Running title: Mechano-Perception in Characeae

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Title: Ionic Mechanism of Mechano-Perception in Characeae

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Abbreviations: APW, artificial pond water; [Ca²⁺], cytoplasmic free calcium concentration; $E_m$, membrane potential; $\Delta E_m$, receptor potential; $(\Delta E_m)_s$, $\Delta E_m$ at the start of stimulation; $(\Delta E_m)_e$, $\Delta E_m$ at the end of stimulation; $(\Delta E_m)_{0.1}$, $\Delta E_m$ on the stimulation for 0.1 s; $(\Delta E_m)_L$, $(\Delta E_m)_E$ on the stimulation at L mm apart from the node; 55KCl-APW, APW containing 55 mM KCl; 100KCl+10CaCl₂-APW, APW containing 100 mM KCl and 10 mM CaCl₂; 100sorbitol-APW, APW containing 100 mM sorbitol; PM, photomultiplier
Abstract: Characean internodal cells generate receptor potential in response to mechanical stimuli. We studied responses of internodal cells upon long-lasting stimulus and the results were as follows. (1) The cell generated receptor potential at both moments of compression and decompression. (2) The receptor potential ($\Delta E_m$) was significantly larger at the moment of decompression than at the compression. (3) The longer the duration of stimulus, the larger the magnitude of $\Delta E_m$ at the moment of decompression was. (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 the compression, as well as the case in $\Delta E_m$.

It was suggested that the amplitude of the receptor potential had tight correlation with degree of membrane deformation. We discussed the ionic mechanism of mechano-perception under long-lasting stimulus in relation to mechanosensitive activation of Ca$^{2+}$ channels at the plasma membrane.

Keywords: Ca$^{2+}$, Characeae, mechanosensitive ion channel, membrane deformation, membrane potential, receptor potential
Introduction

All plants perceive and adequately respond to various mechanical stimuli. For example, plant roots generally recognize the gravity, and they grow along the gravitational vector (Morita and Tasaka 2004). Ethylene, one of plant growth hormones, regulates elongation of cells in response to physical stresses because its biosynthesis is enhanced by the physical stresses (Goeschl et al. 1966). Thus, plants that often meet with strong wind or animal trails will become shortened and expanded. Mechano-perception should be located on the first step of the above reactions. In the case of rapid bending of the pulvinus in *Mimosa pudica*, of trap lobes in *Aldrovanda vescicuosa* and *Dionaea muscipula*, their mechanically induced motions are triggered by action potential. Electrophysiological studies on *Dionaea* and *Aldrovanda* revealed that mechanical stimulus applied to the sensory hair induces receptor potential there. If the receptor potential is sufficiently large, action potential is induced at the sensory hair in *Dionaea* and at the leaf epidermal cells in *Aldrovanda*, which propagates the whole leaf and triggers a rapid closure of the trap lobes (Sibaoka 1991). Thus, we assumed that generation of receptor potential is the first step of mechano-perception in plants and focused our interests on the generation of receptor potential.

Higher plants are not suitable materials to perform cellular level studies of mechano-perception because they are composed of multicellular system and the cells are generally very small. In this study, we used characean internodal cells as materials for studying mechano-perception because they are composed of much simpler system and have very large size.

Kishimoto (1968) reported that characean cells generate receptor potential in response to mechanical stimuli, which were realized by striking them. The characteristics of receptor potential were as follows; the magnitude of receptor potential ($\Delta E_m$) was dependent on the intensity of stimulus and became larger upon repeated stimuli. Shimmen (1996, 1997a, 1997b) reported that activation of Cl$^-$ channels is involved in generation of receptor potential. Kaneko et al. (2005) demonstrated that not only activation of Cl$^-$ channels but also that of Ca$^{2+}$ channels are involved in generation of the receptor potential. They discussed the ionic processes of receptor potential as the following order; (1) mechanosensitive activation of Ca$^{2+}$ channels takes place at the plasma membrane upon mechanical stimulus, (2) Cl$^-$ channels are activated by cytoplasmic Ca$^{2+}$ which is raised by its flow into the cytoplasm from external medium through the Ca$^{2+}$ channels (Kaneko et al. 2005). According to Sibaoka (1991), action potential in *Dionaea* and *Aldrovanda* may be a Ca$^{2+}$ spike, though that in *Mimosa* may be a Cl$^-$ spike.

It has not been made clear how mechanical stimulus activates Ca$^{2+}$ channels. It may be possible that, for example, membrane deformation or change in turgor pressure of the cell during mechanical stimulus activates the Ca$^{2+}$ channels. We studied how the mechanical stimulus is
perceived and activates the Ca\(^{2+}\) channels in characean cells.

