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Membrane Stretching Triggers Mechanosensitive Ca^{2+} Channel Activation in *Chara*

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Abstract In order to confirm that mechanosensitive Ca^{2+} channels are activated by membrane stretching, we stretched or compressed the plasma membrane of *Chara* by applying osmotic shrinkage or swelling of the cell by varying the osmotic potential of the bathing medium. Aequorin studies revealed that treatments causing membrane stretching induced a transient but large increase in cytoplasmic concentration of Ca^{2+} ($\Delta[Ca^{2+}]_c$). However, the observed $\Delta[Ca^{2+}]_c$ decreased during the treatments, resulting in membrane compression. A second experiment was carried out to study the relationship between changes in membrane potential (ΔE_m) and stretching or compression of the plasma membrane. Significant ΔE_m values, often accompanied by an action potential, were observed during the initial exchange of the bathing medium from a hypotonic medium to a hypertonic one (plasmolysis). ΔE_m appears to be triggered by a partial stretching of the membrane as it was peeled from the cell wall. After plasmolysis, other exchanges from hypertonic to hypotonic media, with their accompanying membrane stretching, always induced large ΔE_m values and were often accompanied by an action potential. By contrast, action potentials were scarcely observed during other exchanges from hypotonic to hypertonic solutions (=membrane compression). Thus, we concluded that activation of the mechanosensitive channels is triggered by membrane stretching in *Chara*.

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Abbreviations

AP	Action potential
APW	Artificial pond water
$[Ca^{2+}]_c$	Cytoplasmic level of free calcium ion
$\Delta[Ca^{2+}]_c$	Transient increase in $[Ca^{2+}]_c$
ΔD_m	Membrane deformation
E_m	Membrane potential
ΔE_m	Change in membrane potential
55KCl	55 mM KCl containing 0.1 mM $CaCl_2$
PM	Photomultiplier
150sorbitol etc.	APW containing sorbitol in 150 mM etc. and 1 mM $CaCl_2$

Introduction

Plants respond to various environmental stimuli, such as light, temperature and water. Likewise, mechanical stimuli are also very important signals. For example, mechanical stresses make plants short and thick through enhancing ethylene biosynthesis (Goeschl et al. 1966). Mechanical bending of sensory hairs in the lobe induces action potential (AP), which triggers lobe closing in Venus flytrap (Sibaoka 1991). Tendrils of *Pisum sativum*, likewise, show coiling in response to touch signals (Engelberth 2003). Geotropism may also be another important example of mechanosensing in plants.

Kishimoto (1968) and Shimmen (1996) studied the ionic mechanism of mechanical signal perception at the cellular level in characean plants. Kishimoto (1968) reported that changes in membrane potential (ΔE_m) occur in response to mechanical stimuli. Amplitude of ΔE_m is dependent upon the intensity of the stimulus, suggesting that it may act as a receptor potential (Kishimoto 1968; Shimmen 1996). Shimmen (1996, 1997a, b) reported that the ionic mechanism of the receptor potential is activation of Cl^- channels at the plasma membrane. Since the ΔE_m was not influenced by lowering Ca^{2+} outside the cell, he suggested little contribution of Ca^{2+} to the generation of the ΔE_m.

Kaneko et al. (2005) demonstrated, however, that transient increases in cytoplasmic Ca^{2+} concentrations ($\Delta[Ca^{2+}]_c$) took place at the very beginning of the receptor potential and that the amplitude of $\Delta[Ca^{2+}]_c$ was also dependent on the strength of mechanical stimuli, as was the case in the amplitude of the receptor potential. Thus, they suggested that Ca^{2+} channel activation may be the first step in mechanoperception. Since Cl^- channel activation in the depolarizing phase of the AP takes place depending on $\Delta[Ca^{2+}]_c$ (Kikuyama and Tazawa 1998; Kikuyama 2000), Kaneko et al. (2005) suggested the following order for the ionic processes in the generation of a receptor potential, according to an analogous discussion of the AP: (1) activation of Ca^{2+} channels by mechanostimulation, (2) increased influx of Ca^{2+} from the environment, (3) transient $\Delta[Ca^{2+}]_c$ and (4) Cl^- channel activation in a $[Ca^{2+}]_c$-dependent manner.

Iwabuchi et al. (2005) showed that the amplitude of the ΔE_m was closely related to the degree of membrane deformation (ΔD_m), concluding that ΔD_m is the main activator of Ca^{2+} channels. What, then, is the physical basis of ΔD_m? Cellular or membrane deformation should be tightly correlated with changes in membrane curvature and tension at the membrane. Since some patch-clamp studies showed stretch-activated ion channels (SA channels) in plants (Dutta and Robinson 2004), in animals (Popp et al. 1992) and in *Saccharomyces* (Kanzaki et al. 1999), the physical basis of ΔD_m may be membrane stretching or changes in membrane tension during mechanical stimulus. We strongly suggested in a previous report that changes in membrane tension, rather than membrane tension itself, may be the major factor to induce receptor potential or activation of Ca^{2+} channels in *Chara* (Iwabuchi et al. 2008).

