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Short Communication

Evidence for the Occurrence of, and Possible Physiological Role for, Cyanobacterial Calmodulin[1]

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ABSTRACT

Phosphate uptake by the blue-green alga *Oscillatoria limnetica* Lemmerman is stimulated by micromolar concentrations of Ca^{2+}. The calmodulin antagonists 4-(3-[2(trifluoromethyl)phenylthiazin-10-yl]propyl)-1-piperazine ethanol-HCl and its monofluoro-analog inhibit orthophosphate uptake of *Oscillatoria limnetica* by over 97% implying involvement of calmodulin in this process. A calmodulin-like protein was quantitated in cell-free extracts from *O. limnetica* by radioimmunoassay.

Phosphate uptake has been investigated in a range of organisms including bacteria, algae, fungi, and higher plants (2, 5, 12, 14, 18–22). While stimulation of Pi uptake by various cations has been widely reported (5, 11, 18–21, 23), our work has concentrated on the effect of Ca^{2+} on Pi uptake. Low concentrations (2 μM) of Ca^{2+} have been shown to stimulate Pi uptake by the blue-green alga *Anacystis nidulans* (UTEX 625) (5, 20). In our continuing studies, we have found Ca^{2+} stimulation of Pi uptake in another blue-green alga *Oscillatoria limnetica* (Lemmerman) (11). The stimulation was observed at micromolar levels of Ca^{2+} and Pi and inhibited by CaM[2] antagonists, suggesting a possible involvement of CaM in this process. Calmodulin or CaM-like proteins seem to be ubiquitous in animal and plant cells (3, 9) and there is a report of a CaM-like protein in *Escherichia coli* (8). Herein, we suggest the occurrence of a CaM-like protein in a photosynthetic prokaryote and propose a possible physiological role for this regulator.

MATERIALS AND METHODS

Oscillatoria limnetica was isolated from Lake Opinicon, Ontario, by Karen Jones. Stocks were maintained at 25°C in medium modified by Daley and Brown (4) from Hughes *et al.* (6). Experimental cells were grown in half-strength medium bubbled with 0.5% CO_2-enriched air and harvested in mid-log phase (36 h after inoculation). Illumination of stock and experimental cells was provided by 3 Gro-Lux and 1 Warm White fluorescent tubes and was 110 μE m^{-2} s^{-1}.

Cells were harvested by centrifugation at 4000*g* for 5 min and washed in 5 mM Tris-H_2SO_4 buffer (pH 8.0 at 25°C). The cells were then Pi-starved by resuspension in half-strength growth medium lacking Pi. After 4 h, cells were harvested, washed, and resuspended to an A_{660nm} of 0.05 in 5 mM Tris-H_2SO_4 buffer.

Initial rates of Pi uptake were determined using a light table described elsewhere (19) that provided illumination of 100 μE m^{-2} s^{-1}. Aliquots of 25 ml of the cell suspension were put into 50-ml Erlenmeyer flasks on top of the light table, and bubbled with moist air. Studies on the kinetics of Pi uptake were initiated by the addition of ^{32}Pi solutions to the flasks. Ca^{2+} (as $CaCl_2$ was added 1 min prior to the Pi solutions. Uptake was terminated by filtering the cells from the medium using a vacuum filtration system and 0.4-μm Metricel filters (Gelman, Ann Arbor, MI), a procedure that took 3 to 5 s. The filters and samples were placed in low-K glass scintillation vials containing 15 ml of 5% (w/v) $K_2S_2O_8$ (to decolorize the cells). The extent of Pi uptake was determined by measuring the Cerenkov radiation in a liquid scintillation counter.

Fluphenazine-HCl was added to the cells to a concentration of 50 μM 5 min prior to addition of the Pi solutions. The cells were collected after 1 min of exposure to the labeled Pi and Pi uptake was determined as above.

For the quantitation of the CaM-like protein, *O. limnetica* cells were suspended in homogenization buffer (125 mM borate buffer with 75 mM NaCl and 1 mM EGTA, pH 8.4), and broken in a French pressure cell. The homogenate was then rapidly brought to a boil, quenched on ice, then centrifuged at 10,000*g* for 30 min. RIA was performed according to the manufacturers directions (Caabco). Briefly, aliquots of the supernatant along with CaM standards were mixed with assay buffer (homogenization buffer plus 0.02 g/l BSA), ^{125}I CaM, and sheep anti-rat testis CaM antibodies, and incubated for 18 h at 25°C. Next, staphylococcus-A cells were added to all tubes which were then mixed and incubated for 30 min at 25°C. The tubes were centrifuged at 1000*g* for 10 min. The activity of ^{125}I in each pellet was determined in a gamma counter and corrected for nonspecific binding. The values were divided by the activity in total bound samples (which contained no CaM) and expressed as a percentage of the total bound. A standard curve of CaM concentration was prepared by plotting the values of the per cent total bound of the CaM standards as a function of their CaM concentration on log/logit paper.

