Modulation of Ca^{2+} Influx into a Single Rice Cell with an Electric Signal

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A pulsing electric signal was applied to a target rice cell with a pair of Pt plate electrodes to modulate the Ca^{2+} influx into the cells. The change of intracellular concentration of Ca^{2+} was measured continuously. The cross membrane potential ($|V_{\text{CMP}}|$) generated at the cell membrane was analyzed on the basis of the measured data of potential gradient at the very position of the cell. When $|V_{\text{CMP}}|$ was no higher than 1.0 mV, no appreciable influx of Ca^{2+} was observed. In contrast, when $|V_{\text{CMP}}|$ was higher than 1.9 mV, a marked increase in the Ca^{2+} influx occurred. This influx was inhibited by verapamil, a Ca^{2+} channel blocker, which suggested the involvement of Ca^{2+} channel. Therefore, the Ca^{2+} influx into a rice cell can be modulated with a pulsing electric signal via potential sensitive Ca^{2+} channels.

Key Words : Ca^{2+} Influx, Electric Signal, Cross Membrane Potential

1 Introduction

When a stress signal is applied to a particular site of a plant, the signal is translocated throughout the whole body of the plant. Consequently the cellular response to the stress signal occurs not only at the site where the signal is applied directly but also at remote sites. Such a response of a plant as a whole body is called the systemic response in plant.¹⁻⁵⁾ In the relevance to this systemic response, the potential role of an electric signal has attracted much attention since the report by Wildon *et al.*¹⁾ They suggested that the expression of proteinase inhibitor gene (*pin*) in tomato plant could be induced by an electric signal. The role of an electric signal in triggering *pin* was demonstrated by a more direct experiment in which a pulsing electric signal was applied to the stem of a tomato plant with a pair of silver electrodes to cause an action potential and then the expression of *pin* at a remote site.⁴⁾

In spite of these pioneering works, however, it is still unclear what intensity of an electric signal is effective for the gene expression. In order to clarify this question, the single cell method is necessary for the estimation of $|V_{\text{CMP}}|$. Before analyzing the effect of an electric signal on the gene expression, we intended to analyze its effect on the cell membrane because an electric signal should be concentrated on the cell membrane at first. Among many membrane-associated phenomena, we have focused our attention on the Ca^{2+} entering through the cell membrane, because Ca^{2+} is well known to play an important role in various cellular signaling including the expression control of stress response genes.⁶⁻¹¹⁾

In this study, a pulsing electric signal was applied to rice single-cells, and the potential gradient around the cell was measured to estimate $|V_{\text{CMP}}|$. Based on this, a relationship between $|V_{\text{CMP}}|$ and Ca^{2+} influx was investigated. The involvement of Ca^{2+} channel in this electri-

cally modulated Ca^{2+} influx was also discussed.

2 Experimental

2.1 Plant material

Cultured cells of rice derived from *Oryza sativa* L. japonica cv. Nipponbare were cultured in N6 medium¹²⁾ containing 1 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D). The cells were cultured in 24 cm^3 medium in a 100- cm^3 Erlenmeyer flask at 25°C with 100 rev min^{-1} shaking in the dark and maintained by regular transfer of 10 cm^3 of 7-day-old calli into 14 cm^3 of fresh medium.

2.2 Preparation of single rice cells

Single rice cells were prepared from cultured cells of rice.¹³⁾ Cultures were used 4 days after the last transfer and passed through a 100 μm nylon mesh. One gram of filtered cells was suspended in a 10 cm^3 enzyme solution consisting of 0.05% (w/v) Pectolyase Y-23 (Kikkoman Corporation, Tokyo), 2% (w/v) Cellulase Onozuka RS (Yakult Honsha Co. Ltd., Tokyo), 0.01% (w/v) CaCl_2 , 0.1% (w/v) K-dextran sulfate, and 9% (w/v) mannitol and being adjusted at pH 5.6. The suspension was incubated for 1 h on a shaker (30 rpm, 30°C), followed by incubation without shaking for 2 h at 30°C. Single cells were separated from layer lumps of calli by passage through a 30 μm mesh sieve and collected by centrifugation (900 rpm, 2 min). Single cells were washed twice with a mannitol solution and finally suspended in a modified MS medium¹³⁾ containing 2 mg dm^{-3} 2,4-D (MS-D medium).

2.3 Measurement of the potential profile in the medium between Pt electrodes

As shown in Fig. 1, a pair of Pt plate electrodes (L and R) were set in MS-D medium in a plastic dish (3.0 cm^2) on the microscope stage. The Pt electrodes were connected to an isolator and then to an electric stimulator. An electric voltage ($V_{\text{LR}} = V_{\text{L}} - V_{\text{R}}$) was applied to the Pt electrode terminals. The Pt electrodes were covered with

Fig. 1 Experimental setup for the electric signal application and the potential distribution measurement.