Shimmen (1996, 1997a, 1997b) studied the mechanism of receptor potential in characean cells upon mechanical stimuli, which were realized by dropping a glass tubing onto the cell. In this method, the momentary velocity or kinetic energy when the glass tubing hits the cell can be exactly estimated but the duration of stimulus should be limited only for a moment. Kaneko et al. (2005) adopted another stimulation method in which the cell was compressed using a unique apparatus made by modifying a rotary solenoid driven electrically. Although they compressed the cell only for 0.1 s (Kaneko et al. 2005), the apparatus is able to stimulate by other ways, for example by compressing the cell for a longer time or by hitting the cell for many times, etc. The major subject of the present study is to obtain, by compressing the cell for a long time, some information whether the receptor potential is generated at the start of stimulation (= at the moment of compression) or at the end of stimulation (= at the moment of decompression) or at the both.

We demonstrate that both receptor potential and transient increase in cytoplasmic Ca\(^{2+}\) concentration (\(\Delta[Ca^{2+}]\)) take place not only at the moment of compression but also at the moment of decompression, and that the responses at the moment of decompression were significantly larger than those at the compression.

**Results**

*Receptor potential in response to a long-lasting stimulus*

In the present study, we compressed the cell for T seconds and, for convenience, the stimulus will be shown as Ts-stimulus. As already shown in the previous papers (Shimmen 1996, 1997a, 1997b, 1997c, Kaneko et al. 2005), the cell generated a receptor potential (\(\Delta E_m\)) upon 0.1s-stimulus (\((\Delta E_m)_{0.1}\)) (Fig. 1A). By contrast, when the 10s-stimulus of the same amplitude (30 g) was applied, the cell generated \(\Delta E_m\) not only at the moment of compression but also at the moment of decompression (Fig. 1B). The \(\Delta E_m\) at the moment of decompression (\((\Delta E_m)_{D}\)) was significantly larger than that at the moment of compression (\((\Delta E_m)_{S}\)). This was the case in all *C. corallina* cells tested (\(n > 30\)), though the amplitude of the stimulus to cause the response was widely different from experiment to experiment.

\(\Delta E_m\) in response to 10s-stimuli in other characean plants

To study whether the above response to the 10s-stimuli in *C. corallina* is a general phenomenon in *Characeae* or not, the same study was also carried out in *Nitella flexilis*, *N. axilliformis* and *Nitellopsis obtusa*. Fig. 2 shows typical examples of the \(\Delta E_m\) in response to the 10s-stimulus in
each species. Clearly, the fact that \((\Delta E_m)_E\) is significantly larger than \((\Delta E_m)_S\) is generally observed in all four characean species tested.

**Relationship between stimulated portion of the cell and the amplitude of \(\Delta E_m\)**

In order to examine whether or not the amplitude of \(\Delta E_m\) is dependent on the stimulated portion in the cell, the following experiments were performed in *C. corallina*. Intact internodal cells with nodal complexes at both ends were set in the measuring chamber and mechanically stimulated at the cell portion \(L\) mm apart from the nodal complex in pool B (\(L = 1, 2, 3, 5\) and 10). The symbol \((\Delta E_m)_L\) is the \((\Delta E_m)_E\) upon the stimulus applied at the portion \(L\) mm apart from the node. In order to outline the relation between \(L\) and \((\Delta E_m)_L\), we plotted \(\log(\Delta E_m)_L/(\Delta E_m)_0.1\) against \(L\) in Fig. 3A because not only each \((\Delta E_m)_L\) but also the ratio \((\Delta E_m)_L/(\Delta E_m)_0.1\) scattered about two orders from cell to cell. In spite of large scattering of each value, it is clear that the value of \(\log(\Delta E_m)_L/(\Delta E_m)_0.1\) was larger at the portion closer to the nodal complex (\(n = 10\)). This result may suggest that the stimulus close to the nodal complex induces \(\Delta E_m\) not only at the internodal cell but also at nodal cells through cellular deformation there. To confirm this, similar experiments were carried out on internodal cells from which the nodal complexes had been removed by ligation followed by cutting off them, and the results are shown in Fig. 3B. In this case, \((\Delta E_m)_L\) was nearly independent of the stimulated portion. In spite of this, it should be noted that the \(\Delta E_m\) was significantly larger when the stimulus was applied just onto the cell end (\(L = 0\) mm, \(n = 11\)). Simultaneous measurements of \(\Delta E_m\) and membrane deformation, \(\Delta D_m\), in internodal cells without nodal complex revealed that stimulation to the cell end induced \(\Delta D_m\) of similar amplitude compared with that in response to stimulations at the other portions (data not shown), although that to the cell end gave the maximum value in \(\Delta E_m\) (cf. Fig. 3B).

