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Mechanism for the Change of Cytosolic Free Calcium Ion Concentration by Irradiation of Red Light in Oat Cells

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Abstract: In our previous studies (Chae *et al.*, 1990; Chae *et al.*, 1993), we found that a phytochrome signal was clearly connected with the change in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in oat cells. It was determined that the $[Ca^{2+}]_i$ change occured both by mobilization out of the intracellular Ca^{2+} store and by influx from the medium. The specific aim of this work is to elucidate the processes connecting Ca^{2+} mobilization and influx. The cells treated with thapsigargin (increasing $[Ca^{2+}]_i$ by inhibition of the Ca^{2+}-ATPase in the calcium pool) in the presence of external Ca^{2+} showed the same increasing pattern (sustained increasing shape) of $[Ca^{2+}]_i$ as that measured in animal cells. Red light irradiation after thapsigargin treatment did not increase $[Ca^{2+}]_i$. These results suggest that thapsigargin also acts specifically in the processes of mobilization and influx of Ca^{2+} in oat cells. When the cells were treated with TEA (K^+ channel blocker), changes in $[Ca^{2+}]_i$ were drastically reduced in comparison with that measured in the absence of TEA. The results suggest that the change in $[Ca^{2+}]_i$ due to red light irradiation is somehow related with K^+ channel opening to change membrane potential. The membrane potential change due to K^+ influx might be the critical factor in opening a voltage-dependent calcium channel for Ca^{2+} influx.
Key words: efflux, Fluo-3/AM, influx, mobilization, red-light.

Calcium ion plays an important role as a second messenger in the signal transduction process of various organisms (Marme, 1989; Minta *et al.*, 1989; Schaller *et al.*, 1992). Homeostasis of cytosolic calcium ion is the result of the activity of passive and active transporters located in the plasma membrane as well as in the membranes of different calcium pools. Regulation of cytoplasmic calcium ion concentration is achieved through these complex transport systems.

Thapsigargin (T_g) is known as an inhibitor of the Ca^{2+}-ATPase in the Ca^{2+} pools (Thastrup *et al.*, 1990) and it appears to elevate cytosolic $[Ca^{2+}]$ in cells by a mechanism involving mobilization of intracellular Ca^{2+} stores (Takemura *et al.*, 1989). It is also reported that thapsigargin is first active in the mobilization of Ca^{2+} from the calcium pool and the loss of an intracellular Ca^{2+} pool can serve as a signal for Ca^{2+} entry (Thastrup *et al.*, 1989; Thastrup *et al.*, 1990; Michael *et al.*, 1991; Combettes *et al.*, 1994).

Roger *et al.* (1990) reported that changes in cytoplasmic free Ca^{2+} concentration were involved in phytochrome-mediated chloroplast movement, and these changes were connected with a calcium-activated potassium channel in the algal plasma membrane. Some researchers (Miller, 1987; Clementi *et al.*, 1988; Mihara *et al.*, 1991) found that influx of extracellular K^+ induced activation of a Ca^{2+} channel. Michael *et al.* (1991) reported Ca^{2+} permeability of plasma membrane was increased by release of Ca^{2+} from intracellular calcium pools. Crain *et al.* (1992) reported that depolarization of intracellular space by treatment of exogenous high concentration of K^+ induced change in membrane potential. This change of membrane potential may activate the voltage-sensitive Ca^{2+} channel.

According to our previous results (Chae *et al.*, 1990; Chae *et al.*, 1993), a phytochrome signal regulated the cytoplasmic Ca^{2+} concentration of oat cells via the processes of mobilization and influx. We also found that red light irradiation on oat cells increased the amount of IP_3 with the activation of phospholipase C (PLC) activity (Chae *et al.*, 1992; Chae *et al.*, unpublished results). Nonetheless, the regulation mechanism involved in the processes of mobilization and influx has not been elucidated yet.

In this paper, we propose that depolarization induced by K^+ influx after Ca^{2+} mobilization by red light opens a voltage dependent Ca^{2+} channel.