The present study was carried out to investigate whether or not changes in membrane tension are the major factor activating Ca^{2+} channels in *Chara* by directly stretching or compressing the plasma membrane. This was realized by osmotically expanding or shrinking the protoplast, which was made by plasmolysing the cell. If the mechanosensitive Ca^{2+} channels in *Chara* are also a stretch-activated, both the membrane depolarization and an increase in cytoplasmic Ca^{2+} concentration should be evident upon membrane stretching, while not occurring during membrane compression. It was clearly demonstrated in the present study that membrane stretching—namely, increasing membrane tension—triggers the activation of mechanosensitive Ca^{2+} channels in *Chara*.

Materials and Methods

Plant Material

Chara corallina cultured in the laboratory was used throughout the experiment. Single internodes, isolated from neighboring leaflets and internodes, were kept in artificial pond water (APW; 0.1 mM each of KCl, NaCl and $CaCl_2$) for more than a day before use.

Electrical Measurements

E_m of *C. corallina* was measured by an ordinary microelectrode method. A glass micropipette, filled with saturated KCl and connected to a $Hg–Hg_2Cl_2$ half-cell, was inserted into the vacuolar space of an internodal cell of *C. corallina*. The potential difference between the microelectrode and a reference electrode outside the cell was amplified and recorded with a memory oscilloscope (PowerLab/800; ADInstruments, Nagoya, Japan) and processed using KaleidaGraph (Hulinks, Tokyo, Japan).

Calcium Transient

$\Delta[Ca^{2+}]_c$ was measured by the same method as previous reports (Kikuyama and Shimmen 1997; Kikuyama and Tazawa 1998 2001; Kaneko et al. 2005; Iwabuchi et al. 2005, 2008). Aequorin, which emits light in the presence of free Ca^{2+}, was used as a Ca^{2+} indicator. An aequorin solution, composed of 100 mM KCl, 6 mM $MgCl_2$, 0.1 mM EGTA and approximately 1 mg ml^{-1} aequorin, was microinjected into the cytoplasm of an internodal cell of *C. corallina*. The volume of injected aequorin solution was approximately 1 nl, which is $<0.1\%$ of total cytoplasmic volume.

The internodal cell loaded with aequorin was set in the measuring chamber and placed over a photomultiplier (PM) tube (R1924P; Hamamatsu Photonics, Hamamatsu, Japan) in a dark box (Fig. 1b). Light emission from aequorin was measured by the PM. The PM current, which reflects $[Ca^{2+}]_c$, was treated in the same manner as the E_m.

At the end of each measurement, we cut the cell in the dark box using a razor blade. With this, all aequorin remaining in the cell emitted light by reacting with Ca^{2+} in the bathing medium. This was carried out to check whether

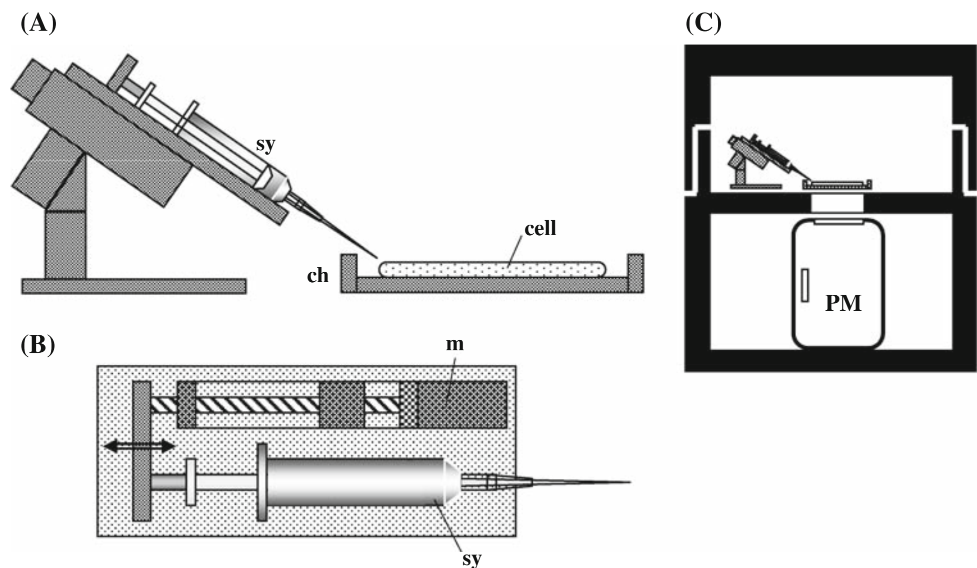

Fig. 1 Experimental setup for the measurement of $\Delta[Ca^{2+}]_c$ by stretching or compressing the membrane. **a** An internodal cell of *Chara* which had been microinjected with aequorin was set in a measuring chamber (ch). Stretching or compression of the membrane was carried out by adding appropriate medium using a syringe (sy). **b** The syringe was operated by a motor system (m), which moves unidirectionally. **c** The set shown in (**a**) was placed in a dark box equipped with a PM, and $\Delta[Ca^{2+}]_c$ was measured as the change in light emission from aequorin

or not enough aequorin was present in the cell after the measurement (Kikuyama and Tazawa 1983) because aequorin was used up during each measurement. We accepted only results in which enough aequorin was present after the measurement.

