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Role of calcium and calmodulin antagonist in photosynthesis and salinity tolerance in *Chlorella vulgaris*

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Abstract

To cast light upon the role of Ca^{2+} and calmodulin on photosynthetic rate (P_N), dark respiration (R_D) and amino acid and protein contents in salinity stressed and non-stressed *Chlorella* cultures, the Ca^{2+} chelator EGTA [ethylene glycol-bis-(2-aminoethyl ether)-*N,N*-tetraacetate] and the calmodulin antagonist TFP (trifluperazine) were used. TFP markedly inhibited P_N while EGTA exerted a slight, if any, effect on P_N. NaCl tolerance, on the other side, was markedly abolished by TFP that inhibited P_N and lowered rate of proline accumulation. Calmodulin might be involved in osmoregulation and salt tolerance of *Chlorella*. R_D, however, was markedly enhanced by EGTA and Ca^{2+}-free medium and hence the Ca^{2+} deprivation increased stress severity exerted by NaCl. Combinations of Na^+ and Ca^{2+} enhanced P_N, decreased R_D and proline content in comparison with an osmotically equivalent reference culture containing only NaCl. Addition of Ca^{2+} to TFP treated cultures failed to reactivate calmodulin for proline synthesis. However, when Ca^{2+} was added to EGTA-treated cultures, only relatively reduced proline contents were recorded.

Introduction

The regulation of calcium level is a major mechanism of plant cell metabolism. Ca^{2+} has a role in the maintenance of membrane integrity (Leopold and Willing 1984), cell elongation (Pickard 1970), amylase secretion (Jones and Jacobsen 1983), delay of senescence (Ferguson 1984), and several other processs. In addition, Ca^{2+} regulates various biochemical processes after binding to calmodulin and related proteins (Cheung 1980). Photosynthesis and respiration has also been correlated with Ca^{2+} and calmodulin (Bangerth *et al.* 1972, Woodrow and Rowan 1979, Jarett *et al.* 1982, Muto *et al.* 1982, Burris *et al.* 1983, Ferguson 1984). Ca^{2+} may be involved in the conformational changes in the thylakoids and in the coupling of electron flow (Gross *et al.* 1976). In addition, Ca^{2+} is an important factor in the resistance of plants to salinity (Lahye and Epstein 1971, Greenway and Munns 1980, Kent and Läuchli

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1985). Abdel-Basset (1986) found that Ca^{2+} in certain ratios to Na^+ (Na^+/Ca^{2+} of 13.5) reversed most of NaCl stress symptoms in *Chlorella vulgaris*. This ratio improved the growth and photosynthetic rate (P_N), and lowered both the respiration rate (R_D) and proline accumulation rates of salinized *Chlorella* cultures (stress relief).

The aim of this work was to follow the response of *Chlorella vulgaris* to NaCl in calmodulin inhibited cultures (using TFP) or in Ca^{2+}-chelated cultures (using EGTA). In addition, the effects of isoosmotic Na^+-Ca^{2+} combinations were investigated. P_N, R_D and protein, proline and other free amino acids contents were analysed in short-term experiments.

Material and methods

Unialgal cultures of *Chlorella vulgaris* Beijerinck (isolated from the river Nile) were grown in batch cultures using the Beijerinck's nutritive medium (Stein 1966) under continuous irradiation (5.9 W m^{-2}) and aeration at temperature 25 ± 1 °C. This medium contains 3.6 g(Ca^{2+}) m^{-3} and 103 g(P) m^{-3}. The aliquots of algal suspension were studied without dilution and at the same conditions of growth under the following treatments:

EGTA [ethylene glycol-bis(2-aminoethyl ether)-N,N-tetraacetate (*Serva*, Heidelberg, Germany)] : 2, 4 or 7 mM was applied to check the responsiveness of P_N and R_D to Ca^{2+} chelation.

Ca^{2+} free medium: Algal cells were washed twice in the Beijerinck's medium free of Ca^{2+} and were allowed to grow for further 48 h to determine the effects of Ca^{2+} deprivation (or at least dilution).

