Distinctive inhibition of the lysosomal targeting of lysozyme and cathepsin D by drugs affecting pH gradients and protein kinase C

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INTRODUCTION

The targeting of soluble lysosomal enzymes, such as cathepsin D (EC 3.4.23.5), to lysosomes is mediated by mannose 6-phosphate residues and specific receptors (Kornfeld, 1992). Several observations indicate that in mammalian cells other, as yet unknown mechanisms, participate in the transport of the soluble enzymes to lysosomes (von Figura and Hasilik, 1986). In Saccharomyces cerevisiae, protein structure signals are responsible for the targeting of proteases to the vacuole (Paravicini et al., 1992). In human cells, lysozyme (EC 3.2.1.17) may be a candidate for carbohydrate-independent lysosomal targeting. During the 70 years of research since its discovery (Fleming, 1922), lysozyme has become one of the most studied proteins (Jolles and Jolles, 1984; McKenzie and White, 1991). Nevertheless, little is known about its biological actions or its intracellular transport. Lysozyme is considered to be a secretory product of various epithelial cells and leukocytes. However, it is also present in leucocytes where it is associated with secretory granules and lysosome-like organelles (Baggiolini et al., 1969; Miyachi et al., 1985; Cramer and Breton-Gorius, 1987; Mapp and Revell, 1987; Ohtani and Sasano, 1988; Mutasa, 1989).

Ralph et al. (1976) have reported that, in the human promonocytic cell line U937, approx. two-thirds of the activity of lysozyme is found in the medium and the remainder in the cells. In our previous work reporting on a subcellular fractionation of U937 cells, lysozyme had been found in the same fractions as typical lysosomal marker enzymes (Gupta et al., 1985). We have used 1,25-di-hydroxycholecalciferol (calcitriol)-treated U937 cells, in which the detection of both cathepsin D and lysozyme is facilitated owing to an enhanced rate of transcription (Redecker et al., 1989). We present immunocytochemical evidence that lysozyme and cathepsin D localize to the same organelles and, further, results on metabolic labelling which show that the transport of these two enzymes to lysosomes is distinctly inhibited in the presence of agents known to affect the acidification of intracellular compartments or the activity of protein kinase C.

MATERIALS AND METHODS

Cells, culturing and metabolic labelling

Human promonocytic U937 cells were cultured in RPMI 1640 medium (Gibco BRL, Eggenstein, Germany) that was supplemented with 10% (v/v) heat-inactivated fetal-calf serum (bio pro, Karlsruhe), 100 units/ml penicillin and 100 μg/ml streptomycin under air/CO₂ (19:1, v/v). All experiments were performed with cells which were cultured (starting at a density of 1.5 x 10⁶ cells/ml) in the presence of 0.5 μM calcitriol for 3 days. Calcitriol was donated by Dr. M. R. Uskokovic (Hoffmann La Roche, Nutley, NJ, U.S.A.). 4β-Phorbol 12-myristate 13-acetate (4β-PMA; ‘TPA’) was from Sigma Chemie (Deisenhofen, Germany) and R-59022, an inhibitor of protein kinase C, was from Biomol (Hamburg, Germany). Metabolic labelling was performed in the above medium, lacking methionine or methionine and cysteine, in the presence of 0.4–2 MBq/ml of [³⁵S]methionine (specific radioactivity 40 TBq/mmol, from Amersham-Buchler, Braunschweig, Germany) or Tran³⁵S-label (specific radioactivity 40 TBq/mmol, from ICN Biomedicals).

Immunoprecipitation and separation of the labelled proteins

The preparation of extracts of cell and growth medium, immunoprecipitation with affinity-purified antibodies and purified goat anti-(rabbit IgG) antibody-coated Eupergit CIZ (Röhm Pharma, Weiterstadt, Germany), washing and solubilization of the immunoprecipitates were all performed as described previously (Grässel et al., 1989). The labelled proteins were separated in SDS/PAGE (Laemmli, 1970). The standards used were phosphorylase b, BSA, ovalbumin, carbonic anhydrase and cyto-
chrome c with the nominal molecular masses, in kDa, of 97.4, 69, 46, 30 and 12.3 respectively. The radioactivity was detected by fluorography (Laskey and Mills, 1975) and, in selected experiments, it was quantified by scintillation counting (Waheed et al., 1982) and densitometry using a laser densitometer (Ultroscan model from Pharmacia).

