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THE EFFECTS OF HIGH CONCENTRATIONS OF SODIUM OR CALCIUM IONS ON THE LIPID COMPOSITION AND PROPERTIES OF *TETRAHYMENA* MEMBRANES

SHARON M. MATTOX and GUY A. THOMPSON, Jr.

Department of Botany, The University of Texas, Austin, TX 78712 (U.S.A.)

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Summary

Tetrahymena pyriformis cells have been grown in media varying in NaCl concentration from 3.7 mM (normal medium) to 0.3 M and varying in $CaCl_2$ from 0.2 mM (normal medium) to 0.1 M. *Tetrahymena* grown in 0.3 M NaCl showed relatively few alterations in phospholipid composition, with significant changes being found only in the cell surface membranes (pellicle), which increased in phosphatidylethanolamine content from 39% (low Na^+) to 48% (high Na^+) of the total phospholipids. The small decrease in fatty acid unsaturation and increase in shorter chain fatty acids in pellicle phospholipids were not statistically significant. No significant changes in phospholipid head group composition or fatty acid distribution were observed in high Ca^{2+}-grown cells. Complementary studies of membrane fluidity, as inferred from freeze-fracture electron microscopy analysis, indicated that membranes of high Na^+-acclimated cells were similar to those of control cells, when each was measured in its respective medium. However, the outer alveolar membrane of the pellicle and the food vacuolar membrane were considerably less fluid in high-Ca^{2+} cells. The lower fluidity in vacuolar membranes may have been responsible for alterations in the cells' capacity to form food vacuoles.

Living organisms can adapt to a wide variety of adverse environmental factors. The best-documented cases of this acclimation process involve the response to temperature change [1]. For a wide range of poikilothermic organisms [2—4], the key to survival at low temperature is the ability to increase the unsaturation of their membrane lipids, thus restoring a functional degree of fluidity to these important structures.

In many natural situations, salinity poses an even greater threat to survival than do harsh temperatures. Yet relatively little effort has been directed towards learning what changes in membrane lipids might accompany the acclimation of cells to saline environments, despite the fact that varying cation levels are known to significantly alter the physical properties of artificial membranes [5,6].

We have examined the response of the free-living protozoan *Tetrahymena pyriformis* to levels of Na^+ and Ca^{2+} that might be encountered in natural areas of high salinity. Because *Tetrahymena* is among those organisms which respond to changes in environmental temperature by making large alterations in their membrane lipid composition [2], it seemed feasible to anticipate that high cation levels might also modify its membrane lipid pattern. Information regarding any such changes in lipid composition would be useful in understanding the strategies utilized by this species during acclimation.

Although *Tetrahymena* strains have been reported to occur in brackish water [7,8], they are normally considered to be fresh-water organisms. We found our strain NT-1 unable to survive for more than a few hours when inoculated into enriched proteose peptone medium (normally 3.7 mM in Na^+ [9]) supplemented with 0.3 M NaCl. However, by raising the concentration of Na^+ every few days by steps of 0.05 M, it was possible to acclimate *Tetrahymena* to grow in 0.3 M NaCl-supplemented medium within a period of approx. 2 weeks.

Following acclimation, the cell's generation time reached a plateau of 10 h vs. the 3.5 h generation time of control cells. The final stationary phase cell density attained in 0.3 M NaCl medium was $3—4 \cdot 10^5$ cells/ml (as determined with a Coulter Counter, Model B), approximately half that reached by controls.

Although cells fully acclimated to 0.3 M NaCl-supplemented medium were more rounded than control cells, as determined by phase microscopy, and averaged 40% smaller in volume, they appeared normal in other respects *. Lipids were extracted by the procedure of Bligh and Dyer [10] and phospholipids were separated according to Rouser et al. [11]. The phospholipid composition of the whole logarithmic phase cells grown in high-Na^+ medium showed small but not statistically significant differences from the lipid pattern in control cells from normal proteose peptone medium (see Ref. 12 for compositional data for controls). However, the small differences observed in whole cell extracts were magnified in the case of pellicles, which contain the surface membranes of the cell [13]. Here the largest change was a rise of phosphatidylethanolamine from 39% of total lipid phosphorus in control cells to 48% in Na^+-acclimated cells (Table I). Compensating for this rise was a drop in the level of 2-aminoethylphosphonolipid and a marginal decrease in phosphatidylcholine of the Na^+ cells. Cardiolipin, which is present as a minor phospholipid component (3—5%), showed no significant change.