**Relationship between \(\Delta E_m\) and the duration of stimulus**

Mechanical stimuli with various duration (0.1, 1, 3, 5, 10 and 30 s) but fixed magnitude (45 g) were applied to each internodal cell in *C. corallina* at the portion 10 mm apart from the node (Fig. 4). As shown in the previous reports (Shimmen 1996, 1997a, 1997b, Kaneko et al. 2005), \(\Delta E_m\) had a single peak upon 0.1s-stimulus (Fig. 1A). Long-lasting stimulus, however, induced \(\Delta E_m\) not only at the start of stimulation but also at the end as shown in Fig. 1B. It should be noted that \((\Delta E_m)_E\) is significantly larger than \((\Delta E_m)_S\) and this was confirmed in four Characeae internodal cells (Figs. 1B, 2). Since the values of \((\Delta E_m)_E\) were highly diverse from cell to cell, we described these values as \((\Delta E_m)_E/(\Delta E_m)_{0.1}\) of each cell, where \((\Delta E_m)_{0.1}\) is the \(\Delta E_m\) upon 0.1s stimulus onto the same cell before Ts stimuli. Fig. 4 shows relationship between logarithm of the duration of stimulus (log t) and \((\Delta E_m)_E/(\Delta E_m)_{0.1}\). Clearly, the \((\Delta E_m)_E/(\Delta E_m)_{0.1}\) was significantly dependent on the duration t, i.e., the
longer stimulation caused the larger magnitude of \((\Delta E_m)_s/(\Delta E_m)_o\) \((n = 15)\). By contrast, \((\Delta E_m)_s/(\Delta E_m)_o\) was almost independent of \(t\) (data not shown).

The experiments showed that mechanical stimuli of long duration or of large strength often induced action potentials which were triggered by large receptor potentials as pointed out by Shimmen (1996). Thus the all following experiments were performed in APW supplemented with 100 mM KCl and 10 mM CaCl\(_2\) (100KCl+10CaCl\(_2\)-APW) in both pools (Fig. 10A), because cells become inexcitable in such high [K\(^+\)] solutions (Shimmen et al. 1976, Shimmen 1997a).

**Effect of Cl\(^-\) in the bathing medium on \(\Delta E_m\) under long-lasting stimulus**

In the previous papers in which duration of mechanical stimulus was very short, Shimmen (1997a) showed that generation of receptor potential was explained as Cl\(^-\) channel activation because \(\Delta E_m\) was highly depended on Cl\(^-\) concentration in the extracellular medium. Kaneko et al. (2005) showed that Ca\(^{2+}\) channel activation may also occur during \(\Delta E_m\) because cytoplasmic Ca\(^{2+}\) level transiently increased in a mechanosensitive manner. Thus, we studied whether both Cl\(^-\) channel activation and Ca\(^{2+}\) channel activation take place in \((\Delta E_m)_s\) and \((\Delta E_m)_e\) or not under long-lasting stimulus (10s-stimulus).

An internodal cell of *Nitella flexilis* was immersed in 100KCl+10CaCl\(_2\)-APW and stimulated with 10s-stimulus of 85 g. As shown in Fig. 5, \((\Delta E_m)_s\) was toward positive but \((\Delta E_m)_e\) was toward negative. Although values of \((\Delta E_m)_s\) and \((\Delta E_m)_e\) were highly different from cell to cell, seven cells out of nine also showed positive \((\Delta E_m)_s\) while negative \((\Delta E_m)_e\) as shown in Fig. 6. In two exceptional cells, both \((\Delta E_m)_s\) and \((\Delta E_m)_e\) were toward negative (cell 6 and 8 in Fig. 6).

**Change of cytoplasmic Ca\(^{2+}\) level**

Since activation of Ca\(^{2+}\) channels was involved in receptor potential under short-term stimulation, we studied whether Ca\(^{2+}\) channel activation is also involved in \((\Delta E_m)_s\) and/or \((\Delta E_m)_e\) or not by measuring light emission of aequorin microinjected into the cytoplasm. Changes of cytoplasmic Ca\(^{2+}\) level and \(\Delta E_m\) were simultaneously measured by the same manner as the previous paper (Kaneko et al. 2005). Upon the 10s-stimulus, transient increase in \([\text{Ca}^{2+}]_c\) \((\Delta[\text{Ca}^{2+}]_c)\) took place at both moments (Fig. 7). It should be noted that the amplitude of \(\Delta[\text{Ca}^{2+}]_c\) was smaller at the moment of compression (corresponding to \((\Delta E_m)_s\)) than that at the moment of decompression (corresponding to \((\Delta E_m)_e\)), and these were the case in all 9 cells tested. Namely, \(\Delta[\text{Ca}^{2+}]_c\) at the compression was not detected in 7 cells because it was as small as noise level of the PM current. Other two cells showed detectable values of \(\Delta[\text{Ca}^{2+}]_c\) \((3.8 \pm 2.6 \text{ nA}, \text{mean} \pm \text{SE})\). On the other hand, all nine cells showed detectable values of \(\Delta[\text{Ca}^{2+}]_c\) at the decompression, in which the maximum and the minimum values were 1117.9 and 6.2 nA, respectively, and the mean \(\pm\) SE was
31.6 ± 14.8 nA.

