Exocytosis, endocytosis and membrane recycling in

*Tetrahymena thermophila*

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Summary

Mutant and wild-type cell lines of *Tetrahymena thermophila* were used to investigate a possible connection between acid hydrolase secretion and the major processes through which membranes are recycled in this ciliated protozoon. These processes consist of food vacuole formation (endocytosis), and food vacuole egestion and mucocyst release (both exocytosis). We have found that a mutant (MS-1, sec−) blocked in hydrolase secretion is not blocked in either food vacuole formation or egestion and that it has normal mucocyst exocytosis. Another line of experiments with wild-type cells showed also that hydrolase secretion and endocytosis are independent of each other. Thus, sucrose (0.1 M) did not interfere with hydrolase secretion, but blocked food vacuole formation. Furthermore, release of acid hydrolases was selectively stimulated by dibucaine without any effect on food vacuole egestion. Finally, exocytosis of mucocysts could occur without simultaneous release of acid hydrolases, as when cells were exposed to 0.15 M NaCl, which evokes a massive secretory response of mucocysts. Our results demonstrate that formation and egestion of food vacuoles and exocytosis of mucocysts are unrelated to secretion of acid hydrolases. Furthermore, they suggest that secretion of acid hydrolases is not a secondary effect of membrane recycling through these processes.

Key words: membrane recycling, secretion, acid hydrolases, *Tetrahymena thermophila*.

Introduction

Release of acid hydrolases to the extracellular medium by the ciliate *Tetrahymena* has been the object of many studies (Blum & Rothstein, 1975; Müller, 1971; Nilsson, 1979). Müller (1972) found that released acid hydrolases originate from the denser of two populations of lysosomes and considered this release to be secretion. We have recently substantiated this view and shown that enzyme release is enhanced by Ca^{2+}-mobilizing agents and that the hydrolases are liberated without loss of other cytoplasmic components (Tiedtke et al. 1984).

The cellular route of the hydrolases is unknown. To date, lysosomal enzymes are not known to pass the membrane of the lysosome (Creek & Sly, 1984) and therefore vesicles that contain lysosomal enzymes should fuse with the plasma membrane before release of enzymes. Here, we investigate a possible connection of this process with other events taking part in membrane traffic in *Tetrahymena*.

Materials and methods

Strains

The wild-type strains B11 and B VII, and the mutant MS-1 of *Tetrahymena thermophila* blocked constitutively in release of lysosomal enzymes (Hünseler et al. 1987a), were used in this study. The phenotype of MS-1 is caused by a recessive single gene mutation (Tiedtke et al. 1987).

Culture conditions

The cells were grown in PPYS medium: 1% proteose peptone (Difco), 0.1% yeast extract (Difco), 0.003% sequestrene (Geigy). Cell cultures were inoculated at initial concentrations of 2×10^6 cells ml^{-1} and grown at 30°C in a shaker.
Evaluation of egestion rates

Cells in PPYS medium were labelled with India ink (0.05% final concn) for 20 min at 30°C. During this pulse the cells ingested India ink particles and contained many black food vacuoles. The cells were then washed twice with PPYS medium to remove remaining India ink particles, resuspended in fresh PPYS medium and incubated at 30°C for 30 min. At the end of this chase period many labelled food vacuoles were observed in the posterior parts of the cells ready for egestion through the cytoproct (Fig. 1A–C). In this way, egestion could be followed from the start of the experiment. The wild-type (B VII) and the mutant MS-1 cell suspensions were adjusted to 8 × 10^6 cells ml^-1 following the pulse-labelling with India ink. At each time point indicated four samples of cells were removed and 0.5 ml of each sample was fixed for food vacuole countings, the remaining cell suspensions were centrifuged and the cell-free supernatants were collected on ice for assays of enzyme activity. Out of each fixed sample 60 cells were chosen randomly and the number of labelled food vacuoles per cell was found.

To measure the action of dibucaine on the egestion of labelled food vacuoles, wild-type cells (B III) were washed after a chase of 30 min in PPYS medium into 10 mM-Tris·HCl, pH 6.0. The cells were exposed to the drug at 25°C for 15 min and then processed for food vacuole counting and enzyme assays as described above for control cells not treated with the drug.

