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Mechanosensitive Ion Channels in *Chara*: Influence of Water Channel Inhibitors, $HgCl_2$ and $ZnCl_2$, on Generation of Receptor Potential

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Abstract Characean internodal cells generate receptor potential (ΔE_m) in response to mechanical stimuli. Upon a long-lasting stimulus, the cells generated ΔE_m at the moment of both compression and decompression, and the amplitude of ΔE_m at the moment of decompression, $(\Delta E_m)_E$, was larger than that at compression. The long-lasting stimulus caused a membrane deformation (ΔD_m) having two components, a rapid one, $(\Delta D_m)_{rapid}$, at the moment of compression and a slower one, $(\Delta D_m)_{slow}$, during the long-lasting compression. We assumed that $(\Delta D_m)_{slow}$ might have some causal relation with the larger ΔE_m at $(\Delta E_m)_E$. We treated internodal cells with either $HgCl_2$ or $ZnCl_2$, water channel inhibitors, to decrease $(\Delta D_m)_{slow}$. Both inhibitors attenuated $(\Delta D_m)_{slow}$ during compression. Cells treated with $HgCl_2$ generated smaller $(\Delta E_m)_E$ compared to nontreated cells. On the other hand, cells treated with $ZnCl_2$ never attenuated $(\Delta E_m)_E$ but, rather, amplified it. Thus, the amplitude of $(\Delta D_m)_{slow}$ did not always show tight correlation with the amplitude of $(\Delta E_m)_E$. Furthermore, when a constant deformation was applied to an internodal cell in a medium with higher or lower osmotic value, a cell having higher turgor always showed a larger $(\Delta E_m)_E$. Thus, we concluded that changes in tension at the membrane may be the most important factor to induce activation of mechanosensitive Ca^{2+} channel.

Keywords Ca^{2+} · *Chara* · Mechanosensitive ion channel · Membrane deformation · Receptor potential · Stretch-activated channel · Water channel inhibitor

Introduction

Perceptions and responses to various mechanical stimuli, such as gravity, osmotic change, touch and so on, are crucial events for plants to live in fluctuant circumstances. Mechanical stimuli induce various responses such as morphological changes or organelle movements in plants. Plant roots generally recognize gravity, and they grow along the gravitational vector (Morita & Tasaka 2004). In the fern *Adiantum capillus-veneris* protonemal cells, chloroplast movement is induced by mechanical stimulation (Sato, Kadota & Wada, 1999; Sato, Wada & Kadota, 2001). Mechanically induced movement of chloroplasts in the fern was inhibited by Gd^{3+} and La^{3+}, a stretch-activated channel inhibitor and a calcium channel blocker, respectively, suggesting that activation of mechanosensitive Ca^{2+} channel is involved in the initial step of signal transduction in chloroplast movement (Sato et al., 2001). *Dionaea muscipula*, known as a sensitive plant, has specialized bilobed leaves with trigger hairs along the ventral surfaces to trap and

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digest insects as nutrients (Braam, 2005). When an insect crawls on the ventral leaves and brushes the trigger hairs, the hairs generate receptor potential. If the receptor potential is sufficiently large, action potential is induced, is propagated and consequently gives rise to the closure of trap lobes (Sibaoka, 1991; Simons, 1981). Thus, generation of receptor potential is a significant step for recognition of mechanical stimulus, generation of action potential and thus sequential signal transduction. Mechanosensitive channels could also be involved in this response.

Mechanosensitive channels are widely present in all living organisms from prokaryotes to eukaryotes. One of the most analyzed channels may be MscL in bacteria. Under voltage-clamp patches in *Escherichia coli*, a large channel current was observed when the membrane was stretched by applying negative pressure in the suction pipette (Martinac et al., 1987) or by diluting the bathing medium (Martinac et al., 1992). Moreover, MscL was encountered in a survey of cloning and crystallization, and a camera-iris model has been proposed as the opening mechanism on MscL (Sukharev et al., 2001).

Presence of mechanosensitive ion channels has been reported in various plants (Cosgrove & Hedrich, 1991; Ding, Badot & Pickard, 1993; Ding & Pickard 1993a, 1993b; Falke et al., 1988; Nakagawa et al., 2007). Cytoskeletons, especially microtubules, were suggested to have an important role for the activation of the channels upon mechanical stimulus. Engelberth (2003) reported that disruption of cytoskeletal microtubules by colchicine led to total inhibition of thigmic responses in tendrils of *Pisum sativum* and *Bryonia dioica*. On the other hand, in mechanosensitive Ca^{2+} channel (*MCA1*) from *Arabidopsis thaliana*, it was suggested that the major factor in channel activation is membrane stretching (Nakagawa et al., 2007).

Characean plants, which are thought to be an ancient family of land plants, are composed of a much simpler system and have very large internodal cells compared to those of higher plants with multicellular systems. Thus, this plant is one of the best materials for studying various physiological phenomena at the cellular level. We have adopted this as an experimental material to study mechanisms of receptor potential or of activation of mechanosensitive ion channel(s) upon mechanical stimuli (Kaneko et al., 2005; Iwabuchi, Kaneko & Kikuyama, 2005).

Characean cells generate receptor potential (ΔE_m) in response to mechanical stimuli (Kishimoto, 1968; Shimmen, 1996; Shephard, Shimmen & Beilby, 2001; Shephard, Beilby & Shimmen, 2002). Shimmen (1997a, 1997b) reported that the activation of Cl^- channels is involved in generation of ΔE_m. Kaneko et al. (2005) demonstrated that activation of not only Cl^- channels but also Ca^{2+} channels is involved in the generation of ΔE_m. They discussed that the ionic processes of ΔE_m occur in the following order: (1) mechanosensitive activation of Ca^{2+} channels takes place at the plasma membrane upon mechanical stimulus and (2) Cl^- channels are activated by cytoplasmic Ca^{2+}, which is raised by its flow into the cytoplasm from the external medium through the Ca^{2+} channels (Kaneko et al., 2005). By applying a long-lasting stimulus to characean internodal cells, we characterized ΔE_m as follows (Iwabuchi et al., 2005).

1. The cell generated ΔE_m at the moment of both compression and decompression, which was observed in all four characean species tested, *Chara corallina*, *Nitella flexilis*, *Nitella axilliformis* and *Nitellopsis obtusa*.
2. ΔE_m was significantly larger at the moment of decompression than at compression.
3. The longer the duration of stimulus, the larger the magnitude of both ΔE_m at the moment of decompression and membrane deformation (ΔD_m), suggesting a tight correlation between ΔE_m and ΔD_m.
4. Aequorin studies revealed that the increase in $[Ca^{2+}]_c$ ($\Delta[Ca^{2+}]_c$) took place at both moments.
5. The amplitude of $\Delta[Ca^{2+}]_c$ was larger at the moment of decompression than at compression, as was the case for ΔE_m.

