J. Biochem. Mol. Biol. Vol. 28, No. 5 pp. 382~386 September 30, 1995

Light Effects on the Membrane Potential in Oat Cells

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(Received April 12, 1995)

Abstract: One of the reaction pathways in light-invoked signal transduction can be initiated through ion fluxes across the plasma membrane in higher plants. We isolated protoplasts from oat coleoptile and examined the effects of light on the membrane potential using a membrane potential-sensitive fluorescent probe (bisoxonol). Both red and far-red light initially induced a hyperpolarization in oat cells. Red light-induced hyperpolarization was effectively dissipated by 100 mM K^+, but the hyperpolarization induced by far-red light was not depolarized by any of the cations (K^+, Ca^{2+}, Li^+, Na^+) tested. The depolarization induced by red light and K^+ was inhibited by 200 mM TEA, which is a K^+ channel blocker. These results suggest that K^+ influx through the inward K^+ channel may be a depolarization path in the phytochrome-mediated signal transduction.

Key words: bisoxonol, inward K^+ channel, membrane potential, phytochrome, voltage-dependent Ca^{2+} channel.

Light is one of the most important environmental signals regulating plant growth and development(Kendric and Kronenberg, 1986; Furuya, 1987; Chasan, 1993). Among the photoreceptors perceiving the light signals, the red light receptor phytochrome is the most intensively studied molecule.

Lew *et al.* (1990) have published a series of studies documenting R/FR reversible changes in the activity of a K^+ uptake channel in *Mougeotia* by using patch-clamping techniques. In many plant tissues, light elicits small changes in the surface potential or the cell-membrane potential. These changes are indicative of an altered transport of one or more ionic species across the plasma membrane. For example, if light activated the H^+ pump (Serrano *et al.*, 1988), the plasma membrane would be hyperpolarized (the electrical potential inside the cell becomes more negative). In contrast, activation of K^+ channels or Cl^- channels would typically depolarize the membrane by bringing the potential closer to the Nernst reversal potential for K^+ or Cl^-. Chae *et al.* (1990) reported that red light could induce an increase of $[Ca^{2+}]_{cyt}$ in oat cells but far-red light had no effect. Shacklock *et al.* (1992) had also shown that red light stimulated a transient rise in $[Ca^{2+}]_{cyt}$ in wheat protoplasts. As reviewed by Tretyn *et al.* (1991), these results indicate that a key step in the phytochrome signal transduction pathway is an increase in $[Ca^{2+}]_{cyt}$, and that this step can be induced either chemically or by light. However, it is difficult to say what other processes are required as events preliminary to increasing $[Ca^{2+}]_{cyt}$, and also difficult to differentiate between primary transduction events and their subsequent effects (Short and Briggs, 1990).

Three factors, H^+ efflux (Serrano *et al.*, 1988), K^+ efflux (Schroeder *et al.*, 1987) or Ca^{2+} efflux (Fallon *et al.*, 1993) are known to induce membrane hyperpolarization in plant cells. But the possibility of membrane hyperpolarization mediated by phytochrome signal transduction has not been reported yet.

In the present paper we report on a phytochrome-mediated membrane potential change and the factors involved in membrane potential changes in oat protoplasts.

Materials and Methods

Oat seedlings

Oat seeds (*Avena sativa* L. Agriculver, INC. NY, USA) were soaked at 4℃ for 24 h in darkness. Soaked seeds were spread on wet vermiculite (650 g/tray) and perlite (90 g/tray) in aluminum trays (40×52 cm) and the trays were put in an oat-growing box made of wood. The oats were grown at temperatures ranging between 21 and 29℃ for 6~7 days in complete darkness. Plants were watered daily. The upper 2~3 cm of oat coleoptile tips were used for protoplast preparation.

Chemicals and solutions

Onozuka cellulase R-10 was purchased from Yakult Honsha (Yakult Honsha Co., Tokyo, Japan). Bisoxonol

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was purchased from Molecular Probes (Molecular Probes INC., Eugene, USA). BSA, Mes, and TEA were purchased from Sigma (Sigma Chemical Co., St. Louis, USA). Bisoxonol was dissolved in DMSO at 1 mg/ml.