Simultaneous recordings of $\Delta E_m$ and $\Delta D_m$ under long-lasting stimulus

An internodal cell of *C. corallina* was immersed in 100KCl+10CaCl$_2$-APW and mechanically stimulated with 30s-stimulus of 45 g, and the $\Delta E_m$ and degree of membrane deformation ($\Delta D_m$) were simultaneously recorded. The $\Delta D_m$ was measured with an apparatus (Fig. 10) as shown in Materials and Methods. Fig. 8 is a representative example of $\Delta E_m$ and $\Delta D_m$ and shows that $\Delta E_m$ was toward positive but $\Delta E_m$ was toward negative as is the case in Figs. 5 and 6. The $\Delta D_m$ rapidly increased at the moment of compression, followed by a gradual increase in $\Delta D_m$ during the compression (Fig. 8). At the moment of decompression, the $\Delta D_m$ rapidly recovered a little, then further recovered gradually. Repeated experiments showed that the cellular responses accompanying compression always occurred in the similar fashion as shown in Fig. 8 but those accompanying decompression did not. Namely, how rapidly $\Delta D_m$ returned to the original level was significantly dependent on the osmotic value in the bathing medium (data not shown), and will be reported elsewhere.

Relationship between $\Delta E_m$ and $\Delta D_m$

As will be shown in Discussion, we hypothesized that the amplitude of $\Delta E_m$ is dependent on the degree of membrane deformation, $\Delta D_m$. To obtain supporting evidence for this hypothesis, we studied how $\Delta E_m$ and $\Delta D_m$ are affected by the duration of stimulus. Namely, we applied stimuli with various durations (0.1, 1, 3, 5, 10 and 30 s) in turn to each internodal cell of *C. corallina* and simultaneously measured the $\Delta E_m$ and $\Delta D_m$ (Fig. 9). Since values of $\Delta E_m$ and $\Delta D_m$ just before the end of stimulation (they are shown as $\langle \Delta E_m \rangle_E$ and $\langle \Delta D_m \rangle_E$, respectively) were significantly different from cell to cell, these values were respectively normalized against $\langle \Delta E_m \rangle_{0.1}$ and $\langle \Delta D_m \rangle_{0.1}$, which were $\Delta E_m$ and $\Delta D_m$ for 0.1s-stimulus in each cell ($n = 20$). Fig. 9A shows relationship between $\langle \Delta D_m \rangle_E / \langle \Delta D_m \rangle_{0.1}$ and logarithm of the duration of stimulation (log t), and Fig. 9B shows that between $\langle \Delta E_m \rangle_E / \langle \Delta E_m \rangle_{0.1}$ and log t. Clearly, both values of $\langle \Delta D_m \rangle_E / \langle \Delta D_m \rangle_{0.1}$ and $\langle \Delta E_m \rangle_E / \langle \Delta E_m \rangle_{0.1}$ are highly dependent on the duration t, suggesting that $\Delta E_m$ may be dependent on $\Delta D_m$. To confirm the high correlation between $\langle \Delta D_m \rangle_E / \langle \Delta D_m \rangle_{0.1}$ and $\langle \Delta E_m \rangle_E / \langle \Delta E_m \rangle_{0.1}$, we further plotted mean ± SE of $\langle \Delta D_m \rangle_E / \langle \Delta D_m \rangle_{0.1}$ against those of $\langle \Delta E_m \rangle_E / \langle \Delta E_m \rangle_{0.1}$, as Fig. 9C. The one order regression gives a formula $y = 10.4 x - 9.8$. This indicates that there is a kind of threshold in $\langle \Delta D_m \rangle_E / \langle \Delta D_m \rangle_{0.1}$ around 1 for generating receptor potential and this is well coincident with the fact that a mechanical stimulus of very small strength (maybe corresponding to a very little $\langle \Delta D_m \rangle_E / \langle \Delta D_m \rangle_{0.1}$) did not cause any $\Delta E_m$ (data not shown). The value of correlation coefficient was 0.95, which strongly suggests a high correlation between these two parameters.
**Discussion**