Stretching and Compressing the Plasma Membrane

Stretching and compression of the plasma membrane during the E_m measurements were achieved by exchanging the bathing medium from APW to a sorbitol-containing APW and vice versa.

During the $\Delta[Ca^{2+}]_c$ measurement, an internodal cell was placed in the measuring chamber as shown in Fig. 1a, c. First, the APW in the chamber was manually removed under ambient room light. After closing the dark box, the extracellular space in the measuring chamber was filled with APW containing 400 mM sorbitol and 1 mM $CaCl_2$ (*400sorbitol*) using a motor-driven syringe (sy in Fig. 1a, b). Deplasmolysis was achieved by exchanging the bathing medium from *400sorbitol* to *150sorbitol*. First, the *400sorbitol* outside the cell was manually removed under ambient room light, then the *150sorbitol* was added into the chamber in the darkness. The osmotic value of a *C. corallina* internode was around 230 mOsm, which was measured using the "turgor balance" method of Tazawa (1957).

Results

$\Delta[Ca^{2+}]_c$ upon Membrane Stretching

An internodal cell was first injected with a $[Ca^{2+}]_c$ indicator, aequorin, and placed in a chamber filled with APW. The chamber was placed over a PM in a dark box (Fig. 1). We measured the PM current reflecting $[Ca^{2+}]_c$ for several minutes in the darkness to ensure its stability; the APW was exchanged with *400sorbitol* and the $\Delta[Ca^{2+}]_c$ measured. A representative result is shown in Fig. 2. The hypertonic medium, *400sorbitol*, was added at t = 0 (lower trace). This caused a significant $\Delta[Ca^{2+}]_c$ (trace 1 in Fig. 2) about 10 s after the addition. The $\Delta[Ca^{2+}]_c$ was transient and exhibited only a single peak. Next, the bathing medium was exchanged again from *400sorbitol* to *150sorbitol* in the same manner. The second treatment should have caused membrane stretching through osmotic expansion of the protoplasm, and a significant $\Delta[Ca^{2+}]_c$ was likewise seen

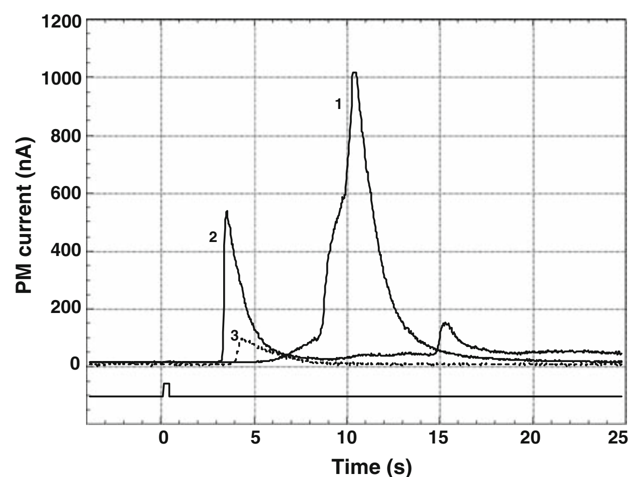

Fig. 2 A typical example of $\Delta[Ca^{2+}]_c$ in hyper- and hypotonically treated cells. First, hypertonic treatment was carried out by replacing the APW with *400sorbitol* (*trace 1*). Next, the *400sorbitol* was exchanged for *150sorbitol* (*trace 2*). Then, the *150sorbitol* was exchanged for *400sorbitol* (*trace 3*). Each medium was added into the measuring chamber at time zero, as shown by the *lower trace*. *Upper traces* are $\Delta[Ca^{2+}]_c$ shown as changes in the PM current

Table 1 Comparison of peak values (nA) of $\Delta[Ca^{2+}]_c$ among the first, second and third exchanges of bathing medium

Cell	Exchange		
	1	2	3
1	>1,000	536	93
2	157	70	23
3	30	514	23
4	14	165	203
5	>1,000	33	24
6	>1,000	366	477
7	433	>1,000	16

Values are peak PM values measured. Results shown in cell 1 correspond to those shown in Fig. 3

(trace 2 in Fig. 2). Although $\Delta[Ca^{2+}]_c$ for the second treatment was significantly smaller than at the first one in this case, this is not always the case (Table 1). The bathing medium was again exchanged from *150sorbitol* to *400sorbitol* (third treatment) to induce shrinkage of the protoplasm (=membrane compression). In this case, the observed $\Delta[Ca^{2+}]_c$ was much smaller than those in the first and second exchanges of the bathing medium (trace 3 in Fig. 2). Similar results were confirmed in seven experiments, although values were greatly different from cell to cell. Table 1 shows each peak value of $\Delta[Ca^{2+}]_c$ after the first exchange ($\Delta[Ca^{2+}]_{1st}$) from APW to *400sorbitol* (plasmolysis), the second exchange ($\Delta[Ca^{2+}]_{2nd}$) from *400sorbitol* to *150sorbitol* (deplasmolysis) and the third exchange ($\Delta[Ca^{2+}]_{3rd}$) from *150sorbitol* to *400sorbitol* (membrane compression) on each cell. In cases when $\Delta[Ca^{2+}]_{2nd}$ was larger than $\Delta[Ca^{2+}]_{3rd}$, the ratios were 5.8, 3.0, 22.3, 1.4 and >62.5. On the other hand, in the reversed cases, the ratios were 0.81 and 0.77, showing that the difference of $\Delta[Ca^{2+}]$ between the second and third treatments was very small in the case of $\Delta[Ca^{2+}]_{2nd} < \Delta[Ca^{2+}]_{3rd}$. Similarly, $\Delta[Ca^{2+}]_{1st}$ values were much larger than $\Delta[Ca^{2+}]_{3rd}$ values except for cell 4. Thus, it should be concluded that the maximum values of $\Delta[Ca^{2+}]_c$ in the third treatment were smaller than those for the first and second treatments.