A fatty acid analysis revealed no appreciable differences between phospholipids of control and Na^+-acclimated whole cells. Small differences were found

* Cell volume was calculated from length and width measurements of cells freshly fixed in 1% HCHO, considering the shape to approximate a prolate spheroid.

TABLE I

PHOSPHOLIPID COMPOSITION OF PELLICLES ISOLATED FROM CONTROL AND SODIUM-ACCLIMATED *TETRAHYMENA*

Pellicles were prepared by the cell fractionation scheme of Nozawa and Thompson [13]. Data are expressed as percent of the total lipid phosphorus and are averages ± S.D. of three experiments. Minor phospholipid components are not shown.

Lipid species	Lipids from:	
	Control cells	Na^+-acclimated cells
Phosphatidylcholine	19.6 ± 1.5	15.9 ± 3.8
Phosphatidylethanolamine	38.6 ± 1.4	47.6 ± 1.2 *
2-Aminoethylphosphonolipid	29.3 ± 1.9	25.0 ± 3.4 **
Cardiolipin	5.9 ± 4.1	5.6 ± 3.8

* Significantly different from control, $P > 0.01$.
** Significantly different from control, $P < 0.2$.

in the fatty acid composition of purified pellicles (Table II). A slight reduction in unsaturated bonds (115 double bonds per 100 fatty acid molecules in Na^+ pellicles vs. 137 in controls) was correlated with an increase in saturated fatty acids shorter than 18 carbons (39% in sodium pellicles vs. 24% in controls).

In contrast to the adverse effect of sudden exposure to Na^+ on cell viability, *Tetrahymena* transferred from normal medium (0.02 mM in Ca^{2+}) to 0.1 M Ca^{2+} medium resumed growth after a lag of only 1—2 h despite the fact that the ionic strength differential was as great as in case of 0.3 M NaCl *.

After growing in an elevated concentration of Ca^{2+} for some days, the cells developed a long slender appearance and became reduced in volume to 45% of the control value. The generation time of 4 h was only slightly longer than that of control cells.

The phospholipid compositions of Ca^{2+}-acclimated whole cells and purified pellicles showed only marginal differences from control preparations. The fatty acid compositions of both whole cell and pellicle phospholipids were very similar to those of controls, with fatty acids of the Ca^{2+}-grown pellicles displaying a small, statistically insignificant increase in shorter chain fatty acids and a 10% decrease in total double bonds.

Because the cells exposed to high-Ca^{2+} or high-Na^+ medium regained the ability to grow at a stable rate for long periods of time, one may assume that some type of acclimation had taken place. It was of interest to determine whether such an acclimation involved alterations in membrane structure and/or function.

We examined the physical properties of several *Tetrahymena* membranes by freeze-fracture electron microscopy of cells acclimated to grow under the high-Na^+ or high-Ca^{2+} conditions. By quickly chilling aliquots from each type

* In order to prevent calcium phosphate precipitates, flasks containing 100 ml of normal enriched proteose peptone medium were autoclaved and diluted prior to cell inoculation with an equal volume of autoclaved 0.2 M $CaCl_2$.

TABLE II

FATTY ACID COMPOSITION OF TOTAL PHOSPHOLIPIDS FROM CONTROL AND SODIUM-ACCLIMATED *TETRAHYMENA* PELLICLES

Data represent average weight% ± S.D. of at least three experiments, as determined by gas-liquid chromatography [7]. Trace components are not shown.

	Fatty acid								
	12:0 *	14:0	16:0	16:1	18:0	18:1	18:2 (Δ6,11)	18:2 (Δ9,12)	18:3
Lipids from control cells	1.4 ± 0.5	10.4 ± 3.7	11.9 ± 0.8	13.2 ± 0.6	1.7 ± 0.5	10.2 ± 3.0	4.7 ± 1.7	11.0 ± 2.9	25.8 ± 1.5
Lipids from Na^+-acclimated cells	6.3 ± 1.4 **	13.5 ± 1.3	18.9 ± 1.0 ***	4.8 ± 2.5 **	2.7 ± 1.1	7.2 ± 2.1	2.4 ± 0.7	14.7 ± 3.6	23.0 ± 2.3

* The figure preceding the colon represents the number of carbon atoms while that following the colon represents the number of double bonds.