Preparation of protoplasts

The preparation of protoplasts was carried out as previously described (Chae *et al.*, 1990) with several modifications. The tissue (coleoptile tips) was sterilized in a 70% ethanol solution for 5 sec, and the ethanol was completely removed by washing three times with distilled water. The washed tissue was cut into 1 mm pieces, and these were added to the enzyme medium (0.6 M sorbitol, 1 mM $CaCl_2$, 0.05% BSA, 5% cellulase, pH 6.5). Digestion proceeded for about 4 h at 25℃ with gentle rotary shaking at 40 rpm. Digested oat tissue, after being filtered through a 100 μm nylon mesh, was centrifuged at 1,600 rpm for 8 min. The enzyme solution was then replaced with a washing solution (0.6 M sorbitol, 1 mM $CaCl_2$, 0.05% BSA, pH 6.5). After removal of the supernatant, the pellet was suspended in washing solution. After washing the protoplasts three times with washing solution, the pellet was resuspended in 20 mM Mes buffer (pH 6.5) containing 600 mM sorbitol, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM KCl and 0.2 mM DTT. Protoplasts were sedimented by centrifugation at 200×*g* for 8 min, the supernatant was decanted, and the cells were resuspended in the buffer described above. The number of protoplasts was adjusted to be 3×10^5/ml assay medium.

Light treatments

Protoplasts were irradiated for 1 min with the light source of a slide projector (NiKolet Co., Ltd). Red and far-red light was obtained using a 660 nm filter (Eailing, 35-4233 DHE 2-5) and a 730 nm filter (Eailing, 35-4019 DKH 10-4), respectively. The photon fluence rates, measured with a LI-185-B Quantum/Radiometer/Photometer (Li-Cor, INC.) were 280 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for red light, 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for far-red light. Manipulations conducted during the dark period were performed under dim green safelight. After the light treatment, the protoplasts were gently mixed and changes in fluorescence were measured.

Measurements of relative membrane potential

Changes in protoplast membrane potential were monitored by the change of intensity of fluorescence emission of the membrane potential-sensitive dye, bisoxonol (Bronner and Landry, 1991). The assay medium contained of 20 mM Mes (pH 6.5), 600 mM sorbitol, 200 mM NaCl, 1 mM $CaCl_2$, 1 mM KCl and 3×10^5 protoplasts/ml. Bisoxonol was added to the assay medium to a final concentration of 0.2 μM and the suspension was incubated for 1 h at 25℃ to allow stabilization of the fluorescence intensity. Fluorescence intensity was measured with a Hitachi F-3000 fluorescence spectrophotometer. The bandwidths for excitation at 540 nm and emission at 580 nm were 5 and 10 nm, respectively (Bronner and Landry, 1989). Although the fluorescence excitation at 540 nm is of low intensity, the possibility exists for the induction of a change in membrane potential. We measured the fluorescence intensity intermittently rather than continuously (Kim *et al.*, 1992). Discontinuous illumination also minimizes possible photodamage to cells during fluorescence measurements (Waggoner, 1979).

Results and Discussion

Changes in the membrane potential of oat cells were measured using the slow (seconds)-responding fluorescent dye (bisoxonol) which is a lipophilic anion (Rink *et al.*, 1989; Taglialatela *et al.*, 1990). Fluorescent intensity changes of bisoxonol reflect a rapid potential-dependent redistribution of the dye between cell interior and exterior. Hyperpolarization (when the inside becomes negative) causes a decrease in fluorescent intensity, whereas depolarization causes an increase in fluorescence intensity. Depolarization of the membrane transfers dye from the external solution onto binding sites inside the cells, thus increasing the net fluorescence.

Fig. 1 shows the membrane potential changes in oat cells induced by irradiation of red light. The changes of fluorescence intensities were observed at 1 min intervals after irradiation by red light on the oat protoplasts. As seen in Fig. 1, hyperpolarization was observed soon (>1 min) after irradiation by red light and then depolarization occurred. When oat protoplasts were kept in constant darkness, no change of membrane potential was observed. The dark-adapted cells did not depolarize even when 100 mM K^+ was added. Cells irradiated with red light were depolarized upon treatment with K^+, but this was not observed in the absence of K^+. For clear identification of K^+ as the depolarization factor, the effects of red light were investigated after the treatment of cells with the K^+ channel blocker (20 mM TEA). When TEA was present, fluorescence intensity was not increased with K^+ treatment. This result indicates that the K^+ influx must be a depolarization factor under red light irradiation.

