Release of lysosomal enzymes in *Tetrahymena*: a Ca^{2+}-dependent secretory process

A. TIEDTKE[1], L. RASMUSSEN[2], J. FLORIN-CHRISTENSEN[3] and M. FLORIN-CHRISTENSEN[3]

[1]*Zoological Institute, University of Münster, Schloßplatz 5, D-4400 Münster, FRG*
[2]*Institute of Anatomy and Cytology, University of Odense, Campusvej 55, DK 5230 Odense M, Denmark*
[3]*Center for Parasite and Vector Research, National University of La Plata, Calle 2, No. 584, 1900 La Plata, Argentina*

Summary

The ciliate *Tetrahymena thermophila* releases lysosomal enzymes into nutrient and starvation media. We show here that this process occurs selectively, i.e. without leakage of cytoplasmic components, as indicated by lack of release of isocitrate dehydrogenase, a cytosolic enzyme with high activity in *Tetrahymena*. The role of intracellular Ca^{2+} in the process was also investigated. The Ca^{2+} ionophore A23187 has strong stimulatory effects on this release. Ionophore stimulation is maximal in the presence of extracellular Ca^{2+} but can occur also in its absence. Quin 2 fluorescence measurements indicate that intracellular Ca^{2+} increases in both cases. Mg^{2+} completely prevents the stimulatory effects of A23187. Ionomycin, another Ca^{2+} ionophore, also stimulates lysosomal enzyme release with a maximal response in the presence of extracellular Ca^{2+}. Measurements of extracellular isocitrate dehydrogenase showed that ionophore-stimulated lysosomal enzyme release can take place without leakage of cytoplasmic components. The observations that divalent cation ionophores stimulate selective lysosomal enzyme release and that this effect is strongest in the presence of external Ca^{2+} indicate that this cation plays a crucial role in the control of this process in *Tetrahymena*. Together with other observations they support the view that a subpopulation of *Tetrahymena* lysosomes has properties like those of secretory vesicles.

Key words: lysosomal enzymes, secretion, *Tetrahymena thermophila*.

Introduction

Eukaryotic cells export materials contained in secretory vesicles, mainly by exocytosis. In this process, the membrane of the vesicle fuses with the plasma membrane, leading to discharge of its contents into the extracellular medium. Two features are fundamental to this process. First, it is selective, i.e. only the contents of the vesicle are released and no leakage of cytoplasmic components takes place (Rubin, 1984). Second, it is stimulated by an increase in the concentration of cytoplasmic free Ca^{2+} (Plattner, 1981; Douglas, 1981).

Release of lysosomal enzymes (RLE) into the medium in *Tetrahymena*, a ciliated protozoon, has often been studied (for reviews, see Blum & Rothstein, 1975; Nilsson, 1979). Müller's (1972) study is particularly relevant and supports the idea that the process is not a consequence of cell disintegration. He showed that the release took place without obvious decrease in cell numbers and found that the enzymes originated from the denser of two populations of acid-hydrolase-containing particles. In addition, he observed that the specific activities of the lysosomal enzyme were higher in the medium than in cell homogenates, suggesting that the release was selective for these enzymes. Demonstration of the secretory nature of RLE, however, requires direct evidence of selectivity and Ca^{2+} dependency. First of all, it should be proved that cytoplasmic markers are not leaked during the process. An enzymic assay could provide the required information. Second, Ca^{2+} dependency can be studied by the use of divalent cation ionophores, i.e. agents that greatly increase permeability of membranes to these ions.

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In this study we have found isocitrate dehydrogenase to be a cytoplasmic marker for *Tetrahymena* and we have investigated Ca^{2+} dependency of RLE using the two ionophores, A23187 and Ionomycin.

