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Involvement of the Plasma Membrane ATPase in the Osmoregulatory Mechanism of the Alga *Dunaliella salina*

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ABSTRACT

The unicellular halotolerant alga *Dunaliella salina* recovers normally from a hypertonic shock even when suspended in NaCl and buffer only. Furthermore, addition of Cu^{2+}, valinomycin and KCl, or permeable ions such as methyltriphenylphosphonium or thiocyanate, do not affect the recovery. However, treatment with two specific inhibitors of the plasma membrane adenosine triphosphatase (ATPase), diethylstilbestrol, or vanadate, fully inhibit the recovery. The inhibition is manifested by the inability of the cells to both synthesize glycerol and return to their original volume. The inhibitions are nonlethal, reversible and equally effective in the dark or the light. Since the plasma membrane ATPase is the only enzyme known to be inhibited by both diethylstilbestrol and vanadate, it is concluded that its activity is essential for the recovery of *Dunaliella* from a hypertonic shock. Mechanisms by which the plasma membrane ATPase may participate in the activation of glycerol production in the algae are discussed.

Members of the genus *Dunaliella* are motile unicellular algae, which lack a rigid polysaccharide wall. Instead, the algae are natural protoplasts enclosed by an elastic plasma membrane (15). *Dunaliella* is distinguished by the very wide range of salt concentrations tolerated by its species. *Dunaliella salina* can grow in salinities ranging from 0.1 M to saturated NaCl solutions (7).

Dunaliella adjusts to an osmotic shock by first shrinking or swelling, in response to an upshock or a downshock, respectively, behaving like a perfect osmometer. This is followed by a period of metabolic adjustment which lasts about 2 h, at which time the cells return to approximately their original volume. The major osmolyte responsible for this adjustment is glycerol, which is synthesized or eliminated during this period (4).

Several novel enzymes have been characterized in *Dunaliella* (5, 19, 29) and a metabolic cycle of glycerol synthesis and elimination has been proposed (8, 30). However, little is known about the control sites of glycerol metabolism, or about the initial triggering signal which induces the metabolic recovery from an osmotic shock.

In this communication we describe attempts to identify the signal which triggers glycerol metabolism during recovery from an osmotic shock. We show that inhibition of the plasma membrane H^+-ATPase, previously characterized in *Dunaliella* (18, 22, 28), inhibits recovery of the algae from a hypertonic shock. This suggests that the activity of the plasma membrane ATPase is an essential feature of the signal initiating recovery from shock.

MATERIALS AND METHODS

Growth Conditions

Dunaliella salina were grown in media containing 1 M NaCl as previously described (9). For experiments in which vanadate was added, the algae were first transferred from the regular medium containing 0.2 mM phosphate to one containing no phosphate for 24 h. As will be shown this markedly increases the effect of vanadate, probably by decreasing the intracellular concentration of phosphate which competitively interferes with the action of vanadate (16). For experiments involving a hypertonic shock in the dark, the algae were first grown for 2 d in a medium containing 0.5 mM instead of 5 mM KNO_3 in order to increase the starch content of the cells (10).

Hypertonic shocks were from 1 M to 2 M NaCl and were performed by diluting cells, suspended in either 1 M NaCl + 10 to 50 mM Hepes (pH 7) or 1 M NaCl medium, at a volume ratio of 1:1 with a 3 M NaCl solution containing 10 to 50 mM Hepes (pH 6.8–7.0).

Assays

Cell volume was determined using a Coulter counter ZM, glycerol concentration as described by Ben-Amotz and Avron (6), O_2 evolution with an oxygen electrode, and intracellular ATP level with purified luciferase and luciferin using the Lumac/3M Biocounter.

Treatment with DES[1]

Cells were centrifuged and resuspended in 1 M NaCl + 10 mM Hepes (pH 7) to a final concentration of 2×10^6 cells $\times$ mL^{-1}. Following a 15 min incubation in the light, different DES concentrations were added to the algae. It was found that prolonged incubation with DES, even at very low concentrations (10 μM) was lethal for *Dunaliella*. Therefore the cells were incubated with DES for only 20 min in the light. The DES was then removed by centrifugation and resuspension in the same medium, the cells incubated for 15 min and osmotic shocks performed.

