In Vivo pH Regulation by a Na⁺/H⁺ Antiporter in the Halotolerant Alga Dunaliella salina

Adriana Katz, Michal Bentai, Hadassah Degani, and Mordhay Avron*

Departments of Biochemistry (A.K., M.A.) and Isotope Research (M.B., H.D.), The Weizmann Institute of Science, Rehovot 76100, Israel

ABSTRACT

Na⁺/H⁺ exchange activity in whole cells of the halotolerant alga Dunaliella salina can be elicited by intracellular acidification due to addition of weak acids at appropriate external pH. The changes in both intracellular pH and Na⁺ were followed. Following a mild intracellular acidification, intracellular Na⁺ content increased dramatically and then decreased. We interpret the phase of Na⁺ influx as due to the activation of the plasma membrane Na⁺/H⁺ antiporter and the phase of Na⁺ efflux as due to an active Na⁺ extrusion process. The following observations are in agreement with this interpretation: (a) the Na⁺ influx phase was sensitive to Li⁺, which is an inhibitor of the Na⁺/H⁺ antiporter, did not require energy, and was insensitive to vanadate; (b) the Na⁺ efflux phase is energy-dependent and sensitive to the plasma membrane ATPase inhibitor, vanadate. Following intracellular acidification, a drastic decrease in the intracellular ATP content is observed that is reversed when the cells regain their neutral pH value. We suggest that the intracellular acidification-induced change in the internal Na⁺ concentration is due to a combination of Na⁺ uptake via the Na⁺/H⁺ antiporter and an active, ATPase-dependent, Na⁺ extrusion. The Na⁺/H⁺ antiporter seems, therefore, to play a principal role in internal pH regulation in Dunaliella.

Estimations of intracellular pH in Dunaliella indicate that the cytoplasmic pH is maintained at a constant value, around 7.4, in a wide range of extracellular pH (5.0–9.0), suggesting the existence of an efficient pH control mechanism (8, 9).

Na⁺/H⁺ antiporters are ubiquitously distributed in bacterial, plant, and animal plasma membranes and have been suggested to be involved both in the regulation of intracellular pH and Na⁺ concentration (14, 19).

Plasm membrane vesicles isolated from the halotolerant unicellular alga Dunaliella salina were found to contain an active Na⁺/H⁺ antiporter (12). The antiporter was highly specific to Na⁺ and competitively inhibited by Li⁺ or amiloride. It was solubilized and reconstituted into active proteoliposomes (13). Dunaliella can grow in media containing a very wide range of salt concentrations (0.1–5.5 mM), but the intracellular Na⁺ concentration under all these conditions is low (10–100 mM) (2, 5, 7, 11). It was therefore suggested that the antiporter in Dunaliella may play a major role in the intracellular regulation of Na⁺ concentration.

In the present paper, we report on an investigation examin

1 In memory of Professor Jacob B. Biale.

MATERIALS AND METHODS

Culture of the Algae

Dunaliella salina were grown in media containing 1 M NaCl and 25 mM NaHCO₃ as described previously (4). Cultures were grown under continuous illumination at 26°C with slow continuous shaking and under pH stat control. The pH was maintained at 7.0 by addition of gaseous CO₂.

Lowering Intracellular pH and Measurements

Algae in the log phase were centrifuged and resuspended at a cell density of 3 to 5 x 10⁸ cells/ml in a reaction medium composed of the growth medium, buffered with 50 mM Mes and 20 mM Mops at pH 5.5 (or as indicated). The suspension was preincubated for 30 min in the light, and then the desired weak acid, adjusted to pH 5.5 (or as indicated), was added. At the times indicated, 0.1 mL of the suspension was applied to a 5-mL column of Dowex-Tris for estimation of intracellular Na⁺ as described previously (20). Elution from the Dowex columns was with 4 mL of 1.6 M glycerol, 5 mM Mops-Tris (pH 5.5) at 0°C. Na⁺, K⁺, and Li⁺ content were determined in a flame photometer. Cell number and volume were measured in a model ZM Coulter counter. O₂ evolution was measured with a Rank Brothers electrode.

LiCl Treatment

Cells were centrifuged, resuspended in growth medium in which 1 M LiCl replaced the NaCl, and incubated for 2 h in the light (21). They were then centrifuged, resuspended in the reaction medium, and treated with a weak acid as described above. The treatment with Li did not affect the ability of the cells to grow following resuspension in the regular growth medium.