From the above results, we discussed that activation of mechanosensitive Ca^{2+} channel occurs depending on the degree of ΔD_m (Iwabuchi et al., 2005) because larger ΔE_m always reflected larger activation of Ca^{2+} channels (Kaneko et al., 2005).

We also reported previously that characean cells generate ΔE_m not only at the start of stimulation but also at the end of it and that the ΔE_m at the end of stimulation, $(\Delta E_m)_E$, was larger than the ΔE_m at the start (Iwabuchi et al., 2005). Since compression of a cell necessarily heightens cell turgor, a long-lasting mechanical stimulus should cause water outflow from the cell through water channels in the plasma membrane. Thus, ΔD_m under constant force would show further increase during compression. According to the results of our previous report (Iwabuchi et al., 2005), the larger ΔD_m at the end of stimulation may be a major factor to induce a larger $(\Delta E_m)_E$. Thus, $(\Delta E_m)_E$ may become smaller if we can suppress the increase in ΔD_m during compression. Since it is expected that treatment of cells with water channel inhibitor should decrease ΔD_m during compression, $(\Delta D_m)_{slow}$, we treated cells with water channel inhibitors to determine whether $(\Delta D_m)_{slow}$ is the most important cause of the generation of ΔE_m or not.

In characean cells, $HgCl_2$ and $ZnCl_2$ inhibit hydraulic conductivity of the plasma membrane maximally by about 95% and 90%, respectively (Tazawa, Asai & Iwasaki, 1996). It is expected that inhibition of water flow by water

channel inhibitors should suppress $(\Delta D_m)_{slow}$, consequently resulting in smaller $(\Delta E_m)_E$. Here, we discuss the generation of ΔE_m or the activation of mechanosensitive Ca^{2+} channel in relation to the deformation of the plasma membrane and conclude that changes of tension at the plasma membrane may be the most important factor in the activation of Ca^{2+} channels.

Materials and Methods

Plant Materials

Internodal cells of *C. corallina* were used as materials. They were cultured in the laboratory under 14 h light/10 h dark a day with two 20-W fluorescent lamps about 30 cm over the water surface. After isolating single internodes from neighboring leaflets and internodes, they 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 Measurement

Membrane potential (E_m) was measured with the "K-anesthesia method" as reported previously (Shimmen, Kikuyama & Tazawa, 1976; Shimmen, 1996; Kaneko et al., 2005; Iwabuchi et al., 2005). Briefly, an internode was placed in a measuring chamber composed of two pools (Fig. 1a). After electrically insulating the pools at the partition wall with Vaseline, both pool A and pool B were filled with APW supplemented with 100 mM KCl and 10 mM $CaCl_2$ (100K-10Ca-APW). The reason we used a medium containing 100 mM KCl as the bathing medium was as follows. Application of mechanical stimulus in the APW often induced an action potential (Shimmen, 1997a), which covers the receptor potential. To inhibit generation of action potential, we used the medium containing 100 mM KCl. The use of 100K-10Ca-APW as the bathing medium depolarized the E_m to very close to 0, and the cell never generated action potential; but mechanical stimulus induced negative ΔE_m because equilibrium potential for Cl^- is around -40 mV in 100 mM KCl (cf. Shimmen, 1997a; Kanko et al., 2005; Iwabuchi et al., 2005). In experiments of Figure 8 (*see below*), however, APW was used as the bathing medium in both pools because the osmotic value of the APW is very close to 0 and was very easily heightened by adding sorbitol.

The potential difference between the two pools, the E_m, was measured with a pair of Hg-Hg_2Cl_2 electrodes through a 10-mM KCl agar-salt bridge. The E_m was recorded with a memory oscilloscope (PowerLab/800; ADInstruments, Nagoya, Japan) and processed with computer software (KaleidaGraph; Hulinks, Tokyo, Japan).

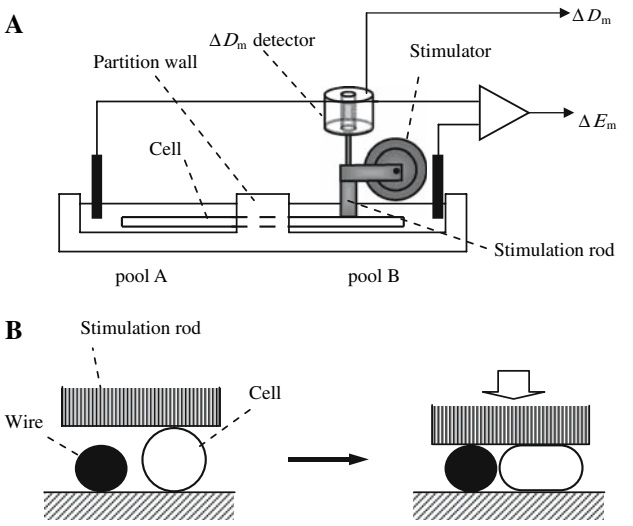

Fig. 1 A schematic representation of the experimental system. **a** A system used for ΔE_m and ΔD_m measurement. Pool A and pool B separated by a partition wall were filled with experimental solution. Mechanical stimulation was applied to the cell portion in pool B by a stimulator, which was a rotary solenoid driven with an electric current. ΔE_m was measured as a change of potential difference between pools A and B through a pair of Hg-Hg_2Cl_2 electrodes, each of which was connected with a 10-mM KCl agar-salt bridge and placed in each pool. A ΔD_m detector was placed on the stimulation rod because displacement of the rod should reflect the membrane deformation, ΔD_m. **b** A scheme for constant ΔD_m experiments. A piece of stainless-steel wire was placed beside the cell. By applying a sufficiently large current to the stimulator, the stimulation rod compresses the cell to a degree of the diameter of the wire

Measurement of ΔD_m

The ΔD_m upon mechanical stimulation was measured with a detector (differential transformer, DTD 3, ST-3; Seiyu Electronics, Kanagawa, Japan) as shown in Figure 1a. A movable ferrite core of the detector was set on a stimulation rod. When a mechanical stimulus was applied to the cell, the ferrite core of the detector lowered its position together with the stimulation rod. The differential transformer generates voltage signals, which are proportional to the positional change of the ferrite core. Thus, the output voltage from the detector relatively reflects the degree of compression, ΔD_m. However, it is stressed that this method is able to measure the degree of vertical deformation against the longitudinal axis of internodal cells but does not detect any information about the shape of the membrane upon mechanical stimulation.