[1] Abbreviation: DES, diethylstilbestrol.

Treatment with Vanadate

Following the incubation in the phosphate-free medium, the pH of the cell suspension was adjusted to 6.8, 50 mM Hepes (pH 6.8) added, and the cells incubated for about 15 min in the light. The desired concentrations of vanadate were added to the suspension, and incubation continued for another 50 to 60 min in the light. Shocks were then performed without removing vanadate from the medium.

RESULTS

Components That Do Not Inhibit Recovery

In an attempt to identify the signal which triggers recovery of *Dunaliella* from osmotic shocks, we tried first to inhibit recovery by eliminating different components from the reaction medium during the shocks. The possibility that calcium influx is an initial signal for recovery from a hypoosmotic shock, was suggested, based on reports by Riisgard (24, 25) that showed that low concentrations of copper (about 20 μM) inhibited recovery of *Dunaliella marina* from a hypoosmotic shock. However with *D. salina*, addition of copper up to 0.2 mM, lack of calcium in the outer medium and addition of EGTA (2 mM), or addition of ions competing with calcium such as La^{3+} and Cd^{2+} (23) at concentrations of 100 μM had no influence on the ability of *D. salina* to recover from a shock (data not shown).

The possibility that other components of the medium are needed for recovery from hypertonic or hypotonic shocks, was eliminated when it was found that *Dunaliella* recovers normally from either type of shock when suspended in NaCl (or choline-chloride, Fig. 1) and buffer only.

Another possible signal may be changes in the membrane potential across the plasma membrane of the algae during shock. However, addition of valinomycin and KCl (5 μM and 30 mM, respectively) or permeable ions such as methyltriphenylphosphonium (100 μM) and SCN^- (50 mM) to algae suspended in NaCl and buffer did not affect the recovery from either a hypertonic or a hypotonic shock (data not shown).

Clearly *Dunaliella* possesses a very sturdy mechanism for recovery from osmotic shocks even under extremely stressful conditions. In some of the conditions described above, the algae cannot survive for more than a few hours, but nevertheless they recover normally from an osmotic shock. However, the recovery from an osmotic shock is metabolic in nature, since transfer to low temperature (4°C) completely prevents recovery (data not shown).

Inhibition of Recovery by DES

DES, a specific inhibitor of the plasma membrane H^+-ATPase (13, 21, 26, 27), was found to inhibit the volume recovery of *Dunaliella* cells exposed to a hypertonic shock (Fig. 2). The extent of inhibition was dependent on the concentration of DES with which the cells were pretreated. Shrinkage of the cells upon transfer from 1 M NaCl to 2 M NaCl was not affected, but the metabolic recovery to their original volume was totally inhibited by 20 μM DES (Fig. 2A).

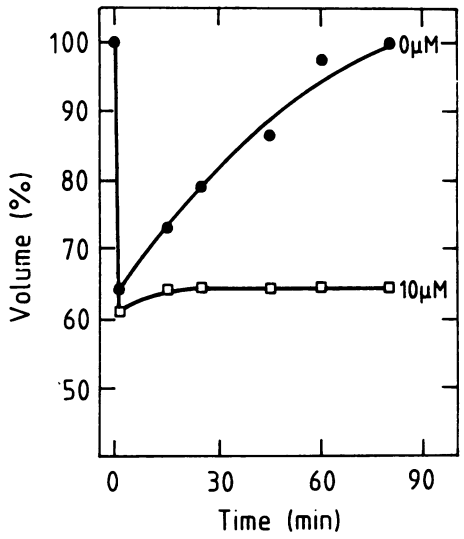

Figure 1. Effect of vanadate on recovery from a hypertonic shock in choline-chloride media. Change in volume during recovery from a hypertonic shock from 1 M to 2 M choline chloride, in *D. salina*. Where indicated 10 μM vanadate were added as described under "Materials and Methods." The algae were transferred by centrifugation and resuspension, to a choline chloride solution and 50 mM Hepes (pH 6.8) 5 min before the shock.

