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THE EFFECT OF PHYTOCHROME ACTION ON THE ACTIVITY OF CYTOSOLIC CHOLINESTERASE IN OAT CELLS

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SUMMARY : Cholinesterases in the oat cell were found to be distributed in the cell wall (50%) and cytoplasm (42%). Activity of the cytosolic enzyme was inhibited about 80% by 1 mM Ca^{2+}. The enzyme activity was also inhibited by Mn^{2+}, but no inhibition by Mg^{2+} was observed. Effects of red light and calcium ion on the enzyme activity were investigated in vivo to confirm the involvement of phytochrome action in the regulation process of this enzyme via Ca^{2+}. It was observed that inhibition by red light only occurs when external Ca^{2+} existed in the cell medium. Based on a previous report(8) that red light stimulates the influx of Ca^{2+} into the cytosol of oat cell, inhibition of the enzyme activity by irradiation of red light can be suggested to occur via the influx of Ca^{2+}. © 1990 Academic Press, Inc.

Acetylcholine is a well-known neurotransmitter in animal cells. However, in plant cells, the roles of acetylcholine are yet not well known(1). Jaffe(2) first reported that acetylcholine in plant cells may be a primary messenger in some photomorphogenic processes mediated by the red light receptor phytochrome. In this report he mentioned that red light (660 nm) irradiation increased the level of cytosolic acetylcholine and far-red light (730 nm) irradiation decreased it. The enzyme that controls the level of cytosolic acetylcholine is known to be a cholinesterase rather than an acetylcholine transferase (2,3).

After discovering that calcium plays an important role in regulating plant growth and developments mediated by phytochrome action (4-7), a few studies have been performed to investigate the red light effect on cytosolic

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Abbreviations: PEI, Polyethyleneimine; KPB, Potassium Phosphate Buffer; IAA, Indole-3-Acetic Acid.

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Ca^{2+} concentration changes (8-11) and the biochemical roles of this second messenger Ca^{2+} (12). Because the relationship between the generation of acetylcholine and the phytochrome action has not fully been investigated, in this report, we have investigated the phytochrome effect on the activity of cholinesterase.

MATERIALS AND METHODS

Materials

Acetylthiocholine chloride, DTNB (5,5'-dithiobis-2-nitrobenzoic acid), bovine serum albumin, ATP, ascorbic acid, and IAA (indole-3-acetic acid) were purchased from Sigma Chemical Co. Polyethyleneimine (PEI) was obtained from Kasei, Japan, and the other reagents were of the highest grade of purity available.

Oat tissue

Oat seeds (Avena sativa L. cv Garry) were purchased from Stanford Seed Co., Buffalo, NY, U.S.A., and they were planted in moist vermiculite (100g/ tray) and grown at 25°C for 5 days in complete darkness.

Extraction and assay of cholinesterase

Extraction of cholinesterase was carried out at 4°C under a green safety light by following the method of Ernst and Hartmann(13). The harvested oat tissues were frozen with liquid nitrogen, and the frozen tissues were homogenized with a mortar and pestle in the two volumes(v/w) of homogenizing buffer (4%$(NH_4)_2SO_4$, 10mM KPB, pH 7.0). The crude homogenate was stirred for 30min and then filtered through two layers of nylon mesh (55μm). Nucleic acids, pectins, and acidic proteins were precipitated by adding 1 ml of 10% (v/v) PEI solution(pH 7.0) to each 100 ml of the filtrate. After stirring for 15min, the filtrate was centrifuged for 30min at 40000g, and the pellet was discarded. Ammonium sulfate(40% to 80%) precipitation was carried out, and the pellet was dissolved in 10 mM KPB(pH 7.0). Centrifugation at 40000g was carried out again for 30 min, and the supernatant was dialyzed in 10 mM KPB(pH 7.0, 4°C) for 24 hrs. Assay of cholinesterase was performed by following the methods of Riov and Jaffe(14). Acetylthiocholine chloride was used as a substrate, and the absorbance of thiocholine at 412 nm was monitored to estimate the enzyme activity. Protein was determined by the method of Lowry et al. (15) with bovine serum albumin as a standard.

Cell fractionation

Etiolated oat tissues were homogenized with a mortar and pestle in two volumes(v/w) of 0.05 M KPB(pH 7.4) containing 0.44M sucrose. The crude homogenate was filtered through two layers of nylon net, and the filtrate was fractionated using sucrose discontinuous gradient centrifugation as described by Fluck and Jaffe (16).

The light effect on the cholinesterase activity in vivo

The light effect on the cholinesterase activity in vivo was examined by Jaffe's method (2) with minor modifications. Oat tissues were placed in a sandwich made of two glass slides. The end of the sandwich was placed in a bathing chamber. The bathing solution was composed of 4 μM Na_2ATP, 1 μM ascorbic acid, 1 X 10^{-4}μM IAA, 1mM $MnCl_2$, 1mM $MgCl_2$, 1mM $CaCl_2$, and 7 X 10^{-2}mM HCl. After irradiation at 25°C for 4 min, the tips were cut from the tissues and frozen with liquid nitrogen for extraction. Irradiation of light was performed by the previously described method(17). The monochromatic lights used were generated by a Xenon lamp in the spectrofluorometer (Hitach MPF 3000). The frozen tips were homogenized with a mortar and pestle in two

volumes of 10 mM KPB, pH 7.0. The homogenate was centrifuged for 30 min at 40000g, and the supernatant was used for the determination of cholinesterase activity.