Materials and methods

Materials

Tetrahymena thermophila strain BIII (Nanney & McCoy, 1976) was used in this study. Proteose peptone and yeast extract were from Difco (MI, USA), Sequestrene was from Ciba Geigy (Basel, Switzerland). A23187, Quin 2, Quin 2-AM and isocitric acid were from Sigma Chemical Co. (MO, USA). Ionomycin was from Calbiochem (CA, USA). *p*-Nitrophenylphosphate and NADP were from Boehringer-Mannheim (Mannheim, FRG). EGTA and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were from Serva (Heidelberg, FRG). Solutions were made with double-distilled water. All other chemicals were of analytical grade.

Methods

Cell cultures. *T. thermophila* was cultivated with stirring at 25°C in medium containing 1% proteose peptone, 0·1% yeast extract and 0·003% Sequestrene.

Exposure to ionophores. Cells from log cultures were washed twice in 10 mM-Tris·HCl, pH 7·0, and finally resuspended in 20 mM-Tris·HCl, 125 mM-sucrose, 1 mM-EDTA, pH 7·0 (TSE buffer), at 5×10^5 cells ml^{-1}. This buffer was used because it was found that sucrose greatly prevented the cell swelling that occurred when the cells were exposed to A23187 in 10 mM-Tris·HCl, pH 7·0; 1 mM-EDTA, on the other hand, was included in order to have defined conditions with respect to otherwise uncontrolled Ca^{2+} concentrations, due to contamination from glassware and buffer substances. Addition of 2 mM-$CaCl_2$ or -$MgCl_2$ resulted in $\simeq$1 mM free Ca^{2+} or Mg^{2+}. Ionophore solutions were added to these suspensions using a Hamilton microsyringe of adequate size, while stirring. Controls were treated with the same concentrations of solvents as those in which the ionophores were dissolved. At 0 min and after incubation at 25°C for the indicated times, the cell suspensions were centrifuged and the supernatants were kept for enzyme assays. When indicated, $CaCl_2$ or $MgCl_2$ was added to the cell suspensions from 100 mM stock solutions before addition of the ionophores.

Cell suspensions were sonicated at setting 3 in an Ultrasonics sonifier, using three pulses of 10 s for determination of enzyme concentrations. This fully released the enzyme activities.

A23187 was dissolved in ethanol/dimethylsulphoxide (DMSO), 3:1 (v/v), to a 6 mM stock solution and kept at −20°C. Inomycin was dissolved in DMSO to a 10 mM stock solution and kept at 4°C. All experiments were carried out in parallel duplicates and repeated at least three times.

Fluorescence measurements in Quin-2-loaded cells. Cells were washed twice in 10 mM-Tris·HCl, pH 7·0, and resuspended in the same buffer. Quin 2-AM (10 mM stock solution in DMSO) was added to a final concentration of 100 μM. The same amount of DMSO was added to another equal portion of the cell suspension to be used as blank for cell autofluorescence and fluorescence due to A23187. After incubation for 90 min at 25°C, the cells were washed in 10 mM-Tris·HCl, pH 7·0; and finally resuspended in TSE buffer. Less than 20% of the initial Quin 2-AM remained in the supernatant of the loading mixture. A relatively high concentration of Quin 2-AM, as well as a prolonged period of exposure were required to obtain signals sufficiently high to be clearly distinguished from fluorescence changes associated with the addition and incorporation of A23187.

Only part of the Quin 2-AM taken up by the cells was hydrolysed to free Quin 2; although fluorescence (F 339→492) increased throughout incubation, emission spectra (F 339→λ) did not reach the peak at 492 nm characteristic of pure Quin-2 free acid. The presence of the indicator form, Quin 2, however, was verified by the large change in fluorescence in cell homogenates upon addition of $CaCl_2$ or EGTA. Addition of Ca^{2+} to Quin 2-AM, on the other hand, had no effect on fluorescence.

Fluorescence was measured in an Aminco Bowman spectrofluorometer in 3 ml Quartz cuvets, thermostatically controlled at 25°C, and under continuous stirring. Fluorescence was excited at 339 nm and read at 492 nm (F 339→492). Recordings were made with an LKB 2000 recorder.