Measurement of Cytoplasmic Free Ca^{2+}

Changes in cytoplasmic free calcium level ($\Delta[Ca^{2+}]_c$) were measured in the same manner as reported previously (Kikuyama & Shimmen, 1997; Kikuyama & Tazawa, 1998; Kaneko et al., 2005; Iwabuchi et al., 2005). Aequorin,

which emits light depending on Ca^{2+} concentration, was used as a $[Ca^{2+}]_c$ indicator. Internodal cells which had been microinjected with aequorin solution (100 mM KCl, 6 mM $MgCl_2$, 0.5 mM ethyleneglycoltetraacetic acid [EGTA], 1 mM piperazine-1,4-bis(2-ethanesulfonic acid) [PIPES], 0.5 mg ml^{-1} *fch*-aequorin) were placed in a dark box, and the aequorin luminescence was measured with a photomultiplier (PM) tube (R1924P; Hamamatsu Photonics, Hamamatsu, Japan). Aequorin luminescence was monitored as PM current, which reflects $[Ca^{2+}]_c$. In the dark box, E_m and aequorin luminescence were measured simultaneously.

Mechanical Stimulation

Mechanical stimulation onto the cell portion in pool B (Fig. 1a) was carried out in the same manner as reported previously (Iwabuchi et al., 2005). The cell part in pool B was around 3 cm in length, and the width of the stimulation rod was 2 mm; thus, about 7% of the cell part was compressed in this study, but we did not measured actual contact area between the cell surface and the stimulation rod. Forces for compressing the cell are linearly dependent on the amplitude of applied voltage.

Constant ΔD_m Experiment

Constant ΔD_m experiments were carried out as follows using stainless-steel wire (Fig. 1b). A piece of stainless-steel wire (0.4–0.5 mm in diameter) was placed beside the cell portion under the stimulation rod. By applying a sufficiently large electric current to the stimulator (a rotary solenoid), the cell was compressed to a degree of the diameter of the wire; thus, the same value of ΔD_m was realized in one and the same cell. Each experiment was performed in the following order. First, a piece of the wire having a smaller diameter than that of the cell was used as a spacer. Then, both pools A and B were filled with APW, and ΔE_m was measured under a sufficiently strong stimulation for 10 s. Next, the solution in both pools was replaced with APW supplemented with 200 mM sorbitol (200sorbitol-APW), and the second recording was carried out in the same manner as the first. Use of thicker wire as a spacer caused smaller ΔD_m and smaller or sometimes non-detectable ΔE_m, especially in 200sorbitol-APW. On the other hand, larger ΔD_m with use of thinner wire caused larger ΔE_m and often an action potential. Thus, we chose appropriate wire in each measurement to cause detectable ΔE_m but not action potential.

Treatment of $HgCl_2$ and $ZnCl_2$

At first, each cell was stimulated for 10 s (except for in Fig. 6 below). Then, the solution (100K-10Ca-APW) in both pools A and B was replaced with 100KCl-APW containing 0.1 mM $HgCl_2$ or that containing 5 mM $ZnCl_2$ and 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES, pH 7.4 with NaOH). After treatment for a few minutes, the solution was replaced again with the initial solution (100K-10Ca-APW), and ΔE_m and ΔD_m were also measured. The control cells were also treated quite similarly as described above, although the 100KCl-APW contained neither $HgCl_2$ nor $ZnCl_2$.

Results

Receptor Potential (ΔE_m) and Membrane Deformation (ΔD_m) in Response to a Long-Lasting Stimulus in Internodal Cells Treated with $HgCl_2$

To verify whether the ΔE_m depends on the degree of ΔD_m or not, cells treated with $HgCl_2$ were simultaneously measured as follows. First, an internodal cell was mechanically stimulated in a bathing medium with 25 g for 10 s. After the first stimulation, the cell was treated with 0.1 mM $HgCl_2$ solution for 5 min (Hg treatment), then $HgCl_2$ solution was replaced with the bathing medium without $HgCl_2$. About 5 min after the replacement, the cell was stimulated again in the same manner, 25 g for 10 s. Figure 2 shows a representative result. After the treatment, $(\Delta E_m)_E$ was significantly suppressed (Fig. 2b) compared with that before the treatment (Fig. 2a). Amplitude of ΔD_m at the moment of compression, $(\Delta D_m)_{rapid}$, was not influenced by the Hg treatment at all (Fig. 2a, b). On the other hand, $(\Delta D_m)_{slow}$, which is the ΔD_m during stimulation, was significantly suppressed in its amplitude by Hg treatment (Fig. 2b). This indicates that $(\Delta D_m)_{slow}$ reflects water loss from the cell because a significant decrease of $(\Delta D_m)_{slow}$ was caused by a water-channel inhibitor, $HgCl_2$. In order to evaluate the effect of $HgCl_2$ on $(\Delta E_m)_E$ etc., we took each ratio of $(\Delta E_m)_E$, $(\Delta D_m)_{rapid}$ and $(\Delta D_m)_{slow}$ before and after Hg treatment. Mechanical stimulus and Hg treatment were applied in the same manner as in Figure 2 except for the duration of Hg treatment (1–20 min). In the control experiment, both pools of a measuring chamber were replaced with the bathing medium which had no inhibitor, kept for 5 min and stimulated again in the same manner as the first. By dividing the values of $(\Delta E_m)_E$, $(\Delta D_m)_{rapid}$ and $(\Delta D_m)_{slow}$ after Hg treatment by those before treatment, relative values of these parameters were obtained. They are plotted against the duration of Hg treatment in Figure 3. It is clear that $(\Delta E_m)_E$ and $(\Delta D_m)_{slow}$ become smaller with longer duration of treatment (Fig. 3a, b; $n = 5$). By contrast, $(\Delta D_m)_{rapid}$ was almost independent of Hg treatment (Fig. 3a).