TFP [trifluperazine, (*Fluka*, Buchs, Germany)]: 20, 40 and 100 µM were applied to check the responsiveness of P_N and R_D to calmodulin antagonists.
Salinization: 100, 200 and 300 mM NaCl were applied. To clarify the role of Ca^{2+} and calmodulin with respect to salt tolerance EGTA (7 mM) or TFP (0.1 mM) was added to variously salinized cultures of *Chlorella*.

Na^+-Ca^{2+} combinations: Isoosmotic combinations with Na^+/Ca^{2+} of 5, 10 and 15 were used (Ca as $CaCl_2$). These were osmotically equivalent to the moderate salinization (reference culture contained only 200 mM NaCl). The Na^+-Ca^{2+} combinations were also applied in the presence of TFP to understand the role of calmodulin.

P_N and R_D were measured under the various EGTA or TFP concentrations immediately after their addition. In salinized cultures, the assay and analysis were measured after 3 h (short-term experiments) with NaCl, $CaCl_2$, EGTA and TFP. The mean values of three replicates were presented. P_N (oxygen evolution) and R_D (oxygen uptake in darkness) were determined polarographically using an oxygen electrode (*Schott Geräte GmbH*, Germany) under the same irradiance as the growth (5.9 W m^{-2}). The chlorophyll content was determined spectrophotometrically in acetone extracts using the equations recommended by Metzner *et al.* (1965).

The proline was extracted by sulphosalicylic acid and then determined colorimetrically after Bates *et al.* (1973). The total free amino acids were estimated by the Lee and Takahanshi (1966) method. The soluble proteins were determined in the boiling water extracts according to the procedure described by Lowry *et al.* (1951).

Results

Ca^{2+} free medium reduced P_N of *C. vulgaris* (Table 1). EGTA reduced P_N only at the highest concentration (7 mM), but lower concentrations of EGTA enhanced it (Table 1). However, at much lower concentrations than EGTA, TFP markedly inhibited P_N as soon as it was added.

Table 1. Photosynthetic oxygen evolution (P_N) and respiratory oxygen consumption (R_D) of *Chlorella vulgaris* cultures as influenced by EGTA or TFP immediately after addition expressed as percentage of the control (without salinity). P_N and R_D of the control culture were 15.8 and 1.9 mmol(O_2) kg^{-1}(Chl) s^{-1}, respectively.

Treatment	P_N	R_D	P_N/R_D
control	100	100	9.1
-Ca	67	171	4.2
EGTA 2 mM	112	129	8.1
EGTA 4 mM	112	171	6.3
EGTA 7 mM	88	157	5.6
TFP 20 μM	56	86	6.3
TFP 40 μM	56	200	3.3
TFP 100 μM	46	243	2.5

After being salinized for 3 h, P_N was lowered more at a higher NaCl concentration (Table 2). TFP treated cultures exhibited a markedly inhibited P_N with a net O_2 uptake occurring in the light. However, EGTA enhanced P_N of variously NaCl-salinized *Chlorella* cultures.

At a Na^+/Ca^{2+} ratio of 10, P_N was higher in comparison with the isoosmotic reference culture containing only NaCl. However, TFP treated cultures exhibited a markedly lowered P_N in the presence of these combinations (Table 2).

R_D was affected by all treatments. Ca^{2+}-free medium, EGTA and TFP caused an enhanced R_D (Table 1) immediately after addition. NaCl also enhanced R_D after 3 h. Chelation of Ca^{2+} by EGTA in salinized cultures resulted in higher R_D than in the control or salinized cultures not treated with EGTA. However, TFP markedly lowered R_D of salinized cultures (Table 2). The combinations of Na^+ and Ca^{2+} lowered R_D compared with the control (Table 2). However, TFP markedly elevated R_D of the cultures treated with these combinations.

The P_N/R_D ratio was lowered with increasing NaCl level, while it increased at Na^+/Ca^{2+} of 10. TFP markedly lowered this ratio to minimal values.

Table 2. Photosynthetic oxygen evolution (P_N) and respiratory oxygen consumption (R_D) of *Chlorella vulgaris* cultures as influenced by salinization treatments (NaCl or NaCl + $CaCl_2$) in the presence of 7 mM EGTA or 100 μM TFP expressed as percentage of the control (without salinity). P_N and R_D of the control culture were 15.8 and 2.2 mmol(O_2) kg^{-1}(Chl) s^{-1}, respectively.