At the end of the experiments maximum fluorescence was read after sonication of the cells in the presence of 0·02% Triton X-100 and addition of 2 mM-$CaCl_2$. The latter was omitted in the cases where 2 mM-Ca^{2+} was already present in the incubation medium. Minimum fluorescence was measured after adding 3 mM-EGTA and 30 mM-Trizma base to the same cell sonicate. Maximum fluorescence corresponds therefore to a free Ca^{2+} concentration of about 1 mM, while minimal fluorescence corresponds to less than 1 nM-Ca^{2+} (Tsien *et al.* 1982). From these values, fluorescence due to A23187 measured in non-loaded cells was subtracted.

Enzyme assays. The volume activities found in cell free supernatants were measured for acid phosphatase (EC 3.1.3.2) and *N*-acetyl-β-D-glucosaminidase (β-hexosaminidase, EC 3.2.1.30) as described by Tiedtke (1983). Isocitrate dehydrogenase (IDH, EC 1.1.1.42) was selected as cytoplasmic marker for its very high activity in the cytosol. Furthermore, the enzyme showed great stability: a cell homogenate obtained by sonication did not show changes in IDH activity after 6 h at 30°C. IDH was assayed spectrophotometrically as described in Biochemica Information II, Boehringer-Mannheim. All enzyme assays were linear with respect to time and enzyme concentration. The assays were not affected by the drugs and salts used throughout this study. To prevent inhibitory action of $CaCl_2$ on IDH activity, 0·8 mM final concentration of K_2EGTA was added to the assay mixtures to compensate for free Ca^{2+} present. Model experiments showed that this amount of K_2EGTA had no inhibitory effects *per se* on the activity, whether or not Ca^{2+} was present.

The activities of the released enzymes are expressed as percentages of the corresponding total cellular activities after subtracting the values corresponding to time 0, at which incubations were started. Enzyme assays were carried out in duplicate. All results correspond to mean values of two parallel experiments with duplicate enzyme determinations ± standard deviation. Data were analysed statistically using Student's *t*-test.

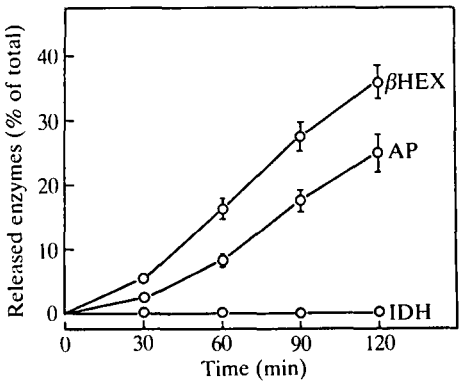

Fig. 1. Extracellular activities of two lysosomal enzymes, β-hexosaminidase (βHEX) and acid phosphatase (AP), and the cytoplasmic marker isocitrate dehydrogenase (IDH), under starvation conditions. Cells were transferred to 10 mM-Tris·HCl, pH 7·0, and incubated at 25°C at time zero.

Results

Release of acid hydrolases in *Tetrahymena* takes place without concomitant release of isocitrate dehydrogenase (IDH), a cytoplasmic enzyme. Fig. 1 shows released activities of β-hexosaminidase, acid phosphatase and IDH as functions of time after transfer to a starvation medium.

The ionophore A23187 markedly enhances lysosomal enzyme release. This effect is shown in Fig. 2A,B, in which the results of exposure to 3 μM-A23187 are depicted. The effect is greatest in the presence of external Ca^{2+}, but also takes place when Ca^{2+} is not added to the TSE medium. Addition of 2 mM-Sr^{2+} has effects similar to those of Ca^{2+} (not shown). Mg^{2+}, on the other hand, completely prevents stimulation. Stimulated release of hydrolases is not accompanied by leakage of IDH (Fig. 2C). This activity becomes significantly different from the low control values only at 20 min of incubation of the cells with A23187 in the absence of $CaCl_2$. Observation of the cells under light microscopy showed a small fraction (less than 2%) of swollen cells in this case. Incubation with A23187 in the presence of Ca^{2+} decreased cell motility, with up to 20% of the cells immobilized at 10 min. At 20 min, however, motility was recovered in most cells. Higher concentrations of A23187 (>5 μM) resulted in release of large amounts of IDH and cell lysis.