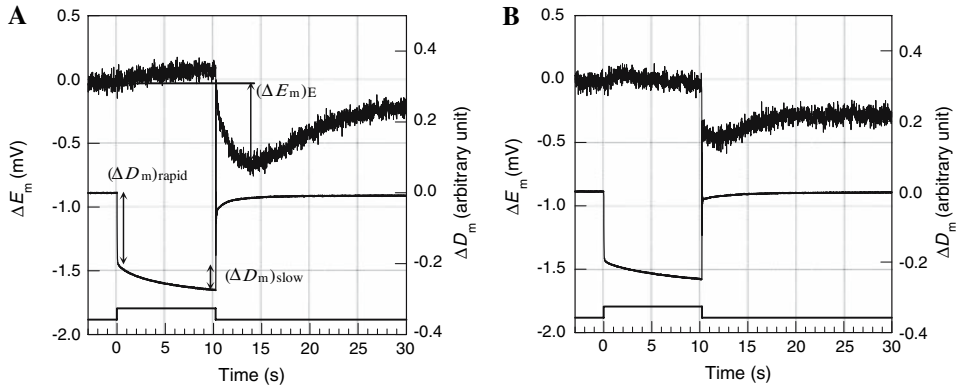

Fig. 2 Receptor potential (ΔE_m) and membrane deformation (ΔD_m) in response to a stimulus for 10 s before and after $HgCl_2$ treatment. A stimulus of 25 g was applied to the cell portion of pool B in 100K-10Ca-APW. Both cell portions in pools A and B were then treated with $HgCl_2$ solution for 5 min, and the cell was stimulated again with another stimulus of the same duration and amplitude. **a** ΔE_m and ΔD_m before $HgCl_2$ treatment. *Upper, middle* and *lower traces* indicate ΔE_m, ΔD_m and stimulation marker, respectively. **b** ΔE_m and ΔD_m after $HgCl_2$ treatment. Each trace has the same meaning as in **a**

Effect of Another Water Channel Inhibitor, $ZnCl_2$, on ΔE_m and ΔD_m

The fact that $HgCl_2$ suppressed not only $(\Delta D_m)_{slow}$ but also $(\Delta E_m)_E$ (Fig. 3) may coincide well with our assumption that ΔD_m causes ΔE_m. However, this result may not come from the Hg^{2+} effect on the water channel alone but from its side effect(s), as shown in "Discussion." Thus, we examined another inhibitor, $ZnCl_2$.

Figure 4 represents a typical example of ΔE_m and ΔD_m before and after treatment with 5 mM $ZnCl_2$ for 20 min at pH 7.4 (see "Materials and Methods"). Amplitude of the stimulus was kept at 25 g for 10 s. As in the Hg treatment (Fig. 2), Zn treatment did not affect $(\Delta D_m)_{rapid}$ but significantly suppressed $(\Delta D_m)_{slow}$ (Fig. 4b). Unexpectedly, Zn treatment never decreased the amplitude of $(\Delta E_m)_E$ compared to that of nontreated cells. Moreover, the time to reach the peak of $(\Delta E_m)_E$ was not significantly different from that in nontreated cells (3.0 ± 0.3 s in nontreated cells, 2.3 ± 0.3 s in treated cells, n = 20). On the other hand, the time for the recovery of $(\Delta E_m)_E$ toward a resting potential, which was taken as $t_{1/2}$ (time from the peak of $[\Delta E_m]_E$ to the half-amplitude of $[\Delta E_m]_E$), was significantly shorter than that of nontreated cells (18.7 ± 3.0 s in nontreated cells, 4.1 ± 0.7 s in treated cells, n = 20). The relationship between the duration of Zn treatment and the ratio of $(\Delta E_m)_E$ and ΔD_m before and after treatment is shown in Figure 5, which clearly demonstrates that $(\Delta D_m)_{slow}$ was highly dependent on Zn treatment; the longer treatment significantly decreased $(\Delta D_m)_{slow}$.

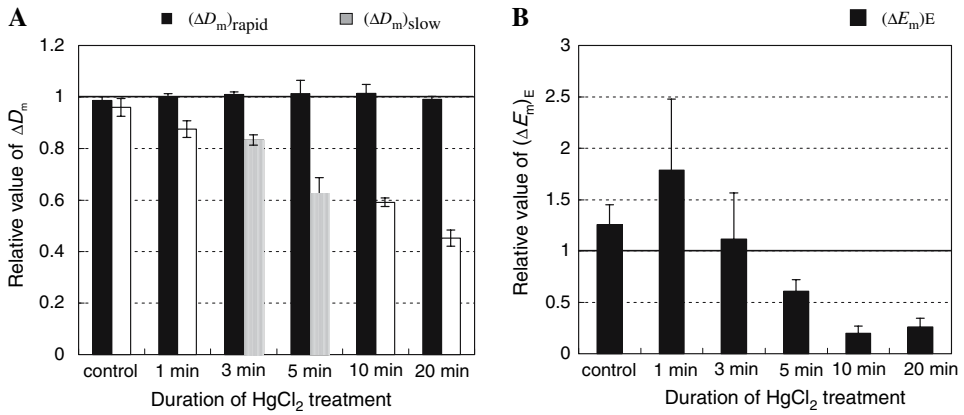

Fig. 3 ΔE_m and ΔD_m in cells before and after $HgCl_2$ treatment of various durations. A stimulus for 10 s was applied to each internodal cell before and after $HgCl_2$ treatment. Each cell was given the stimulus, then treated with $HgCl_2$ for various durations with APW containing 100 mM KCl. At the end of treatment, the $HgCl_2$ solution was exchanged again with the first bathing medium. Five minutes after the exchange, the cell was given the second stimulus. For the control experiment, the bathing medium was exchanged two times: one just after the first stimulus and the second 5 min after the first exchange. Five minutes after the second exchange, control cells were given the second stimulus. The ordinate in **a** and **b** shows each ratio of ΔD_m and ΔE_m in treated cells to those in pretreated cells, respectively. Each bar shows the mean ± standard error (n = 5)

However, the instantaneous deformation, $(\Delta D_m)_{rapid}$, was almost independent of Zn treatment. These results were very similar to those with Hg treatment (Fig. 3a). In contrast to the results of Hg treatment (Fig. 3b), however, $(\Delta E_m)_E$ in Zn-treated cells was never smaller but, rather, larger than that of control cells (Fig. 5b). Namely, the relative values obtained on $(\Delta E_m)_E$ were 1.2 ± 0.08 (n = 25) for control and 1.53 ± 0.16 (n = 20), 1.31 ± 0.11 (n = 20) and 1.47 ± 0.13 (n = 20) for 5, 10 and 20 min of Zn treatment, respectively. We assume the above fact, that the effects of these two inhibitors were quite different from each other, may be attributed to some side effect(s) of $HgCl_2$ other than water channels, as discussed later. Thus, we will hereafter focus on the results of Zn treatment.

