Hydrolysis of Polyphosphates and Permeability Changes in Response to Osmotic Shocks in Cells of the Halotolerant Alga Dunaliella

Meira Weiss, Michal Bental, and Uri Pick*

Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel (M.W., U.P.); and Department of Physiology, University of Pennsylvania, 37th Street, Philadelphia, Pennsylvania 19104 (M.B.)

ABSTRACT

The effects of osmotic shocks on polyphosphates and on the vacuolar fluorescent indicator atebrin have been investigated to test whether acidic vacuoles in the halotolerant alga Dunaliella salina have a role in osmoregulation. Upshocks and downshocks induce different patterns of polyphosphate hydrolysis. Upshocks induce rapid formation of new components, tentatively identified as 5 or 6 linear polyphosphates, formed only after upshocks with NaCl and not with glycerol, indicative of compartmentation of Na+ into the vacuoles. Conversely, downshocks induce a slower transient accumulation of tripolyphosphates, indicating activation of a different hydrolytic process within the vacuoles. Osmotic shocks do not lead to release of atebrin from acidic vacuoles, indicating that they do not induce a major intravacuolar alkalinization. However, osmotic shocks induce transient permeability changes measured by amine-induced atebrin release from vacuoles. Hypoosmotic shocks transiently increase the permeability (up to 20-fold), whereas hyperosmotic shocks induce a rapid drop in permeability. Electron micrographs of osmotically shocked cells also reveal transient changes in the surface and internal organelles of D. salina cells. It is suggested that hyperosmotic and hypoosmotic shocks induce different changes within acidic vacuoles and in the organization and/or composition of the plasma membrane in Dunaliella.

The aim of the studies described herein was to find out whether acidic vacuoles in the halotolerant alga Dunaliella have a role in osmoregulation. This possibility seemed feasible because (a) recent studies in plants have demonstrated a correlation between exposure to high NaCl in salt-tolerant species and activation of vacuolar ion transport systems involved in Na+ sequestration (8, 18); (b) x-ray microanalysis studies indicated that in Dunaliella tertiolecta, Na+ is compartmentalized into vacuoles (10); and (c) we have recently observed a rapid Na+ influx into Dunaliella cells after hyperosmotic shocks, which seems to result from rapid cytoplasmic acidification (20). A likely possibility to explain this phenomenon is a H+ efflux from the vacuoles to the cytoplasm coupled to Na+ elimination into the vacuoles. The presence of acidic vacuoles in Dunaliella has been recently demonstrated from 31P-NMR and staining with cationic dyes (13).

Two probes were utilized to test whether salt induces changes in acidic vacuoles. First, the fluorescent indicator atebrin (6-chloro-9[4-(diethylamine)-1-methylbuthylamino]-2-methoxyacridine), previously used to label acidic vacuoles in yeast and algae (12, 19), was used to label acidic vacuoles in Dunaliella. We have recently demonstrated that atebrin accumulates specifically in acidic vacuoles in D. salina in response to internal acidification, and as such serves as an indicator for intravacuolar pH changes (21). The dye can be released by amines, and the rates of its entry and release are limited by the permeability of the cell membrane and by the capacity of the acidic vacuoles (16). Second, polyphosphates that are located within acidic vacuoles in Dunaliella and are hydrolyzed in response to intracellular pH changes (17) were utilized to probe changes within the vacuoles. The effect of salt was tested by comparison of cells that have been adapted to different salinities and by inducing osmotic upshocks or downshocks to D. salina cells.

MATERIALS AND METHODS

Atebrin Uptake and Release from D. salina Cells

D. salina cells were cultured in 0.1 to 4.0 mM NaCl medium as previously described (4). Incubation with atebrin was performed in cell suspensions containing 1 to 3 x 10⁶ cells/mL, 20 mM Tris-Cl, pH 9, 5 mM KCl, 5 mM MgCl₂, NaCl (as indicated), and 3 μM atebrin hydrochloride at 24°C. For atebrin release experiments, cells were preloaded for 20 min as described above, centrifuged for 10 min at 2000 rpm, and resuspended in fresh medium without atebrin. Atebrin fluorescence was measured in a Perkin-Elmer MF44A spectrofluorimeter with the excitation and emission wavelengths set at 359 and 505 nm, respectively (21).