Increasing Ca^{2+} concentrations in the incubation medium enhances stimulation. Fig. 3 shows the effects of 2 μM-A23187 in TSE and in TSE with the addition of 2 mM- and 3 mM-$CaCl_2$. A23187 concentrations of 1 μM or less were without effect, regardless of whether Ca^{2+} was added to the medium or not.

We have used Quin 2-AM to obtain an indication of the changes in intracellular free Ca^{2+} concentrations induced by application of A23187 in TSE, with and without added Ca^{2+}. Typical recordings of the changes of fluorescence in Quin-loaded and control cells upon exposure to A23187 are depicted in Fig. 4. The results show that following addition of A23187, a distinct transient change in fluorescence takes place in Quin-loaded cells in excess of the increases due to A23187 alone, which can be followed in non-loaded cells. The changes in Quin-loaded cells are more rapid but of similar magnitude in the presence of external free Ca^{2+}. Fluorescence variations in non-loaded cells are due to the intrinsic fluorescence of A23187, which is affected by divalent cations and attachment to membranes. When 1 μM-A23187 was added to Quin-loaded cells, fluorescence changes were less than one fifth of those shown in Fig. 4.

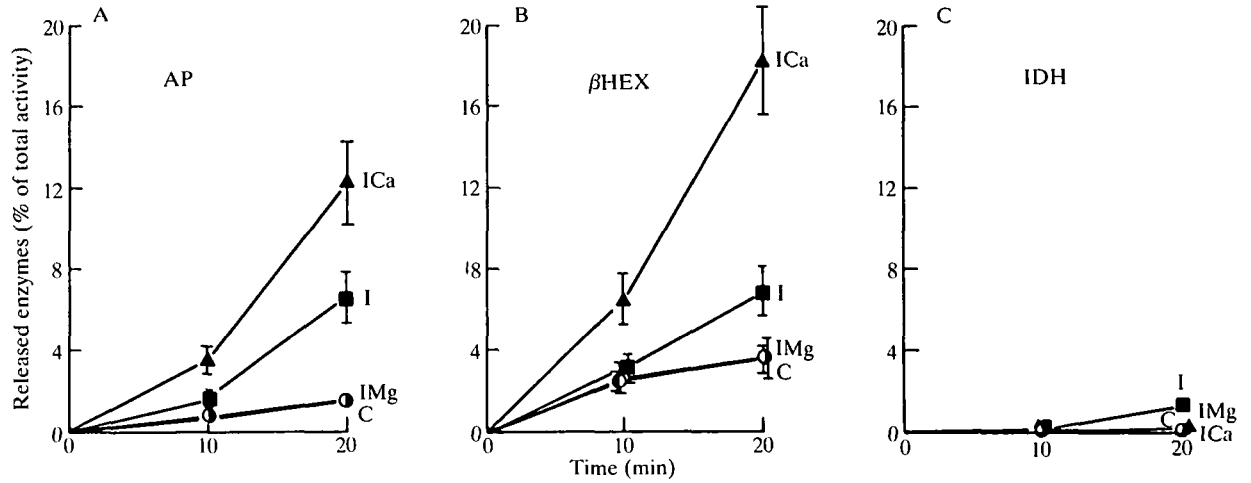

Fig. 2. Release of three enzymes induced by 3 μM-A23187 in the presence and absence of Ca^{2+} and Mg^{2+}. Cells were incubated at 25°C in Tris/sucrose/EDTA buffer with addition of ionophore solvent (control, C, (○)), 3 μM-A23187 (I, (■)), 3 μM-A23187+2 mM-$CaCl_2$ (ICa, (▲)) or 3 μM-A23187+2 mM-$MgCl_2$ (IMg, (●)). Samples were withdrawn at 10 and 20 min for assay of extracellular enzyme activities.