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Materials and Methods

Materials

Sorbitol, EGTA, BSA, thapsigargin, $LaCl_3$, tetraethylammonium chloride (TEA), vanadate, nifedipine and Fluo-3/AM were purchased from Sigma. Cellulase for the preparation of oat protoplasts was obtained from Yakult Honsa, Japan. Oat seed (*Avena sativa*, L. cv Garry) was purchased from Agriculver Inc. USA.

Oat seedlings

Oat seed (50 g) was soaked at 4℃ for 24 h in complete darkness. Soaked seeds were spread on wet vermiculite (100 g/tray) in aluminum trays (35×45 cm). They were grown at 25℃ for 5 days in complete darkness. The tips of the coleoptile (about 2 cm) were harvested for the preparation of protoplasts.

Protoplast isolation

Isolation of protoplasts was carried out as described in the references (Chae *et al.*, 1990; Chae *et al.*, 1993). Oat tissue (60 g) was sterilized in a 70% ethanol solution for 5 sec and the tissue was washed completely with distilled water. The washed tissue was cut into 1 mm pieces and added to the enzyme solution (0.6 M sorbitol, 1 mM $CaCl_2$, 0.05% BSA, 5% cellulase, pH 6.5) for 6 h at 25℃ with gentle round shaking. After the reaction was finished, the reaction mixture was diluted with the washing solution (0.6 M sorbitol, 1 mM $CaCl_2$, 0.05% BSA, pH 6.5) and filtered with nylon mesh (100 μm). The filtered sample was centrifuged at 1,600 rpm and supernatant was discarded. The pellet was washed in washing solution three times.

Loading of Fluo-3/AM into the protoplasts

Loading of Fluo-3/AM into the cytosol of oat protoplasts was carried out using the method described in the previous paper (Chae *et al.*, 1990; Chae *et al.*, 1993). Fluo-3/AM (1 mM) dissolved in dimethylsulfoxide (DMSO) was added into the protoplast suspension medium (final conc. 15 μM) and incubated for 1.5 h at 25℃. After incubation, centrifugation at 1,600 rpm was performed for 8 min and the supernatant was discarded carefully. For the removal of external Fluo-3/AM or free Fluo-3, the precipitate was washed three times with washing solution. Protoplasts loaded with Fluo-3 were suspended in assay buffer (0.6 M sorbitol, 0.05% BSA, 1 mM $CaCl_2$, 1 mM KCl, 1 mM $MgCl_2$, pH 6.5).

Determination of relative fluorescence intensity

The cytosolic calcium ion concentration was calculated from fluorescence intensity. Measurement of the fluorescence intensity was carried out with time scanning at 25℃. Fluorescence intensity of the Fluo-3-Ca^{2+} complex was monitored with excitation at 490 nm. The emission wavelength was fixed at 534 nm.

Relative intensity was measured as;

$$\text{relative intensity } (\%) = \{(F - F_o)/F_o\} \times 100$$

where F_o is the fluorescence intensity of control and F is the change of fluorescence intensity induced by red light irradiation.

The light source was an optic fiber illuminator equipped with a red filter. The protoplasts were maintained in the darkness and irradiated with red light of 7.4 W/m^2 for 1 min.

Treatment of various chemicals

Thapsigargin was dissolved in DMSO to 100 μM and 10 μl of stock solution (final conc. 1 μM) was added to the protoplast suspension medium. EGTA (0.2 M), KCl (2 M), TEA (1 M), Vanadate (10 mM), and $LaCl_3$ (10 mM) were dissolved in deionized water. Ten μl of each stock solution was then added to the assay medium. Nifedipine stock solution (2 mM) was prepared in ethanol. All chemicals were immediately added to the protoplast suspension medium 5 min before the reaction was initiated.

Results and Discussion

We previously reported that red light activated phospholipase C in oat cells, and enhanced the formation of IP_3. We also determined that red light irradiation on oat cells changed the $[Ca^{2+}]_i$ *via* the processes of mobilization and influx.