Vanadate Treatment

Cells were treated with vanadate as described previously (17). The cells were transferred to phosphate-free growth medium and incubated under growth conditions for 24 h.
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The reaction conditions described in the cell were continuously applied. The cells were harvested and trapped inside agarose beads (3% mass/volume) at a concentration of around $7 \times 10^8$ cells/ml beads, as described previously (6). The trapped cells carried out all vital functions, including osmoregulation, with rates comparable with cells in suspension. The beads were incubated for approximately 24 h under the growth conditions prior to the NMR experiment. During in vivo NMR experiments, the cells in the beads were perfused continuously at a rate of 1 mL/min, with the reaction medium described above, but lacking phosphate, iron, and the microelements, at pH 5.5 saturated with 95% $O_2$ and 5% $CO_2$ (6). For adding or removing acetate, the perfusion medium was replaced by one containing or lacking acetate. Intracellular pH was calculated from the shift in the resonance position of Pi, by comparison with calibration curves of Pi in solution.

Statistics

Each experiment was repeated at least once. Each data point is an average of two duplicate samples.

RESULTS

The Effect of Lowering Intracellular pH on the Intracellular Na+ Content

The intracellular pH can be lowered to the extent desired by incubation with a weak acid of an appropriate pK at a suitable extracellular pH (3, 15). In algae like *Dunaliella*, the cell membrane contains an active Na*/H* antiporter, this could result in Na* intake in exchange for proton efflux as one means of regulating the intracellular pH.

Figure 1 demonstrates that treating *Dunaliella* with acetate at concentrations of 15 to 70 mM at an external pH of 5.5, indeed caused a temporal increase in the intracellular Na* concentration. The extent of the influx of Na* was proportional to the concentration of acetate employed and reached very large values. The effect was specific to Na* since intracellular K* levels were hardly affected (Fig. 2). It was previously demonstrated that the Na*/H* antiporter of *Dunaliella* was incapable of transporting K* (12). The phenomena were independent of the cellular membrane potential, since they were unaffected by the presence of valinomycin (5 $\mu$M) and K* (100 mM). These reagents were previously shown (18) to completely relax the transmembrane potential in *Dunaliella*.

No significant change in cellular volume (less than 10%) accompanied the acetate-induced Na* movements. As can be seen (e.g., Figs. 1 and 2), Na* influx is followed by efflux. Thus, following the acid-induced Na*/H* exchange, the cell seems to activate mechanisms that decrease the intracellular Na* concentration.

Acetate could be replaced by other weak acids. In Figure 3,

NMR Measurements and Data Analysis

NMR measurements were performed at 26°C, in the dark, in a Bruker AM-500 NMR spectrometer. $^{31}$P spectra were recorded at 202.5 MHz by applying 60° pulses with a repetition time of 1 s. Composite pulse proton decoupling was continuously applied. The cells were harvested and trapped inside agarose beads (3% mass/volume) at a concentration of around 7 $\times$ 10$^8$ cells/ml beads, as described previously (6). The trapped cells carried out all vital functions, including osmoregulation, with rates comparable with cells in suspension. The beads were incubated for approximately 24 h under the growth conditions prior to the NMR experiment. During in vivo NMR experiments, the cells in the beads were perfused continuously at a rate of 1 mL/min, with the reaction medium described above, but lacking phosphate, iron, and the microelements, at pH 5.5 saturated with 95% O$_2$ and 5% CO$_2$ (6). For adding or removing acetate, the perfusion medium was replaced by one containing or lacking acetate. Intracellular pH was calculated from the shift in the resonance position of Pi, by comparison with calibration curves of Pi in solution.

![Figure 1](image-url) Effect of intracellular acidification on the Na* concentration of *Dunaliella* salina. Acetate at the indicated concentrations was added to a suspension of 4 $\times$ 10$^8$ cells/ml in the light at pH 5.5 as described under "Materials and Methods." Samples were removed at the times indicated and the intracellular Na* content and cell volume were measured.

Vanadate (10 $\mu$M) was added and the suspension incubated further for 2 h. The cells were then centrifuged, resuspended in the reaction medium, and treated with a weak acid as described above.

![Figure 2](image-url) Effect of intracellular acidification on the cellular K* and Na* concentrations. Conditions as described in Figure 1, except that 50 mM acetate was used.

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of the concentrations employed. However, when the external pH is reduced to 5.2, photosynthetic oxygen evolution is strongly inhibited, presumably due to the intracellular acidification, and the order of effectiveness of the different acids is the same as that observed in Figure 3 in their ability to elicit Na⁺ influx.