** Significantly different from control, $P > 0.01$.

*** Significantly different from control, $P < 0.001$.

of culture to various subambient temperatures and then fixing the cells with glutaraldehyde, the characteristic temperature at which phase separation of the membrane lipid components occurs can be determined by noting the highest temperature at which particle-free domains appear in the freeze-fracture replicas [14,15]. These properties are closely correlated with membrane fluidity as estimated by fluorescence polarization [16].

The temperatures of incipient lipid phase separation were compared in several membranes of cells fixed in vivo in high-Na^+, high-Ca^{2+}, or regular medium. There were no detectable differences between controls and Na^+-acclimated cells. The Ca^{2+}-acclimated cells, on the other hand, showed signs of lipid phase separation 5—6°C higher than controls in two locations, the outer alveolar membrane, which lies immediately below and in close contact with the plasma membrane (and, along with the plasma membrane and inner alveolar membrane, constitutes the pellicle), and the food vacuolar membrane. Averages from several replicates, measured as described by Kitajima and Thompson [15], indicated that particle-free domains appeared in Ca^{2+}-adapted outer alveolar membranes at 28 vs. 23°C in controls. Likewise, particle-free areas appeared in food vacuoles of Ca^{2+}-grown cells at 7 vs. 0—0.5°C in controls. Thus these two surface-oriented membranes may be judged to be significantly more rigid in Ca^{2+}-acclimated cells than in normal cells. Unfortunately, the plasma membrane itself cannot be analyzed by this method because its high content of sterol-like lipids prevents any detectable phase separation, even at very low temperatures [15].

The fluid quality of the outer alveolar membrane can also be gauged by a related measurement made on the freeze-fracture electron micrographs. The extent of a low temperature-induced lateral movement of the intramembranous particles has been quantified to provide a measure of this membrane's fluidity [14]. At seven fixation temperatures, ranging from 28 down to 10°C, where particle aggregation was almost complete, aggregation in the outer alveolar membrane of Ca^{2+}-acclimated cells (data not shown) was always greater than in controls, again indicating more rigid lipids in the high-Ca^{2+} membranes.

A number of efforts were made to evaluate the effects on the intramembranous particle aggregation of transferring Ca^{2+}-acclimated cells to normal medium or vice versa. However, equivocal results were obtained because osmotic effects that followed the sudden transfers seriously interfered with the measurements of particle aggregation by freeze-fracture electron microscopy.

In view of the apparent change in physical properties of the food vacuolar membranes of *Tetrahymena* acclimated to growth in high Ca^{2+}, the influence of Ca^{2+} on the rate of vacuole formation was tested with India ink, as previously described [17]. Cells of this strain grown in the low-Ca^{2+}, control medium formed an average of ten vacuoles in a 20 min period. These cells, when challenged with increasing levels of added Ca^{2+}, decreased their rate of vacuole formation until at 0.1 M Ca^{2+} no vacuoles at all were produced within 20 min. However, within approx. 2 h following transfer to 0.1 M Ca^{2+}, the competence to form vacuoles slowly began to return, and cells fully acclimated to high Ca^{2+} had totally recovered their vacuole-forming capacity.

Because it seemed possible that the inhibition of vacuole formation upon transferring cells into 0.1 M Ca^{2+} might simply be an osmotic effect, a number

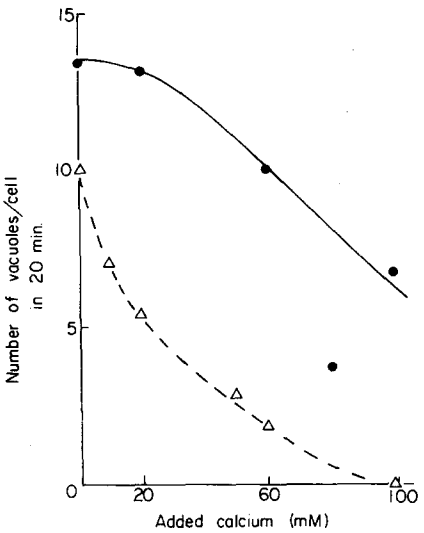

Fig. 1. The effects of increasing concentrations of $CaCl_2$ on vacuole formation by control cells (△) and cells acclimated to growth in 0.1 M $CaCl_2$ and then incubated in normal, low-Ca^{2+} medium for 1 h before testing (●). Values represent averages of at least 20 cells.

of variations of the experiment were made. For example, cells acclimated to the high-Ca^{2+} medium were transferred back into low-Ca^{2+} medium and, after allowing them a period of 60 min to reover fully from any osmotic shock, they were tested for their capacity to ingest India ink in various concentrations of Ca^{2+}. Fig. 1 illustrates that these cells retained an enhanced ability to form food vacuoles in concentrations of Ca^{2+} that strongly inhibited the process in control cells.