1; measuring microelectrode, 2; reference electrode (Ag/AgCl), 3; Pt wire, 4; dialysis membrane, 5; acrylic plate, 6; Pt plate electrode (L), 7; Pt plate electrode (R), 8; MS-D medium, 9; electric stimulator, 10; isolator, 11; electrometer.

a dialysis membrane in order to prevent bubbles generated by the electrochemical water splitting from mechanically stirring rice cells. The potential distribution in the medium between the Pt electrodes was obtained by scanning a measuring microelectrode. The reference electrode (Ag/AgCl) was set near the Pt electrode R. The analytical procedure to determine $|V_{CMP}|$ from the potential gradient is described in details in (14).

2. 4 Measurement of cytoplasmic Ca^{2+}

Single rice cells were incubated with $1 \mu\text{mol dm}^{-3}$ Fluo-3-AM for 1 h at 30°C . During the incubation, Fluo-3-AM entered the cells through the cell membrane due to its hydrophobic property and was hydrolyzed to free acid, Fluo-3, by intracellular esterase. Since Fluo-3 was a hydrophilic molecule, it could hardly permeate the cell membrane and therefore entrapped within the cell. Fluo-3 could reversibly bind to Ca^{2+} and become a fluorescent conjugate. The Fluo-3 loaded cells thus prepared were placed between the Pt electrodes. Under the excitation with blue light, the fluorescence of Fluo-3- Ca^{2+} conjugate was detected with a CCD camera cooled with liquid nitrogen (LN/CCD-576E, Princeton Instruments, Inc., Trenton, NJ).

3 Results and Discussion

3. 1 Potential distributions obtained under various V_{LR} conditions and estimated values of $|V_{CMP}|$

Figure 2(A, B) shows potential profiles obtained for various values of V_{LR} . As shown in Fig. 2(A), large part of V_{LR} was distributed to the surface potential of the both Pt electrodes. In the medium outside of the dialysis membrane, the potential seemed to be constant. Actually, however, the potential changed linearly and therefore the electric field intensity was constant, as depicted in Fig. 2 (B). Moreover, the potential gradient increased with the increase in V_{LR} . Therefore we analyzed $|V_{CMP}|$ according to the constant electric field intensity model. As illustrated in Fig. 2(C), the potential at the cell surface varies along the cell meridian. The maximum potential difference that the cell undergoes can be given by the product: [potential gradient] $\times$ [cell diameter]. This potential

Fig. 2 Potential distribution in the medium between Pt plate electrodes.

(A); Portion of surface potential, (B); Linear gradient in the middle area in medium. $V_{LR} = 5 \text{ V}(\times)$, $10 \text{ V}(\square)$, $20 \text{ V}(\circ)$, $30 \text{ V}(\triangle)$, respectively. The potential is expressed by $V - V_{2.5}$, where $V_{2.5}$ indicates the potential at the middle point (2.5 mm from both electrodes). (C); V_{CMP} determination according to the constant electric field intensity model.

difference is assumed to be concentrated on the cell membrane and then shared by 2 membranes (L side and R side). When V_{LR} was 30 V , the potential gradient at the cell position was estimated as 550 mV mm^{-1} . Assuming the cell diameter to be $20 \mu\text{m}$, $|V_{CMP}|$ was determined as 5.5 mV .

3. 2 Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) change in response to an electric signal

Before applying an electric signal, the $[\text{Ca}^{2+}]_i$ of a target cell was measured for 4.5 min to obtain a steady value of $[\text{Ca}^{2+}]_i$. Then a rectangular pulsing electric signal (pulse height: $V_{LR} = 30 \text{ V}$, pulse width; 30 sec) was applied to the cell. The changing patterns of $[\text{Ca}^{2+}]_i$ ob-

tained with 43 cells were classified into 3 patterns as shown in Fig. 3. The pattern most frequently observed is that of Fig. 3(A). In this pattern, the $[Ca^{2+}]_i$ initially decreased slightly and then immediately increased markedly. After reaching a maximum, it decreased gradually again and finally reached the initial level after 10 min. The patterns of Fig. 3(B) and (C) are apparently different from Fig. 3(A). However they are substantially same in the sense that $[Ca^{2+}]_i$ increased transiently.

One possible reason for such a variety of $[Ca^{2+}]_i$ changing pattern is thought to be the difference of the density of Ca^{2+} channel and the activity of each channel in respective cells.