**Enzyme assays**

The activity of β-hexosaminidase and β-glucuronidase was determined with the corresponding p-nitrophenyl glycoside substrates. The samples were filled up to 50 μl with 0.14 M NaCl and the assay was started by adding 50 μl of a 10 mM substrate solution prepared in 0.2 M Na/citrate buffer (pH 4.6)/0.2% (v/v) Triton X-100/0.04% (w/v) NaN3/0.2% (w/v) BSA. After an incubation at 37 °C the reaction was stopped by adding 0.5 ml of 0.4 M glycine/NaOH buffer, pH 10.4. The absorbance was determined at 405 nm. One unit was calculated as 1 μmol of substrate utilized/min (ε 18.4 mM⁻¹ cm⁻¹).

**Electron microscopy**

The cells were fixed with 2% (w/v) paraformaldehyde in 50 mM sodium phosphate buffer, pH 7.4. Ultra-thin cryosections of 50-100 nm thickness were prepared and subjected to double labelling (Geuze et al., 1981) with affinity-purified antibodies against lysozyme and cathepsin D, and 5- and 9-nm-diam. Protein A-coated gold particles. Then, the sections were stained with uranyl acetate and embedded in methylenchlorose (Geuze et al., 1981). To estimate the area of lysosomes the profiles were treated as ovals and circles and the lengths of their axes or diameters were measured.

**Statistics**

The activity of β-hexosaminidase was determined in each cell homogenate and medium in triplicate. Data from separate cultures are given as means ± S.D. (number of cultures). Data from radioactive labelling of lysozyme were calculated as percentages of that recovered from the cells and the medium in each culture. At most two cultures with the same treatment were studied in parallel. Differences between mean values were analysed by two-sided Student’s t-test (level of significance P < 0.05).

**RESULTS**

**Localization of lysozyme and cathepsin D in lysosomes**

To determine the intracellular localization of lysozyme, ultra-thin frozen sections of fixed U937 cells were prepared and lysozyme was detected by sequential incubations with specific antibodies and Protein A-coated gold particles of different sizes. In Figure 1 a representative ultra-thin section is shown in which lysozyme and cathepsin D are detected using small and large gold particles respectively. Most of the lysozyme molecules detected by the antibody localize to organelles that also contain cathepsin D, and vice versa.

For a statistical evaluation the gold particles were counted in five fields from two sections. Altogether 99 lysosomes were identified as labelled round or oval vesicles circumscribed by a single membrane. Their average area was (33 ± 14) × 10⁻ⁱ⁵ m² [range (10–92) × 10⁻¹⁵ m²]. All of them (n = 99) contained particles of both sizes. The average scores were 19 ± 11 for cathepsin D and 11 ± 5 for lysozyme. The average ratio of cathepsin D to lysozyme counts was 1.98 ± 1.13. Furthermore, cathepsin D and lysozyme were found in small vesicles and tubules. The vesicles contained a smaller number of particles, often of only one kind. However, the scarcity of the labelling of the tubules and vesicles precluded a statistical evaluation for these compartments. It can be concluded that lysozyme is delivered to the same organelles as cathepsin D. This is supported by previous results on subcellular fractionation of U937 cells, in which lysozyme has been found in the same fractions as cathepsin D and β-hexosaminidase (Gupta et al., 1985).

**Lysosomal targeting of lysozyme and cathepsin D and its sensitivity to 4β-PMA and NH₄Cl**

In most cells in which we can detect lysozyme, such as human colon carcinoma Caco-2, hepatoma HepG2, promonocytic THP-1 cells and in baby hamster kidney cells transfected with human lysozyme cDNA (Horst et al., 1991), more than 90% of lysozyme is secreted. In contrast, the promonocytic U937 cells package a relatively large proportion of lysozyme into lysosomes. For the following reason we suggest that this packaging is intracellular. We have shown previously (Gupta et al., 1984) that U937 cells endocytose cathepsin D in a mannos 6-phosphate-sensitive manner. Now, under the same conditions, we have examined the uptake by U937 cells of cathepsin D and lysozyme from a secretion of metabolically labelled cells. At a concentration of 1 × 10⁶ cells/ml the clearance rate of lysozyme from the medium is 1.4 μl/mg of protein per 2 h (mean of two measurements). It is more than an order of magnitude less than the clearance rate of cathepsin D (results not shown). Under the conditions used it could account for ≤ 0.3% of the produced lysozyme being delivered to lysosomes via secretion and endocytosis.