The inhibition of volume recovery and glycerol content were closely correlated (Fig. 2B), supporting the prevailing notion that volume recovery is dependent upon the cells' ability to synthesize glycerol (7).

DES inhibition was found to be reversible. When algae treated with 20 μM DES, that did not recover from an upshock, were incubated in fresh medium overnight, they recovered fully and then continued to grow and multiply normally (data not shown). The DES inhibition is therefore relatively mild, specific, and not lethal.

Recent reports suggest that DES may not be totally specific to the plasma membrane ATPase (2, 21) and may inhibit other ATPases in the cell. In order to check that the inhibition of recovery is not merely a result of the inhibition of photosynthetic ATP synthesis, we tested the effect of exposing DES treated algae to a hyperosmotic shock in the dark. Since *Dunaliella* needs high intracellular concentrations of starch in order to recover from an osmotic upshock in the dark, the algae were pregrown in a nitrate limited medium, which enhances starch accumulation (see "Materials and Methods"). Recovery was not complete, but was clearly as sensitive to DES in the dark as it was in the light (Fig. 3). The inhibition by DES in the dark was also reversible when the inhibited cells were transferred to normal growth conditions (data not shown). Treatment with 10 μM DES had no influence on the rate of respiration of *Dunaliella* (data not shown). The ATP concentration of DES treated cells in the light was not significantly different from that of untreated algae, and remained so for at least two hours after treatment (not shown). Thus the inhibition of osmoregulation by DES does not seem to be due to a general membrane permeability effect or to metabolic inhibition of photosynthetic ATP production.

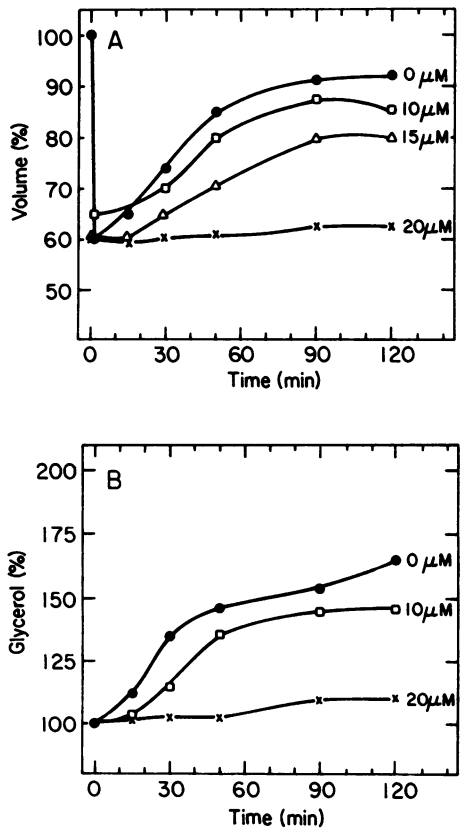

Figure 2. Effect of DES on recovery from a hypertonic shock in the light. Change in volume (A) and glycerol content (B) during recovery from a hypertonic shock from 1 M to 2 M NaCl with 20 mM hepes (pH 7.5), *D. salina* treated with the indicated concentrations of DES, as described under "Materials and Methods." Volume of 100% and glycerol correspond to 110 fL and 15 pg/cell, respectively.