Characterization of the Na⁺ Transport System Activated by Intracellular Acidification

pH Dependence

As was the case for photosynthetic oxygen evolution, a strict dependence was found between the Na⁺ influx and the external pH (Fig. 5). With 50 mM acetate, at pH 6.5 there is no significant increase in the intracellular Na⁺ concentration, whereas at pH 5.0, massive Na⁺ influx is observed.

Effect of Light

When the cells were treated with acetate in the dark (Fig. 6), the rate of influx and the total amount of Na⁺ accumulated was higher than in the light. The decrease in the intracellular Na⁺ concentration that follows the acetate-induced Na⁺ influx is clearly observed in the light but is much reduced in the dark. Thus, it would seem that the observed rate of influx of Na⁺ in response to the internal acidification is a balance between the acid-induced Na⁺ uptake process and an active Na⁺ extrusion process. In the dark, the Na⁺ extrusion process is much slower, leading to a higher accumulation of Na⁺. Thus, the active efflux reactions would seem to depend on an energetic driving force, like ATP, which is synthesized in the light.

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**Figure 3.** Effect of several weak acids on the intracellular accumulation of Na⁺. Conditions as described in Figure 1 except that the acids and the concentrations employed are as indicated.

**Figure 4.** Effect of several weak acids on photosynthetic oxygen evolution at different external pH. O₂ evolution was measured in a suspension containing 1 x 10⁷ cells/ml in the reaction medium at pH 5.2 or 7.0. The weak acids were adjusted to the desired pH before addition. 100% refers to 70 to 80 μmol O₂ x mg-Chl⁻¹·h⁻¹.

**Figure 5.** pH dependence of the intracellular acidification induced Na⁺ uptake. Conditions as described in Figure 1, except that 50 mM acetate was added at the indicated pHs. Preincubation of the cells was at the same pH as indicated.
Effect of Inhibitors

LiCl. It was previously shown that the Na⁺/H⁺ antiporter of *Dunaliella* is inhibited by Li⁺ (12). Following preincubation with Li⁺, under conditions in which it was shown to penetrate the cells and inhibit the antiporter in vivo (21), we checked the effect of Li⁺ on the weak acid-induced sodium influx.

Figure 7b indicates that following preincubation with LiCl, the acetate-induced Na⁺ influx was severely inhibited both in rate and extent. This supports the suggestion that Na⁺ uptake is mediated via the Na⁺/H⁺ antiporter.

Figure 7a demonstrates that the LiCl-treated cells contained about 70 mM Li⁺. A similar inhibition of acid-induced Na⁺ uptake by Li⁺ was observed when cells incubated in a medium containing 0.5 mM NaCl and 0.5 mM LiCl were compared with cells incubated in 0.5 mM NaCl and 0.5 mM choline-chloride (not shown).

Vanadate. Vanadate is a potent inhibitor of the plasma membrane ATPase in *Dunaliella* (17). Since the ATPase may be involved in the mechanism of Na⁺ extrusion from the cells, we investigated its effect on the weak acid-induced Na⁺ movements. As can be seen in Figure 8, pretreatment with vanadate increased the rate and extent of Na⁺ influx, and decreased the following rate of Na⁺ efflux. This is in agreement with the suggestion that Na⁺ efflux is mediated by the energy-independent Na⁺/H⁺ antiporter, whereas Na⁺ extrusion is an active process in which the plasma membrane ATPase participates.

Reversibility

The weak acid-induced Na⁺ influx was rapidly reversed if the pH of the suspension was changed to 7.0 (Fig. 9), indicating that the process was fully and rapidly reversible. Similarly, removing the acetate from the reaction medium (see below, Figs. 10 and 11) also fully reversed the effect.
Figure 9. Reversibility of the weak acid-induced Na⁺ accumulation. Conditions as described in Figure 1, except that 45 mM acetate was used, and that unbuffered Tris was added at the arrow to the samples indicated by dashed lines to bring the extracellular pH to 7.0.

Figure 10. Kinetics of changes in intracellular pH induced by addition of acetate at different concentrations. Conditions as described under "Materials and Methods" using perfused algae entrapped in agarose beads within the NMR tube. Intracellular pH was determined from the chemical shift of the intracellular Pi signal. Each value represents the average of at least 300 scans accumulated over at least 5 min. Acetate was removed by replacing the acetate-containing perfusion medium with one that lacked acetate.