Our previous reports (Chae *et al.*, 1990) described that red light irradiation on oat cells increased the cytosolic free Ca^{2+} concentration. According to these reports, an increase of Ca^{2+} concentration was observed from two different processes, mobilization and influx. The rela-

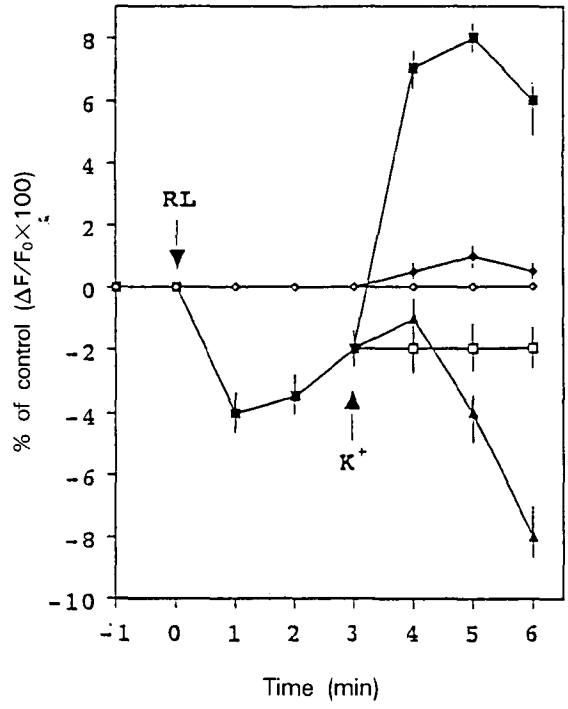

Fig. 1. Time-dependent changes in the red light-induced membrane potential in oat (*Avena sativa*) protoplasts. Fluorescence intensities of 3×10^5 protoplasts/ml, which were pre-equilibrated with 0.2 μM bisoxonol were measured at 1 min intervals. Red light was irradiated for 1 min at the time indicated by the arrow. At the indicated time, K^+ was added to a final concentration of 100 mM. TEA (20 mM) was added 30s before the addition of K^+. (◇) represents the membrane potential change in darkness, (◆) for dark-K^+, (□) for red light irradiation, (■) for red light-K^+, and (▲) for red light-TEA-K^+. The symbols (■) between 1 and 3 min overlap with ▲ and □. Error bars indicate the range of values obtained in three separate experiments. $\Delta F=F_t-F_0$.

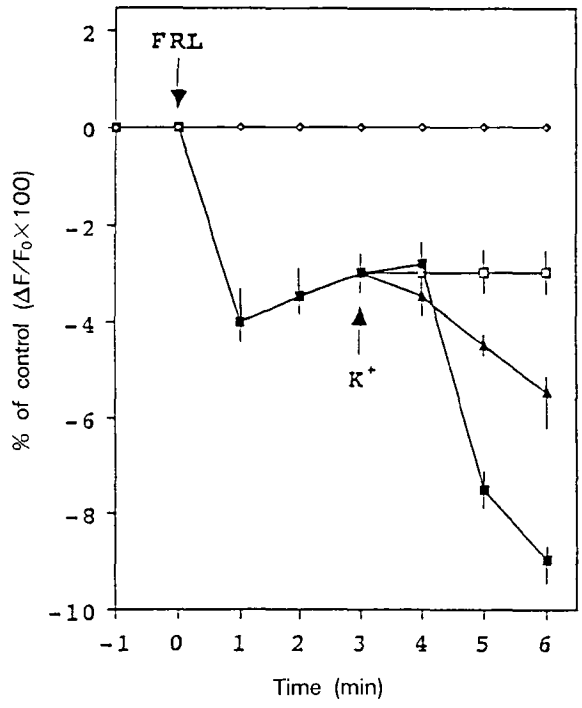

Fig. 2. Time-dependent changes in the far-red light-induced membrane potential in oat (*Avena sativa*) protoplasts. Fluorescence intensities of 3×10^5 protoplasts/ml, which were pre-equilibrated with 0.2 μM bisoxonol were measured at 1 min intervals. Far-red light was irradiated for 1 min at the time indicated by the arrow. At the indicated time, K^+ was added to a final concentration of 100 mM. TEA (20 mM) was added 30s before addition of K^+. (◇) represents the membrane potential change in darkness, (□) for far-red light, (■) for far-red light-K^+, and (▲) for far-red light-TEA-K^+. The symbols(■) between 1 and 3 min overlap with ▲ and □. Error bars indicate the range of values obtained in three separate experiments. $\Delta F=F_t-F_0$.

tionship between mobilization and influx can not be explained at the moment.

In this paper, we tried to examine the possibility that the depolarization induced by the K^+ influx could be a signal to open a voltage-sensitive Ca^{2+} channel. As seen in Fig. 1, membrane depolarization induced by the addition of a high concentration of K^+ does not show up after treatment by TEA, which would have blocked the opening of voltage-sensitive Ca^{2+} channels. The above assumption is proposed on the basis that most of the voltage-sensitive channels are opened by the membrane potential change induced by the depolarization (Armstrong and Eckert, 1987; Alberts *et al.*, 1994; Suh and Kim, 1994).