A search for agents that would interfere selectively with the targeting of either lysozyme or procathepsin D we have observed that 4β-PMA at a concentration as low as 2 nM stimulates preferentially the secretion of the former. Maximum secretion is observed at 0.1–1 μM 4β-PMA. In the experiment shown in Figure 2 the effects of 0.5 μM 4β-PMA on the secretion of lysozyme and procathepsin D are compared with those of 10 mM NH₄Cl. In the presence of 4β-PMA (lane 3) the apparent intracellular retention of lysozyme is strongly inhibited and that of cathepsin D only partially so. In contrast, in the presence of

![Figure 1](image-url) **Co-localization of lysozyme and cathepsin D in U937 cells**

A representative ultra-thin cryosection of calcein-treated U937 cells that was processed for electron microscopy is shown. Lysozyme and cathepsin D were immunolabelled with small (5 nm, arrowhead) and large (9 nm, arrow) Protein A-coated gold particles respectively. Note the presence of both antigens in the lysosomal profiles. The bar corresponds to 200 nm.
Calcitriol-treated U937 cells were subjected to 9 h pulse/3 h chase labelling with Tran35S-label in the presence or absence of 10 mM NH4Cl as indicated. Cathepsin D and lysozyme were immunoprecipitated from extracts of cells and medium. The labelled polypeptides were separated by SDS/PAGE and visualized by fluorography. The fluorograms show lysozyme (L) and the precursor (P), intermediate (I) and large mature (LM) polypeptides of cathepsin D. The small mature chain of cathepsin D is not detected, because of its low content of methionine and cysteine. The samples from the cultures treated with NH4Cl show representative the distribution of cathepsin D and lysozyme, although the apparent decrease in the total labelling of lysozyme as seen in lanes 5 and 6 has not been observed in most other experiments with this substance.

NH4Cl the fractional rate of secretion of lysozyme is not significantly changed, whereas that of procathepsin D is strongly enhanced (Figure 2, lane 6). The stimulation of the secretion of procathepsin D by 4β-PMA and NH4Cl is additive (Figure 2, lane 8). When applied separately, NH4Cl stimulates the fractional secretion of procathepsin D and 4β-PMA preferentially stimulates that of lysozyme. There is very little secretion of the mature form of cathepsin D in the 4β-PMA-treated cells, which indicates that secretion of lysozyme in the presence of 4β-PMA is not due to an exocytosis of the contents of mature lysosomes.

From a quantification of the radioactivity present in the cell-associated and secreted cathepsin D in two experiments examining the effect of NH4Cl (results not shown) it can be calculated that the drug enhances the mean fractional rate of the secretion of cathepsin D from 15% to 62%. Interestingly, the efficiency of the NH4Cl-resistant lysosomal targeting of procathepsin D is very similar to that of lysozyme, which by itself is not sensitive to NH4Cl.

Our data on the distribution of metabolically labelled lysozyme between cells and the medium are compatible with those of Ralph et al. (1976), who have examined non-differentiated U937 cells and reported that approx. 35% of the enzymic activity of lysozyme is associated with cells and the remainder with the medium.

To examine at what stage the transport of lysozyme is perturbed in the presence of 4β-PMA, pulse–chase labelling of U937 cells was performed at 20 °C. At this temperature the exit of proteins from the Golgi apparatus is inhibited (Pelham, 1991). After the temperature is raised to 37 °C the transport ensues and its sensitivity to 4β-PMA can be examined. In Figure 3 results of two experiments with the temperature shift are presented. During a 2-h-long labelling and a 4-h-long chase at 20 °C there is no secretion of lysozyme and procathepsin D, and also the maturation of the latter in the cells is prevented (results not shown). In Figure 3(a) it is shown that after warming the labelled cells to 37 °C approx. half of the accumulated radioactive lysozyme is secreted. In the presence of 4β-PMA the secretion is stimulated. It appears to take less than 20 min for half of the secretable portion of lysozyme to become released into the medium at 37 °C. This rate is compatible with that of the transport of secretory proteins from the trans-Golgi apparatus to the plasma membrane (Fries and Lindström, 1986).