Initiation of $\Delta[Ca^{2+}]_c$

As shown in Fig. 2, $\Delta[Ca^{2+}]_c$ in the second (from *400sorbitol* to *150sorbitol*) and third (from *150sorbitol* to *400sorbitol*) treatments started earlier than in the first treatment. We summarized the $\Delta[Ca^{2+}]_c$ initiation time for each treatment in Fig. 3 ($n = 7$). In the first treatment, causing plasmolysis, the start of $\Delta[Ca^{2+}]_c$ was much later (6.7 ± 1.4 s) than in the second and third treatments (1.8 ± 0.4 and 2.1 ± 0.5 s, respectively). The difference

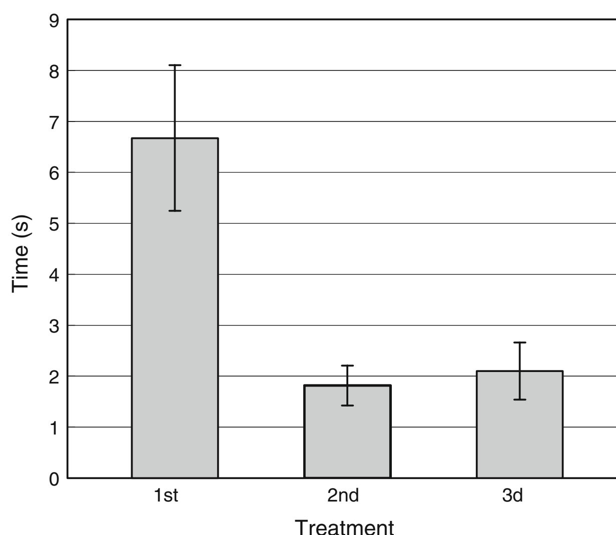

Fig. 3 Relationship between the period before the start of $\Delta[Ca^{2+}]_c$ and the number of treatments. Values are means ± SE ($n = 7$)

in times between the first and the other treatments was statistically significant ($P < 0.03$).

Initiation of Plasmolysis

We measured the time when plasmolysis initiated after addition of *400sorbitol* to the internodal cell. The cell was observed through a microscope equipped with ×4 (objective) and ×10 (ocular) lenses, and *400sorbitol* was added in the same manner as above, using the motor-driven syringe. The initiation of plasmolysis was judged by formation of many small concaves at the cell surface. The initiation time was different from measurement to measurement, the most rapid being 8.9 s, the latest being 20.0 s and the mean ± SE being 12.4 ± 1.4 s ($n = 10$).

ΔE_m upon Stretching and Compressing the Membrane

We did not repeat $\Delta[Ca^{2+}]_c$ measurements more than three times, as shown above, because aequorin is consumed during $\Delta[Ca^{2+}]_c$ measurement, especially when $\Delta[Ca^{2+}]_c$ is large. Thus, we measured ΔE_m instead as Ca^{2+} channel activation because Ca^{2+} channel activation should necessarily cause membrane depolarization. First, a glass micropipette electrode was inserted into an internodal cell of *C. corallina* in APW. After checking that E_m was stable and around −170 mV or more negative, the bathing medium, APW, was exchanged with, e.g., *300sorbitol*. It should be noted that plasmolysis of the cell did not occur in media containing less than 200 mM sorbitol but did in media containing more than 250 mM sorbitol as the osmotic value of the *C. corallina* internodal cells was around 230 mOsm.