**Internodal cells with or without nodal complex** Characean internodal cell generates receptor potential in response to mechanical stimuli applied to the cell for a short time (Kishimoto 1968, Shimmen 1996, 1997a, b, Shepherd et al. 2001, Kaneko et al. 2005). The receptor potential, $\Delta E_m$, was accounted for as an activation of Cl$^{-}$ channels because $\Delta E_m$ was highly dependent on [Cl$^{-}$] in the bathing medium (Shimmen 1997a) and the membrane resistance decreased during the $\Delta E_m$ (Shimmen 1997b). In the above experiments, $\Delta E_m$ was measured in intact internodal cells with nodal complexes at both ends. Thus, the measured $\Delta E_m$ may not be that of the internodal cell alone but the summation of those from internodal and nodal cells, and this was confirmed in the present study. Fig. 3B, in which each internodal cell without nodal complex was stimulated, clearly shows that internodal cell itself can generate receptor potential upon mechanical stimuli. This is coincident with the previous report which demonstrated that [Ca$^{2+}$]$_c$ of internodal cell increased upon mechanical stimulus (Kaneko et al. 2005). Fig. 3B further shows that $(\Delta E_m)_L / (\Delta E_m)_0$ was almost independent of L except when L = 0. By contrast, $(\Delta E_m)_L / (\Delta E_m)_0$ was larger for the smaller value of L in Fig. 3A, in which internodal cells with nodal complex at both ends were stimulated. Since the difference between these two data should originate from the presence of nodal cells, Fig. 3A and B may indicate that the nodal cells also respond to the mechanical stimuli. The fact that nodal complex can respond to mechanical deformation was also suggested in relation to generation of “death message” in *Chara* (Shimmen 2002, 2003). However, it is not clear why $(\Delta E_m)_L / (\Delta E_m)_0$ was larger for the smaller value of L in Fig. 3A and why it was significantly large only when the cell end was stimulated (Fig. 3B). In order to explain these, we hypothesize that the amplitude of receptor potential, $\Delta E_m$, is dependent on the degree of membrane deformation, $\Delta D_m$. According to this hypothesis, the phenomena in Figs. 3A and B may be explained as follows.

Stimulus closer to the node may induce larger $\Delta D_m$ in the nodal complex and subsequently larger $\Delta E_m$ in the node. Since the measured $\Delta E_m$ is a sum of that from the node and that from the internode, stimulus closer to the node is expected to induce larger $(\Delta E_m)_L$. Thus, $(\Delta E_m)_L / (\Delta E_m)_0$ should be larger for the smaller value of L. In the case of internodal cells without nodes, $(\Delta E_m)_L / (\Delta E_m)_0$ was almost independent of L except when L = 0 (Fig. 3B). This may coincide with the above assumption because nodal complexes are absent in this case. Although $(\Delta E_m)_L / (\Delta E_m)_0$ was significantly large (Fig. 3B), values of $\Delta D_m$ in response to the stimuli at L=0 were similar to those in response to the stimuli onto the other portions (data not shown). This fact may also support the above assumption, dependence of $\Delta E_m$ on $\Delta D_m$, because stimulation to the cell end may induce larger membrane deformation (curvature?) even under the same value of $\Delta D_m$. Or it may
be that the stimulus directly applied onto the ligated cell end induce larger membrane deformation even under the same value of $\Delta D_m$ at the injured part where cell wall may be thinner than other intact parts. We do not deny a possibility, however, that membrane property may be more or less changed by physical damages accompanying ligation.

$\Delta E_m$ and $\Delta D_m$. In this study, we demonstrate that characean cells generate receptor potential at both moments of compression and decompression on long-lasting stimulus (Figs. 1, 2, 5, 6). It is worthy to note that the $\Delta E_m$ at the end of stimulation, $(\Delta E_m)_E$, was significantly larger than that at the start of stimulation, $(\Delta E_m)_S$, and this was generally observed in all four characean species tested (Figs. 1, 2).

According to the above assumption that $\Delta E_m$ is dependent on $\Delta D_m$, the reason why $(\Delta E_m)_E$ is larger than $(\Delta E_m)_S$ may be explained as follows. Before the compression, water potential in the cell should be equal to that outside the cell. Upon the compression, water potential inside the cell becomes higher than that outside the cell through the increase of internal pressure. The higher water potential inside the cell should induce net efflux of water. As a result, internal pressure once rises at the beginning of compression should gradually recover through the water loss. Thus, it is expected that the $\Delta D_m$ will increase during the long-lasting compression even when the compression force is kept constant, and this is demonstrated in the present study (Fig. 8). Thus, the fact that longer duration of stimulus resulted in larger $(\Delta E_m)_E$ (Figs. 4, 9B) is also explained by the dependence of $\Delta E_m$ on $\Delta D_m$ because the longer duration of stimulus will cause larger $\Delta D_m$ (Fig. 9A).

Fig. 9C strongly suggests a tight correlation between the amplitude of $\Delta E_m$ and $\Delta D_m$. Shepherd et al. (2002) reported larger $\Delta E_m$ in a medium of high osmotic value was also reported. This may also suggest that $\Delta D_m$ is the major factor to affect the amplitude of $\Delta E_m$ because $\Delta D_m$ in a medium of high osmotic value should be larger than that in APW of low osmotic value.