Dibucaine treatment

Solutions of 6 mmol l^-1 of dibucaine (Sigma) in 10 mM-Tris·HCl, pH 6.0, were freshly prepared before each experiment. At pH 6.0 but not at higher pH values dibucaine was readily soluble. No meaningful pH variations were observed during the experiments in spite of the reduced buffer capacity of Tris·HCl at pH 6.0. Cultures of the wild type (B III) were adjusted to 1.5 × 10^6 cells ml^-1 after the end of the 30-min chase period and samples of one ml (containing cells with labelled food vacuoles) were added to 15 ml centrifugation tubes containing 2 ml of the corresponding drug solution. The drug concentrations before addition of the cells were 1.5 times the final concentration indicated in Results. Control experiments were performed identically using the corresponding buffer without drugs. The tubes with the cells were placed in a tilted rack to ensure aeration of the cell suspension. Immediately before the end of the incubation periods small samples were withdrawn and the status and behaviour of the drug-treated cells were controlled under a microscope. Samples for food vacuole countings and enzyme assays were withdrawn as described above. The data were statistically analysed with the help of Student's t-test.

Alcian Blue-induced mucocyst exocytosis

The method is based on previous observations of the secretagogue activity of this dye (Tiedtke, 1976). It consisted of exposing cell suspensions starved for 2h to 0.1% Alcian Blue and observing in the light microscope the blue-stained mucocyst aggregates around the cells and in the medium. The question whether the Alcian Blue treatment also caused an enhanced secretion of acid hydrolases was not investigated: the Alcian Blue remaining in the supernatant interacts strongly with proteins and prevents meaningful enzyme determinations.

Exposure to 0.1 M-sucrose

Wild-type cells (B III) were washed twice in 10 mM-Tris·HCl, pH 7.4, and the washed cell suspension adjusted to 10^6 cells ml^-1 was divided into two portions. One was mixed with an equal volume of 0.2 M-sucrose in 10 mM-Tris·HCl, pH 7.4, while the other received the same volume of buffer without sucrose. Each of these suspensions was immediately divided in two. One was used to measure released enzyme activities and the other to follow food vacuole formation after addition of India ink (0.05%, final concn). The cells were incubated at 25°C and samples were withdrawn at 30 and 60 min for food vacuole countings and enzyme assays.

Exposure to 0.15 M-NaCl

In this experiment washed B III cells in 10 mM-Tris·HCl, pH 7.4, were either mixed with an equal volume of the same buffer or the same buffer supplemented with 0.3 M-NaCl, under continuous stirring. Suspensions were allowed to stand at 25°C for 2 min and centrifuged. Supernatants were collected for enzyme assays and mucocyst inspection.

Inspection of mucocysts

Samples of the supernatants of both control and NaCl-treated cells were exposed to cationized ferritin after addition of an equal volume of 2 mM-MgCl2. Observation of mucocysts stained in this way was performed using a Zeiss Photomicroscope. For electron microscopy (EM), the samples were spread on carbon-coated grids, negatively stained with 1% aqueous solution of uranyl acetate and observed with a Siemens Elmiskop at 60 kV.
Enzyme assays

Enzyme activities of the cell-free supernatants were assayed at 37°C, using \( p \)-nitrophenyl-conjugated substrates for acid phosphatase (EC 3.1.3.2), N-acetyl-\( \beta \)-D-hexosaminidase (EC 3.2.1.52), \( \alpha \)-mannosidase (EC 3.2.1.24) and \( \alpha \)-glucosidase (EC 3.2.1.20) at final concentrations of 10 mmol l\(^{-1} \), according to Tiedtke (1983a). One milliunit (1 mU) is defined as the amount of enzyme that releases 1 nmol of \( p \)-nitrophenol per min at 37°C. Isocitrate dehydrogenase (IDH, EC 1.1.1.42) was assayed colorimetrically according to the supplier's information (Biochemica Information II, Boehringer-Mannheim). Isocitrate dehydrogenase, a sensitive cytosolic marker enzyme of *Tetrahymena* (Borden et al. 1975), was measured during the drug experiments to exclude cell lysis as a source of released lysosomal enzymes.

Results

**Acid hydrolase secretion (AHS) and egestion of marked food vacuoles in the wild-type and a mutant blocked in AHS**

Food vacuoles of wild-type (B VII) and mutant (MS-1) cell lines were pulse-labelled with India ink. After the 30-min chase period that allows the food vacuoles to collect at the posterior end, the cells were incubated for 3 h at 30°C in nutrient medium. Samples were removed every 30 min for determinations of enzyme activity and every 60 min for estimation of number of food vacuoles per cell. The results are shown in Fig. 2. It can be observed that the mean number of food vacuoles decreased gradually in the wild type and in the mutant MS-1 blocked in AHS. Although the initial mean number of food vacuoles was slightly higher in the wild type (10 per cell) than in the mutant (9 per cell) both strains released a similar number of marked food vacuoles per unit of time, indicating that MS-1 was not impaired in egestion of food vacuoles. In spite of this the mutant did not release \( \beta \)-hexosaminidase and acid phosphatase (and other lysosomal enzymes, data not shown) above the background levels characteristic for this mutant.

