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Effects of cytochalasin B on Na^+ content and cell volume of *Entamoeba histolytica*

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Cells of *Entamoeba histolytica* accumulated K^+ and extruded Na^+ compared to the concentrations of those ions present in the growth medium. Pinocytic activity, measured by the uptake of horseradish peroxidase of ^{125}I-polyvinylpyrrolidone, was high (up to 0.3 ml/ml cells per h). Upon addition of cytochalasin B, at a concentration (20 μM) that completely blocked pinocytosis, cells lost up to 40% of their Na^+ content within 90 min; K^+ content was not affected or increased slightly compared to control cells without the inhibitor. Cation loss was associated with cell shrinkage. The dose-response curves for the effects of cytochalasin B on pinocytosis and Na^+ content were identical. These data provide direct evidence that pinocytosis is an important component of the homeostatic system for Na^+.

Introduction

In the course of our investigations of *Entamoeba histolytica* [1] it struck us that surprisingly little is known about the pump(s) responsible for the ionic well-being of this organism. For instance, do they possess a Na^+ pump, like animal cells, and/or a proton pump, like yeasts? The presence of a Ca^{2+}-dependent ATPase activity associated with the membrane fraction [2] could be indicative of a Ca^{2+} pump, but the existence of such a device in *E. histolytica* has not been investigated on the cellular level.

The usual approach to such a problem complex basically involves two kinds of measurements: determination of the (electro) chemical gradients of the ions that are candidates for being pumped, and a measurement of the unidirectional fluxes of those ions. When we set out to apply these methods to *E. histolytica*, we soon realized that the interpretation of the data was complicated by the existence of a very active pinocytic pathway (see Refs. 3 and 4 and Results). For instance, under normal conditions over 85% of unidirectional Na^+ uptake was through pinocytosis (unpublished data); also, because of the appreciable size of the endocytic compartment, ion concentrations in the cytoplasm could not simply be calculated from the overall cellular ionic content.

Against this background we decided to inhibit pinocytosis with cytochalasin B, an inhibitor of actin polymerization [5]. The results presented here show that the interference of pinocytosis with ion transport studies actually is much more fundamental than we realized at first. They indicate that pinocytic vesicles or the vacuoles derived from them contain in their membranes part of the pump- and leakways that help shape the ionic composition of the cytoplasm. For macrophages and fibroblasts Steinman et al. [6,7] have earlier come to a similar conclusion based on purely-stereological studies.

Abbreviations: bis-Tris propane, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; ^{125}I-PVP, ^{125}I-polyvinylpyrrolidone.

Methods and Materials

E. histolytica strain HM1 : NIH was grown axenically at 36°C in TYI-S medium [8] supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were harvested by chilling and by centrifugation at 400 × *g*, and washed twice in incubation saline. This saline had the same Na^+- and K^+ content, pH and osmolarity as the growth medium, and consisted of (mM): NaCl, 100; KCl, 30; $MgSO_4$, 1; $CaCl_2$, 1; ascorbic acid, 10; cystein, 6; sorbitol, 100; 1,3-bis[tris(hydroxymethyl)methylamino]propane (bis-Tris propane), 25. Bovine serum albumin (Sigma, Fraction V) was added at 1 mg/ml. pH was adjusted to 6.8 with HCl. Final osmolarity (determined with a Vogel OM 801 freeze-point osmometer) was 420 mosM.

The washed cell pellet was suspended in incubation saline to a final density of 20–50 μl packed cells/ml suspension. Pinocytic activity was followed by the uptake of ^{125}I-polyvinylpyrrolidone (^{125}I-PVP; final activity, 0.5 μCi/ml; Amersham) or horseradish peroxidase (1 mg/ml, Sigma Type II). In *E. histolytica*, both these markers are taken up in the fluid phase, judged from the following criteria (results not shown; see also Ref. 3): (i) uptake of either substrate was not affected by a 100-fold change in its concentration, (ii) horseradish peroxidase did not affect the uptake of ^{125}I-PVP and vice versa, and (iii) pinocytosis as calculated from the uptake of horseradish peroxidase was equal to that calculated from the uptake of ^{125}I-PVP. At the indicated time points, duplicate 0.2-ml samples in 1-ml plastic tubes were spun for 1 s in a Beckman microcentrifuge. The supernatant was sampled and aspirated and the amoebal pellets washed twice with 1 ml ice-cold washing saline ($MgCl_2$, 100 mM; sorbitol, 100 mM; bis-Tris propane, 5 mM, brought to pH 6.8 with HCl). Subsequently, samples containing ^{125}I-PVP were directly counted in a Philips P4600 γ-counter. Samples containing horseradish peroxidase were dissolved in Triton X-100, 1 mg/ml, and peroxidase activity spectrophotometrically determined with pyrogallol as a substrate (Sigma Technical Bulletin 7/82). Na^+- and K^+ content of the cells were determined after precipitation of protein with trichloroacetic acid (50 mg/ml).