The above discussion is simply based on the amplitude of $\Delta D_m$. According to Fig. 8, however, it may be reasonable to assume that the very rapid $\Delta D_m$ at the moment of compression corresponds to the elastic expansion of cell wall in response to the applied force, and that the following slow $\Delta D_m$ does to the water loss during the compression during which the amplitude of fast $\Delta D_m$ should decline. Similarly, rapid $\Delta D_m$ at the moment of decompression and the following slow one may respectively correspond to elastic contraction of cell wall and the recovery to the initial cell volume through water absorption in response to the release of compression force. Amplitude of fast $\Delta D_m$ at the decompression is significantly smaller than that at the compression. This was commonly observed in the present study and may be explained as follows. The water loss during the long lasting compression should cause gradual increase in slow $\Delta D_m$ on one hand and decrease in fast $\Delta D_m$ on the other hand, thus the amplitude of fast $\Delta D_m$ at the moment of decompression was larger.
than that at the compression ( \( \Delta D_m \) at the decompression was larger than that at the compression). We tentatively assume that some membrane state just before decompression, which had been established after a water movement across the membrane, may be responsible for the larger \( \Delta E_m \) upon decompression after long-lasting compression. Preliminary experiments using water channel inhibitor, HgCl\(_2\), also support the assumption. Namely, HgCl\(_2\) treatment significantly decreased not only slow \( \Delta D_m \) during compression but also \( (\Delta E_m)_E \) upon decompression (data not shown), and this will be reported elsewhere in detail.

Although no systematic study has been carried out yet, high osmotic value in the bathing medium may increase amplitude of slow \( \Delta D_m \) after the decompression (data not shown), probably reflecting smaller motive force for water influx after decompression in such a medium. On the other hand in APW of low osmotic value, the slow \( \Delta D_m \) at the decompression was not always significantly larger than that at the compression, although \( (\Delta E_m)_E \) was always larger than \( (\Delta E_m)_S \) (data not shown). Thus, it is not clear yet whether the slow \( \Delta D_m \) is actually responsible for \( \Delta E_m \) or not.

**Ion channel activations**

Assuming that cytoplasmic \([Ca^{2+}]_c\) is around 10\(^{-7}\) M (Williamson & Ashley 1982), equilibrium potential for Ca\(^{2+}\) is more positive than +50 mV both in 100sorbitol-APW and in 100KCl+10CaCl\(_2\)-APW. By contrast, equilibrium potential for Cl\(^-\) is about +110 mV in 100sorbitol-APW and -35 mV in 100KCl+10CaCl\(_2\)-APW, because cytoplasmic \([Cl^-]\) is around 30 mM (Tazawa et al. 1974). Thus, activation of Ca\(^{2+}\) and Cl\(^-\) channels should cause positive and negative changes in \( \Delta E_m \), respectively. Actually, the \( \Delta E_m \) in 100KCl+10CaCl\(_2\)-APW at the moment of compression and decompression was toward positive and negative, respectively (Figs. 5, 6). This indicates that, at the start of stimulation, significant activation of Cl\(^-\) channel does not occur but that of Ca\(^{2+}\) channel does. Furthermore, little activation of Cl\(^-\) channel means a little \( \Delta[Ca^{2+}]_c \) because Cl\(^-\) channel activation is dependent on \( \Delta[Ca^{2+}]_c \) (Kaneko et al. 2005, Kikuyama 2001). At the end of stimulation, however, significant activation of Cl\(^-\) channels should occur because \( (\Delta E_m)_E \) was toward negative, indicating that \( \Delta[Ca^{2+}]_c \) is also larger at the end of stimulus. These are experimentally supported by the aequorin study (Fig. 7).

The fact that \( \Delta E_m \) depends on \( \Delta D_m \) strongly suggests that membrane deformation, \( \Delta D_m \), firstly induces activation of mechanosensitive Ca\(^{2+}\) channel in characean plants. On the other hand, Qi et al. (2004) reported stretch activated anion channel in protoplasts from *Arabidopsis* mesophyll cells by patch-clamp study. The channel activation occurs depending on the membrane deformation. Although activation of Cl\(^-\) channels during receptor potential in Characeae may be dependent of \([Ca^{2+}]_c\) as discussed above, it may be possible that a part of Cl\(^-\) channel activation is directly dependent on \( \Delta D_m \).
Materials and Methods

Plant materials

Four characean species, Chara corallina, Nitella flexilis, N. axilliformis and Nitellopsis obtusa were used as materials. They were cultured in the laboratory under 14 h illumination 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 CaCl₂) for more than a day before use.

Electrical measurement

Membrane potential ($E_m$) was measured with “K-anesthesia method” as reported previously (Shimmen et al. 1976, Shimmen 1996, Kaneko et al. 2005). Briefly, an internode was placed in a measuring chamber composed of two pools (Fig. 10A). After electrically insulating the pools at the partition wall with Vaseline, pool A was filled with 55KCl-APW and pool B was filled with 100sorbitol-APW isotonic to 55KCl-APW (Figs. 1, 2, 3). In other experiments, both pools were filled with 100KCl+10CaCl₂-APW. Potential difference between the two pools was measured with a pair of Hg-Hg₂Cl₂ electrodes through 10 mM KCl agar-salt bridge. Under such conditions, the $E_m$ at the cell portion in pool B can be measured without inserting microelectrode into the cell (“K-anesthesia method”, Shimmen et al. 1976). The $E_m$ was recorded with a memory oscilloscope (PowerLab/800, ADInstruments, Nagoya) and processed with a computer software (KaleidaGraph, HULINKS, Tokyo).