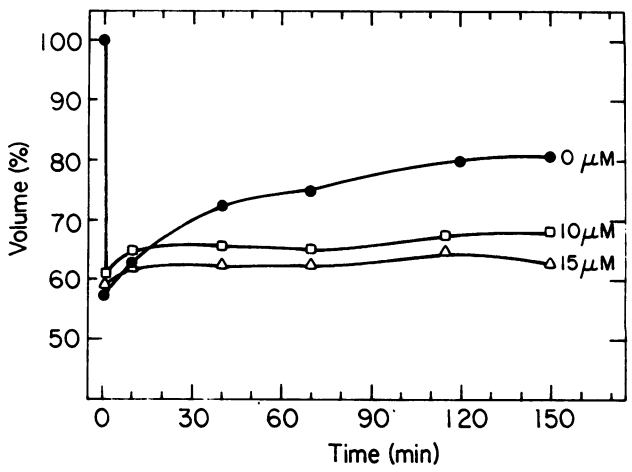

Figure 3. Effect of DES on recovery from a hypertonic shock in the dark. Change in volume during recovery from a hypertonic shock from 1 M to 2 M NaCl in the dark after treatment of *D. salina* with DES, at the indicated concentrations, as described under "Materials and Methods." Volume of 100% corresponds to 115 fL/cell. To enable the algae to recover from an upshock in the dark, their starch content was increased by growing them in a nitrate limited medium (see "Materials and Methods").

Inhibition of Recovery by Vanadate

Vanadate was shown to inhibit the plasma membrane ATPase by forming a stable vanadate complex that locks the enzyme in one conformation (20). Since vanadate inhibition is competitive with phosphate (16), and since the active site of the ATPase is within the cell, it is necessary to prestarve the algae for phosphate prior to treatment for maximal inhibition (see below). Vanadate uptake into the cells is presumably via the phosphate uptake system. After 24 h of starvation for phosphate the cells looked normal and recovered normally from an upshock (Fig. 4). Under these conditions, very low concentrations of vanadate inhibited the volume recovery (Fig. 4). Inhibition of 50% of the volume recovery was effected by around 2 μM vanadate. Inhibition due to vanadate treatment correlated with inhibition of glycerol synthesis, as was found with DES (Fig. 4).

The inhibition by vanadate does not depend on Na^+. *Dunaliella* cells exposed to an upshock in a choline chloride solution recover normally and vanadate inhibited this recovery equally well (Fig. 1).

Vanadate inhibition is also reversible. When treated algae were incubated for 24 to 48 h in a normal medium containing 200 μM phosphate and pH 8.2, they recovered fully. This was true even when the cells were treated with very high concentrations of vanadate (up to 200 μM) and vanadate was not removed from the medium (data not shown).

The concentration of vanadate needed to inhibit the recovery from shock was dependent on the pretreatment of the cells to lower their internal phosphate (Table I). When *Dunaliella* were preincubated in 20 μM phosphate for 24 h (1/10 the concentration in normal medium), 50 μM vanadate were required to cause the same effect as 2 μM in cells preincubated for 24 h with no phosphate. Cells preincubated in normal medium (200 μM phosphate) were not inhibited by concentrations of vanadate up to 500 μM.

Dunaliella treated with vanadate contained a higher ATP concentration than untreated cells (Fig. 5). The ATP content increased by up to 25%, 90 min after the addition of vanadate. This is in agreement with the notion that the plasma membrane ATPase is inhibited and is a major user of the available ATP.

The rate of photosynthesis of *Dunaliella* treated with vanadate decreased with time (Fig. 6). However, recovery from an upshock was as sensitive to vanadate in the dark as it was in the light (data not shown). The inhibition of photosynthesis occurred in two phases: a rapid decrease of about 30% followed by a slower decrease. The first phase may be a result of the rapid immobilization of the algae upon vanadate addition. This immobilization may be due to inhibition of the flagella ATPase (16) and is less dependent on the internal phosphate concentration than the inhibition of the plasma membrane ATPase (data not shown). The second phase correlates with the increase in the intracellular ATP concentration (Fig. 5). It appears, therefore, that the inhibition of photosynthesis may be a secondary result of the increase in cellular ATP concentration. The rate of respiration was not affected in vanadate treated cells, and did not change with time (Fig. 6).