Analysis of Polyphosphate Hydrolytic Products

Labeling of cells with 32Pi, extraction, and analysis of polyphosphates was performed essentially as described in the preceding paper (17). In brief, cells were cultured for 2 to 3 d with 32Pi, extracted with 2 M formic acid. Lyophilized extracts were separated on PEI-cellulose plates, developed in 1.5 M KH₂PO₄, pH 3.5, and the plates were either analyzed by autoradiography or by cutting out individual spots for measurement of 32P content. It should be noted that this extraction

---

1 Abbreviations: PEI, polyethyleneimino; 5–6 PP, 5 or 6 linear polyphosphates.
procedure extracts only polyphosphates of intermediate chain length and not the long-chain polyphosphates.

RESULTS

Effects of NaCl Concentration and of Osmotic Shocks on Polyphosphates in Dunaliella

In a previous work, we demonstrated that amines at alkaline pH induce the hydrolysis of long-chain polyphosphates to tripolyphosphate in response to intravacuolar pH changes in *D. salina* (17). Analysis of acid extracts of $^{32}$P-labeled *D. salina* cultures adapted to a wide range of NaCl concentrations (0.1–3 M) demonstrates that ammonia at pH 9 induces the appearance of tripolyphosphate, and of a few minor short-chain polyphosphates, at all salt concentrations (Figure 1). However, additional polyphosphate components, which seem unrelated to the amine stress, are present in high-salt cultures.

Components that comigrate with 5 or 6 linear polyphosphate chains, as well as longer polyphosphates that remain at the origin (n ≥ 10), are apparent in extracts of cells cultured in 2 or 3 M NaCl. To test whether changes in salt concentration induce the hydrolysis of polyphosphates, $^{32}$P-labeled cells were exposed to a hyperosmotic shock. As is demonstrated in Figure 2A, an osmotic shock from 0.5 to 1.25 M NaCl induces a rapid appearance of hydrolytic products (completed within 2 min) similar to those present in high-salt adapted cells, without osmotic shocks (Fig. 2B). Conversely, hyperosmotic shock of the same magnitude carried out in a glycerol medium does not induce polyphosphate hydrolysis (Fig. 2C). It may be noted that the permeability of *Dunaliella* cells to glycerol is exceptionally low, as might be expected for an organism that accumulates masses of glycerol in the cytoplasm for osmotic regulation (3). Therefore, glycerol is an adequate

Figure 1. Effect of NaCl concentration on amine-induced polyphosphate hydrolysis. *D. salina* cultures that have been adapted for several weeks to 0.1 to 3 M NaCl were labeled for 3 d with $^{32}$P and incubated for 1 h with or without 20 mM ammonium chloride in suspension medium adjusted to pH 9. Polyphosphates were extracted with formic acid, separated on PEI-cellulose plates, and analyzed by autoradiography as described in "Materials and Methods." 3PP, 4PP, 5PP, and 6PP denote the location of linear polyphosphates containing 3, 4, 5, and 6 phosphates.

Figure 2. Polyphosphate hydrolysis in response to hyperosmotic shocks. *D. salina* cultures that have been adapted to 0.5 M (A, C) or 1.5 M NaCl (B) were labeled with $^{32}$P and osmotically shocked by addition of NaCl to a final concentration of 1.25 M (A) or 3.7 M (B). In C, cells were suspended and osmotically shocked in glycerol:NaCl, 9:1 mixtures, osmotically equivalent to 0.5 and 1.25 M NaCl, respectively. At the indicated times after osmotic shocks, samples of cells were extracted and analyzed as in Figure 1.
choice to induce osmotic shocks in this algae. These results indicate that it is the salt, and not the osmotic shock itself, that induces rapid hydrolysis of polyphosphates within the vacuoles.

Hypooosmotic shock, generated by a 2.5-fold dilution, induces different changes in D. salina polyphosphates: a slower and smaller accumulation of a shorter polyphosphate, which comigrates with tripolyphosphate, similar to but smaller in quantity than the component induced by amines, is demonstrated in Figure 3. The accumulation of this putative tripolyphosphate appears to be transient, and it may be hydrolyzed further to pyrophosphate and inorganic phosphate (Fig. 4). These results indicate that increase or decrease in salinity induce different changes inside the vacuoles of Dunaliella. It may be noted that hyperosmotic and hypooosmotic shocks also induce opposite changes in cellular ATP (decreased by upshocks, increased by downshocks, compare Figs. 1 and 2 with Figs. 3 and 4) in agreement with previous in vivo NMR measurements (1).

