Evidence for a Vacuolar-Type Proton ATPase in *Entamoeba histolytica*

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Z. Naturforsch. **45c,** 229–232 (1990); received December 12, 1989

Proton Pump, ATPase, H^+- (vacuolar), Endocytic Vesicle, *Entamoeba histolytica*

Entamoeba histolytica is a primitive eukaryote that lacks mitochondria, Golgi and a well-developed endoplasmic reticulum. Close to half of the cell volume is occupied by pinocytic vesicles, which are in continuous turnover with the plasma membrane and perform functions that in higher eukaryotic cells are taken over by lysosomes. Similar to the latter, the amebal vesicles are acidified. We report here that bafilomycin A_1, a specific inhibitor of vacuolar-type (V-) ATPases, suppressed this acidification at submicromolar concentrations; concomitantly, it inhibited pinocytosis. These results strongly suggest the presence of a V-ATPase in pinocytic vesicles of *E. histolytica,* and thereby support the notion that the V-ATPases in the organelles of higher eukaryotes are derived from an archaic plasma membrane-bound form.

Introduction

Entamoeba histolytica has been classified [1] as an Archezoon: a primitive eukaryote lacking mitochondria, Golgi and a well-developed endoplasmic reticulum [2]. In agreement with its simple morphology, there is functional evidence for only two intracellular organelles: a pinocytic/digestive compartment that performs at least some of the functions taken over by lysosomes in higher cells [3] and a non-pinosomal compartment that, based on its ability to incorporate [^{3}H]mannose into protein, can be classified as endoplasmic-reticulum-like (our unpublished observations). This communication will deal with the former compartment.

E. histolytica constitutively exhibits a high pinocytic activity, taking up close to 30% of its own volume per h by fluid-phase pinocytosis [4, 5]. This process, together with the reverse process denoted regurgitation [4], results in a steady state in which pinocytic vesicles occupy up to 40% of the cell volume [5].

Similar to the internal compartments in higher eukaryotes [6], pinocytic vesicles in *Entamoeba* are acidified [7]. We here show that this acidification was suppressed by very low concentrations of bafilomycin A_1, a specific inhibitor of vacuolar-type (V-) ATPases in higher eukaryotes [8]. This strongly suggests that *Entamoeba,* as do higher eukaryotic cells, contains in its internal membranes a V-type H^+-ATPase. We will discuss this finding in terms of its evolutionary implications.

Abbreviation: FITC-dextran, fluorescein isothiocyanate-conjugated dextran.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/90/0300–0229 $ 01.30/0

Materials and Methods

Cells

Entamoeba histolytica strain HM 1:IMSS, clone A [9] was grown axenically at 36 °C in TYI-S medium [10] 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. Unless indicated otherwise this saline consisted of: 100 mM NaCl, 30 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 100 mM sorbitol and 10 mM Pipes. pH was adjusted to 7.0 with Tris base. The washed cell pellet was suspended in incubation saline.

Pinosomal pH

Pinosomal pH was determined fluorimetrically by the method of Ohkuma and Poole [11]. To this end, cells were allowed to pinocytize fluorescein isothiocyanate-conjugated dextran (FITC-dextran, 2 mg/ml; Sigma) for the indicated periods of time. Cells were then washed three times in ice-cold saline and resuspended in incubation saline at a final protein concentration of about 1 mg/ml (corresponding to 5×10^5 cells/ml [5]). Pinosomal pH was estimated from the ratio of fluorescence produced by excitation at 495 nm to that produced by excitation at 450 nm, at an emission wavelength of 520 nm; solutions of FITC-dextran in saline

buffered at different pH values were used for the construction of a standard curve.

Pinocytosis

As a quantitative marker for fluid-phase pinocytosis we employed horse-radish peroxidase (0.1 mg/ml; Sigma) [4]. After the cells were allowed to pinocytize the marker for the indicated periods, they were washed three times in ice-cold saline and lysed in saline containing Triton X-100 (2 mg/ml). Aliquots of extracellular medium and cell lysate were assayed for horse-radish peroxidase activity using hydrogen peroxide and tetramethylbenzidine [12] as substrates. Values are expressed as ml extracellular medium taken up per ml packed cells. To this end, we estimated the pellet volume from the protein content of the suspensions, taking 1 ml packed cells to correspond to 0.10 g cell protein [5].

Protein

Protein was determined by the method of Lowry *et al.* [13] as modified by Markwell *et al.* [14].

Bafilomycins

Bafilomycins A_1 and B_1 were kindly provided by Drs. K. Altendorf and A. Siebers, University of Osnabrück. Stock solutions were prepared in dimethyl sulfoxide and stored at −20 °C. The actual concentrations of the stock solutions were determined spectrophotometrically [15]. The final concentration of dimethyl sulfoxide in the incubations, including the control incubations, was 0.2% (v/v).