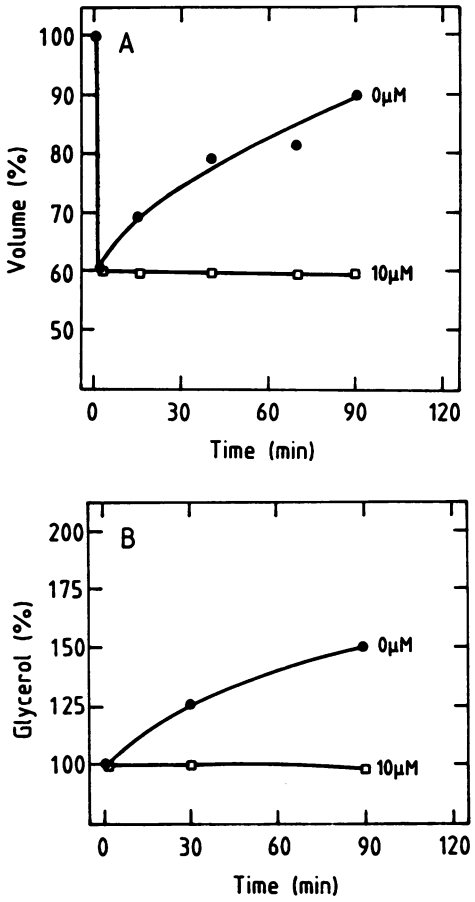

Figure 4. Effect of vanadate on recovery from a hypertonic shock in the light. Change in volume (A) and glycerol content (B) during recovery from a hypertonic shock from 1 M to 2 M NaCl, in *D. salina* treated with 10 μM vanadate, as described under "Materials and Methods." Volume of 100% and glycerol correspond to 100 fL and 16 pg/cell, respectively.

Table I. *Dependence of Vanadate Inhibition on the Phosphate Concentration in Which the Cells Are Pregrown*

D. salina were grown for 24 h in complete media containing no phosphate, 20 μM or 200 μM phosphate (normal medium concentration), and then treated with different concentrations of vanadate as described in "Materials and Methods." The algae were exposed to a hypertonic shock from 1 M to 2 M NaCl and the percent volume recovery after 2 h is indicated: 100% corresponds to 105 fL.

Phosphate Concentration during Pretreatment	Recovery with Vanadate Concentration (μM):				
	0	2	10	50	200
μM			%		
0	98	50	0	0	0
20	95	95	61	42	25
200	93	96	95	100	98

DISCUSSION

D. salina can recover from an osmotic shock under extremely stressful conditions. Apparently no specific external component is needed for recovery, since *Dunaliella* can recover normally when suspended in either buffered NaCl or in buffered choline chloride. Moreover, various agents which supposedly affect the transmembrane electrical potential did not inhibit the recovery from shock.

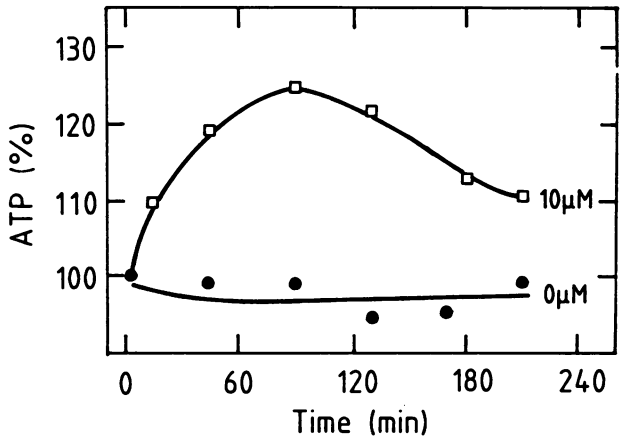

Figure 5. Effect of vanadate on intracellular ATP concentration. ATP concentration of *D. salina* was determined at the times indicated with and without treatment with 10 μM vanadate as described in "Materials and Methods." One hundred percent = 3 mM ATP.