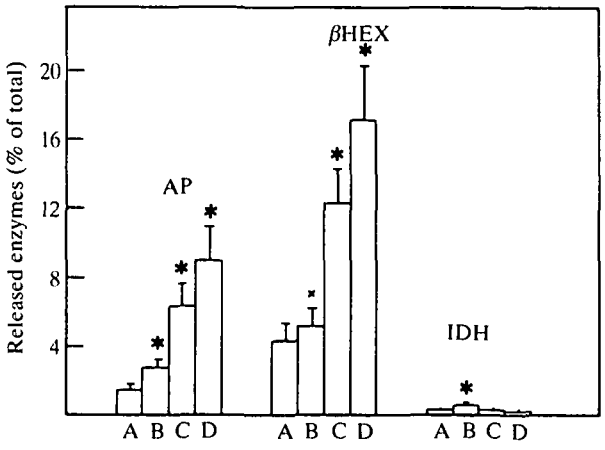

Fig. 3. Effects of increasing Ca^{2+} concentrations on A23187-induced enzyme release. Released enzymes were assayed after incubation of the cells for 20 min at 25 °C in Tris/sucrose/EDTA buffer with addition of: A, ionophore solvent; B, 2 μM-A23187; C, 2 μM-A23187+2 mM-$CaCl_2$; D, 2 μM-A23187+3 mM-$CaCl_2$. In experiments not shown we found that addition of 2 mM- and 3 mM-$CaCl_2$ to cells in TSE buffer without A23187 did not stimulate enzyme release. AP, acid phosphate; βHEX, β-hexosaminidase; IDH, isocitrate dehydrogenase. * and × mark values that differ from controls, with $P < 0.05$ and $P < 0.1$, respectively.

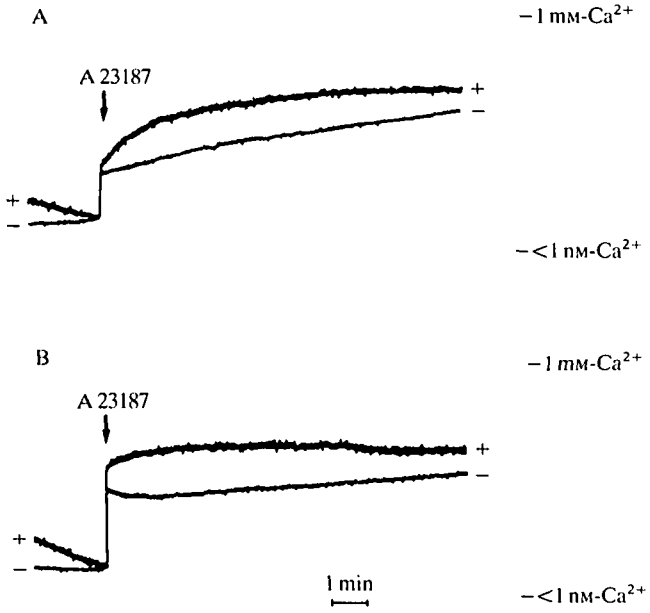

Fig. 4. Fluorescence changes in Quin-2-loaded and control cells upon exposure to 3 μM-A23187 in Tris/sucrose/EDTA buffer (A) and the same buffer plus 2 mM-$CaCl_2$ (B). + and − refer to recordings in presence and absence of Quin, respectively. Fluorescence values at the moment of A23187 application were subtracted in all cases to facilitate comparison of the curves. The marks <1 nM-Ca^{2+} and 1 mM-Ca^{2+} indicate calibration points obtained as described in Materials and methods.