Previous 31P-NMR studies have demonstrated an extracellular tripolyphosphate signal in Dunaliella (9). To test whether

Figure 3. Polyphosphate hydrolysis in response to hypooosmotic shocks. D. salina cells cultured, adapted to 1 m NaCl, and labeled with 32P were diluted to a final NaCl concentration of 0.5 m and, at the indicated times after the shock, samples were extracted and analyzed as in Figures 1 and 2.

Figure 4. Time course of polyphosphates, ATP and Pi changes following hypooosmotic shocks. Spots corresponding to 3-PP, PPI, ATP, and Pi were cut out of the PEI-cellulose plates (Fig. 3) and analyzed for 32P content in scintillation counter.

Figure 5. Effect of osmotic shocks and benzylamine on release of atebrin and of polyphosphates. A. D. salina cells (0.5 m NaCl cultures) labeled with 32P were shocked osmotically or with benzylamine. To test for the release of polyphosphate from the cells, samples (2–3 x 10^6 cpm/sample) were centrifuged at the indicated time after the shock through silicon-oil (16) and the extracellular 32P remaining in the upper layer was determined. B. D. salina cell cultures (0.5 m NaCl) were preloaded with atebrin, incubated in suspension medium containing 0.5 m NaCl, and buffered to pH 9. At 0 time, cells were either osmotically shocked by twofold dilution (down), addition of NaCl up to 1 m (up), or addition of 20 mM benzylamine. Atebrin fluorescence changes were monitored as described in "Materials and Methods."
The polyphosphate hydrolytic products after osmotic shocks are not generated or excreted out of the cells. \(^{32}\)P-prelabeled cells were separated after osmotic or amine shocks by centrifugation through silicon-oil layers and the extracellular \(^{32}\)P content in the upper-silicon oil layer was determined. As demonstrated in Figure 5A, there is no detectable increase in extracellular \(^{32}\)P following osmotic or amine-induced shocks (about 0.1% of cellular \(^{32}\)P), indicating that all the polyphosphate hydrolytic products remain inside the cells.

**Effect of NaCl Concentration on Atebrin Uptake and Release**

To test whether salt induces pH changes in acidic vacuoles in *Dunaliella*, we have utilized the fluorescent amine indicator atebrin, which accumulates in the vacuoles in response to the pH gradient across the vacuolar membrane, and therefore may be used as a probe for intravacuolar pH (21). A comparison of atebrin uptake and release by benzylamine from cells adapted to 0.5 to 3 mM NaCl is demonstrated in Figure 6. Neither the rate of atebrin uptake nor its release from acidic vacuoles, as reflected by the atebrin fluorescence changes, is greatly affected between 0.5 and 4 mM NaCl, indicating that there are no major intravacuolar pH differences between cells grown at different salinities.

To test whether osmotic shocks induce changes in intravacuolar pH, cells were preloaded with atebrin and osmotically shocked. Figure 5B demonstrates that neither upshock nor downshock induce atebrin fluorescence enhancement. As a reference, the effect of benzylamine is shown, which leads to a rapid intravacuolar alkalization, expressed by a biphasic atebrin fluorescence enhancement (16). These results indicate that, within the limits of sensitivity of this method, osmotic shocks do not seem to induce a major decrease of the pH gradient across the vacuolar membrane.

Figure 5A and B also exclude the possibility of fusion of acidic vacuoles with the plasma membrane following osmotic shocks, which should be manifested by atebrin fluorescent enhancement and exocytosis of polyphosphate hydrolytic products. Such a mechanism has been proposed earlier, in order to explain the large increase in surface area after hypotonic shock (14); however, the present results indicate that the acidic vacuoles are not involved in such a mechanism.

**Effect of Osmotic Shocks on the Permeability of the Plasma Membrane**

Although osmotic shocks do not induce the release of atebrin from acidic vacuoles, they greatly affect the kinetics of atebrin release by benzylamine (Fig. 7), which reflect changes in membrane permeability (16, 21).