From the above results it may be conceivable that there is a rather short period during which lysozyme can be prevented from undergoing lysosomal targeting. In the other experiment...
using the temperature shift (Figure 3b) this is shown by determining the amount of lysozyme that is secreted during a 90 min period, which is started either immediately after the temperature shift or after a delay of up to 80 min. The results show that both the basal and the 4β-PMA-stimulated secretion of the radioactive lysozyme cease within 1 h of incubation at 37 °C. The highest rate of both is observed in the first period (Figure 3b, lanes 1 and 2). After an 80 min delay there is much less secretion (lanes 9 and 10); although, a large amount of the radioactive lysozyme remains within the cells (Figure 3, lanes 11 and 12). Obviously, by this time lysozyme has reached compartments from which little secretion can take place whether 4β-PMA is present or not.

**Stimulation of the secretion of lysosomal glycosidases in the presence of 4β-PMA**

In order to see if 4β-PMA affects the segregation of other lysosomal enzymes that are known to depend on mannose 6-phosphate in their lysosomal targeting we measured the secretion of β-hexosaminidase in control and 4β-PMA-treated cells. Data in Table 1 show that during an 18 h period of incubation with the drug the secretion of β-hexosaminidase is stimulated approx. 5-fold, being increased from 0.40±0.12 munit/ml to 2.18±0.15 munits/ml. While the latter value corresponds to 15% of the total, the activity associated with the cells is not appreciably changed in the presence of 4β-PMA. The effect resembles that observed with procathesper D (Figure 2), indicating that 4β-PMA stimulates the secretion of the newly synthesized enzyme. It is not clear, however, whether the effect of 4β-PMA on secretion is long-lasting. On the one hand, after 18 h much less β-hexosaminidase is found in the medium than would be expected from the activity which is released after 1 h. This may be explained by a temporary nature for the stimulation and also by binding to cells and by endocytosis of the released enzyme. On the other hand, when the incubation for 18 h, with or without 4β-PMA, is followed by that for 1 h without 4β-PMA significantly more enzyme activity is released from those cells which have been preincubated with the drug than from those without (sample numbers 3 and 4, P<0.01). In this case the difference may be due partly to a long-lasting effect of the drug on the secretion, but also due to release of the enzyme from a cell-surface pool that may have built up during the preincubation with 4β-PMA. The presence of β-hexosaminidase at the cell surface of endothelial and other human cells has been demonstrated previously (Hasilik et al., 1981).

A qualitatively similar effect of 4β-PMA can be observed with β-glucuronidase, although, the basal rate of the secretion of this enzyme is lower than that of β-hexosaminidase. After an 18 h incubation with or without 4β-PMA the medium contains 0.026 munit/ml and 0.062 munit/ml of β-glucuronidase activity (mean of two experiments). The latter value is near to 5% of the total activity in the culture. Other human monocytic cells such as THP-1, which show a higher basal rate of the secretion of these lysosomal glycosidases, respond similarly to the treatment with 4β-PMA (results not shown).

**Indirect evidence for a role of protein kinase C in the stimulated secretion of lysozyme**

In U937 cells 4β-PMA has been shown to cause rapidly an enlargement of multivesicular bodies (Nilsson et al., 1989). Commonly, the rapid onset effects of 4β-PMA are considered to result from an activation of one or more protein kinase C isoforms. Also, in calcitriol-treated U937 cells, protein kinase C becomes attached to membranes within a few minutes of the addition of 4β-PMA (Rubin et al., 1991). To see if protein kinase C is involved in the stimulation of lysozyme secretion we have compared the effect of 4β-PMA with that of two related drugs.

In Figure 4, we show that in the presence of the 4α-isomer of 4β-PMA, which is known not to stimulate protein kinase C, the secretion of lysozyme is not changed. In contrast, R-59022, an inhibitor of diacylglycerol kinase (Ederveen et al., 1990), which has potential for indirect stimulation of protein kinase C, enhances the fractional rate of the secretion of lysozyme. The interpretation of these data is complicated by the fact that there is a decrease in the total radioactivity incorporated into lysozyme in the presence of R-59022. However, the total amount of lysozyme in either the