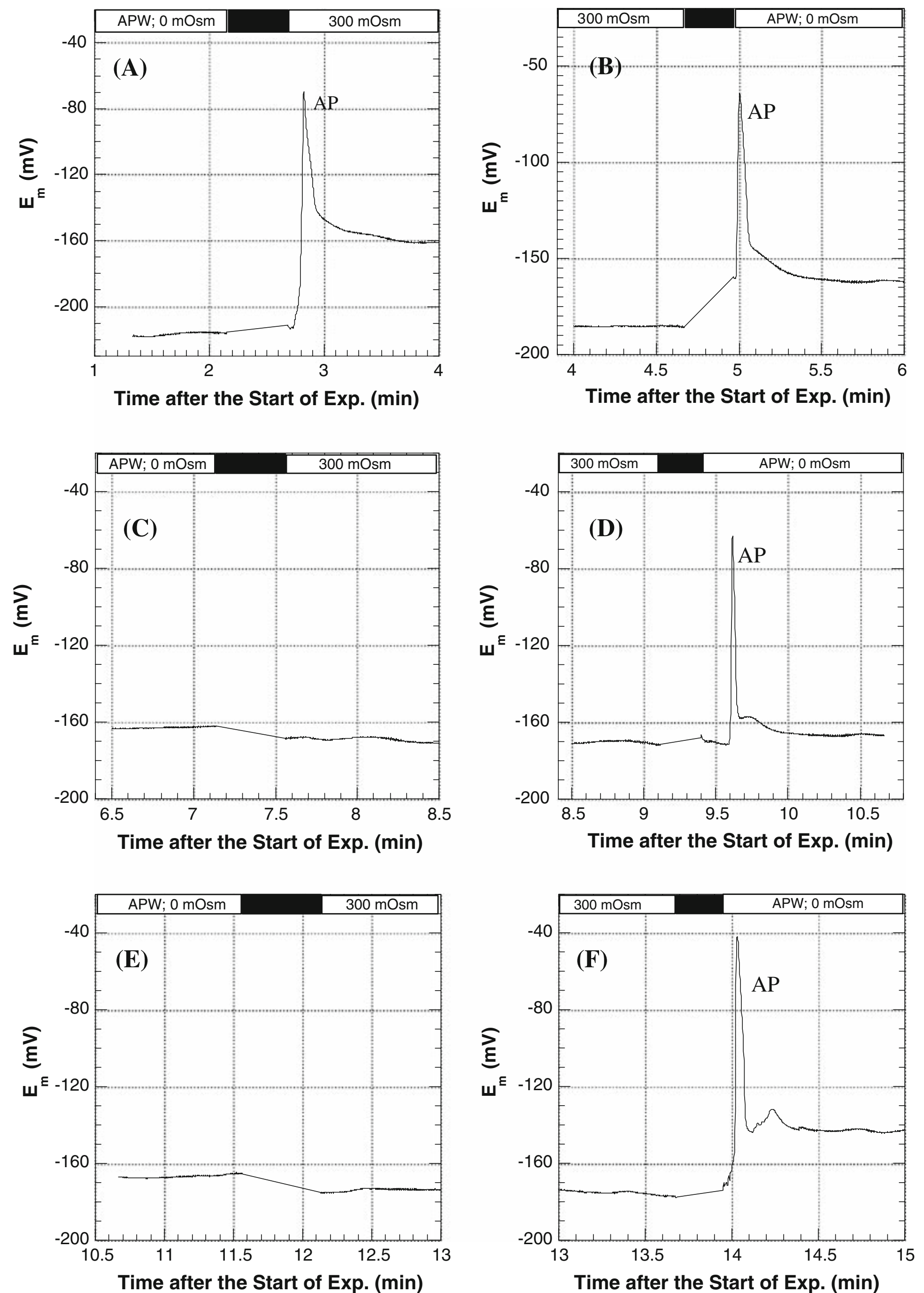

Fig. 4 Relationship between E_m and membrane stretching. Osmotic tension was applied to the plasma membrane by changing the bathing medium from APW to *300sorbitol*. **a** The first exchange of the bathing medium from APW to *300sorbitol*. This treatment plasmolysed the cell, which generated an AP. **b**, **d**, **f** Exchanges from *300sorbitol* to APW, which should cause membrane stretching, induced an AP. **c**, **e** Exchanges from APW to *300sorbitol*, which should cause membrane compression, did not induce any AP or any ΔE_m. *Upper black box* in each record shows the period of medium exchange, and the E_m record during this period is omitted because E_m was not measured during this period

A representative result is shown in Fig. 4, and all data are summarized in Table 2. During the first exchange of the bathing medium from APW to *300sorbitol*, 12 out of 16 cells generated an AP (Fig. 4a, exchange 1 in Table 2), suggesting that a ΔE_m larger than the threshold for AP took place in these cells. The other four cells showed a ΔE_m only, which was defined in the present study as a change >1 mV. On other exchanges accompanying membrane stretching—namely, exchanges from *300sorbitol* to APW—many cells also generated ΔE_m or AP (Fig. 4b, d, f, and exchanges 2, 4, 6 and 8 in Table 2). Conversely, the majority of cells did not generate ΔE_m or AP (Fig. 4c, d, and exchanges 3, 5 and 7 in Table 2) when APW was exchanged for *300sorbitol*, which should cause membrane compression.

A similar experiment, in which exchange of the bathing medium between APW and *200sorbitol* was also carried out, is summarized in Table 3. It should be noted that plasmolysis did not occur in this series because *200sorbitol* is hypotonic to *Chara* cells. It is clear that many cells also

Table 2 Effect of membrane stretching and compression on AP and ΔE_m

Exchange	Π_o (mOsm)	Cells tested (*n*)	Cells with AP	Cells with ΔE_m	Cells with AP or ΔE_m (%)
1	0 → 300	16	12	4	100
2	300 → 0	15	11	1	80
3	0 → 300	12	2	0	17
4	300 → 0	11	9	0	82
5	0 → 300	7	1	0	14
6	300 → 0	7	7	0	100
7	0 → 300	2	0	0	0
8	300 → 0	2	2	0	100