Results

In agreement with results published by other investigators [7], we found that pinocytic vesicles in *E. histolytica* are acidified: as shown in Fig. 1 vesicles took on an acidic pH (approx. pH 5.5) within 1 min after pinocytic membrane invagination, both in growth medium (circles) and in incubation saline (squares). They then kept this acidic pH over a time course of minutes (Fig. 1) to hours (not shown).

Vesicle pH was independent of the extracellular pH between pH 5 and 8 (not shown), but was influenced by the salt content of the extracellular

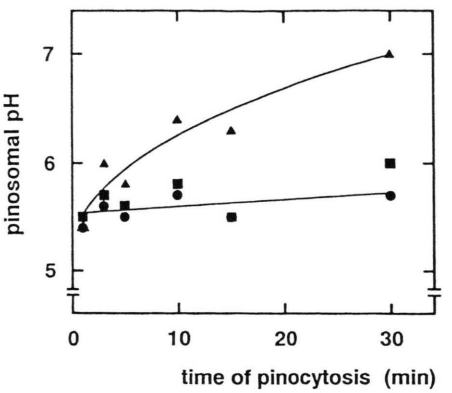

Fig. 1. Time course of the effect of deletion of extracellular salt on pinosomal pH. After the cells were preincubated for 10 min at 36 °C in the indicated media, FITC-dextran was added at 2 mg/ml. At the indicated time points, cells were washed and pinosomal pH was determined from the fluorescence of FITC-dextran as described in Materials and Methods. ●, TYI-S medium [10]; ■, incubation saline; ▲, salt-depleted medium consisting of 366 mM sorbitol and 10 mM Pipes brought to pH 7.0 with Tris base.

medium: specifically, the virtual absence of permeant anions and -cations during pinocytosis rapidly raised the pinosomal pH to that of the medium (Fig. 1, triangles). This observation is compatible with the mechanism of acidification established [6, 16] for the organelles of higher eukaryotic cells, namely, electrogenic proton pumping followed by secondary ion movement (Cl^- influx and/or Na^+ efflux). An increase in pinosomal pH was also observed in the presence of weak bases (data not presented, see also [7]); as we have shown previously [17], pinocytosis is blocked under these conditions, as it is in higher eukaryotic cells [18].

Organelles in the latter are known to be acidified by a special class of H^+-ATPases, the V-ATPases [6]. It has recently been shown [8] that V-ATPases are very specifically inhibited by the bafilomycins. With this in mind we set out to test the effect of bafilomycins on pinosomal pH in *E. histolytica*. In preliminary experiments we established that both bafilomycin A_1 and bafilomycin B_1 affected the cells, the former being the most effective inhibitor. We therefore performed the following experiments with bafilomycin A_1.

Fig. 2 shows the effect of bafilomycin A_1 as a function of inhibitor concentration on pinosomal pH (circles) and pinocytosis (squares). Clear ef-

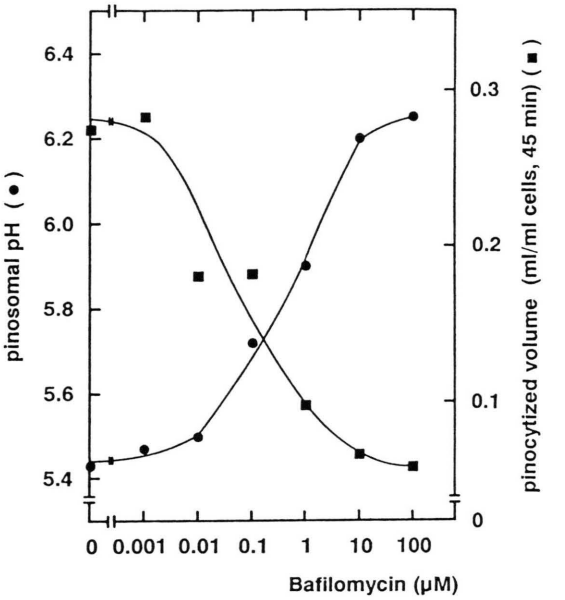

Fig. 2. Effects of bafilomycin A_1 on pinosomal pH and pinocytosis as a function of inhibitor concentration. Cells were incubated for 2 h at 36 °C in saline containing FITC-dextran (2 mg/ml). Then they were washed, resuspended to a protein concentration of 1.41 mg/ml, and incubated for 45 min at 36 °C in the indicated concentrations of bafilomycin A_1. Horse-radish peroxidase (0.1 mg/ml) was added as a marker for pinocytosis, and the suspensions were incubated for another 45 min at 36 °C. Subsequently pinosomal pH (●) and pinocytosis (■) were determined as described in Materials and Methods.