Michael *et al.* (1991) reported that endomembrane Ca^{2+} stores were functionally coupled to the Ca^{2+} permeablility of the plasma membrane. However, it is not clear how information is flowing from the internal Ca^{2+} stores to the extracellular surface. Nonetheless, two mechanisms have been suggested. Some researchers suggested that the increase of Ca^{2+} permeability of plasma membrane was responsive to the elevated cytoplasmic calcium ion concentration released from the endomembrane Ca^{2+} stores (Ng *et al.*, 1990; Michael *et al.*, 1991). Others proposed that plasmalemmal Ca^{2+} permeability increased in response to the Ca^{2+} content of the calcium pools, regardless of the cytosolic Ca^{2+} concentration (Putney, 1986; Takemura *et al.*, 1989).

Thapsigargin (an inhibitor of endomembrane Ca^{2+}-ATPase) appears to elevate cytosolic Ca^{2+} concentration in various cells by a mechanism involving mobilization of intracellular Ca^{2+} stores. Due to the existence of an endogenous leak, Tg induces depletion of the

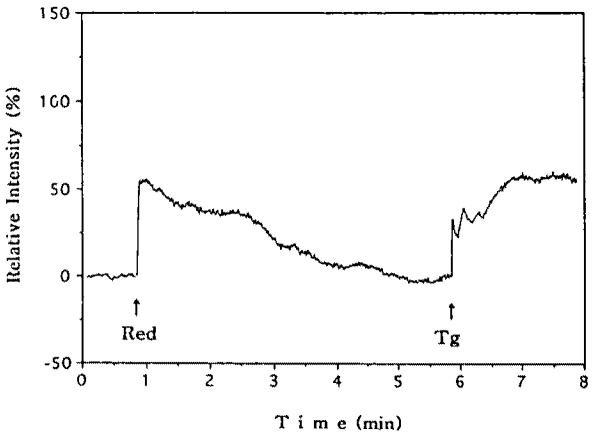

Fig. 1. Time-dependent fluorescence intensity change of the intracellular Fluo-3-Ca^{2+} complex in oat protoplasts treated by red light and thapsigargin. The first arrow is fluorescence intensity changed by irradiation of red light for 1 min, second arrow is fluorescence intensity change by Tg. External calcium ion concentration was 1 mM. Excitation wavelength and emission wavelength were fixed at 490 nm and 526 nm, respectively. Bandwidths of excitation and emission were 10 nm, respectively. After 1 min of red light irradiation on the sample, fluorescence intensity change was monitored (first arrow). At the second arrow 1 μM Tg was added to the protoplast suspension medium.

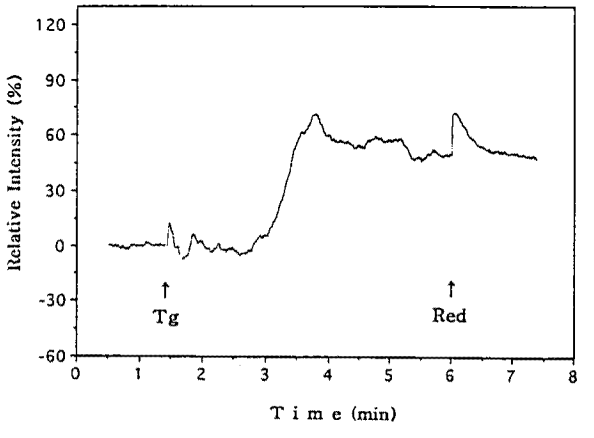

Fig. 2. Red-light effect on Tg-stimulated changes of cytoplasmic calcium ion concentration in oat protoplast suspension medium. After the treatment with 1 μM Tg (first arrow), the fluorescence intensity of intracellular Fluo-3-Ca^{2+} complex was reached at maximum and sustained it for a while, an assay medium containing protoplast (1×10^6/ml) was immediately irradiated with red light at the second arrow for 1 min.