RESULTS AND DISCUSSION

Since Jaffe(2) first reported evidence suggesting that acetylcholine is a biochemical messenger of phytochrome action ($P_r \underset{730nm}{\overset{660nm}{\rightleftharpoons}} P_{fr} \ldots\rightarrow$ Cell Responses), various studies have been carried out to determine how the intracellular level of acetylcholine is controlled by phytochrome (18,19). There are reports that the intracellular level of acetylcholine is controlled by regulation of cholinesterase activity rather than by acetylcholine transferase(2,3). We first analyzed the cellular distribution of cholinesterase in oat cells whose cytoplasm contains a fairly high amount of phytochrome in comparison with other plant cells. Table 1 shows the distribution of cholinesterase activities in different subcellular fractions. As we expected, oat cells contain a relatively higher amount of cholinesterase in their cytosol. In contrast to the oat cell, other plant cells have a higher amount of this enzyme in the cell wall than the cytosol(16). This may indicate that in oat cells, an interaction between phytochrome and this enzyme would be easier, because phytochrome is located initially in the cytosol. However, details on how phytochrome action may be connected to the regulation of this enzyme activity have not yet been reported.

TABLE 1. Distribution of cholinesterase activity in oat cells

Fraction	Cholinesterase Activity(%)	
	Control	4%(w/v) $(NH_4)_2SO_4$
Residue	49.8 ± 0.25*	37.0 ± 1.56
Filtrate		
Supernatant	42.5 ± 2.62	57.9 ± 2.86
Interface fraction	7.7 ± 1.62	5.1 ± 1.33
Pellet	0	0

*SD

Subcellular fractions were prepared by sucrose discontinuous density gradient centrifugation. Enzyme activity was expressed as relative activity against the total enzyme activity.

Recently, a few articles have mentioned that cytosolic free Ca^{2+} plays an important physiological role as a second messenger for phytochrome action(18). Chae et al. reported that cytosolic Ca^{2+} concentration was increased in oat cells by irradiation of red light(8). Remembering that red light irradiation enhances the level of acetylcholine in bean cells(2, 19) and Ca^{2+} inhibits the activity of cholinesterase(13), we can speculate that phytochrome action can be connected with the regulation of cholinesterase activity via the second messenger Ca^{2+}. When we examined the effects of divalent cations(Ca^{2+}, Mn^{2+}, Mg^{2+}) on the activity of oat cholinesterase, Ca^{2+} and Mn^{2+} showed an inhibitory effect(for 1 mM Ca^{2+} : 70 % inhibition, for 1 mM Mn^{2+} : 50 % inhibition), but Mg^{2+} did not reveal any inhibitory effect. In order to confirm that phytochrome action is connected with the regulation of this enzyme activity via Ca^{2+}, we investigated the effects of light(red and far-red) and divalent cations on the enzyme activity in vivo(Table 2). While a drastic inhibition by red light was observed in the presence of three cations (Ca^{2+}, Mn^{2+},Mg^{2+}), almost no change in the enzyme activity occurred under far-red light even with these three cations. However, when we add only two cations, the cases containing Ca^{2+} show a significant inhibitory effect with the red light irradiation, but no inhibition occurrs without Ca^{2+}. One interesting observation is that

TABLE 2. Effects of light and divalent cations on the cholinesterase activity

Light	Cations	Cholinesterase Activity	
		nmoles/min-g tissue	%
Dark (Control)	Ca^{2+}+Mn^{2+} + Mg^{2+}	1.59 ± 0.09	100
Red Light	Ca^{2+}+Mn^{2+} + Mg^{2+}	0.30 ± 0.10	19
	Mn^{2+}+Mg^{2+}	1.34 ± 0.10	84
	Ca^{2+}+Mg^{2+}	0.71 ± 0.14	45
	Ca^{2+}+Mn^{2+}	0.73 ± 0.16	46
Far-red Light	Ca^{2+}+Mn^{2+} + Mg^{2+}	1.46 ± 0.10	92

Concentration of Ca^{2+}, Mn^{2+}, and Mg^{2+} : 1mM,respectively.

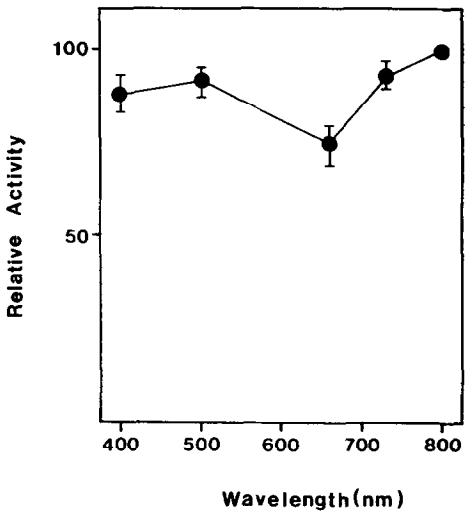

Figure 1. Wavelength dependency of the cholinesterase activity by the irradiation of light. The enzyme was solubilized in 0.05 M KPB containing 0.44 M sucrose, pH 7.4.

Mn^{2+} itself inhibits the enzyme activity *in vitro*, but its influx does not occur with the irradiation of red light *in vivo*. A possible reason for this is that no inhibition of the enzyme activity is observed by irradiation of the red light in the presence of Mn^{2+} and Mg^{2+} *in vivo*. These results suggest to us that phytochrome action is very likely to be related to the regulation of cholinesterase activity through cytosolic Ca^{2+} as a second messenger of the phytochrome signal transduction. Another observation that can support the hypothesis of the involvement of phytochrome in the regulation of cholinesterase activity is the wavelength-dependent inhibition curve shown in Figure 1. The highest inhibition was observed at the wavelength of 660 nm, which is the absorption maximum of the phytochrome P_r form. This implies that the physiologically active phytochrome P_{fr} acts for the influx of Ca^{2+} and the increased cytosolic Ca^{2+} ions participate in the inhibition process of the cholinesterase activity.

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