For determination of cell pellet water and extracellular space, ^3H_2O (0.5 μCi/ml; Amersham) and [^{14}C]sorbitol (0.1 μCi/ml; Amersham) were added to the cell suspension. Duplicate 0.2-ml samples were pipetted into 1-ml plastic tubes containing 0.2 ml of a 1 : 1 mixture of silicone oil and dinonylphthalate, final density 1.025, and the cells immediately spun through the oil layer by a 10-s centrifugation in a Beckman microcentrifuge. Samples of the supernatant and of the trichloroacetic acid-extracted pellet were counted in scintillation fluid (Hydroluma, Baker) in a Packard dual-channel scintillation counter. Extracellular (sorbitol) space was normally 8–11% of total cell pellet water.

Protein content of the cells was determined according to Lowry et al. [9], after washing the cell samples twice in washing saline. Protein content usually ranged between 0.13 and 0.20 g/ml cells.

For the determination of cell water content, cells were spun down and concentrated to a density of 0.1–0.2 ml packed cells/ml. Triplicate 0.4-ml samples were pipetted into preweighed plastic tubes, and spun for 10 s in a Beckman microcentrifuge. The supernatant and the uppermost layer of the cell pellet were aspirated, and pellet wet weight (immediately after centrifugation) and dry weight (after incubation at 95°C overnight) determined on an analytical balance.

Cytochalasin B, fluid-phase markers, ^3H_2O and [^{14}C]sorbitol were added just before the start of the experiment. Experiments were started by transferring cells from room temperature (occasionally from 0°C) to a 36°C rotary shaker water bath.

Cytochalasin B (Sigma) was added from a 5-mM stock solution in ethanol; an equivalent volume of ethanol was added to the control incubation.

Results are expressed per ml cells. This refers to the packed cell volume as measured with ^3H_2O at the start of the experiment, unless indicated otherwise. Differences between duplicates or standard deviations of triplicates were around 5% for the measurements of pinocytosis and ^3H_2O space, and below 2% for the determination of Na^+- and K^+ content and the ratio of wet weight to dry weight.

Results

Freshly-harvested cells of *E. histolytica* had a K^+ content exceeding that of the growth medium, and a Na^+ content lower than that of the growth medium (Fig. 1A; in a series of experiments, both K^+- and Na^+ content ranged between 50 and 70 μmol/ml cells, compared to concentrations of those ions in the growth medium of 30 and 100 mM, respectively). During subsequent incubation in a simple saline that matched the growth medium in Na^+- and K^+ concentration and osmolarity, Na^+- and K^+ content did not change appreciably over 90 min (less than 10%; Fig. 1A). In this saline, cells maintained a high pinocytic activity (Fig. 1B; between 0.10 and 0.30 ml/ml cells per h in different batches of cells).

Addition of cytochalasin B at 20 μM completely blocked pinocytosis (Fig. 1B). Also regurgitation of the fluid-phase marker was largely suppressed (Fig. 2). At the same time, Na^+ content started to drop, whereas K^+ content remained constant (Fig. 1A) or even increased slightly compared to the control (Fig. 3). After 90 min, cytochalasin B-treated cells had lost between 30 and 40% of their original Na^+ content, or up to 20% of their original monovalent cation content.

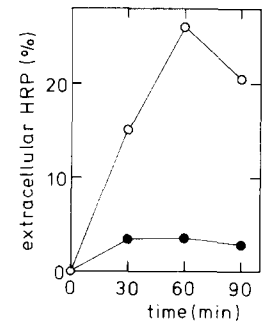

Fig. 2. Effect of cytochalasin B on regurgitation of fluid-phase marker. Cells were pulsed for 15 min at 36°C with horseradish peroxidase (20 mg/ml). Subsequently they were washed twice with ice-cold incubation saline, and reincubated at 36°C plus or minus cytochalasin B. At the indicated time points, duplicate samples were spun in a microfuge and the peroxidase activity in the supernatant determined as described in Methods. Results are expressed as percentage of cellular peroxidase content at $t = 0$; the latter corresponded to a fluid-phase uptake of 36 μl/ml cells. ○, control; ●, plus cytochalasin B (20 μM). HRP, horseradish peroxidase.