Chl *a* was determined by the method of Rigby (19), and protein was quantitated by the method of Lowry *et al.* (15), using BSA as the standard. The radioisotope ^{32}Pi (carrier free as orthophosphoric acid in 20 mM HCl) was purchased from New England Nuclear. Fluphenazine-HCl was the kind gift of Dr. P. Abadie (Squibb Canada, Montreal). The CaM RIA kit was obtained from Caabco Inc. (Houston, Texas). Deionized H_2O was prepared by passing distilled H_2O through a Barnstead Ultrapure deionizer cartridge. The specific resistance was always greater

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[2] Abbreviations: CaM, calmodulin; fluphenazine, 4-(3-[2-(trifluoromethyl)phenylthiazin-10-yl]propyl)-1-piperazine ethanol; RIA, radioimmunoassay.

than 1 megaohm/cm^3. All other chemicals were analytical grade (J. T. Baker).

RESULTS

The effect of 2 μM Ca^{2+} on Pi uptake (at 4 μM) of *O. limnetica* is shown in Figure 1. At these concentrations the stimulation of Pi uptake was approximately 35%.

Cells were treated with fluphenazine-HCl and the effect on Pi uptake is shown in Table I. Treated cells had rates of Pi uptake less than 3% of control cells in both the presence and absence of added Ca^{2+}. When fluphenazine treated cells were washed by two cycles of pelleting and resuspension they remained photosynthetically competent, albeit at rates lower than those of control cells. This suggests that the fluphenazine results are not simply a general toxic response. Two phenothiazine drugs used in this study (fluphenazine and its monofluoro-analog) gave identical results. Calcium plus 1 mM EGTA resulted in rates equal to the control minus $CaCl_2$.

The standard RIA curve is shown in Figure 2. The presence of a CaM-like protein in *O. limnetica* was always indicated by RIA. The concentration of CaM-like protein in *O. limnetica* was

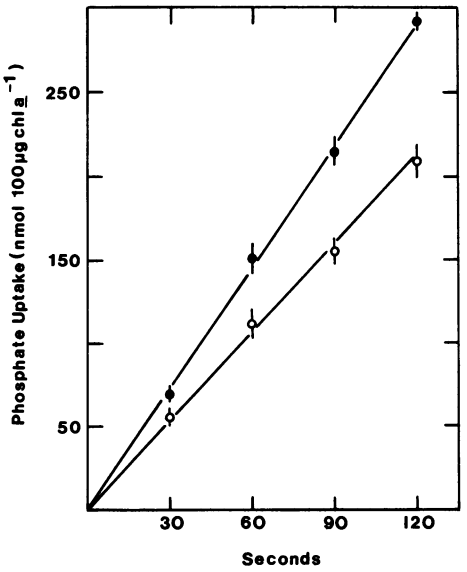

FIG. 1. The effect of Ca^{2+} on Pi uptake. Log phase *O. limnetica* cells were suspended in 5 mM Tris-H_2SO_4 buffer (pH 8.0 at 25°C) to a concentration of 0.17 μg Chl *a*/ml. Uptake of ^{32}Pi was determined in the presence (●) and absence (○) of 2 μM $CaCl_2$. The Pi concentration was 4 μM. When present, the $CaCl_2$ was added 1 min before the Pi. The bars represent the SE; $n = 5$.

Table I. *Effect of Fluphenazine on Pi Uptake by O. limnetica*

Cells were suspended in Tris-H_2SO_4 as described in Figure 1. Phosphate upake was initiated 1 min after addition of $CaCl_2$ and 15 min after addition of fluphenazine-HCl and EGTA. Control cells were exposed to Pi only. The concentrations were: 4 μM Pi; 20 μM Ca^{2+}; 50 μM fluphenazine-HCl; and 1 mM EGTA. The Pi uptake values are the mean ± SE; $n = 5$.