The osmotic value of the bathing medium (Π_o) was changed between 0 and 300 mOsm. Odd numbers in the first column show exchanges from APW to *300sorbitol*, while even ones show exchanges from *300sorbitol* to APW, as shown in the second column. The number of cells tested gradually decreased on repeated exchanges because repeated exchanges often depolarized E_m and depolarized cells (E_m was more positive than –170 mV) were discarded. ΔE_m, a change in $E_m > 1$ mV

Table 3 Effect of stretching and compressing the membrane on AP and ΔE_m

Exchange	Π_o (mOsm)	Cells tested (*n*)	Cells with AP	Cells with ΔE_m	Cells with AP or ΔE_m (%)
1	0 → 200	10	4	6	100
2	200 → 0	10	0	8	80
3	0 → 200	10	0	1	10
4	200 → 0	8	0	7	88
5	0 → 200	7	0	1	14
6	200 → 0	7	0	6	86
7	0 → 200	6	0	2	33
8	200 → 0	6	0	6	100

The osmotic value of the bathing medium (Π_o) was varied between 0 and 200 mOsm. Odd numbers in the first column are exchanges from APW to *200sorbitol*, while even ones are exchanges from *200sorbitol* to APW as shown in the second column. The number of cells tested gradually decreased on repeated exchanges because repeated exchanges often depolarized E_m and depolarized cells (E_m was more positive than −170 mV) were discarded. ΔE_m, a change in $E_m > 1$ mV

generated ΔE_m upon exchange from APW to *200sorbitol* (exchange 1 in Table 3). Other exchanges from *200sorbitol* to APW (exchanges 2, 4, 6 and 8) also induced ΔE_m. When APW was exchanged for *200sorbitol* (exchanges 3, 5 and 7), however, only a small number of cells showed ΔE_m and no cell generated AP, except during the first exchange.

Another experiment, in which plasmolysed protoplasts were osmotically expanded and shrunk, was carried out. An internodal cell, into which a glass micropipette electrode had been inserted, was first plasmolysed by exchanging the bathing medium from APW to *450sorbitol*. The extracellular medium was then exchanged from *450sorbitol* to *250sorbitol* (Table 4). The first exchange resulted in plasmolysis and induced AP in all cells tested (exchange 1 in Table 4). Other exchanges accompanying membrane compression rarely induced ΔE_m (exchanges 3 and 5), but those accompanying membrane stretching resulted in ΔE_m (exchanges 2, 4 and 6). No generation of ΔE_m or AP was observed at exchanges 7 and 8, probably reflecting deterioration of the plasmolysed protoplasts during repeated osmotic treatment.

Discussion

Iwabuchi et al. (2008) indicated that changes in the tension (but not the tension itself) of the plasma membrane may be instrumental in activating mechanosensitive Ca^{2+} channels in *Chara*. In order to confirm this, other experiments in which the membrane was directly stretched or compressed were carried out in the present study. In animal cells, the cell membrane can be easily stretched or compressed by pushing or pulling the elastic sheet on which the cells were grown (e.g., Hayakawa et al. 2001; Nakagawa et al. 2007). It is almost impossible, however, to apply this method to intact plant cells because they are encased in the cell wall.

Table 4 Effect of stretching and compressing membrane on AP and ΔE_m in plasmolysed cells

Exchange	Π_o (mOsm)	Cells tested (*n*)	Cells with AP	Cells with ΔE_m	Cells with AP or ΔE_m (%)
1	0 → 450	16	16	0	100
2	450 → 250	13	4	3	54
3	250 → 450	11	0	1	9
4	450 → 250	11	1	2	27
5	250 → 450	8	0	0	0
6	450 → 250	8	1	1	25
7	250 → 450	4	0	0	0
8	450 → 250	4	0	0	0

The osmotic value of the bathing medium (Π_o) was changed by 200 mOsm in each treatment. Odd numbers in the first column are exchanges from *250sorbitol* to *450sorbitol* (except exchange 1), while even ones are changes from *450sorbitol* to *250sorbitol* as shown in the second column. The number of cells tested gradually decreased on repeated exchanges because repeated exchanges often depolarized E_m and depolarized cells (E_m was more positive than −170 mV) were discarded. ΔE_m, a change in $E_m > 1$ mV

Thus, we developed a method to use plasmolysed cells, allowing us to compress or to expand the plasma membrane by exchanging the bathing medium.

Changes of osmotic potential in the bathing medium necessarily cause water flow across the plasma membrane. It is known that, during transcellular osmosis, inflow of water causes a large depolarization in *Nitella flexilis* but outflow of water does not, indicating that inflow of water can affect the membrane potential. However, the inflow of water scarcely affected the membrane potential of *C. corallina* (Hayama et al. 1979). Tazawa et al. (1994) reported in *N. flexilis* that rapid inflow of water during transcellular osmosis induced $\Delta[Ca^{2+}]_c$, which was explained as follows. The inflow rapidly decreases the osmotic value of the cytoplasm and causes osmotic expansion of putative intracellular Ca^{2+} store(s). The osmotic expansion of the store(s) induces Ca^{2+} release, probably due to activation of SA Ca^{2+} channels there (Kikuyama and Tazawa 2001). This phenomenon is generally seen in four characean species, although this is much smaller in *C. corallina* than in *N. flexilis* (Shimada et al. 1996). Thus, it may be possible that small $\Delta[Ca^{2+}]_c$ and ΔE_m during deplasmolysis in this study reflect Ca^{2+} release from the intracellular Ca^{2+} store(s). In spite of this, $\Delta[Ca^{2+}]_c$ and ΔE_m during the first treatment, causing plasmolysis, should not reflect the Ca^{2+} release because osmotic expansion of intracellular Ca^{2+} store(s) never occurs by this treatment. Thus, it should be the stretching of the plasma membrane that induced $\Delta[Ca^{2+}]_c$ and ΔE_m during plasmolysis, as shown in the next paragraph. Since the degree of Ca^{2+} release from the store(s) was very small in *C. corallina* (Shimada et al. 1996), it may be plausible that the $\Delta[Ca^{2+}]_c$ and ΔE_m during deplasmolysis in this study come from Ca^{2+} influx through SA channels at the plasma membrane, rather than release from internal stores.