Treatment	P_N	R_D	P_N/R_D
control	100	100	8.8
100 mM NaCl	69	100	6.4
100 mM NaCl + EGTA	159	83	16.6
100 mM NaCl + TFP	0	43	1.0
200 mM NaCl	65	113	5.4
200 mM NaCl+ EGTA	133	150	8.2
200 mM NaCl + TFP	-3	57	0.5
300 mM NaCl	58	125	4.6
300 mM NaCl + EGTA	133	167	7.5
300 mM NaCl + TFP	-5	57	0.3
Na^+/Ca^{2+} = 5	53	75	6.5
Na^+/Ca^{2+} = 5 + TFP	8	186	1.4
Na^+/Ca^{2+} = 10	95	50	15.8
Na^+/Ca^{2+} = 10 + TFP	-9	186	0.5
Na^+/Ca^{2+} = 15	32	63	5.0
Na^+/Ca^{2+} = 15 + TFP	-20	286	0.4

Table 3. Contents of proline, other free amino acids and soluble and insoluble proteins related to chlorophyll content [g kg^{-1}(Chl)] as influenced by various salinization treatments in the presence of 7 mM EGTA or 100 μM TFP.

Treatment	Proline	Amino acids	Soluble proteins	Insoluble proteins	Total proteins	Ratio sol./insol.
control	35	109	1717	3902	5619	0.44
100 mM NaCl	39	57	1172	2930	4102	0.40
100 mM NaCl + EGTA	89	53	1663	2148	3811	0.77
100 mM NaCl + TFP	24	66	747	5078	5825	0.13
200 mM NaCl	80	61	1235	3125	4360	0.40
200 mM NaCl+ EGTA	107	67	1484	2474	3958	0.60
200 mM NaCl + TFP	33	72	619	4688	5307	0.13
300 mM NaCl	59	65	1953	4102	6055	0.48
300 mM NaCl + EGTA	113	80	1328	3051	4379	0.44
300 mM NaCl + TFP	29	72	765	4427	5192	0.17
Na^+/Ca^{2+} = 5	58	73	931	4011	4942	0.23
Na^+/Ca^{2+} = 5 + TFP	31	109	322	4688	5010	0.07
Na^+/Ca^{2+} = 10	53	81	846	3646	4492	0.23
Na^+/Ca^{2+} = 10 + TFP	31	121	699	2865	3564	0.24
Na^+/Ca^{2+} = 15	50	85	804	3464	4268	0.23
Na^+/Ca^{2+} = 15 + TFP	23	59	547	4818	5365	0.11

Proline was markedly accumulated in the NaCl-salinized *Chlorella* cultures. More or less the same trend but with higher contents of proline was found in the salinized cultures treated with EGTA (Table 3). However, the NaCl in combination with $CaCl_2$ lowered the proline contents compared with that ones in the isoosmotic reference culture containing only 200 mM NaCl. TFP markedly lowered the proline accumulation (Table 3).

The contents of other free amino acids were generally lowered by NaCl. Both EGTA and TFP reduced the amino acid contents of salinized cultures. However, relatively high concentrations of amino acids were induced by Na^+-Ca^{2+} combinations.

Similarly, the contents of total protein was variably lowered under most of the treatments used (Table 3). A drastic lowering of the protein concentration was observed in EGTA treated cultures under NaCl, while the soluble/insoluble protein ratios were highest in these cultures. However, the combinations of NaCl and $CaCl_2$ did not exert drastic changes in the protein content compared with those of the corresponding isoosmotic reference culture containing only 200 mM NaCl.

Discussion

P_N of *C. vulgaris* cultures was reduced in Ca^{2+}-free medium or by the highest EGTA concentration used (7 mM). At much lower concentrations than EGTA, TFP markedly inhibited P_N as soon as it was added. Similar results were obtained by Barr *et al.* (1982) and Burris *et al.* (1983) who agreed that TFP markedly inhibited photosynthesis. The inhibition by TFP implies the involvement of calmodulin in photosynthesis (Barr *et al.* 1982). A calmodulin-like protein was found in the stroma of pea chloroplasts (Jarret *et al.* 1982) and a Ca-calmodulin enzyme may be involved in the activation of chloroplast enzymes (Muto *et al.* 1982). Ca^{2+} ions may be involved in a photosynthesis through the a spillover phenomenon (Gross *et al.* 1976), in the conformational changes in the thylakoids and in the coupling of electron flow (Barber 1976).