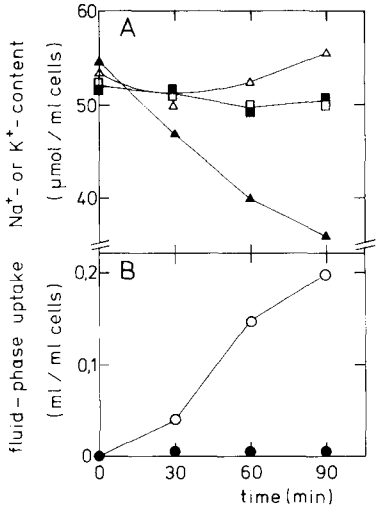

Fig. 1. Time-course of the effects of cytochalasin B on ion content (A) and pinocytic activity (B) in *E. histolytica*. Pinocytosis was measured with horseradish peroxidase (1 mg/ml) as a fluid-phase marker. For experimental details, see Methods. △, ▲, Na^+ content; □, ■, K^+ content; ○, ●, fluid-phase uptake. Open symbols, controls; closed symbols, plus cytochalasin B (20 μM).

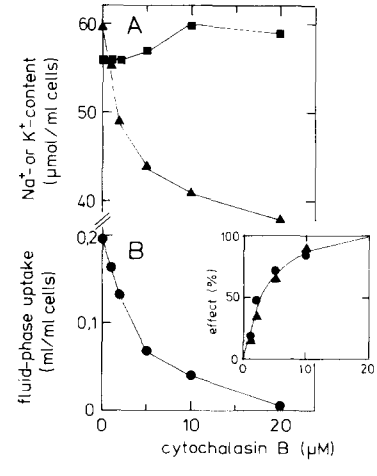

Fig. 3. Ion content (A) and pinocytic activity (B) as a function of cytochalasin B concentration. Cells were incubated for 90 min in the presence of the indicated concentrations of cytochalasin B, with horseradish peroxidase (1 mg/ml) as a fluid-phase marker. For experimental details, see Methods. ▲, Na^+ content; ■, K^+ content; ●, fluid-phase uptake. The inset shows the results expressed as a dose-response curve (percent of maximal effect of cytochalasin B as a function of cytochalasin B concentration).

TABLE I

EFFECTS OF CYTOCHALASIN B ON MONOVALENT CATION CONTENT AND CELL VOLUME

Cells were incubated for 2.5 h in fresh growth medium plus or minus 20 μM cytochalasin B. Ion content is expressed on a protein base. Cell water was both determined gravimetrically (left) and calculated from ^3H_2O-space and protein content (right). All data have been corrected for extracellular space. For experimental details, see Methods.

	Ion content (μmol/g protein)			Cell water	
	Na^+	K^+	$Na^+ + K^+$	g/g dry wt.	ml/g protein
Control	400	429	829	4.10	5.88
Plus cytochalasin B	276	414	690	3.36	4.76
Effect of cytochalasin B (%)	−31	−3	−17	−18	−19

Table I shows that the cytochalasin B-induced monovalent cation loss was associated with an equivalent decrease in cell volume. This indicates that the extruded Na^+ must have been accompanied by an anion (rather than exchanged for a cation), probably mainly by Cl^-.

Under the phase-contrast microscope, cytochalasin B-treated cells looked rounded-off, and slightly more compact than untreated controls (not shown).

Since cytochalasin B is known as a rather specific inhibitor of actin polymerization [5] *, the data suggest that the loss of cell Na^+ and the accompanying volume decrease were secondary to-, or even a direct consequence of-, the inhibition of pinocytic uptake. This conclusion is supported by the observation (Fig. 3) that the dose-response curves for the effects of cytochalasin B on pinocytosis and cellular Na^+ content were identical.

Discussion

Like all other cells investigated so far, *E. histolytica* maintained an ionic steady state characterized by a K^+ content exceeding that of the incubation medium, and a Na^+ content lower than that of the incubation medium. Cytochalasin B, an inhibitor of pinocytosis, disturbed this steady state by causing the cells to lose additional Na^+. Since this Na^+ loss, if anything, must have occurred against the existing Na^+ gradient across the cytoplasmic membrane, it cannot have been due to aspecific membrane damage caused by the drug. Rather, it appeared to be directly related to the concomitant inhibition of pinocytosis.