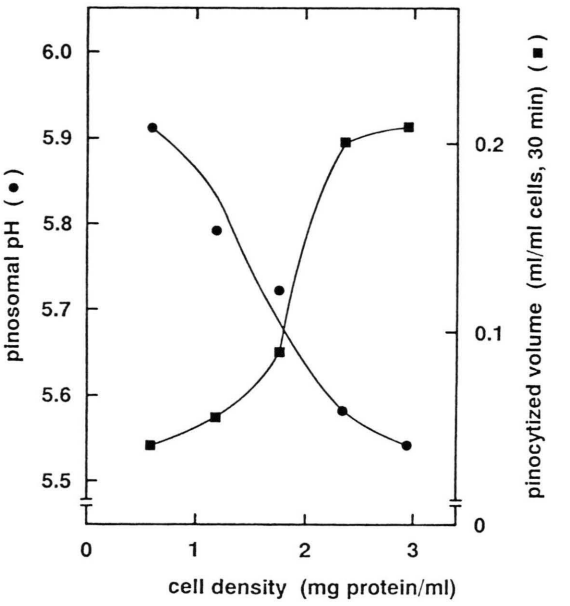

Fig. 3. Effect of a fixed concentration of bafilomycin A_1 on pinosomal pH and pinocytosis as a function of cell protein concentration. Cells were allowed to pinocytize FITC-dextran for 2 h at 36 °C, washed, and resuspended to the indicated protein concentrations. Bafilomycin was added at a concentration of 0.1 nmol/ml, and the suspensions were incubated for 30 min at 36 °C. Then horse-radish peroxidase was added, and the cells were incubated for another 30 min at 36 °C. Cells were washed and pinosomal pH (●) and pinocytosis (■) were determined as described in Materials and Methods.

fects on both processes can be noted at bafilomycin concentrations as low as 0.1 μM; at 10 μM, pH was raised by 0.8 units and pinocytosis was suppressed by over 75%. These observations are in agreement with the notion [20] that the two effects are causally related, in the sense that proton pumping is essential for membrane fusion.

Bafilomycins are lipophilic compounds. Consequently, in other membrane systems their action is directly dependent upon the amount of protein in the assay [8]. Fig. 3 shows that this applies to *E. histolytica* as well: the effects of a fixed concentration of bafilomycin A_1 decreased with increasing cell density. Again, an inverse relationship was found between pinosomal pH and pinocytosis. From Figs. 1 and 2 and an additional experiment (not shown) we calculated the amount of bafilomycin A_1 needed to inhibit pinocytosis by 50% (associated with an increase in pinosomal pH of 0.3 pH units) as between 0.05 and 0.3 nmol/mg cell protein. These values are within the range determined for the inhibition of V-ATPase activity in vacuolar membranes of *Neurospora crassa* and *Zea mays* and membranes of chromaffin granules from bovine adrenal medulla [8]. This constitutes strong evidence for the existence of a V-type ATPase in pinocytic vesicles of *E. histolytica*.

Discussion

In *E. histolytica,* contrary to the situation in higher eukaryotic cells, the protein composition of plasma membrane and pinosomal membranes appears to be very similar if not identical [3, 4]. This may be related to the apparent lack in this ameba [2] of structures such as coated pits and -vesicles, which in higher eukaryotic cells are instrumental in maintaining the protein composition of the different compartments [19].

In the framework of our results, this has an interesting implication: if – as suggested by the data presented above – the pinosomal compartment in *E. histolytica* contains a V-type ATPase, this enzyme must be present in the plasma membrane, too. It has been postulated [20, 21] that the V-ATPase found in the intracellular organelles of higher eukaryotic cells originally resided on the plasma membrane of a protoeukaryote, and was taken up by endocytosis. In this sense, the V-type ATPase of *E. histolytica* could well represent an intermediate step in evolution, in which the enzyme has been internalized but has not yet been exclusively relegated to the internal compartment.

Because of this evolutionary implication it would clearly be important (i) to confirm biochemically that the enzyme is present on the plasma membrane, (ii) to investigate whether it is the sole H^+-ATPase present there, and (iii) to characterize the protein on the molecular level. In our attempts to do so, we have so far been hampered by two experimental problems: (i) the cells possess an extremely active ecto-ATPase that masks any ATPase activity due to ion pumps (manuscript in preparation); and (ii) we have as yet been unable to observe Mg^{2+}-ATP-induced acidification of pinocytic vesicles isolated [5] under different conditions (including the presence or absence of dithioerythritol, Mg^{2+} and ATP).

As we have mentioned above, *E. histolytica* appears to possess an endoplasmic reticulum-like organelle that is separate from the pinocytic compartment. A final question that needs to be answered is whether this organelle contains a V-type ATPase as well, and, if so, whether this ATPase is identical to that present in pinosomes and – presumedly – in plasma membrane.

Acknowledgements

This work has been supported by the Deutsche Forschungsgemeinschaft (SFB 171, Project C2) and by the Fonds der Chemischen Industrie. We thank Drs. Annette Siebers and Karlheinz Altendorf for providing us with bafilomycins and for helpful discussions.

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