calcium stores and the decrease in Ca^{2+} content of the pool leads to the activation of a plasma membrane Ca^{2+} channel through an unknown signaling mechanism. Tg has been known as an useful compound to study the relationship between the processes of mobilization and influx. Therefore, we also hired this compound to get some information about the relationship between the two processes. Red light irradiation for 1 min increased the cytosolic Ca^{2+} concentration by

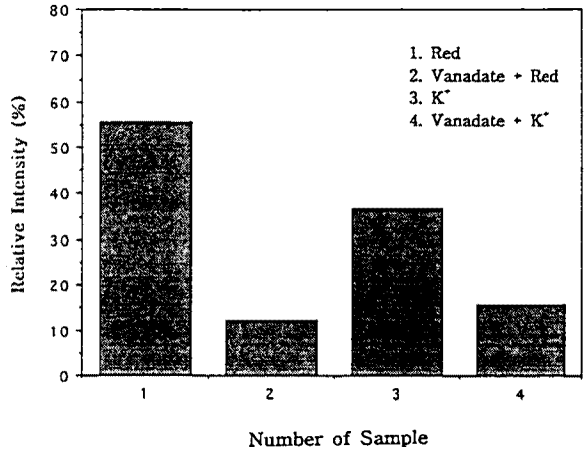

Fig. 3. Effect of Vanadate (ATPase inhibitor) on changes in cytoplasmic calcium ion concentration due to irradiation with red light and treatment with K^+. Vanadate (100 μM) was added to inhibit the efflux of ions through plasmalemmal ATPase. Before oat cells were irradiated with red light, they were treated with vanadate for 5 min. Conditions for red light irradiation were the same as described in Fig. 1. Lane 1 represents the relative fluorescence intensity of oat protoplasts irradiated with red light in absence of vanadate. Lane 2 is the one with red light irradiation in the presence of vanadate. Lane 3 represents the relative fluorescence intensities of oat protoplasts treated with exogenous 20 mM K^+ in the absence of vanadate. Lane 4 is the one with exogenous 20 mM K^+ in the presence of vanadate.

about 60%. When the increase of cytosolic Ca^{2+} concentration by red light returned to the resting level, 1 μM Tg was immediately added to the oat cells. Fig. 1 shows a sustained increase of $[Ca^{2+}]_i$ by Tg which reveals a result similar to animal cells (Thastrup *et al.*, 1990; Michael *et al.*, 1991). If the mobilization process triggered by Tg is not connected with the influx process, the increasing pattern of $[Ca^{2+}]_i$ should be a transient one. In our previous papers (Chae *et al.*, 1990; Chae *et al.*, 1993), we found that red light induced both the mobilization and the influx of Ca^{2+}. Summarizing the above results, Tg also functions properly in the processes of increasing cytosolic $[Ca^{2+}]$ in oat cells.

We then irradiated red light on oat cells after cytosolic $[Ca^{2+}]$ had been increased by Tg (Fig. 2). No change of cytosolic $[Ca^{2+}]$ was observed by irradiation of red light. This result means that Tg alone completed the increase of cytosolic $[Ca^{2+}]$ through the processes of mobilization and influx. Cells need then no more cytosolic Ca^{2+} and red light can not effect any increase of $[Ca^{2+}]_i$. Since it is demonstrated that Tg is the compound acting first in the Ca^{2+} mobilization process and this signal is connected to the Ca^{2+} entry process (Michael *et al.*, 1991), we can say that the same processes occur in the change of $[Ca^{2+}]_i$ by red light in oat cells.