The dependence of these permeability changes on the size of the osmotic shock is demonstrated in Figure 8. Upshocks induce a maximal decrease in permeability of about twofold.
whereas downshocks induce up to a 20-fold increase in permeability. The corresponding changes in cell volume under these conditions are shown in the inset.

To test whether the permeability changes are correlated with volume changes, the time dependence of both parameters after osmotic shocks and benzylamine was compared. As is shown in Figure 9A, the enhanced permeability following downshock is transient and decays within minutes (half-life = 3 min) back to the original permeability before the shock. This rapid decay is much faster than the recovery of cell volume under these conditions (half-life approximately 60 min), which results from metabolic conversion of internal glycerol to starch (6). Conversely, permeability changes after upshocks are fairly well correlated with cell volume changes.

Rapid Morphological Changes after Osmotic Shocks in *D. salina* Cells

The transient permeability changes of *D. salina* cells, after osmotic shocks, may result from rapid changes in the organization of the plasma membrane and/or other organelles inside the cells. In an attempt to identify rapid morphological changes, cells were fixed shortly after osmotic shocks and stained cell slices were analyzed by electron microscopy. To minimize distortion of cellular structures during fixation on handling, all treatments were performed in isotonic media at 4°C.

Figure 10 demonstrates that the morphology of cells fixed immediately after osmotic shocks (Fig. 10A, D) greatly differs from their appearance just 5 min after the shocks (Fig. 10B, E). Cells exposed to downshock have wrinkled surfaces (Fig. 10A), whereas within 5 min they assume evenly rounded shapes (Fig. 10B), and this general structure does not change significantly 30 min after the shock (Fig. 10C). Immediately following upshocks, cells appear unevenly shrunken, having narrow necks connecting the distal chloroplast to the apical nucleus-containing ends (Fig. 10D). Within 5 min, cells appear evenly elongated in shape (Fig. 10E), and after 30 min they partly regain volume and their usual shape (Fig. 10F).

These results clearly suggest that osmotic shocks induce rapid transient changes in the cell shape and surface area.

**DISCUSSION**

The present observations that osmotic shocks induce hydrolysis of polyphosphates, and previous observations from our laboratory of rapid changes in the polyphosphate 31P-NMR spectra following shocks (1), indicate changes within acidic vacuoles of *Dunaliella* in response to osmotic shocks. However, the nature of these changes is not clear.

The rapid production of intermediate polyphosphates, tentatively identified as 5-6 PP, after hyperosmotic shocks probably results from accumulation of Na+ in vacuoles because: (a) only cells adapted to high NaCl, or osmotically upshocked in NaCl but not in glycerol media, accumulate 5-6 PP; and
Figure 10. Morphological changes after osmotic shocks. *D. salina* cells were osmotically shocked from 1 to 0.5 M NaCl (A-C) or from 1 to 2 M NaCl (D-F) and fixed with 1.5% glutaraldehyde (for 2 h at 4°C) either 1 min (A, D), 5 min (B, E), or 30 min (C, F) after the shock. Cell slices were stained with uranyl acetate as described previously (2). V, vacuoles; N, nucleus; Chl, chloroplast; St, starch; M, mitochondria; G, golgi; Fl, flagellum.

(b) the rapid production of 5–6 PP is correlated with the rapid Na⁺ influx after hyperosmotic shocks (within 1–2 min, ref. 20). Therefore, these results indicate that Na⁺ is rapidly compartmentalized into the vacuoles after upshocks. This conclusion is consistent with previous x-ray microanalysis studies in *D. tertiolecta* that estimated intravacuolar Na⁺ concentrations 10 times higher than in the cytoplasm (10). Compartmentation of Na⁺ into plant vacuoles takes place via the tonoplast Na⁺/H⁺ antiporter (8). In *Dunaliella*, there is still no evidence for the existence of a vacuolar Na⁺/H⁺ antiporter, although a similar antiporter has been characterized in *Dunaliella* plasma membrane vesicles (11). It appears likely that Na⁺ is taken up into the vacuoles via a Na⁺/H⁺ antiporter, although the absence of atebrin fluorescence enhancement after upshocks does not support such a mechanism. Therefore, it appears that the activation of polyphosphate hydrolysis after upshocks may be triggered by the increase in Na⁺ concentration within the vacuoles. The 5–6 PP may have a role in binding Na⁺ and in increasing the capacity of the vacuoles to trap Na⁺. Thus, acidic vacuoles in *Dunaliella* may have a role in short-
range compartmentation of Na\(^+\) from the cytoplasm, similar to mechanisms proposed in glyphicophilic plants (8, 18).