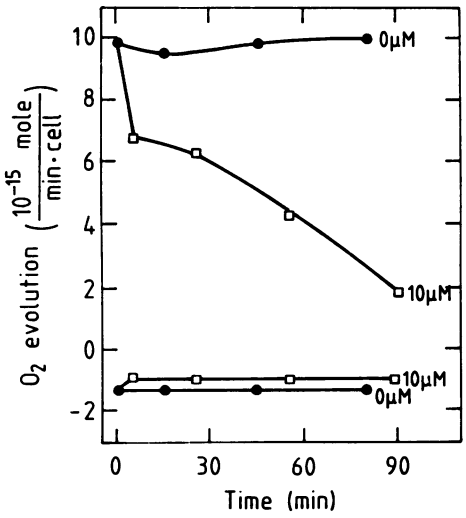

Figure 6. Effect of vanadate on photosynthesis and respiration. The rate of photosynthesis and respiration in *D. salina* treated with 10 μM vanadate, was measured using an O_2 electrode. For photosynthetic oxygen evolution the cell suspension was illuminated by a slide projector through a 550 nm cutoff filter and a heat filter. Incident light intensity was 3.6×10^3 J/m^2·s.

Vanadate-sensitive H^+-ATPases have been demonstrated in plasma membranes of plant, fungal, algal and yeast cells and are considered as the major primary generators of pH and electrical gradients across the cell membranes and thereby of the driving force for accumulation of organic and inorganic metabolites into the cells (26). Existence of a vanadate, DES, and dicyclohexylcarbodiimide sensitive ATPase in plasma membrane preparations from *Dunaliella* has been demonstrated (16, 18, 22, 28). Moreover, inhibitors of the plasma

membrane ATPase induce an increase in intracellular Na^+ and a decrease in intracellular K^+ concentrations suggesting that this enzyme provides the driving force for transport across the cell membrane (22).

In this study we demonstrated that DES and vanadate, both specific inhibitors of the plasma membrane ATPase, severely inhibit recovery of *D. salina* from a hypertonic shock. The inhibitions were reversible and nonlethal, and were therefore not due to a general metabolic inhibition of the cells. Even though these inhibitors are not totally specific for the plasma membrane ATPase, this is the only enzyme known to be inhibited by both. We conclude therefore that the plasma membrane ATPase has a central role in the mechanism of recovery of *Dunaliella* from a hypertonic shock.

It is not clear at present whether activation of the ATPase is required for recovery, or just the presence of an active enzyme. Braun *et al.* (11) showed in roots of higher plants an increase in the activity of the plasma membrane ATPase due to increase in salinity. Assuming that the ATPase is activated in *Dunaliella* due to an osmotic shock, and that this is a primary event in the process of recovery, three alternative mechanisms may be suggested:

1. Activation of proton extrusion from the cells leads to internal alkalinization. Recently, Goyal *et al.* (17) reported that following osmotic upshocks there is indeed a significant internal alkalinization in *Dunaliella.* Furthermore, it was demonstrated that several enzymes in *Dunaliella* which are involved in starch degradation have an alkaline pH optimum. It is conceivable, therefore, that internal alkalinization brought about by activation of the plasma membrane H^+-ATPase may play a role in recovery from osmotic upshocks.

2. The activated plasma membrane ATPase is a major consumer of ATP during recovery; an increase in the local inorganic phosphate concentration may be expected. Goyal *et al.* (17) reported that the enzyme glucan phosphorylase increases in activity as a function of increasing concentrations of inorganic phosphate. This enzyme catalyzes the first step in the synthesis of glycerol from starch during a hypertonic shock (12).

3. The decrease in ATP concentration or an increase in ADP concentration due to activation of the ATPase may stimulate an enzyme in the glycerol synthesis pathway. A decrease in the ATP concentration of the cells following an upshock has indeed been reported (3, 14). The stimulation may be due to the effect of a decreased ATP level, or more effectively by the larger decrease in the "energy-charge" (1) which will be affected by both the decrease in ATP and the increase in ADP and inorganic phosphate concentrations.

It is not clear at present how the plasma membrane ATPase is triggered by an increase in osmolarity. Possibly the enzyme undergoes a conformational change following a physical change in the plasma membrane. Shrinking or swelling following hypertonic or hypotonic shocks involve dramatic changes in the plasma membrane of *Dunaliella* which may be sufficient to affect the activity of this membrane bound enzyme.

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