Relationship between $(\Delta E_m)_E$ and the Duration of the Stimulus in Cells Treated with $ZnCl_2$

We previously reported that the amplitude of receptor potential is larger as the duration of mechanical stimulation is longer (Iwabuchi et al., 2005). To study this in Zn-treated cells, mechanical stimuli with various durations (0.1, 1, 3, 5 and 10 s) but fixed magnitude (25 g) were applied to each internodal cell which had been treated with $ZnCl_2$ for 10 min. As shown in Figure 6, the longer duration of stimulation caused a larger amplitude of $(\Delta E_m)_E$ not only in nontreated cells but also in Zn-treated cells. However, $(\Delta E_m)_E$ of Zn-treated cells was always larger than that of nontreated cells irrespective of the duration of stimulus (Fig. 6). This may indicate that ΔD_m, especially its slow component $(\Delta D_m)_{slow}$, does not have tight correlation with $(\Delta E_m)_E$, as discussed later.

Change of Cytoplasmic Ca^{2+} Level in Cells Treated with $ZnCl_2$

In order to confirm whether Ca^{2+} channel activation also occurs in Zn-treated cells or not, ΔE_m and the change of light emission of aequorin ($\Delta[Ca^{2+}]_c$) microinjected into the cytoplasm were measured simultaneously. As shown in Figure 7, ΔE_m was generated at the moment of both compression and decompression, and $\Delta[Ca^{2+}]_c$ was also observed at both moments. All six cells treated with Zn showed similar results, which were the same as those in nontreated cells as previously reported (Iwabuchi et al., 2005).

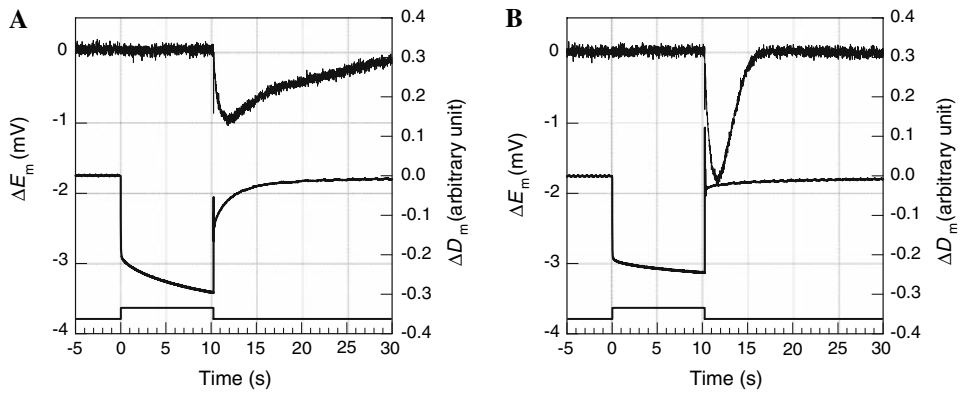

Fig. 4 An example of simultaneous measurement of ΔE_m and ΔD_m in Zn-treated cells. A stimulus (10 s, 25 g) was applied to an internodal cell. **a** ΔE_m and ΔD_m before $ZnCl_2$ treatment. **b** ΔE_m and ΔD_m after treatment for 20 min. *Upper, middle* and *lower traces* are ΔE_m, ΔD_m and mechanical stimulation, respectively

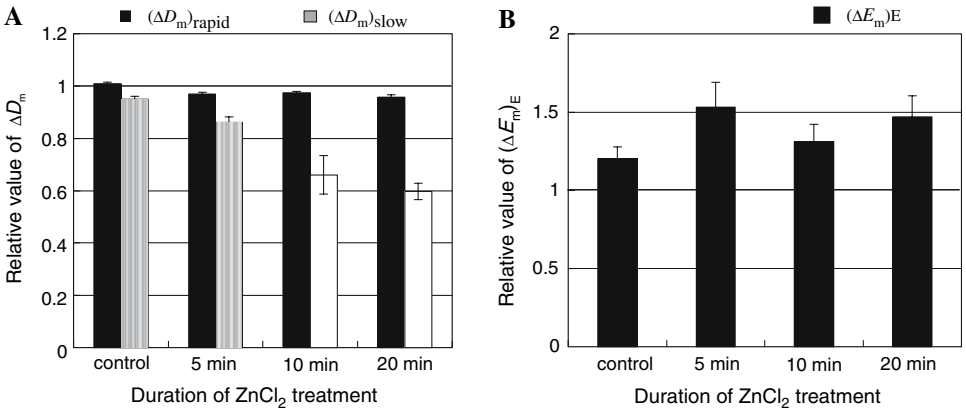

Fig. 5 ΔE_m and ΔD_m before and after $ZnCl_2$ treatment for various durations. The ordinate in **a** and **b** is the same as shown in Figure 3. Each bar shows the mean ± standard error (n = 25 control cells, n = 20 treated cells)

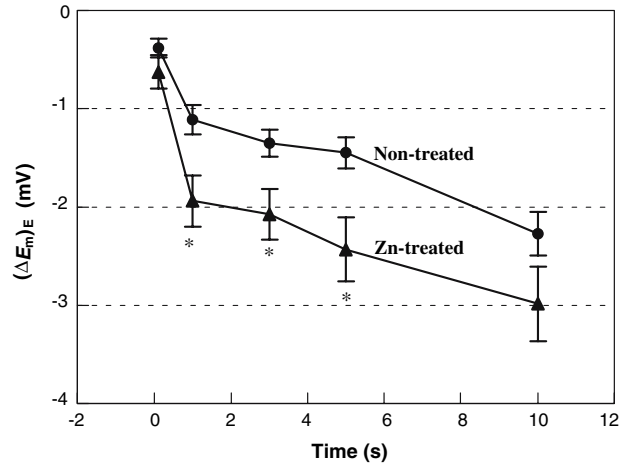

Fig. 6 Relationship between $(\Delta E_m)_E$ and duration of stimulation in Zn-treated cells. Various stimuli for 0.1, 1, 3, 5 and 10 s (25 g) were applied to each internodal cell. Zn-treated cells were treated with $ZnCl_2$ for 10 min before recording and then stimulated in 100K-10Ca-APW. *Circles* and *triangles* correspond to nontreated cells and treated cells, respectively. Each plot shows the mean ± standard error (n = 30). *Values were significantly different from each other ($P < 0.05$ with t-test)