**Mucocyst exocytosis in wild-type and mutant cells**

Mucocyst secretion was induced in wild-type (B VII) and mutant (MS-1) cells by exposure to Alcian Blue. The cell lines were alike in their release of mucocysts, showing that MS-1 has normal mucocyst exocytosis.

**Influence of dibucaine on release of acid hydrolases and egestion of marked food vacuoles**

Table 1 shows the results of exposure of wild-type cells (B VII) to 100 or 200 \( \mu \)M-dibucaine on release of four lysosomal enzymes and egestion of labelled food vacuoles. The drug considerably stimulated the release of acid phosphatase, \( \beta \)-hexosaminidase, \( \alpha \)-glucosidase and \( \alpha \)-mannosidase. The mean number of remaining food vacuoles per cell was, however, similar in control and drug-treated cells showing that food vacuole egestion was not increased by dibucaine. These results point to an uncoupling of AHS and food vacuole egestion.

Dibucaine also stimulated AHS in the mutant MS-1 in about the same proportions as it did in wild-type cells. Mutant cells treated for 15 min with dibucaine (100 \( \mu \)M) released 0.51 ± 0.03 mU ml\(^{-1} \) of acid phosphatase and 0.47 ± 0.027 mU ml\(^{-1} \) of \( \beta \)-hexosaminidase. The activities released by the cells not treated with dibucaine were 0.16 ± 0.01 mU ml\(^{-1} \) and 0.18 ± 0.005 mU ml\(^{-1} \), respectively. This represents a 3.2-fold stimulation of acid phosphatase and a 2.6-fold stimulation of \( \beta \)-hexosaminidase release. The corresponding increases in wild-type cells were 2.97-fold for
Table 1. Influence of dibucaine on the egestion of food vacuoles and the release of acid hydrolases in the wild type (B III) of T. thermophila

<table>
<thead>
<tr>
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<th>Released enzyme activities (mU ml⁻¹) and mean numbers of labelled food vacuoles per cell</th>
<th>Mean number of food vacuoles per cell*</th>
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<tbody>
<tr>
<td></td>
<td>Acid phosphatase</td>
<td>β-Hexosaminidase</td>
</tr>
<tr>
<td>Control</td>
<td>1:07 ± 0:045</td>
<td>1:12 ± 0:07</td>
</tr>
<tr>
<td>100 µM-dibucaine</td>
<td>3:18 ± 0:27</td>
<td>2:3 ± 0:03</td>
</tr>
<tr>
<td>200 µM-dibucaine</td>
<td>5:33 ± 0:11</td>
<td>3:16 ± 0:13</td>
</tr>
</tbody>
</table>

*The mean number of labelled food vacuoles at the beginning of the drug treatment was 11:3 ± 0:3. Release of acid hydrolases by the drug-treated cell suspensions is significantly higher (P < 0:005) than release of acid hydrolases in the untreated control suspension. The mean numbers of food vacuoles per cell are not significantly different from control samples in dibucaine-treated cells. Data were analysed with the help of Student's t-test.

Acid phosphatase and 2:23-fold for β-hexosaminidase (Table 1).

Addition of sucrose and effects on food vacuole formation and AHS

Incubation of wild-type cells (B III) in buffer containing 0:1M-sucrose completely prevented food vacuole formation during a 60-min period. Control cells formed 10:6 ± 0:8 vacuoles per cell within the same period. Release of acid phosphatase and β-hexosaminidase was identical in treated and non-treated cells.

NaCl induced mucocyst exocytosis

Massive exocytosis of mucocysts can be induced by brief (2 min) exposure of the cells to 150 mM-NaCl. Discharged mucocysts stayed in the supernatant after centrifugation. They were detected in the light microscope as rod-like structures after negative staining with cationized ferritin (Fig. 3A). The identity of these rods as decondensed mucocysts matrices was verified by EM. Samples negatively stained with uranyl acetate exhibited the regular fibrillar pattern and uniform size characteristic of decondensed mucocysts matrices (Fig. 3B). Supernatants of control preparations only occasionally contained mucocysts. As summarized in Table 2, the NaCl treatment induced massive exocytosis of mucocysts but did not stimulate AHS. Treatment with 150 mM-NaCl rapidly immobilized the cell and caused osmotic shrinkage. However, the cell recovered from this treatment when diluted 10-fold with 10 mM-Tris·HCl, pH 7·4, at the end of the 2-min incubation. Cells resumed normal shape and motility during the following 2 h.