We will argue here that our data provide evidence for a participation of the endosomal compartment in the generation of the cytoplasmic ionic steady state. The argument goes as follows: The cells were approximately in a steady state for Na^+ and K^+ to start with (Fig. 1), which means that for each of those ions equal amounts were taken up and lost per unit time. Both ion uptake and ion release potentially are through two routes: trans-plasma membrane and enclosed in vesicles (i.e., pinocytic and exocytic). In the simplest model (Fig. 4a), those two pathways are completely independent, i.e. all ions coming in across the plasma membrane also leave that way, and, conversely, all ions coming in through pinocytosis leave through exocytosis. All pinocytically-accumulated Na^+ is obligatorily accompanied by K^+, in a concentration ratio that corresponds to that in the incubation medium (100 mM Na^+ vs. 30 mM K^+). How will cytochalasin B affect the ionic steady state in this model? We have shown that cytochalasin B inhibits both pinocytosis and regurgitation; we do not know whether it inhibits other forms of exocytosis as well. If so, the model of Fig. 4a predicts that cytochalasin B would suppress ion uptake and ion release by the same amount, and thus would not affect the steady state at all; conversely, in case some form of exocytosis persists, the drug would induce a net release of both Na^+ and K^+ in

* In animal cells cytochalasin B is a potent inhibitor of the glucose carrier as well [5]. Also *E. histolytica* contains a passive glucose transport system [4]. However, cytochalasin B has no effect on this system (measured with [^{14}C]xylose as a substrate; data not shown).

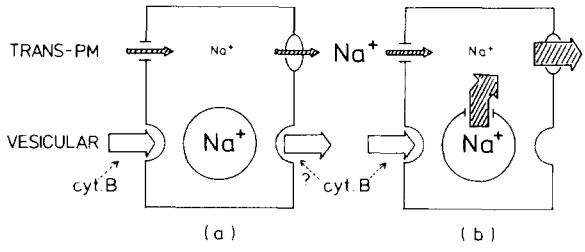

Fig. 4. Models for the homeostatic system for Na^+ in *E. histolytica*. Both uptake and release of Na^+ are the sum of a trans-plasma membrane (trans-PM) and a vesicular component. (a) The two components are fully independent; (b) pinocytically-accumulated Na^+ is extruded by the in-series arrangement of an endocytic membrane 'leak' with a plasma membrane 'pump'. For further details, see text. Sizes of arrows and lettering indicate the relative magnitudes of fluxes and concentrations, respectively. Open arrows, vesicular fluxes; arched arrows, transmembrane fluxes; O, pump; =, leak; cyt. B, cytochalasin B.

a ratio equal to that of the pinocytized fluid, i.e. 100 : 30. However, neither of those two alternatives happened: rather, cytochalasin B caused the cells to lose Na^+ exclusively. With other words, at least part of the pinocytically-accumulated Na^+ left the cells whereas its K^+ counterpart was retained. This selective treatment of Na^+ and K^+ apparently occurred at the level of the endosomal membrane compartment. We therefore must assume that this membrane compartment contains one or more Na^+- and/or K^+-transport system(s) and that, in contrast to the model of Fig. 4a, some or all of the ions taken up by the vesicular route leave the cells through the transmembrane route, or vice versa.

One model that would accomodate our data is presented in Fig. 4b. In this model Na^+ taken up by pinocytosis leaves the cells through a transmembrane pathway. In detail, the plasma membrane contains an outwardly-directed Na^+-'pump'. This pump may either be driven by ATP or by the (electro)chemical gradient of another substance. Primary pinocytic vesicles progress into secondary structures that have a different membrane composition, with a leak pathway rather than a pump for Na^+. As noted by Steinman et al. [6], who proposed a very similar scheme for fibroblasts and macrophages, the net result would be an epithelial-like arrangement of pumps and leaks that would cause a vectorial Na^+ transport from the vesicles through the cytoplasm and out of the cells.

This model predicts that cytochalasin B-induced cell shrinkage is due to a decrease in size of the endocytic, rather than the cytoplasmic, compartment. It may be possible to test this prediction by stereologic techniques [6].

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