Constant ΔD_m Experiment in Cells of Low and High Turgor

Since the present study may suggest that $(\Delta D_m)_{slow}$ itself is not so important on $(\Delta E_m)_E$ as shown above, we performed another experiment in which ΔD_m was almost kept constant while tension at the membrane was widely varied by changing the cell turgor (Fig. 1b). Figure 8a shows a typical example. It should be noted that the ΔE_m in this experiment is a change in the positive direction because $[Cl^-]$ in the APW is low (0.4 mM) (Shimmen, 1997a; Kaneko et al., 2005). The peak value of $(\Delta E_m)_E$ (solid line) measured in APW was highly attenuated in 200sorbitol-APW (broken line). Furthermore, $(\Delta E_m)_E$ once attenuated in 200sorbitol-APW recovered the initial amplitude (dotted line), sometimes followed by an action potential, when the bathing medium was again exchanged from 200sorbitol-APW to APW. Measurements on other cells also showed the same tendency, although the third measurement in APW was not always carried out. The summarized result clearly shows that $(\Delta E_m)_E$ in 200sorbitol-APW (1.3 ± 0.4 mV, n = 8) was significantly smaller than that in APW (9.9 ± 2.0 mV, n = 8) (Fig. 8b). These results may indicate that tension at the membrane or membrane stretch is more responsible for the generation of ΔE_m.

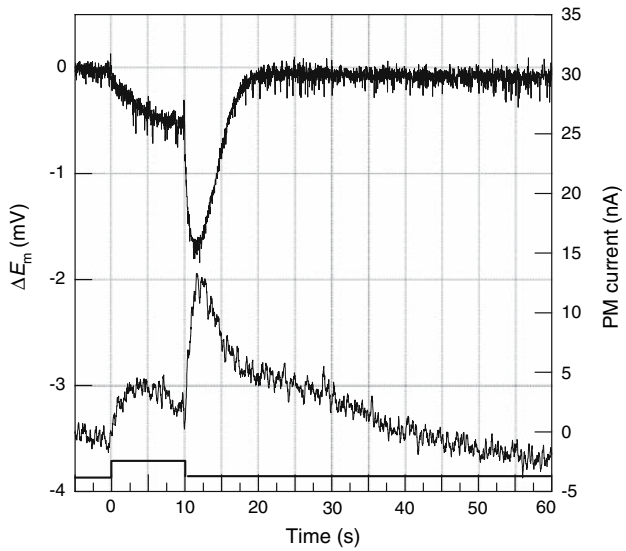

Fig. 7 An example of simultaneous recordings of ΔE_m and $\Delta[Ca^{2+}]_c$. A cell microinjected with aequorin was treated with $ZnCl_2$ for 20 min and stimulated for 10 s (25 g). *Upper, middle* and *lower traces* indicate ΔE_m, PM current ($\Delta[Ca^{2+}]_c$) and mechanical stimulation, respectively

Discussion

Shimmen (1997a, 1997b) reported that activation of Cl^- channels is involved in the generation of ΔE_m. Kaneko et al. (2005) showed that activation of Ca^{2+} channel is also involved in the generation of ΔE_m and that there is a high correlation between the amplitude of ΔE_m and the degree of activation of mechanosensitive Ca^{2+} channel. Iwabuchi et al. (2005) discussed that mechanosensitive Ca^{2+} channel is activated depending on the degree of ΔD_m.

In 100K-10Ca-APW, ΔE_m at the moment of decompression, $(\Delta E_m)_E$, was generated in the negative direction, as previously reported (Iwabuchi et al., 2005). Since cytoplasmic $[Ca^{2+}]$ and $[Cl^-]$ are around 10^{-7} M (Williamson & Ashley 1982) and 20 mM (Tazawa, Kishimoto & Kikuyama, 1974), respectively, the equilibrium potential for Ca^{2+} and Cl^- across the plasma membrane is about +150 and −45 mV in 100K-10Ca-APW, respectively. Thus, results in the present study are similar to those of previous studies which suggested that $(\Delta E_m)_E$ is composed of the activation of Ca^{2+} channels, followed by the activation of Cl^- channels (Kaneko et al., 2005; Iwabuchi et al., 2005).

Although many *Chara* cells used in the present study showed very small ΔE_m at the moment of compression, as shown in Figures 2 and 4, for an unknown reason(s), Iwabuchi et al. (2005) demonstrated that many *Chara* cells showed ΔE_m at both compression and decompression; and it was apparent that $(\Delta E_m)_E$ was significantly larger than ΔE_m at compression. Furthermore, this was also the case in other characean cells, such as *Nitella* and *Nitellopsis* (Iwabuchi et al., 2005). It may be possible to explain the larger $(\Delta E_m)_E$ as follows, if the observed amplitude of ΔE_m is dependent on the stimulated area. Just before the