Discussion

Membrane recycling is the means by which a cell maintains its plasma membrane surface constant in spite of processes that continuously add and withdraw portions of it. This is an obvious need for cells with high rates of endocytosis and exocytosis. We have dealt here with a T. thermophila mutant that is blocked in acid hydrolase secretion (AHS), which can grow and multiply normally (Hünseler et al. 1987a). This mutant...
can proceed without acid hydrolase release (AHS) in cell surface. We used the method described by Gottlieb. We can therefore conclude that membrane recycling and egest food vacuoles, and to discharge mucocysts. via wild-type cells was obtained by exposure to (HM-

face enzymes and found that MS-1 has a significantly deficient dissociation of the enzymes from receptors at the plasma membrane after exocytosis. In such a case, it should be expected that hydrolases accumulate on the cell surface. We used the method described by Gottlieb & Dwyer (1981) for determination of membrane surface enzymes and found that MS-1 has a significantly lower content of acid phosphatase and β-hexosaminidase activities on the cell surface than wild-type cells (unpublished data). Since biosynthesis of lysosomal enzymes is not impaired in this mutant (Hünseler et al. 1987b), the mutation probably affects the behaviour of the vesicles containing acid hydrolases, preventing their fusion with the plasma membrane. This emphasizes the view that AHS is separate from the mainstream of membrane recycling in Tetrahymena. Dissociation of food vacuole formation from AHS in wild-type cells was obtained by exposure to 0.1 mM-sucrose, which blocks the former but not the latter. This result agrees with the study by Silberstein (1979) on a Tetrahymena mutant cell line that is blocked in food vacuole formation but secretes essentially normal amounts of acid hydrolases. It also confirms the report by Müller (1975) of the independence of AHS from the contractile vacuole activity, which, unlike AHS, is inhibited in media with high osmolality.

It was proposed that the acid hydrolases left the cells via the egested food vacuoles. Rothstein & Blum (1974) based this view on a simultaneous stimulation of AHS and food vacuole egestion mediated by catecholamine antagonists. Our results, however, both with the mutant MS-1 and with the wild-type cells exposed to dibucaine, which may enhance enzyme release by an increase in cytoplasmic free Ca²⁺ (Tiedtke et al. 1984), demonstrate that egestion of vacuoles is unrelated to AHS. This seems to be the case also for both Dictostelium (Dimond et al. 1984) and Acanthamoeba (Hohman & Bowers, 1984). We must point out that the secretory mutant MS-1 can grow on bacteria as sole food source as fast as the wild-type cells (Hünseler et al. 1987a). It has, therefore, a normal intracellular digestion process, i.e. fusion of lysosomes with food vacuoles. Such fusion has recently been demonstrated by Fok & Schokley (1985) in Tetrahymena growing in PPY medium. As a consequence, we can conclude that retrieval or inactivation of lysosomal enzymes must take place prior to egestion, since MS-1 normally egests food vacuoles but does not release enzymes. Evidence for retrieval of lysosomal enzymes has been obtained for another ciliate, Paramecium caudatum (Allen & Fok, 1984).

A possible role for mucocyst exocytosis in AHS was suggested by the finding of acid phosphatase activity associated with these organelles (Tiedtke & Görtz, 1983). The method employed, however, permitted no quantification of the amounts of enzyme released in this way. Our present experiments, in which exposure to 0.15 M-NaCl results in massive discharge of mucocysts without enhancement in AHS, show that these two processes are essentially independent. This agrees well with our previous observation that another secreted hydrolase, β-hexosaminidase, is not associated with discharging mucocysts, as shown by immunostaining with specific antibodies against this enzyme (Tiedtke, 1983b). As to the biological role of the secreted hydrolases, their exploitation in extracellular digestion has already been shown for Tetrahymena (Rasmussen et al. 1986). The observations presented here suggest that AHS, at least in Tetrahymena, should be regarded as an independent cellular event and not just as an incidental consequence of membrane recycling, as Hohman & Bowers (1984) proposed for Acanthamoeba castellani.

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<th>Acid phosphatase</th>
<th>β-Hexosaminidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.83 ± 0.07</td>
<td>0.80 ± 0.09</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.55 ± 0.06</td>
<td>0.43 ± 0.04</td>
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</tbody>
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Secretion of acid hydrolases is significantly lower (P < 0.005) in NaCl-treated cells.

References


