apple, Tokyo). To determine the ae-

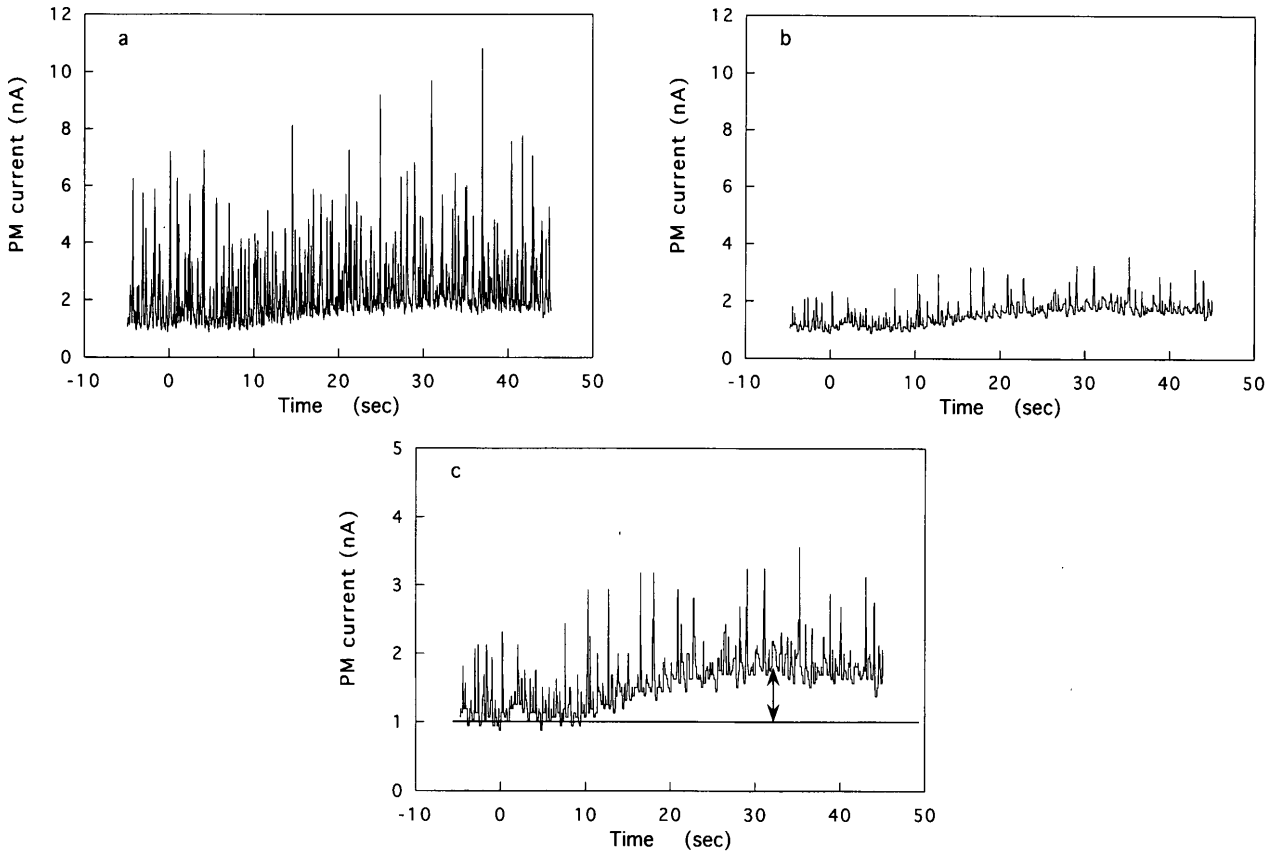

Fig. 2 Separation of the light signal from aequorin from the dark-current noises of the PM. (a) Original data. (b) Aequorin light signals after isolation by processing of the original data as described in the text. (c) Expansion of the ordinate scale; ΔL was obtained as the deflection from the base line, as shown by a double headed arrow. For full details, see the text.

quorin current at a specific time, we isolated the minimum value from among the ten readings obtained in the 0.2 s period prior to this time using Excel software (Microsoft, Tokyo). The recording for the next time point, which was shifted by 0.02 s from the foregoing point, was again determined in the same way as before. A series of minimum readings that had been obtained in this way was plotted against time. The data in Figure 2a (for cell 8 in Table 1) were processed in this manner and the results are shown in Figure 2b. The noise due to the dark current was largely suppressed although the trace of lower points remained essentially the same as that in Figure 2a. After expansion of the ordinate scale, ΔL was obtained as indicated by an arrow in Figure 2c.