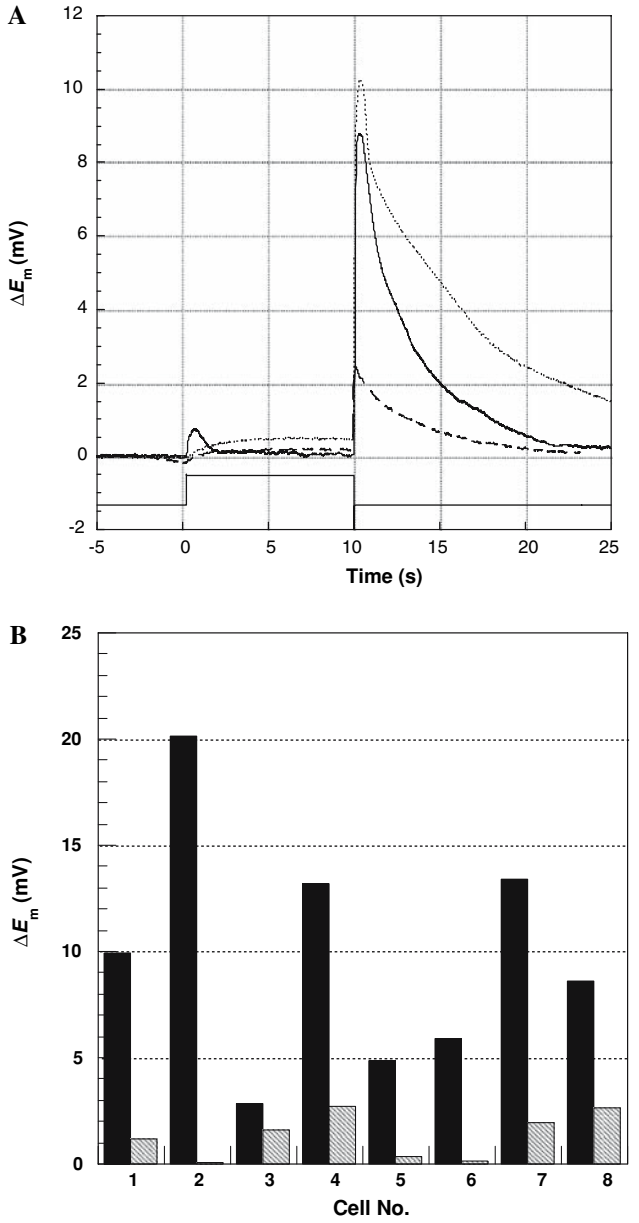

Fig. 8 Effect of cell turgor on ΔE_m. ΔE_m was measured under constant ΔD_m with mechanical stimulation for 10 s. **a** An example of ΔE_m recording. First, pools A and B of the measuring chamber (*see* Fig. 1) were filled with APW, and ΔE_m was measured (*solid line*). The second ΔE_m measurement was performed after replacing APW in both pools with 200sorbitol-APW (*broken line*). The third measurement was done after replacing the solution in both pools with APW again (*dotted line*). **b** Comparison of $(\Delta E_m)_E$ in APW and 200sorbitol-APW. Each $(\Delta E_m)_E$ measured in APW (first measurement) and in 200sorbitol-APW (second measurement) is shown as a *filled bar* and *hatched bar*, respectively

compression, the cell and the stimulation rod contacted at a very small area. Just before the decompression, however, the contact area became larger than that before stimulation because the cell deformation reached $(\Delta D_m)_{rapid}$ + $(\Delta D_m)_{slow}$ at this moment. According to the above assumption that the amplitude of ΔE_m is dependent on the stimulated area, this should cause a larger $(\Delta E_m)_E$. However, this may not be the case because Zn-treated cells showed smaller ΔD_m but larger $(\Delta E_m)_E$ than nontreated ones (Figs. 4 and 5). Another explanation of the larger $(\Delta E_m)_E$ may be water efflux during compression. If water flow across the membrane increases membrane "sensitivity" to mechanical stimulus, $(\Delta E_m)_E$ would become larger because compression necessarily causes water flow out of the cell. According to this assumption, larger $(\Delta D_m)_{slow}$ would have tight correlation with larger $(\Delta E_m)_E$. The present study, however, showed the opposite result in Zn-treated cells: treatment of cells with $ZnCl_2$ made $(\Delta D_m)_{slow}$ smaller and $(\Delta E_m)_E$ rather larger (Figs. 4 and 5).

The fact that $(\Delta E_m)_E$ was significantly larger than ΔE_m at the moment of compression may suggest that some "factor" to activate mechanosensitive Ca^{2+} channel appears or increases during the long-lasting stimulus. The factor may not be an increase in contact area of stimulation rod with the cell or water flow across the membrane, as discussed above. Other effects accompanying ΔD_m may be a change in membrane tension, probably resulting from stretching and/or compression of membrane, or another unknown effect(s). For convenience, possible effects accompanying ΔD_m will be divided in two groups, a change in membrane tension and all other effects; the latter will be simply shown as "deformation" hereafter.

In internodal cells treated with $HgCl_2$, the amplitude of $(\Delta D_m)_{slow}$ was significantly suppressed, as was expected. At the same time, $(\Delta E_m)_E$ also decreased in amplitude (Fig. 2). This may support our hypothesis that ΔD_m is required for the generation of ΔE_m. In spite of this, we do not deny some possible side effects of Hg^{2+} on ΔE_m, e.g., on mechanosensitive Ca^{2+} channels and/or Cl^- channels involved in the generation of ΔE_m, because of the following. It is well known that $HgCl_2$ is not only a water channel inhibitor but also a modulator of the SH group of proteins. Thus, $HgCl_2$ should strongly affect membrane phenomena if the functional part of the membrane protein contains an SH group, as suggested by Lucas & Alexander (1980). Actually, it has been shown that $HgCl_2$ rapidly depolarizes the membrane potential of *C. corallina* cells (Tazawa et al., 1996; Schütz & Tyerman, 1997). The reagent also caused depolarization and an increase of $[Ca^{2+}]_c$ (Liu & Lin-Shiau, 2002) and is known to induce Ca^{2+} release from sarcoplasmic reticulum vesicles isolated from a skeletal muscle (Abramson et al., 1983; Brunder, Dettbarn & Palade, 1988). In the present study, possible side effects of $HgCl_2$ were also observed in many cells when the duration of $HgCl_2$ treatment was more than 5 min, including inhibition of cytoplasmic streaming and instability of the resting potential (*data not shown*). The inhibition of cytoplasmic streaming by $HgCl_2$ may also be

caused by a modulation of the SH group(s) in the motile system of streaming, as indicated by Chen & Kamiya (1975), who inhibited the streaming of *Nitella* by treatment with an SH inhibitor, *N*-ethylmaleimide. Thus, we determined that results from Hg-treated cells are not plausible, though $HgCl_2$ is a well-known inhibitor of water channels. On the other hand, the effect of $ZnCl_2$ may be limited only to the water channels because this did not show any harmful influence on the resting potential or on the cytoplasmic streaming as far as we examined.

In Figure 5, each cell was mechanically stimulated two times: the first stimulus was before Zn treatment and the second one was after Zn treatment of various durations. The amplitude of $(\Delta D_m)_{rapid}$ at the second stimulus was almost equal to that at the first stimulus; namely, the relative values described as $(\Delta D_m)_{rapid}$ at the second stimulus vs. that at the first stimulus were very close to unity (Fig. 5a). On the other hand, those of $(\Delta D_m)_{slow}$ after Zn treatment were significantly smaller than unity, especially when the duration of treatment was longer (Fig. 5a). By contrast, values of $(\Delta E_m)_E$ at the second stimulus were larger than those at the first stimulus; namely, the relative values obtained for $(\Delta E_m)_E$ were never smaller than unity (Fig. 5b). Upon mechanical stimulation, it is assumed that ΔE_m could be generated by two possible factors, membrane "deformation" and changes in membrane tension accompanying ΔD_m, as shown above. In the present study of Zn treatment, ΔE_m was never suppressed in spite of significant decrease of $(\Delta D_m)_{slow}$ (Figs. 4 and 5). This indicates that membrane "deformation" is not essential for the generation of ΔE_m; mechanosensitive Ca^{2+} channel may be activated not by "deformation" but by changes in membrane tension or membrane stretching.