Treatment	Pi Uptake	Control
	nmol/100 µg Chl a·min	%
Control	172 ± 8	100.00
Pi + Ca^{2+}	263 ± 12	152.91
Pi + EGTA	174 ± 5	101.16
Pi + fluphenazine-HCl	5 ± 0.2	2.91
Pi + Ca^{2+} + fluphenazine-HCl	5 ± 0.2	2.91

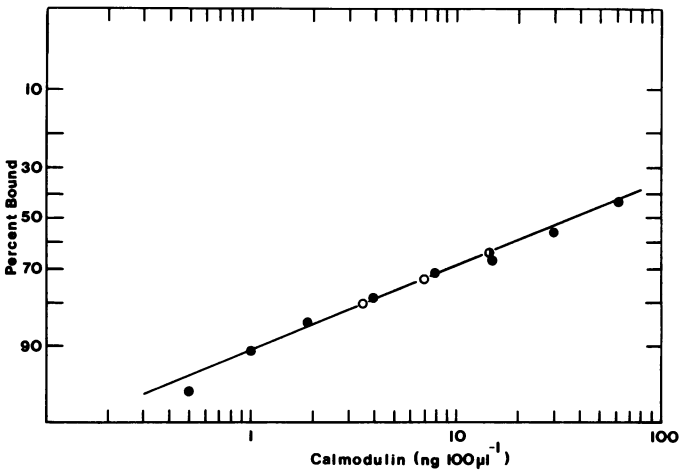

FIG. 2. Calmodulin radioimmunoassay. Standards were from 0.5 to 125 ng bovine testis CaM 100 μl^{-1} (●). The experimentals contained in a constant final volume: (○), 50 or 100 μl *O. limnetica* extract; (◑), 100 μl *O. limnetica* extract + 100 μl bovine testis CaM (7.8 ng ml^{-1}).

6302.5 + 189.0 ng/100 μg Chl *a* or 52.6 + 1.6 ng/μg total protein (the values are means + SE, $n = 4$). Doubling the volume of *O. limnetica* extract in the assay mixture resulted in a doubling of the amount of CaM observed (3.5–7.0 ng). Addition of 100 μl of *O. limnetica* extract (containing 7 ng CaM) to 100 μl of a CaM standard (7.8 ng) gave a very similar result to that observed with 200 μl of the same CaM standard, 14.5 *versus* 15.6 ng.

DISCUSSION

It has been shown that the consistent effect of Ca^{2+} is to both lower the K_m and increase the V_{max} of Pi uptake by *O. limnetica* (10). Stimulations by equimolar $CaCl_2$ and $CaSO_4$ were equal in magnitude, demonstrating that it was Ca^{2+}, and not the counterion, responsible for the stimulation (unpublished results; a detailed characterization of Pi uptake by *O. limnetica* is currently in preparation).

The mechanism by which Ca^{2+} stimulates Pi uptake is unknown although several hypotheses have been proposed (5, 7, 21, 23), one of which is that Ca^{2+} acts by masking cell surface negative changes. This has been shown not to be the case with the blue-green algae *A. nidulans* and *O. limnetica* (11, 20). The stimulation of Pi uptake by low levels of Ca^{2+} suggested a possible involvement of CaM in this process. The antipsychotic phenothiazine drugs are notable CaM antagonists (13) and the effect of fluphenazine-HCl and its monofluoro-analog on Pi uptake (Table I) was consistent with this hypothesis. Additional suggestive data were obtained by RIA of cell-free homogenates. We recognize that data obtained by RIA using antibodies raised against rat testis CaM are not unequivocal. In view of the substantial structural similarity found for all CaMs thus far examined (9, 25) our data are, however, strongly suggestive.

Attempts have been made to quantify the CaM-like protein by CaM-dependent phosphodiesterase activation. Activation was observed but an increase in activation concomitant with an increase of *O. limnetica* extract in the assay was not. Apparently some factor in the extract interferes with the assay above a certain concentration.

A recent report (17) suggests that methods used to quantitate CaM from animal sources may underestimate plant CaM concentrations, presumably due to slight structural differences. It is therefore likely that our results represent a minimum estimate of CaM concentration.

Only a single report of a CaM-like protein in a prokaryote is presently in the literature (8). In light of recent reports on the

localization of CaM in higher plant chloroplasts (10, 17), it is perhaps not surprising that we have found a CaM-like protein present in a blue-green alga. There has accumulated a substantial body of biochemical evidence in support of the theory that chloroplasts evolved by endosymbiotic capture of primitive blue-green algae (16). Consistent with this is the finding that phenothiazine drugs inhibit photosynthesis in chloroplasts and blue-green algae (1, 10; A. G. Miller, personal communication). Chloroplasts and blue-green algae carry out oxygenic photosynthesis in much the same manner, and since CaM has been implicated as having a regulatory role in chloroplast photosynthesis (10), it is not improbable that CaM could serve to regulate several physiological processes, including Pi uptake, in blue-green algae.

Using the cloned electric eel CaM gene, C. Vann and L. Sherman (personal communication) have obtained a positive signal when probing a library of *A. nidulans* R2 DNA, providing additional support for the occurrence of CaM-like proteins in cyanobacteria. When sequence information for the cyanobacterial gene becomes available, the semantic controversy between CaM and CaM-like should be resolved.

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