Tetrahymena has been shown here to grow over a nearly 100-fold concentration range of external Na^+ and a 500-fold range of external Ca^{2+}. No significant changes in whole cell lipid composition were found in cells grown at these extremes. Even purified pellicles, the surface membrane complex, showed only small lipid changes despite the fact that this structure is directly exposed to the external cations.

The only significant changes in lipid composition involved an elevation of phosphatidylethanolamine from 39% of the total phospholipids to 48% and a concomitant reduction of 2-aminoethylphosphonolipid in the high-Na^+ cell pellicles. Any interpretation of the effect of these alterations on the physicochemical properties of the membrane would be premature. In simpler artificial lipid bilayers, elevated Na^+ levels would be expected to increase lipid fluidity significantly be reducing intermolecular bridging involving the more tightly bound but now greatly outnumbered Ca^{2+} [5]. This fluidizing tendency may be counteracted by the rise in phosphatidylethanolamine components, which are known to be a more rigid phospholipid class than the corresponding molecular species of phosphatidylcholine [18]. No pertinent physical studies have yet been carried out on phosphonolipids.

Apart from studies on specially halotolerant bacetria, which can grow in

NaCl concentrations of 1 M or higher [19,20], few reports of salt effects on lipid composition have been published. Phospholipids and their fatty acid components have been analyzed in *Escherichia coli* grown with 0.6 M, 0.3 M or no added NaCl [21]. Although stationary-phase cells exhibited some changes in fatty acid composition, growing cells showed no significant differences in fatty acid content or in proportions of polar head groups. On the other hand, barley seedlings stressed by exposure to 0.2 M NaCl for 4—5 weeks experienced a decrease in phosphatidylethanolamine from 41% of total lipid phosphorus to 32% and a concurrent increase in phosphatidylcholine from 29% to 32% [22].

Too few data are available to permit any meaningful comparisons among species regarding their response to varying cation levels. It has been shown using artificial lipid bilayers that the important competition between divalent cations, especially Ca^{2+}, and monovalent cations is most pronounced when negatively charged phospholipids, such as phosphatidylserine, phosphatidylglycerol, or phosphatidic acid, are present in sizeable proportions [5]. *Tetrahymena* membranes contain a very small percentage of phospholipids carrying a strong negative charge at physiological pH. The most significant of these, cardiolipin, accounts for 5% or less of the total phospholipids. The effect of Ca^{2+} on negatively charged lipids occurring as a small component of neutral phospholipids is generally not detectable in measurements of bulk-phase lipid physical properties [23]. In *Tetrahymena*, the low proportion of lipids carrying a negative charge might explain in part why relatively few charges in lipid composition were found when comparing membranes of high-Na^+ and high-Ca^{2+} cells.

Freeze-fracture analysis of cells grown in high Ca^{2+} did show an apparent alteration in the fluidity of two surface-oriented membranes. The functional capabilities of one of these, the food vacuolar membrane, was also altered. While this change might be due to highly localized modifications in the lipid pattern, an effect of Ca^{2+} on the cell's cytoskeletal components would seem more likely.

Ca^{2+} is a major cation regulating membrane potential in many cells, including *Tetrahymena* and other ciliated protozoa [24], and a complex chain of events involving release of vesicle-bound epinephrine and activation of adenylate cyclase in *Tetrahymena* has recently been found to be triggered by a sudden Ca^{2+} influx [25]. Our laboratory [26] and others [27,28], have recently initiated studies of Ca^{2+} flux across ciliate membranes. It is clear that external monovalent cations such as K^+ and Na^+ are also intimately involved in controlling entry of the all-important Ca^{2+} [28]. The present findings contribute to these ongoing studies by showing that *Tetrahymena* can make any adjustments in cation transport system necessitated by exposure to high extracellular Ca^{2+} or Na^+ without undergoing a major shift in membrane lipid composition.

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