In Zn-treated cells, $(\Delta E_m)_E$ recovered the initial resting level quickly in comparison with nontreated cells (Fig. 4). If the receptor potential of *Chara* has two components, namely ΔE_m at the plasma membrane and that at the tonoplast as is the case in action potential (Findlay & Hope, 1964; Findlay, 1970; Kikuyama & Tazawa, 1976; Kikuyama, 1986, 2001; Shimmen & Nishikawa, 1988), $(\Delta E_m)_E$ of Zn-treated cells might reflect ΔE_m only at the plasma membrane because $ZnCl_2$ blocks activation of Cl^- channels at the tonoplast (Berecki et al., 1999). In spite of this, disappearance of ΔE_m at the tonoplast would never cause rapid recovery of $(\Delta E_m)_E$ but a slower one, as follows. Since equilibrium potential for Cl^- across the tonoplast can be estimated around +52 m*V* (vacuole positive against cytoplasm) according to $[Cl^-]_c$ (ca. 20 mM) and $[Cl^-]_{vac}$ (ca. 160 mM) (Tazawa et al., 1974), inhibition of the tonoplast component in Zn-treated cells would inhibit the positive component of $(\Delta E_m)_E$, resulting in its slower recovery. This goes against the present results (Fig. 4). Thus, the reason for the swift recovery of ΔE_m in Zn-treated cells is unclear at this point.

The amplitude of $(\Delta E_m)_E$ in cells treated with $ZnCl_2$ was larger than that in nontreated cells, irrespective of the duration of mechanical stimulus (Fig. 6). This may be explained as follows. In nontreated cells, when a mechanical stimulus was applied to the cell, the cell turgor should instantly increase concomitantly with ΔD_m. The heightened turgor should increase water potential inside the cell and give rise to outflow of water from the cell, resulting in a gradual decrease of cell turgor. Thus, heightened force against the compression force applied by the stimulator would gradually decrease toward the original turgor level. This could be the reason a gradual increase in $(\Delta D_m)_{slow}$ takes place during long-lasting stimulation, as shown in Figures 2a and 4a. On the other hand, $HgCl_2$ and $ZnCl_2$ treatments would inhibit the gradual increase of $(\Delta D_m)_{slow}$ during the stimulus by inhibition of water channels, and this was experimentally confirmed (Figs. 2, 3a, 4 and 5a). Therefore, heightened turgor by cell compression may be retained for longer periods in Zn-treated cells than in nontreated cells. Since a higher turgor has the same meaning as a higher tension of the membrane according to a physical rule related to surface tension, the fact that the $(\Delta E_m)_E$ of Zn-treated cells was larger than that of nontreated cells (Figs. 4, 5b and 6) strongly supports our new hypothesis that change in membrane tension at the plasma membrane has a more important role in the generation of ΔE_m than in membrane "deformation." This assumption could also be partly supported by Hg-treatment experiments (Fig. 3b), in which treatment for a short period (1 min) showed larger $(\Delta E_m)_E$ though the deviation was large. It may be possible that Hg treatment for 1 min is sufficient to inhibit water channels but insufficient to cause any side effect(s).

Shephard et al. (2001) reported that ΔE_m was larger when cell turgor was lowered by immersing internodal cells in APW containing 50–200 mM sorbitol. They discussed that a given stimulus to a low-turgor cell could cause a larger deformation of some structure, e.g., cell wall–plasma membrane–cytoskeleton complex, if the tension of the plasma membrane decreased along with a decrease of cell turgor. Thus, it may result in more activation of mechanosensory channels. In the present study, however, we applied a constant ΔD_m to the same cell in APW and in 200sorbitol-APW (Fig. 8). Since complete loss of turgor only decreased the cell volume less than 4% in *Nitella flexilis* (Kamiya, Tazawa & Takata, 1963) and did not affect E_m or action potential in *Nitella pulchella* (Tazawa, Kikuyama & Nakagawa, 1975), we assumed that effects other than a change in turgor were not brought about by exchanging bathing medium between APW and 200sorbitol-APW. Figure 8 clearly demonstrates that $(\Delta E_m)_E$ of low-turgor cells was significantly smaller than that of high-turgor cells. Under a constant ΔD_m condition,

the tension at the membrane must be larger in high-turgor cells than in low-turgor cells. Thus, the data of Figure 8 also strongly support our new hypothesis that membrane tension is a more important factor for generating ΔE_m than membrane "deformation."

We discussed in the previous paper that ΔE_m depends on ΔD_m (Iwabuchi et al., 2005). In the present study, however, it was suggested that generation of ΔE_m, the activation of mechanosensitive Ca^{2+} channels, was not directly dependent on ΔD_m, especially on $(\Delta D_m)_{slow}$, in *Chara*. Qi et al. (2004) identified a novel mechanosensitive anion channel in the protoplast of *Arabidopsis thaliana* mesophyll cells using the patch-clamp technique. The channel in the outside–out patches could be activated by positive pressure in the pipette, while negative pressure had no effect. On the other hand, Cosgrove & Hedrich (1991) reported a stretch-activated anion channel in plasma membranes of *Vicia faba* guard cells. The channel could be activated by suction in the pipette (negative pressure in the pipette) with outside–out patches but not by positive pressure. Dutta & Robinson (2004) showed in pollen protoplasts of lily that the channel could be activated by both positive and negative pressure in the patch pipette. Thus, the direction of membrane deformation may be essential for the activation of mechanosensitive channels, and the activation mechanism of mechanosensitive channels would be diverse among plants and/or tissues for their appropriate functions. If the putative mechanosensitive Ca^{2+} channel of *Chara* is also stretch-activated as shown by patch-clamp studies in higher plants, the $\Delta[Ca^{2+}]_c$ should take place during compression. The present study demonstrates, however, that Ca^{2+} channel activation took place only at the moment of compression and decompression but not during compression (Fig. 7). This may suggest that, in *Chara*, activation of Ca^{2+} channels is dependent on a change in membrane tension but not on the tension itself.

The fact that $(\Delta E_m)_E$ was larger than ΔE_m at the start of stimulation (Figs. 2 and 4) (Iwabuchi et al., 2005) is not fully explained; the larger $(\Delta E_m)_E$ in nontreated cells may be explained by a larger tension at the membrane as a result of larger ΔD_m at the end of stimulation than at the start of stimulation. In Zn-treated cells, however, we have no experimental or theoretical bases showing that the tension at the membrane is truly larger at the end of stimulation than at the start of it, and we cannot explain why $(\Delta E_m)_E$ was larger than ΔE_m at the start of stimulation. The decisive factor that activates mechanosensitive Ca^{2+} channels remains to be resolved.

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