Figure 11. 31P-NMR spectra of acetate-treated Dunaliella. The spectra represent averaged signals for the indicated periods, processed with line broadening of 25 Hz from the 40 mM acetate experiment described in Figure 10. A vertical line is drawn at 3 ppm to facilitate observation of the chemical shift of the intracellular Pi signal. A broad polyphosphate signal (6) was present in all these spectra, but is not evident in the narrow range reproduced here. On the control spectrum are the signals due to phosphonomoesters (PME), Pi, glycerophosphorylglycerol (GPG), and the γ-, α- and β-phosphates of ATP.

case with Na⁺ influx (Fig. 1), 10 mM acetate caused only a minor change in the intracellular pH.

Figure 11 shows the phosphorus NMR spectra obtained before the acetate treatment (Fig. 11, top), during the low intracellular pH steady state (Fig. 11, middle) and following recovery (Fig. 11, bottom). In addition to the shift in position of the intracellular Pi, translated to shifts in intracellular pH in Fig. 10, the spectra indicate that ATP is substantially hydrolyzed to Pi and AMP during the acetate treatment (in the dark), and that the process is fully reversible. ATP is resynthesized when the algae regain their normal intracellular pH. This is in agreement with the previous suggestion that ATP is utilized through the plasma membrane ATPase to drive the accumulated Na⁺ outside the cell.

The decrease in ATP content of the cells following the
addition of acetate at pH 5.5 was also followed by measuring the intracellular ATP with the luciferin-luciferase assay (not shown). With this assay, it was apparent that most of the decrease occurred rapidly so that within 5 min after the addition of acetate the intracellular ATP content decreased to less than half of its normal value (around 2 mm).

**DISCUSSION**

Utilizing weak acid-induced intracellular acidification, we demonstrate herein a method to follow the activity of the plasma membrane Na+/H+ antiporter in vivo and show its function in intracellular pH regulation. We could observe both the decrease in intracellular pH (Fig. 10) and the resulting Na+ fluxes (Figs. 1–3, 5–9). Involvement of the plasma membrane antiporter in cytoplasmic pH regulation was previously suggested for animal, plant, and prokaryotic cells (1, 16, 19). A similar Na+ influx due to lowering of intracellular pH by a protonophore at low external pH was recently observed in Dunaliella (21).

We suggest that entry of the undissociated free acid lowers the intracellular pH. This activates Na+/H+ exchange via the Na+/H+ antiporter of the plasma membrane (12, 13). No metabolic energy is required; the process is driven by the pH and the Na+ gradients alone and is an immediate response that tends to increase the lowered internal pH. In parallel, an active Na+ extrusion process is activated in which cellular ATP and the plasma membrane ATPase are involved. Thus, the observed time course of cytoplasmic Na+ and pH changes following the addition of a weak acid, depends on the interplay between three processes: (a) the rate of intracellular acidification due to the uptake of the protonated acid; (b) the rate of passive pH regulation, due to the activation of the plasma membrane Na+/H+ antiporter; and (c) the rate of active Na+ extrusion utilizing ATP and the plasma membrane ATPase.

The following observations support this interpretation: (a) When Dunaliella cells are exposed to a low concentration of a weak acid (10–15 mM; Figs. 1 and 10), a balance is achieved between the three processes and the acidification is regulated very efficiently by the regulatory mechanisms of the cell resulting in no significant change in intracellular Na+ or pH. However, if the concentration of the weak acid is increased (40–50 mM, Fig. 1,10), the intracellular pH decreases, and Na+ enters the cell via the Na+/H+ antiporter. After about 30 min, the rate of H+ extrusion via the Na+/H+ antiporter and the active extrusion process just balances the acid entry and a plateau in both Na+ and intracellular pH is observed. A recovery phase is then apparent when the rate of the regulatory mechanisms of the cell exceeds the rate of acidification. (b) The initial Na+ influx is specific to Na+ (Fig. 2), as is the Na+/H+ antiporter (12). (c) The initial Na+ influx does not require energy (Fig. 6), is insensitive to vanadate (Fig. 8), but is sensitive to Li+ (Fig. 7), as is the Na+/H+ antiporter (12). (d) The Na+ efflux phase is inhibited by lack of energy (Fig. 6) and by vanadate (Fig. 8). (e) Cellular ATP is rapidly hydrolyzed to AMP and Pi following intracellular acidification (Fig. 11).

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**LITERATURE CITED**