Results

Increases in $[Ca^{2+}]_c$ during transcellular osmosis—An internodal cell of *Nitella axilliformis* was loaded with aequorin and placed in the measuring vessel, which contained 1 mM EGTA, as shown in Figure 1. When transcellular osmosis was induced by replacing 1 mM EGTA in one pool with a solution that contained 300 mM sorbitol at time zero, a significant increase in $[Ca^{2+}]_c$ was observed (Fig. 3). The peak PM current was about 17 nA. Since the basal current was 2 nA, ΔL was calculated to be 15 nA (cell 6 in Table 1). When the cell was cut after the measurement, a large PM current was observed (Fig. 3b), showing that a sufficient amount of aequorin had been present in the cell during the experiment. Eight of twelve cells tested yielded measurable increases in $[Ca^{2+}]_c$ (cells 1–8 in 1 mM EGTA, Table 1), while four cells, for which results are not listed in Table 1, showed either an insignificant change (three cells) or no change in ΔL. In another experiments, in which the osmotic gradient was increased from 300 mOsm to 350 mOsm, all six cells tested showed significant increases in $[Ca^{2+}]_c$ (data not shown). Essentially the same results were observed with *Chara corallina* and *Nitellopsis obtusa* upon induction of endoosmosis with an osmotic gradient of 300 mOsm (Table 1).

The transient increase in $[Ca^{2+}]_c$ upon endoosmosis was also observed in a medium that contained 1 mM Ca^{2+} (1 mM $CaCl_2$, 4 mM PIPES, pH 7.0, adjusted with KOH) and these results are also shown in Table 1.

Simultaneous measurements of changes in $[Ca^{2+}]_c$, E_m and g_m during transcellular osmosis—We made detailed measurements of E_m and g_m in APW, together with measurements of the emission of light from aequorin on the endoosmotic side. As shown in Figure 4a, both E_m and g_m of *Nitella axilliformis* changed significantly, in parallel with in-

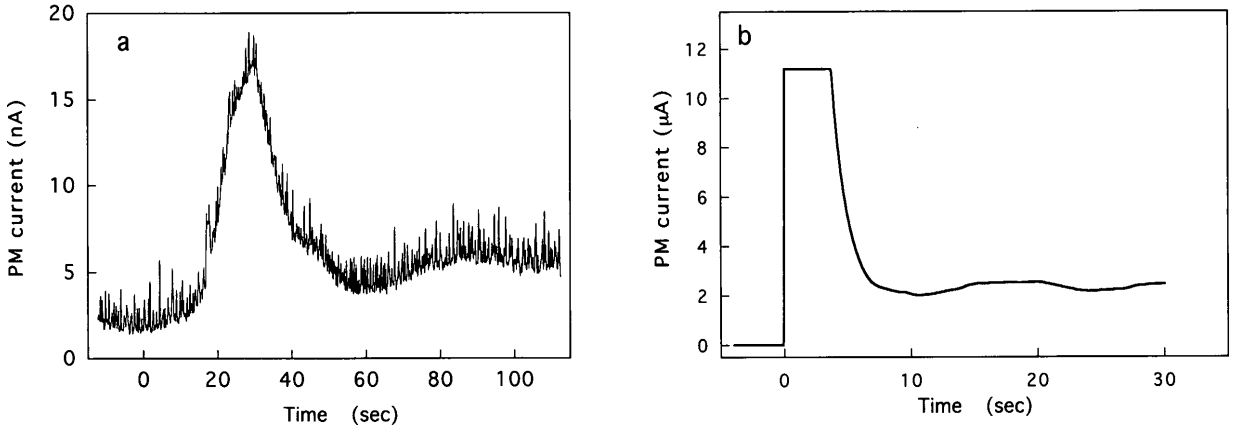

Fig. 3 A change in the PM current during endoosmosis in *Nitella axilliformis* in 1 mM EGTA. Transcellular osmosis was induced by 300 mM sorbitol at time zero (a). When the cell was cut in 1 mM $CaCl_2$, a very large increase in the PM current was observed, indicating that a sufficient amount of aequorin had been present during transcellular osmosis (b).

creases in the PM current. In this case, the membrane depolarization was 27 mV and the increase in g_m was 22% of the value of g_m just before the osmosis (g_{m0}). The changes were much smaller than those in *Nitella flexilis* (Fig. 4b; cf. Hayama et al. 1979), in which the membrane depolarization was 125 mV and the increase in g_m was 82%. In rare cases, *Nitella axilliformis* generated an action potential on the endoosmotic side as does *Nitella flexilis* (Hayama and Tazawa 1978; data not shown).