Rasi-Caldogno *et al.* (1992) reported that Ca^{2+} efflux

through plasma membrane was carried out by the active transporter, Ca^{2+}-ATPase. This Ca^{2+}-ATPase is activated by Mg^{2+}, but inhibited by vanadate. East *et al.* (1992) reported that Ca^{2+}-ATPase existed in one of two conformations E_1 and E_2. E_1 has two binding sites for Ca^{2+} with high affinity and the binding site of E_2 has low affinity to Ca^{2+}. Blatt (1991) reported that H^+-ATPase was involved in generation of hyperpolarization which can activate the voltage-sensitive K^+ channels. To identify the involvement of these ATPases on the change of cytosolic Ca^{2+} concentration, oat cell was pretreated with vanadate (ATPase inhibitor) for 5 min. In the absence of vanadate, cytosolic calcium concentration was increased by about 56% by red light and about 37% by exogenous 20 mM K^+ (lanes 1 and 3 in Fig. 3). But oat cells pretreated with vanadate showed only 12% increase of cytosolic calcium ion concentration by red light (lane 2 in Fig. 3), and 16% increase by treatment of 20 mM K^+ (lane 4 in Fig. 3). We consider that the increase of IP_3 by red light promotes the mobilization of Ca^{2+} from the intracellular calcium pools, and the mobilized Ca^{2+} is effluxed to extracellular space by plasmalemmal Ca^{2+}-ATPase. However, an involvement of H^+ efflux by H^+-ATPase can not be ruled out by the vanadate experiment only. Further studies should be carried out in the near future.

Berridge (1987) and Blatt (1991) reported that the efflux of mobilized Ca^{2+} and intracellular H^+ induced activation of the K^+-inward rectifier. In the *Vicia* guard cell, a K^+-inward rectifier current was promoted by extracellular H^+ concentration. When the pH was changed from 7.4 to 5.5, current was increased about five- to seven-fold at voltage near −200 mV. To investigate an involvement of K^+-inward rectifier in the change of cytosolic Ca^{2+} concentration due to red light, TEA (potassium channel blocker) experiments were carried out (Fig. 4). Oat cells not treated with TEA showed 56% and 37% increases of cytosolic Ca^{2+} concentrations due to the red light irradiation and the exogenous K^+ treatment, respectively (lanes 1 and 3 in Fig. 4). In the presence of TEA, treatment with exogenous K^+ increased cytosolic Ca^{2+} concentration about 13% (lane 4 in Fig. 4). Under the same conditions, a red light signal increased the concentration only 5% (lane 2 in Fig. 4). In the cells treated with TEA, the increase of cytosolic Ca^{2+} concentration due to red light was less than that due to exogenous K^+ treatment. The results suggest that activation of K^+-inward rectifier may be a critical factor in increasing cytosolic Ca^{2+} concentration. Upon recalling that K^+ influx causes the depolarization of the intracellular space and the consequent depolarization of membrane potential (Crain *et al.*, 1992), we can imagine that voltage-dependent K^+ channels could be involved in the influx processes of Ca^{2+}.

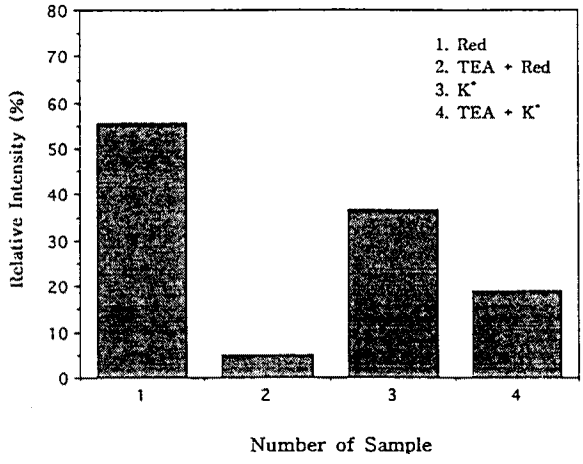

Fig. 4. Effect of tetraethylammonium chloride (TEA) on changes in cytoplasmic calcium ion concentration by treatment with potassium ion and irradiation with red light on oat protoplast suspension medium. TEA (10 mM) was added to block the influx of potassium ions. Experimental conditions were described in Fig. 3. Lane 1 represents the relative fluorescence intensities of oat protoplasts irradiated with red light in the absence of TEA. Lane 2 is the one with light irradiation in the presence of TEA. Lane 3 represents the relative fluorescence intensity of oat protoplasts treated with exogenous 20 mM K^+ in the absence of TEA. Lane 4 is the one with exogenous 20 mM K^+ in the presence of TEA.