Measurement of cytoplasmic free Ca²⁺

Changes in cytoplasmic free calcium level ($\Delta$[Ca²⁺]) were measured in the same manner as reported previously (Kikuyama and Shimmen 1997, Kikuyama and Tazawa 1998, Kaneko et al. 2005). Aequorin, which emits light depending on Ca²⁺ concentration, was used as a Ca²⁺ indicator. The internodal cells which had been microinjected with aequorin solution (100 mM KCl, 6 mM MgCl₂, 0.5 mM EGTA, 1 mM PIPES, 0.5 mg ml⁻¹ fch-aequorin) were placed in a dark box and the aequorin luminescence was measured with a photomultiplier tube (PM; R1924P, Hamamatsu Photonics, Hamamatsu). The aequorin luminescence was monitored as PM current which reflects [Ca²⁺]. In the dark box, the $E_m$ and the aequorin luminescence were measured simultaneously.

Mechanical stimulation

Mechanical stimulation onto the cell portion in pool B was carried out in the same manner as reported previously (Kaneko et al. 2005) by compressing it for a longer duration (Fig. 10A).
Forces for compressing the cell are linearly dependent on the amplitude of electric current (Kaneko et al. 2005).

Measurement of $\Delta D_m$

The $\Delta D_m$ upon mechanical stimulation was measured with an apparatus shown in Fig. 10B. A thin metal plate was attached at the end of an acrylic stick used for mechanical stimulation, and the plate was set between a light source (light emitting diode, LED) and a photodiode so that the plate partly intercept the LED light. When a mechanical stimulus was applied to the cell, the attached plate lowered its position between the LED and the photodiode, so that the plate lessened the amount of light reaching the photodiode. Upon the decompression, the amount of light reaching the photodiode recovered the initial value because the plate came back to the original position. Since the output voltage of the photodiode directly correlates the intensity of the perceived light, the output voltage from the diode reflects the degree of compression, $\Delta D_m$. 
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References


Legends to Figures

Fig. 1  Receptor potential in response to 0.1s-stimulus and 10s-stimulus.  *Chara* internodal cell was stimulated at the portion 10 mm from the node in 100sorbitol-APW.  A and B are representative examples for 0.1s-stimulus and 10s-stimulus, respectively.  Upper trace shows $\Delta E_m$ and lower trace does the mechanical stimulation.  $\Delta E_m$ showed a single peak for 0.1s-stimulus (A) while it did two peaks for 10s-stimulus (B).  $(\Delta E_m)_{0.1}$ is the $\Delta E_m$ for 0.1s-stimulus.  $(\Delta E_m)_S$ and $(\Delta E_m)_E$ in B are the $\Delta E_m$ at the start and the end of stimulation, respectively.  The values were defined as differences between the $E_m$ before stimulation and the each peak of $\Delta E_m$.

Fig. 2  The fact that $(\Delta E_m)_E$ is larger than $(\Delta E_m)_S$ is generally seen in other three characean plants.  Mechanical stimulus was applied in the same manner as Fig. 1B.  Representative results in each characean cells are presented: (A) *Nitella axilliformis*, (B) *N. flexilis*, (C) *Nitellopsis obtusa*.

Fig. 3  Relationship between $(\Delta E_m)_L$ and the stimulated portion, L.  *Chara* cells were stimulated in 100sorbitol-APW with 10s-stimulus whose amplitude was fixed to 21 g.  The L is shown as a length from the nodal complex (in A) or from the cell end (in B).  Since each $\Delta E_m$ was highly different from cell to cell, the $(\Delta E_m)_L$ is normalized against $(\Delta E_m)_{10}$ of each cell and shown as $\log \{(\Delta E_m)_L/(\Delta E_m)_{10}\}$.  A is the relationship in intact internodal cells ($n = 10$) and B is that of cells whose nodal complexes had been removed ($n = 11$).

Fig. 4  Relationship between $\Delta E_m$ and duration of stimulation (t).  Various stimuli for 0.1, 1, 3, 5, 10 and 30 s were applied to each *Chara* internodal cell in 100sorbitol-APW.  The stimuli were applied at the cell portion 10 mm from the node and the intensity was fixed to 45 g.  The abscissa, t, is shown as log t because t ranged in two orders, and the ordinate does $(\Delta E_m)_t/(\Delta E_m)_{0.1}$, where $(\Delta E_m)_E$ and $(\Delta E_m)_{0.1}$ are the $\Delta E_m$ at the end of T$s$-stimulus and 0.1s-stimulus, respectively.  Each plot shows the mean ± SEM of $(\Delta E_m)_t/(\Delta E_m)_{0.1}$ ($n = 15$).

Fig. 5  Receptor potential, $\Delta E_m$, in a medium of high Cl$^-$ concentration.  A 10s-stimulus (85 g) was applied to an internodal cell of *N. flexilis* in 100KCl+10CaCl$_2$-APW.  Upper trace shows the $\Delta E_m$ and lower trace does the mechanical stimulation.