Comparison in terms of the changes in $[Ca^{2+}]_c$ induced by endoosmosis in 1 mM EGTA between Nitella flexilis and other species—Although an increase in $[Ca^{2+}]_c$ was evident in *Nitella axilliformis* during transcellular osmosis (Fig. 3a), as it was in *Nitella flexilis*, cytoplasmic streaming was affected only in *Nitella flexilis* and not in *Nitella axilliformis* (data not shown). These observations might indicate that the increase in $[Ca^{2+}]_c$ was much smaller in *Nitella axilliformis* than in *Nitella flexilis*. To examine the increase in $[Ca^{2+}]_c$ more quantitatively, we normalized the PM current as log ($\Delta L/\Delta L_{max}$), where ΔL and ΔL_{max} denote the maximum change in the PM current during transcellular osmosis and the maximum change in the PM current upon cutting the cell, respectively. Since ΔL_{max} reflects the total amount of aequorin that remains in the cell and since pCa ($-\log [Ca^{2+}]$) is proportional to log ($\Delta L/\Delta L_{max}$) (Shimomura et al. 1988), log ($\Delta L/\Delta L_{max}$) should serve as a measure of the change in the concentration of Ca^{2+} (Allen and Blinks 1979, Kikuyama and Tazawa 1983). As shown in Table 1, log ($\Delta L/\Delta L_{max}$) was much smaller in *Nitella axilliformis, Chara corallina* and *Nitellopsis obtusa* than in *Nitella flexilis*. In *Nitella axilliformis*, there were no significant difference in log ($\Delta L/\Delta L_{max}$) between cells in 1 mM EGTA and those in 1 mM Ca^{2+}.

Increases in $[Ca^{2+}]_c$ of cytoplasmic drops during hydration—The cytoplasm of *Nitella axilliformis* or *Chara corallina* was squeezed out as described in Materials and Methods. When such a cytoplasmic drop was hydrated directly by the addition of hypotonic medium, an increase

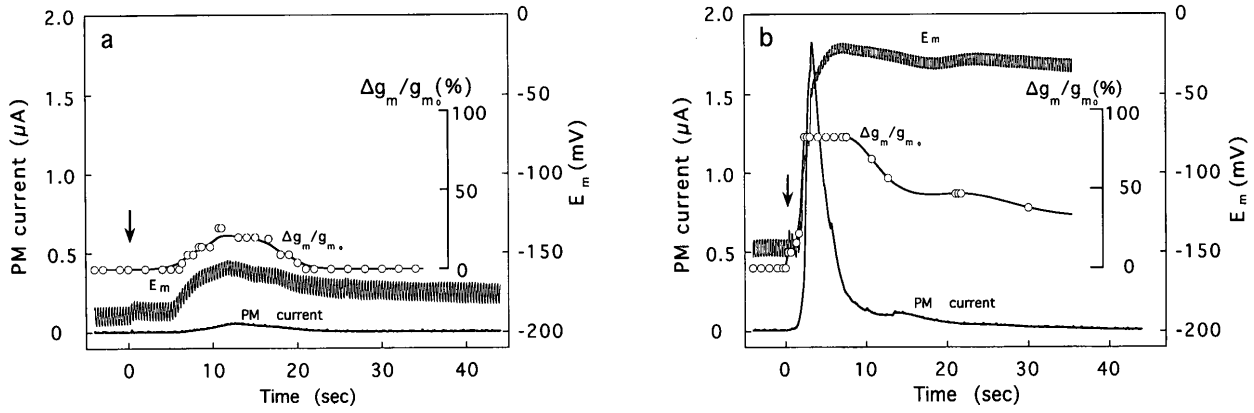

Fig. 4 Changes in the PM current, E_m and g_m on the endoosmotic side. The change in g_m is shown as the ratio between the increment in g_m during transcellular osmosis (Δg_m) and g_m before the osmosis (g_{m0}). Measurements were made in APW. Transcellular osmosis was induced by 250 mM sorbitol at time zero. (a) *Nitella axilliformis*. (b) *Nitella flexilis*.