After being salinized for 3 h, P_N of *C. vulgaris* was lowered and an addition of TFP entirely inhibited it. This reveals that photosynthesis of calmodulin-inhibited *Chlorella* cells is remarkably sensitive to NaCl.

The Na^+/Ca^{2+} ratio of 10 improved P_N compared with that one of the isoosmotic reference culture containing only NaCl. Thus Ca^{2+} may probably repair the NaCl injured thylakoids. A possibility is that Ca^{2+} reduces the Na^+ uptake and thus its toxicity is diminished. The TFP treated cultures had a low P_N. However, Barr *et al.* (1983), working with non-salinized cultures, found that the addition of Ca^{2+} to the TFP-treated cultures relatively protected the spinach chloroplasts and stimulated the electron transport.

On the other hand, R_D was enhanced in Ca^{2+}-free medium by EGTA and TFP immediately and in salinized cultures after 3 h. Higher R_D was observed in the salinized cultures treated with EGTA. However, TFP markedly lowered the R_D of salinized cultures. In this respect, Ca^{2+} deficiency enhanced R_D (Ferguson 1984)

while high Ca^{2+} contents reduced it (Bangerth *et al.* 1972). A high R_D in Ca^{2+}-chelated cultures may indicate that the stress severity of NaCl is elevated. Consequently, a higher energy demand for osmoregulation, repair of injured tissues and active ion uptake (Gale 1975, Schwarz and Gale 1981, Lambers *et al.* 1983) is required. The R_D rise in Ca^{2+}-deficient cells is probably due to the loss of membrane stability (Ferguson 1984) that includes a breakdown of compartmentation and an increase in the contents of respiratory substrates from the vacuole to the cytoplasm; this finally enhances R_D (Woodrow and Rowan 1972, Ferguson 1984). These processes can be retarded by Ca^{2+} (Bangerth *et al.* 1972). In salinized plants, Na^+ displaces Ca^{2+} from membranes (Lynch and Läuchli 1988) and the loss of membrane stability occurs probably because of Ca^{2+} deficiency. The combinations of Na^+ and Ca^{2+} resulted in a remarkably lower R_D than in the control cultures. However, TFP elevated the R_D of the *Chlorella* cultures subjected to Na^+-Ca^{2+} combinations.

Since the nitrogenous compounds are common factors in osmoregulation, the contents of proline, free amino acids and proteins were considered in this work. Proline was accumulated in the *Chlorella* cultures salinized for 3 h and especially in the cultures treated with EGTA. The chelation of intracellular Ca^{2+} thus elevated the stress severity exerted by NaCl, as in the case of elevated R_D. The physiological significance of proline accumulation may be the osmoregulation (Barnett and Naylor 1966), the provision of both carbon and nitrogen for the post-stress relief (Thompson *et al.* 1966), or the removal of ammonia (Henkel 1966). The Na^+-Ca^{2+} combinations lowered the proline contents compared with the isoosmotic reference culture. In this respect, Dreier (1983) and Imamul Huq and Lahrer (1984) found that Ca^{2+} lowered the proline levels of stressed plants. Since the proline accumulation is simultaneously indicative for the stress damage (Stewart and Hanson 1980), Ca^{2+} may play an important role in the NaCl stress counteraction. On the other hand, the inhibition of proline accumulation under stress by TFP may indicate that calmodulin is involved in the synthesis of proline.

The contents of free amino acids and proteins were lowered under various treatments. A drastic lowering of the total protein content was observed in the presence of EGTA while the soluble/insoluble protein ratios were the highest ones. High ratios of sol./insol. proteins may be due to an increased proteolytic activity.

Generally, salt tolerance of *C. vulgaris* might involve Ca^{2+} and calmodulin. The presence of EGTA elevated proline contents and enhanced R_D. TFP turned *Chlorella* cells less salt tolerant. Nevertheless, Ca^{2+} could not recover the activity of calmodulin in the TFP-treated *Chlorella* cultures.

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