When an internodal cell is plasmolysed by a hypertonic treatment, the plasma membrane of the cell should be compressed due to decreases in cell volume through water loss from the cell. In spite of this, some portions of the plasma membrane should be stretched at the points where the membrane was tightly connected to the cell wall since the membrane in these regions will be pulled away from the cell wall during plasmolysis, as pointed out by Hayashi and Takagi (2003) and Hayashi et al. (2006). In other words, plasmolysis may also cause localized membrane stretching. As a result, not only an increase in Ca^{2+} influx but also significant ΔE_m, often followed by an AP, should take place during plasmolysis, as shown in the present study. Initiation of plasmolysis was measured around 12 s, while $\Delta[Ca^{2+}]_c$ started at about 6.5 s after the first treatment with *400sorbitol* (Fig. 3). In Fig. 4a, an AP started at about 5–10 s after the end of medium exchange, which was very close to the 6.5 s in Fig. 3. Since the initiation of plasmolysis was judged by formation of small convexes at the observed area, actual initiation of plasmolysis should be earlier than the measured one. Thus, this strongly supports the above assumption that $\Delta[Ca^{2+}]_c$ took place at the moment of membrane detachment from the cell wall during plasmolysis.

The fact that the $\Delta[Ca^{2+}]_c$ started slower at the first plasmolysing treatment than at the second and third treatments (Figs. 2 and 3) may be explained as follows. When an intact cell in APW is immersed in hypertonic medium, the membrane should start to be compressed due to protoplasmic shrinkage. Given the assumption that membrane stretching causes Ca^{2+} channel activation (Iwabuchi et al. 2008), $\Delta[Ca^{2+}]_c$ is not expected to occur in this compression period. When shrinkage of the protoplasm reaches a certain degree, the membrane should begin to detach from the cell wall. Membrane detachment will cause local stretching of the membrane, as discussed above, and $\Delta[Ca^{2+}]_c$ should start at this time point. During the second and third treatments, however, membrane stretching and

compression should start as soon as the bathing medium is exchanged, which correlates well with the present result that the $\Delta[Ca^{2+}]_c$ started just after the medium was exchanged (Figs. 2 and 3). Thus, the fact that the $\Delta[Ca^{2+}]_c$ started slower at the first hypertonic treatment may also support the above assumption that plasmolysis contains a process causing membrane stretching.

Aequorin studies revealed that a large $\Delta[Ca^{2+}]_c$ took place upon exchanges of the bathing medium, causing plasmolysis and deplasmolysis (traces 1 and 2 of Fig. 2, Table 1), corresponding to membrane stretching. By contrast, the third treatment, which caused membrane compression, caused relatively smaller $\Delta[Ca^{2+}]_c$ (trace 3 in Fig. 2, Table 1). These results suggest that the activation of Ca^{2+} channels is triggered by membrane stretching but not by membrane compression. It was reported in brackish charophyta, *Lamprothamnium succinctum,* that hypotonic treatment induced a large $\Delta[Ca^{2+}]_c$ while hypertonic treatment did not (Okazaki et al. 1987, 2002). In tobacco suspension-cultured cells, hypoosmotic shock also caused $\Delta[Ca^{2+}]_c$ probably through activation of Ca^{2+} channels at the plasma membrane, although a process that requires Ca^{2+}-independent phosphorylation may also be involved in this case (Takahashi et al. 1997). These findings agree with our present results showing that increases in membrane tension induce activation of Ca^{2+} channels.

The E_m studies also showed similar results. Upon exchanges of the bathing medium causing membrane stretching, many cells generated ΔE_m or AP (Fig. 4a, b, d, f and exchanges 1, 2, 4, 6 and 8 in Tables 2, 3, and 4) but did not upon exchanges causing membrane compression (Fig. 4c, e and exchanges 3, 5 and 7 in Tables 2, 3, and 4). The fact that a larger number of cells generated AP on exchanges from APW to *300sorbitol* ($\Delta\Pi = 300$ mOsm) (Table 2) than to *200sorbitol* ($\Delta\Pi = 200$ mOsm) (Table 3) strongly suggests that degree of Ca^{2+} channel activation is dependent on the degree of membrane stretching.