Table 1 Increases in the PM current during transcellular osmosis in 1 mM EGTA or 1 mM Ca^{2+}

Species	Cell no.	ΔL (nA)	ΔL_{max} (nA)	log ($\Delta L/\Delta L_{max}$)
Nitella axilliformis (in 1 mM EGTA)	1	2.3	>11,150[a]	−3.69
	2	1.6	2,412	−3.18
	3	45.1	>11,150[a]	−2.39
	4	10.0	>11,150[a]	−3.05
	5	10.3	>11,150[a]	−3.03
	6	15.4	>11,150[a]	−2.86
	7	2.8	>11,150[a]	−3.60
	8	0.8	>11,150[a]	−4.14
	mean[b]			−3.24±0.19
Nitella axilliformis (in 1 mM Ca^{2+})	1	4.7	11,100	−3.37
	2	8.5	>11,200[a]	−3.12
	3	40.4	6,224	−2.19
	4	3.9	>11,200[a]	−3.46
	5	14.1	>11,200[a]	−2.90
	6	2.0	>11,200[a]	−3.75
	7	8.3	>11,200[a]	−3.13
	mean[b]			−3.13±0.19
Nitella flexilis (in 1 mM EGTA)	1	100	8,400	−1.92
	2	20	3,000	−2.17
	3	66	4,200	−1.80
	mean[b]			−1.96±0.11
Chara corallina (in 1 mM EGTA)	1	1.5	>13,700[a]	−3.96
	2	1.3	6,200	−3.68
	3	0.4	3,400	−3.93
	4	0.6	1,100	−3.26
	5	0.4	4,100	−4.01
	mean[b]			−3.77±0.14
Nitellopsis obtusa (in 1 mM EGTA)	1	0.7	12,800	−4.26
	2	0.8	12,300	−4.19
	mean			−4.23

The osmotic gradient was 300 mOsm for all measurements.
[a] Records were off-scale so that actual values were larger than the values cited.
[b] Mean values are given±SEM.

in $[Ca^{2+}]_c$ was observed in both species. Ten cytoplasmic drops of *Nitella axilliformis* in 14 trials and nine drops of *Chara corallina* in 11 trials emitted light upon hydration. These results are summarized in Table 2. Typical examples of the emission of light from aequorin upon hydration of cytoplasmic drops of *Nitella axilliformis* and *Chara corallina* are shown in Figure 5.

Discussion

Hayama et al. (1979) reported that a large and prolonged depolarization occurred in the endoosmotic region of the cell during transcellular osmosis in *Nitella flexilis* but not in other species of Characeae, such as *Nitella axilliformis, Chara corallina* and *Chara australis*. In the present study, by contrast, we found that both phenomena, namely, an increase in $[Ca^{2+}]_c$ (Fig. 3a) and membrane depolarization (Fig. 4a), also occurred in *Nitella axilliformis* upon hydration of the cytoplasm on the endoosmotic side. It is known that E_m, g_m (Mimura and Tazawa 1983) and cytoplasmic streaming (Tominaga et al. 1983) are all sensitive to $[Ca^{2+}]_c$. Thus, small changes in both E_m and g_m (Fig. 4a) and the absence of inhibition of cytoplasmic streaming upon hydration of the cytoplasm in *Nitella axilliformis* can be explained by the much smaller increase in $[Ca^{2+}]_c$ in *Nitella axilliformis* than in *Nitella flexilis* (Table 1).

Table 2 Increases in the PM current during hydration of cytoplasmic drops

Species	Cell no.	ΔL (nA)	ΔL_{max} (nA)	log ($\Delta L/\Delta L_{max}$)
Nitella axilliformis	1	5.1	11,140	−3.34
	2	14.8	>11,180[a]	−2.88
	3	3.4	>11,180[a]	−3.52
	4	7.1	11,140	−3.20
	5	3.7	11,030	−3.47
	6	1.8	11,140	−3.79
	7	3.1	11,140	−3.56
	8	54.9	11,150	−2.31
	9	6.2	>11,220[a]	−3.26
	10	6.5	>11,220[a]	−3.24
	mean[b]			−3.26±0.13
Chara corallina	1	64.4	>11,180[a]	−2.24
	2	4.1	11,140	−3.43
	3	1.4	>11,150[a]	−3.90
	4	2.1	>11,150[a]	−3.73
	5	5.9	>11,150[a]	−3.28
	6	2.7	11,050	−3.61
	7	1.3	5,090	−3.59
	8	2.4	10,510	−3.64
	9	4.4	11,140	−3.40
	mean[b]			−3.42±0.16

[a] Records were off-scale so that actual values were larger than the values cited.
[b] Means are given±SEM.