Fig. 6  $(\Delta E_m)_S$ and $(\Delta E_m)_E$ of *N. flexilis* in 100KCl+10CaCl$_2$-APW of high Cl$^-$ concentration.  Experiment was carried out with the same manner as in Fig. 5.
Fig. 7 Transient increase of cytoplasmic Ca\(^{2+}\) level (\(\Delta[Ca^{2+}]_c\)) and \(\Delta E_m\) measured in 100KCl+10CaCl\(_2\)-APW. PM current, which reflects [Ca\(^{2+}\)]\(_c\), and the \(\Delta E_m\) were simultaneously measured in \textit{N. flexilis} (\(n = 9\)), and a representative result is shown. The stimulus was applied in the same manner as in Fig. 5. The peak of PM current at the decompression is out of scale, and the entire trace is shown in the insert.

Fig. 8 Simultaneous recordings of \(\Delta E_m\) and \(\Delta D_m\) under long-lasting stimulus. Long-lasting stimulus (30 s, 45 g) was applied to \textit{Chara} internodal cell in 100KCl+10CaCl\(_2\)-APW. Upper, middle and lower traces show \(\Delta E_m\), \(\Delta D_m\) and mechanical stimulation, respectively. The \((\Delta E_m)_{E}\) was toward positive but \((\Delta E_m)_{E}\) was toward negative. The \(\Delta D_m\) gradually became larger during the compression. Rapid recovery of \(\Delta D_m\) at the moment of decompression was also followed by a gradual one. \((\Delta D_m)_k\) in Fig. 9 was defined as shown in the Figure.

Fig. 9 Relationships between \((\Delta E_m)_{E}\) and the duration of compression (t), between \((\Delta D_m)_{E}\) and t, and between \((\Delta E_m)_{E}\) and \((\Delta D_m)_{E}\). Mechanical stimuli of 45 g with various duration (0.1, 1, 3, 5, 10 and 30 s) were applied to each \textit{Chara} internodal cell in 100KCl+10CaCl\(_2\)-APW. A shows \((\Delta D_m)_{E}/(\Delta D_m)_{0.1}\) vs. log t, where \((\Delta D_m)_{E}\) and \((\Delta D_m)_{0.1}\) are the \(\Delta D_m\) just before decompression and that for 0.1s-stimulus measured in each cell, respectively. B shows \((\Delta E_m)_{E}/(\Delta E_m)_{0.1}\) vs. log t, where \((\Delta E_m)_{E}\) and \((\Delta E_m)_{0.1}\) are the \(\Delta E_m\) just before decompression and that for 0.1s-stimulus measured in each cell, respectively. C shows \((\Delta E_m)_{E}/(\Delta E_m)_{0.1}\) vs. \((\Delta D_m)_{E}/(\Delta D_m)_{0.1}\), which gives a linear regression \(y = 10.4x - 9.8\) and the correlation coefficient of 0.95. Bars show the SEM (\(n = 20\)).

Fig. 10 A schematic representation of the experimental system. (A) A setup for stimulating an internodal cell (Cell) and measuring membrane potential \(E_m\) with the "K-anesthesia method" (Shimmen et al. 1976). The cell was placed in a measuring chamber composed of two pools A and B. After electrically insulating the pools at the partition wall with Vaseline, pool A was filled with 55 mM KCl solution and pool B was with 100sorbitol-APW, or both pools were done with 100KCl+10CaCl\(_2\)-APW. Potential difference between the two pools was \(E_m\) at the cell part in B and was measured with a pair of Hg-Hg\(_2\)Cl\(_2\) electrodes through 10 mM KCl agar-salt bridge. The cell part in pool B was stimulated by compressing it with an acrylic stick driven by a rotary solenoid (Stimulator). To detect degree of cell deformation, a metal plate was attached to the acrylic stick and deformation of the cell was measured as \(\Delta D_m\). (B) An enlarged illustration of the \(\Delta D_m\) detector. A metal plate attached at the end of acrylic stick for mechanical stimulation was set between a light source (LED) and a photodiode. Upon application of mechanical stimulus, the plate goes down and intercepts the light from the LED to the photodiode, resulting in the change of
its output-voltage. Thus, the $\Delta D_m$ was measured as the change in output voltage from the photodiode.
Fig. 1   (1.5 column)
Fig. 2   (1 column)
Fig. 3  (1 column)
Fig. 4 (1 column)
Fig. 5
Fig. 6  (1 column)
Fig. 7 (1 column)
Fig. 8  (1 column)
\[ \frac{(\Delta D_m)_E}{(\Delta D_m)_{0.1}} \]

\[ \frac{(\Delta E_m)_E}{(\Delta E_m)_{0.1}} \]

Fig. 9 (1.5 column)
Fig. 10  (1 column)