Activation of mechanosensitive Ca^{2+} channels has been suggested to be highly correlated with membrane stretching or membrane tension. In animal cells, swelling of insulin-secreting cells caused an increase in $[Ca^{2+}]_c$, suggesting a stretch-mediated activation of Ca^{2+} channels (Sheader et al. 2001). Touch and wind signals increased the cytoplasmic level of Ca^{2+} in tobacco plants transformed with aequorin, suggesting a possible contribution of mechanosensitive Ca^{2+} channels (Haley et al. 1995). An anion channel from protoplasts of *Arabidopsis thaliana* mesophyll cells was activated by positive pressure on the membrane, while negative pressure on the membrane had no effect (Qi et al. 2004). Conversely, anion channels in the plasma membrane of *Vicia faba* guard cells were activated by negative pressure on the membrane and were not affected by positive pressure (Cosgrove and Hedrich 1991). Dutta and Robinson (2004) showed in pollen protoplasts of lily that Ca^{2+} channels could be activated by both positive and negative pressures in the patch pipette. Recently, Nakagawa et al. (2007) demonstrated that MCA1, a channel protein of *A. thaliana*, may be a calcium channel and is activated by increasing the membrane tension.

In the $\Delta[Ca^{2+}]_c$ study in this report, cells experienced two different osmotic states, a turgid state in APW or in *150sorbitol* and a turgorless state in *400sorbitol*, and a significantly large $\Delta[Ca^{2+}]_c$ was observed accompanying membrane stretching. It is known that plasmolysed characean cells can survive and show active cytoplasmic streaming for more than several days in $Ca(NO_3)_2$ or in sucrose (Hayashi and Kamitsubo 1959). Tazawa (1964) reported that plasmolysed cells could not survive after vacuolar perfusion followed by a recovery of turgor, while nonplasmolysed cells could survive for a long time (e.g., 30 days). This also suggests that characean cells once plasmolysed were irreversibly damaged after deplasmolysis. In the present study, cells experienced repeated plasmolysis and deplasmolysis. This may be the reason why the number of cells decreased after repeated plasmolysis and deplasmolysis, as shown in Tables 2 and 3. Furthermore, the plasmolysed state itself might have some toxic effect, as pointed out by McCulloch and Beilby (1997). In spite of this, it is clear that membrane stretching induces Ca^{2+} channel activation (Fig. 2, Table 1) and ΔE_m and AP (Tables 2, 4).

In the experiments for which the results are detailed in Table 3, cells were always turgid, although their turgor was varied. Cells showed ΔE_m and AP upon an increase in turgor but did not upon a decrease in turgor; this also agrees with our assumption that an increase in membrane tension activates Ca^{2+} channels because cell turgor has the same meaning as membrane tension according to a physical rule. In the experiments summarized in Table 4, cells were turgorless except in APW before the experiment, and again ΔE_m and AP were observed upon membrane stretching. The results shown in Tables 3 and 4 strongly suggest that increase in turgor, rather than the turgor pressure per se, is the major factor activating mechanosensitive Ca^{2+} channels, concurrent with previous investigations (Iwabuchi et al. 2008).

Iwabuchi et al. (2005) reported that the receptor potential, ΔE_m, at the end of a long-lasting compression of the cell was significantly larger than that at its start. During long-lasting compression, cell turgor once raised by the compression would become close to the initial level through loss of water from the compressed cell. As a result, water potential inside the cell should become smaller at the end of long-lasting compression than that before it. Release of the compression force should induce a larger water inflow than at the start of compression. This may induce a

larger stretching of membrane than that at the beginning of the compression and, according to the present results, may be a reason for the larger ΔE_m at the end of long-lasting compression (Iwabuchi et al. 2005).

When the cell was treated with $ZnCl_2$, a water channel inhibitor, ΔD_m during the compression was significantly suppressed by the inhibitor (Iwabuchi et al. 2008). In this case, smaller ΔE_m should be expected because membrane stretching at the end of long-lasting compression is smaller than that of untreated cells. In spite of this, ΔE_m of $ZnCl_2$-treated cells at the end of long-lasting compression was significantly larger than that of untreated cells (Iwabuchi et al. 2008). This may indicate that some factors other than membrane tension have some role on the generation of receptor potentials or the activation of mechanosensitive Ca^{2+} channels. It may be possible that the inhibitor, $ZnCl_2$, affects some mechanism which transfers information about the change in membrane tension to the ion channel. For example, the cytoskeleton has been proposed to have an important role in mechanosensing. In tendrils of *Bryonia dioica* and *Pisum sativum*, disruption of microtubules by colchicine led to total inhibition of the response to the thigmic stimulus in *Pisum* and to a reduced response in *Bryonia* (Engelberth 2003). In *Vicia* guard cell protoplasts, disruption of actin microfilaments activated the SA Ca^{2+} channels, while stabilizing it blocked the activation (Zhang et al. 2007). These reports suggest that cytoskeletons, microtubules and actin are involved in mechanosensing mechanisms. Conversely, Nakagawa et al. (2007) suggested that the cytoskeletal system does not affect MCA1, an SA Ca^{2+} chananel from *Arabidopsis*. They reported that MCA1 was activated during hypoosmotic stress or with the anionic amphipath trinitrophenol, both of which generate membrane distortion. Furthermore, they showed that MCA1 expressed in Chinese hamster ovary cells was activated by applying stretching force to the cells.

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