In *Nitella flexilis*, an influx of water during endosmosis results in an action potential that is accompanied by a large influx of Ca^{2+} (Fig. 4b; cf. Hayama and Tazawa 1978). In *Nitella axilliformis*, by contrast, the average value of log ($\Delta L/\Delta L_{max}$) in 1 mM Ca^{2+} (3.24±0.19; mean±SEM, n=8) was very close to that in 1 mM EGTA (3.13±0.19; mean±SEM, n=7) (Table 1). This similarity indicates that the release of Ca^{2+} from internal store(s) is predominant contributor to the increase in $[Ca^{2+}]_c$ upon hydration of the cytoplasm, as shown in *Nitella flexilis* (Tazawa et al. 1995). The release of Ca^{2+} from internal store(s) was confirmed by the fact that HICR was also observed in isolated cytoplasmic drops (Fig. 5, Table 2). What is the reason for the difference in the ability of hydration to induce an action potential in the two species of *Nitella*? A key to this problem may mainly be the difference in the capacity for HICR, or in the sensitivity to hydration of cellular organelles that storing Ca^{2+}. In *Nitella flexilis*, the HICR is sufficiently large to elevate $[Ca^{2+}]_c$, which causes depolarization of the membrane to the threshold level required for an action potential, while in *Nitella axilliformis* the HICR is insufficient (Table 1).

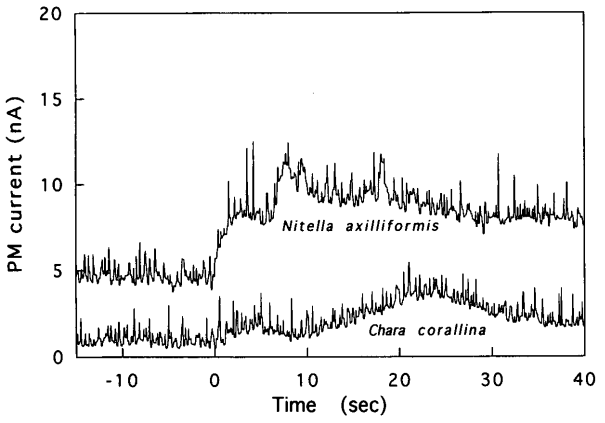

Fig. 5 Changes in the PM current of cytoplasmic drops of *Nitella axilliformis* and *Chara corallina* during hydration, which was induced by the addition of hypotonic medium to drops at time zero, as described in the text.

Experiments in vitro showed that HICR in *Nitella flexilis* involved the release of Ca^{2+} from some cytosolic storage sites (Kikuyama et al. 1995). We also demonstrated the HICR in isolated drops of cytoplasm from other species of Characeae (Fig. 5, Table 2). It seems likely that cytosolic organelles that release Ca^{2+} upon hydration are generally present in Characeae. To identify the cytosolic sites of stor-

age of Ca^{2+} that are responsible for HICR, it will be necessary to separate the various cytosolic organelles and to examine the capacity for HICR of each type of organelle.

In a brackish-water Characeae, *Lamprothamnium succinctum*, hypotonic treatment caused a large membrane depolarization and a large increase in membrane conductance (Okazaki et al. 1984), both of which were closely related to the increase in $[Ca^{2+}]_c$ (Okazaki et al. 1987). Although the observed Ca^{2+} transient during the osmotic expansion of cells is common to freshwater and brackish-water Characeae, the mechanisms are quite different. In the freshwater Characeae, the Ca^{2+} transient is caused not only by an increased influx of Ca^{2+} but also by the release of Ca^{2+} from internal storage sites. By contrast, in the brackish-water Characeae *Lamprothamnium*, the increase in $[Ca^{2+}]_c$ is caused by an influx of external Ca^{2+} (Okazaki et al. 1987). However, it remained to be determined whether or not a small amount of Ca^{2+} is released from internal storage sites upon hypotonic treatment in *Lamprothamnium* also.

In the present study, we demonstrated that the mechanism for HICR exists not only in *Nitella flexilis* but also in other species, namely *Nitella axilliformis, Chara corallina* and *Nitellopsis obtusa*. The sensitivity or the capacity of such a system differs among species. The mechanism for HICR might have developed in each species to a different extent. It is also possible that the mechanism developed extensively in ancestral characeans but became less efficient in some species with time. In a physiological context, the HICR mechanism might, at least in *Nitella flexilis*, be involved in sensing of changes in the osmotic conditions of the surroundings. Since characean plants are assumed to be related evolutionarily to archegoniate plants (Stewart and Mattox 1975), it is worth examining whether or not a mechanism for HICR is also operative in terrestrial plants that are normally exposed to large fluctuations in ambient water potential.

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