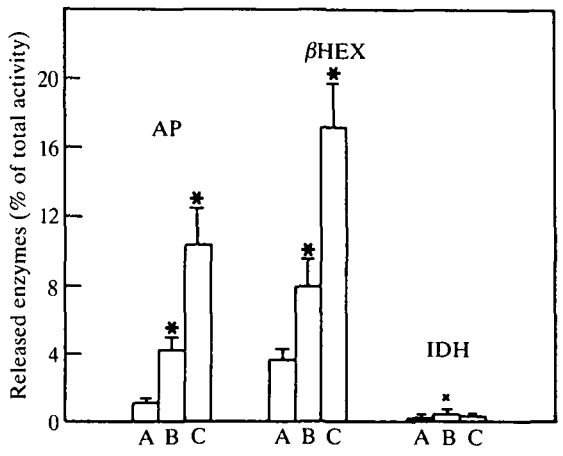

Fig. 5. Effects of the ionophore Ionomycin on enzyme release. Released enzymes were assayed after 20 min incubation of the cells at 25 °C in Tris/sucrose/EDTA buffer with addition of: A, solvent; B, 80 μM-Ionomycin; and C, 80 μM-Ionomycin+2 mM-$CaCl_2$. * and × mark values that differ from controls, with $P < 0.05$ and $P < 0.1$, respectively. See Fig. 3 legend for abbreviations.

A second Ca^{2+} ionophore, Ionomycin, was tested for its effect on enzyme release. Results were comparable to those obtained with A23187 except that much higher concentrations of Ionomycin are required. These data are shown in Fig. 5.

Discussion

This study demonstrates the selectivity of lysosomal enzyme release with respect to cytoplasmic markers in *Tetrahymena*. This information is basic to the elucidation of the nature of this phenomenon.

We also offer the first evidence of the Ca^{2+} dependency of this process. Our results show that the divalent cation ionophore A23187 can selectively stimulate release of lysosomal enzymes in *Tetrahymena*. This effect is more intense in the presence of free Ca^{2+} in the medium but can take place without addition of this cation. Influx of Ca^{2+} from the extracellular fluid can account for the action observed when this cation is available from the incubation medium. When Ca^{2+} is not externally supplied, the ionophore may act by mobilizing this ion from intracellular sources. Our Quin 2 fluorescence measurements strongly suggest that whether Ca^{2+} is added to the medium or not, intracellular Ca^{2+} indeed rises upon exposure to A23187. Mobilization of Ca^{2+} from intracellular sources is accepted to account for the effects of this drug in other cell types (Pozzan *et al.* 1982). In *Tetrahymena* it has been proposed that the alveolar sacs function as intracellular Ca^{2+} reservoirs (Satir & Wissig, 1982). Muto & Nozawa (1985), on the other hand, demonstrated accumulation of Ca^{2+} by *Tetrahymena* microsomal membrane vesicles. The stimulation

of release of lysosomal enzymes by A23187 is prevented by incorporation of Mg^{2+} into the medium. This agrees with results for other Ca^{2+}-dependent processes in *Tetrahymena* and other cells (Matt *et al.* 1978; McMillan *et al.* 1980; Wissig & Satir, 1980).

The results obtained with Ionomycin further support the views presented here, i.e. that intracellular Ca^{2+} plays an important role in the control of release of lysosomal enzymes in *Tetrahymena*.

Lysosomal enzymes are synthesized on membrane-bound ribosomes and are processed, transported and stored in the membrane-delimited compartment of the cell. Lysosomal enzymes have not been reported to pass the lysosomal membranes (Greek & Sly, 1984). Therefore, it is very likely that the observed specific release of lysosomal enzymes to the cell environment is due to an exocytic process. Our observation that this release of lysosomal enzymes can be selectively stimulated by drugs inducing elevation of intracellular free Ca^{2+} concentrations is consistent with this view. It supports the notion that *Tetrahymena* has a population of lysosomes with properties similar to those of classic eukaryotic secretory vesicles.

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