The hyperpolarization pattern formed by irradiation with far-red light was quite similar to the one obtained by irradiation of red light (Fig. 2). However, opposite depolarization patterns were observed between the two irradiations (red and far-red light). When 100 mM of K^+ was added to the sample, fluorescence intensity was drastically increased in the case of red light irradiation, but it drastically decreased in the case of far-red light irradiation (Fig. 1 and 2). These results indicate that the hyperpolarization factors may be an identical one in the two cases, but different factors may be involved for depolarization.

Table 1. Ion specificity of ion-induced depolarization in oat protoplasts. Depolarization was measured as described in Fig. 1. Red light irradiation, which started at 2.5 min before salt treatment, lasted for 1 min. Fluorescence intensities were measured at 1 min after salt treatment. Depolarization is expressed as percent of control. Values are the average of duplicate assays with a variance of less than 1%; these results are representative of at least two independent experiments for each condition

	Depolarization (% of control)	
	RL	FRL
200 mM KCl	7	−4
200 mM NaCl	−25	−15
200 mM LiCl	0	−9
20 mM $CaCl_2$	−3	−1

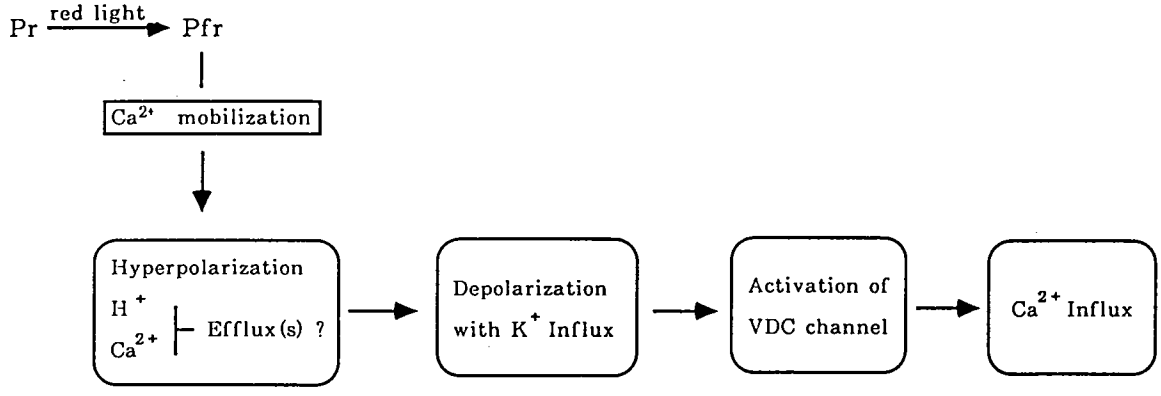

Fig. 3. Model for change of membrane potential by red light irradiation in oat (*Avena sativa*) protoplasts.

Considering the above results of TEA treatment, the depolarization induced by irradiation with red light in the presence of K^+ is quite likely due to K^+ influx. However, hyperpolarization factors during the irradiations (red/far-red) can not be presented at the moment. The possible factors in the hyperpolarization induced by the irradiations are the effluxing cation(s), if no anions are present in the medium. The specific kind of cation(s) for these hyperpolarizations could be deduced by further experiments.

To see whether depolarization could be due to another cation influx, the effects of Na^+, Li^+ and Ca^{2+} on the depolarization of the oat protoplasts were measured (Table 1). Na^+, Li^+ and Ca^{2+} did not depolarize oat protoplasts treated with either red or far-red light. When 200 mM of K^+ was added to the sample, fluorescence intensity was drastically increased by red light irradiation and it was drastically decreased by far-red light irradiation. These results indicate that K^+ influx is likely to be the depolarization factor in the phytochrome-mediated signal transduction.

Fig. 3 shows a hypothetical model for the change of membrane potential by red or far-red light irradiation in oat cells. Hyperpolarization of the cells occurs in response to light (red/far-red) irradiation via the efflux of cation(s), but we do not yet know which cation is effluxed by irradiation of light at the moment.

In the case of red light irradiation, K^+ is a depolarization factor. Most likely, an inward K^+ channel is activated by red light. However, the fact that hyperpolarization is observed under irradiation by far-red light even after the addition of K^+ suggests that some other kind of cation is effluxed or an anion is influxed. Depolarization induced by red light can open the voltage-dependent Ca^{2+} channel. Ca^{2+} influx then starts and cytosolic Ca^{2+} concentration is increased.

Acknowledgement

This work was supported by research grants from the Korea Science and Engineering Foundation (1994) and the Basic Science Research Institute Program from Ministry of Education, Republic of Korea (1995).

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