Table 1. Effect of various chemicals on calcium ion movement by red light irradiation and K^+ treatment in oat protoplasts

Agonist / Blocker	Irradiation of red light for 1 min	Treatment of exogenous K^+
None	100	65.93
Nifedipine	0	–
Vanadate	22.01	28.27
TEA	9.10	22.95
$LaCl_3$+EGTA	15.83	8.30

Relative intensity (%)

Every chemical was added to the protoplast suspension medium and incubated for 5 min. Noises occurring due to treatment with exogenous chemical were removed completely. Oat cells incubated with inhibitor were treated with red light and exogenous potassium ion.
Tetraethylammonium ion, 10 mM; Vanadate, 100 μM; Nifedipine, 20 μM; $LaCl_3$, 100 μM; EGTA, 2 mM.

The effects of various channel blockers on the change in cytosolic Ca^{2+} concentration in oat cells are listed in Table 1. Some researchers reported that nifedipine blocked endomembrane Ca^{2+} channels in various cells (Robert *et al.*, 1992; Winifred, 1982; Carlos *et al.*, 1992). In the case of cells treated with nifedipine, there was no change in cytosolic Ca^{2+} concentration due to red light. This means that nifedipine also acts on

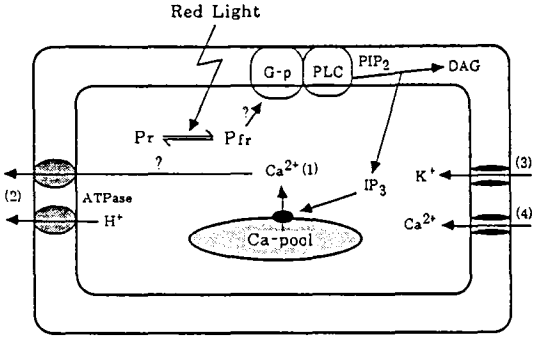

Fig. 5. Hypothetic processes of cytoplasmic calcium ion concentration change induced by irradiation of red light on oat protoplast. 1) Ca^{2+} release from Ca^{2+}-pool (vacuole) by binding IP_3 to its receptor located on the tonoplast. (2) Ca^{2+} and/or H^+ efflux(s) *via* Ca^{2+} -ATPase and/or H^+ ATPase after Ca^{2+} mobilization. (3) K^+ influx *via* K^+-inward rectifier after the efflux(s) of Ca^{2+} and/or H^+. (4) Ca^{2+} influx *via* voltage-dependent Ca^{2+} channel after the plasma membrane is depolarized by the influx of K^+. P_r: red light absorbing form (biologically inactive form); P_{fr}: far-red light absorbing form (biologically active form); G-p: GTP-binding protein; PLC: phospholipase C: PIP_2, phosphatidyl inositol 4,5-bisphosphate; IP_3: inositol 1,4,5-trisphosphate.

endomembrane calcium channels in oat cells. When the oat cells were pretreated with La^{3+} (to block plasmalemmal calcium channels) and EGTA (to remove external Ca^{2+}), cytosolic Ca^{2+} concentration was slightly changed by red light. This small change might be due to the mobilized Ca^{2+} from intracellular calcium pools being mobilized by red light.

From the above results, we propose the following pathway for change in cytosolic Ca^{2+} concentration due to red light (Fig. 5). Red light triggers a cascade of events; (1) increase of IP_3 by red light irradiation, (2) mobilization of Ca^{2+} from the intracellular calcium pools, (3) Ca^{2+} efflux through Ca^{2+}-ATPase, (4) activation of K^+-inward rectifier, (5) depolarization of the membrane, (6) opening of voltage-dependent Ca^{2+} channels, (7) influx of Ca^{2+}.

Recently, Kim *et al.* (1995) proposed that mobilized Ca^{2+} activated the CIF (Calcium Influx Factor) and it could activate the Ca^{2+} entry channel for the acceleration of Ca^{2+} influx. Nevertheless, further studies are required to elucidate clearly the mechanism of change in cytosolic free calcium concentration due to extracellular signals.

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