The significance of the transient accumulation of triphosphates after downshocks is less clear. Our previous observations that amines also induce accumulation of triphosphates, probably by intravacuolar alkalization, may suggest that hypotonic shocks induce similar pH changes within the vacuole. Also, the absence of detectable release of atebrin from the vacuoles under these conditions indicates that if there is a pH change within the vacuole, it is too small to be followed by this technique or that it is counterbalanced by H\(^+\) production resulting from hydrolysis of polyphosphates (17). What seems clear, however, is that the changes within the vacuoles after upshocks and downshocks are different, with respect to both the nature of polyphosphate hydrolytic products and the kinetics of their formation.

The nature of the permeability changes to atebrin are not clear. Because atebrin is released from within acidic vacuoles, the factors that influence its release kinetics may involve changes in the plasma and vacuolar membranes as well as the cytoplasmic and vacuolar pH. Moreover, the complex biphasic kinetics of atebrin fluorescence enhancement, which is induced by benzylamine, further complicates the interpretation because it reflects two separate phenomena: a rapid phase, resulting from internal release of atebrin from vacuoles to the cytoplasm, and a slow phase, reflecting release of atebrin from the cells (16). Nevertheless, several lines of evidence suggest that the rate of uptake and release of atebrin is primarily limited by the permeability of the plasma membrane: (a) In a previous paper, it was demonstrated that modification of the plasma membrane composition by incorporation of a cholesterol derivative or of lysophosphatidylcholine decrease or increase, respectively, the permeability to atebrin (21); (b) The external pH dependence for atebrin uptake (21) and for atebrin release by amines (16) suggests that the diffusion of free amine through the plasma membrane is the rate-limiting step in both atebrin uptake and atebrin release; (c) Atebrin release from vacuoles can be greatly accelerated by selective lysis of the outer membrane, indicating that the vacuolar membrane is far more permeable to amines than the plasma membrane (16). Therefore, it appears that the changes in benzylamine-induced atebrin release reflect primarily changes in the permeability of the plasma membrane, although additional factors, such as changes in cytoplasmic pH and in the vacuolar membrane in response to osmotic shocks, cannot be excluded. This conclusion is consistent with the rapid morphological changes, observed by electron microscopy, and with several previous observations in *Dunaliella* that include: (a) changes in the ordering of lipids in *Dunaliella* membranes after osmotic shocks (5); (b) rapid morphological changes including fusion of internal vesicles with the plasma membrane after downshock (15); (c) accelerated turnover of inositol phospholipids in the plasma membrane after downshock (7).

It is important to note that the large permeability changes to atebrin reported here do not result from rupture of the cell membrane because within the shock limits that *D. salina* cells can withstand (up to 4 to 5-fold for upshocks and 5 to 6-fold for downshocks), there are no significant losses of internal K\(^+\), glycerol, or adenine nucleotides from the cells (20).

The enhanced permeation of atebrin after downshock is too large to be accounted for by the increase in surface area or surface to volume ratio, and is also kinetically distinct from volume changes. In view of the indications for fusion of internal vesicles with the plasma membrane (14) and of the plastic changes in the surface of the cells after downshock (Fig. 10), we suggest that downshock induces a transitory disorganization of the membrane either by segregation of intramembranal particles or that fusion with lipid-rich vesicles temporarily creates lipid-rich patches in the membrane, which contribute to the high permeability to atebrin. Rapid mixing of the membrane components should restore the original low permeability. Such a transient change in the membrane organization may be involved in the recently reported activation of phospholipase C in plasma membrane of *Dunaliella* after osmotic shock (7).

**ACKNOWLEDGMENTS**

We wish to express our thanks to the late Prof. M. Avron for helpful comments and discussions.

**LITERATURE CITED**