The involvement of extracellular Ca^{2+} in the above observed $[Ca^{2+}]_i$ change was ascertained by 2 methods: (1) trapping of Ca^{2+} by EGTA, (2) blocking of Ca^{2+} channel. After suspending the cells in a Ca^{2+} free MSD medium, 5 mmol dm^{-3} EGTA was added to this medium to trap trace amount of Ca^{2+} which might have remained in the Ca^{2+} -free medium. Then an electric signal was applied to the cell and the $[Ca^{2+}]_i$ change was measured. Under this condition, no response of $[Ca^{2+}]_i$ was obtained (Fig. 4(A)).

Blocking of Ca^{2+} channel was done with a well known Ca^{2+} channel blocker, verapamil. When verapamil (0.1 mmol dm^{-3}) was applied to rice cells 10 min before the electric signal, no change of $[Ca^{2+}]_i$ was observed (Fig. 4(B)). These results indicate that the $[Ca^{2+}]_i$ increase triggered by the electric signal should be the Ca^{2+} flow

Fig. 3 Typical patterns of $[Ca^{2+}]_i$ change occurring in response to an electric signal.

ΔF was defined as $FI_{max} - FI_0$, where FI_{max} was the maximum value of the response curve and FI_0 was a steady level before application of the electric signal. Patterns (A), (B), (C) were obtained with 22, 8, 7 cells of 43 cells, respectively. $\square$ indicates the electric signal application period. V_{LR} was 30 V and pulse width was 30 sec. $[Ca^{2+}]_e = 30 \text{ mmol } dm^{-3}$.

Fig. 4 Inhibition of the electric signal induced $[Ca^{2+}]_i$ change by trapping Ca^{2+} in the outside medium or by blocking Ca^{2+} channel.

(A); a Ca^{2+} free medium containing EGTA (5 mmol dm^{-3}), (B); MSD medium (30 mmol dm^{-3} Ca^{2+}) containing verapamil (0.1 mmol dm^{-3}). An electric signal condition was same as Fig. 3. About ΔF , refer to the legend for Fig. 3. Twenty cells were observed in each case.

from outside medium into the cell through Ca^{2+} channels.

In the relevance to the stress gene expression, we formerly observed a quite similar changing pattern to Fig. 3 (A), when an elicitor was applied to a suspension of rice cells to express chitinase gene (unpublished data). Such a transient change of $[Ca^{2+}]_i$ was found essential for successive expression of the chitinase gene. Therefore, under the present condition of an electric signal, it should be probable that the chitinase gene had been expressed, though we have not yet obtained the experimental evidence.

3.3 Modulation manner of Ca^{2+} influx

The dependency of extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) and $|V_{CMP}|$ on $[Ca^{2+}]_i$ change was investigated to evaluate the modulation manner of Ca^{2+} influx. We symbolized the fluorescent intensity change by ΔF (Fig. 3), which corresponded to the amplitude of the $[Ca^{2+}]_i$ change.

As shown in Fig. 5, ΔF increased with increase in $[Ca^{2+}]_e$ in the range below 100 mmol dm^{-3} . This result suggests that the Ca^{2+} influx should have occurred by passive diffusion through Ca^{2+} channels or non-specific pores in the cell membrane. As discussed above, the involvement of the non-specific pores was ruled out by the complete blocking by verapamil.

As far as an electric signal is concerned, it is important to estimate a threshold value of $|V_{CMP}|$ that could open the channel, which should be called the gating potential. As shown in Fig. 6, no appreciable influx occurred when $|V_{CMP}|$ was no higher than 1.0 mV. The extent of Ca^{2+} in-

Fig. 5 Dependence of $[Ca^{2+}]_e$ on the electric signal induced $[Ca^{2+}]_i$ change.

Each plot is expressed by mean $\pm$ SE, where sample number is 5 for every plot. An electric signal: $V_{LR} = 30$ V, pulse width 30 sec. About ΔF , refer to the legend for Fig. 3.

Fig. 6 Relationship between $[Ca^{2+}]_i$ change and $|V_{CMP}|$.

Each plot is expressed by mean $\pm$ SE, where sample number is 21 for 0.6, 1.0, 1.9 mV, and 23 for 5.5 mV. Pulse width was 30 sec. About ΔF , refer to the legend for Fig. 3.

flux sharply increased when $|V_{CMP}|$ exceeded 1.9 mV.

In conclusion, the modulation of Ca^{2+} influx into a rice cell with an electric signal has been demonstrated for the

first time. Experimental results support the specific involvement of potential sensitive Ca^{2+} channel. The quantitative discussion about the effective amplitude of V_{CMP} has provided us useful information in designing an experimental setup for the gene expression control with an electric signal.

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