**Table 1 Effect of 4β-PMA on the apparent secretion of β-hexosaminidase**

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>PMA (period)</th>
<th>β-Hexosaminidase (munit/ml)</th>
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<tr>
<td></td>
<td>18 h</td>
<td>Medium (μ)</td>
</tr>
<tr>
<td>1</td>
<td>_</td>
<td>0.40±0.12 (4)</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>2.18±0.15 (4)</td>
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<tr>
<td>3</td>
<td>_</td>
<td>0.11±0.01 (3)</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>0.49±0.10 (4)</td>
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<tr>
<td>5</td>
<td>_</td>
<td>0.57±0.20 (4)</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>0.49±0.28 (4)</td>
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**Figure 4 Effect of 4β-PMA, its 4α isomer and R-59022 on secretion of lysozyme and cathepsin D**

Calcitriol-treated U937 cells were metabolically labelled in 0.5 ml aliquots, containing 2.5x10^5 cells each, in the presence of 0.2 μM 4β-PMA, 0.2 μM 4α-PMA (4α, 1x10^-5 M R-59022 (R59) or 0.1% (v/v) each of dimethyl sulphoxide and ethanol which were used as solvents (Co), for 18 h at 37 °C. The fluorogram shows the labelled cathepsin D and lysozyme that were immunoprecipitated simultaneously and separated in SDS/PAGE.
control or treated cells is not changed any further if the treated cultures are subjected to a chase incubation for up to 48 h (results not shown). This result suggests that R-59022 partially inhibits the labelling of lysozyme and, simultaneously, stimulates its secretion rather than causing a loss of the cellular enzyme. To examine the significance of the stimulation of the secretion, we have evaluated quantitatively the radioactivity associated with the intracellular and secreted lysozyme using calcitriol-treated cells that had been metabolically labelled for 18 h in the absence or presence of R-59022. For this purpose data were taken from the experiment shown in Figure 4 and from three others. The quantification was performed with six pairs of control and R-59022-treated cells. In the presence of the drug 64.2 ± 1.4% lysozyme was secreted and in the control it was only 50.8 ± 7.3% \((n = 6, P < 0.01)\). Under the same conditions 4β-PMA enhanced the secretion to 88.3 ± 3.4% \((n = 3)\). The effects of the examined drugs suggest that protein kinase C may be the mediator of the strong stimulation of the secretion of lysozyme as observed in the presence of 4β-PMA.

**DISCUSSION**

A distinctive feature of the lysosomal targeting in U937 cells is the sequestration of approx. one-third of the transported lysozyme from the secretory pathway into the lysosomes. In cells treated with 4β-PMA this targeting is strongly inhibited. In contrast, the lysosomal transport of procathepsin D is only partially inhibited. When the cells are treated with NH₄Cl to inhibit the acidification of the lysosomal and prelysosomal compartments, and thus the mannose 6-phosphate-dependent segregation of lysosomal enzyme precursors (von Figura and Hasilik, 1986; Kornfeld, 1992), the rate of the targeting of lysozyme is little affected, whereas that of procathepsin D is suppressed to approximately the same level. Under these conditions the lysosomal targeting of procathepsin D is rather sensitive to 4β-PMA also. The lysosomal transport of lysozyme and the acidification-independent lysosomal transport of procathepsin D may be paradigms of alternative targeting such as are postulated to operate in tissues that are little or not affected by the I-cell mutation (von Figura and Hasilik, 1986; Kornfeld, 1992).

In the presence of 4β-PMA the secretion of β-hexosaminidase is also elevated. Previously, Leoni and Dean (1983) observed no enhancement of the secretion of β-hexosaminidase in U937 cells incubated with 1 nM 4β-PMA. However, this is a much lower concentration of the drug than used in the present work. We have examined the dose dependence of the secretion of procathepsin D and observed that for the maximum effect more than 10 nM 4β-PMA is needed.

In the presence of NH₄Cl the formation in trans-Golgi apparatus of vesicles containing mannose 6-phosphate receptors is not blocked (Braulke et al., 1987). It is the unloading of the phosphorylated ligands from the receptor, but not the packaging or the transport of the receptor, which is inhibited. Therefore, the lack of inhibition of the lysosomal segregation of lysozyme in the presence of NH₄Cl does not preclude a packaging of lysozyme into the same vesicles as cathepsin D. In this case the packaging of lysozyme into these vesicles would be selectively inhibited by 4β-PMA. As an alternative, lysozyme may be packaged into distinct vesicles in an as yet unidentified portion of the Golgi apparatus in a 4β-PMA-sensitive manner. Finally, 4β-PMA may affect a more distal event such as fusion of lysozyme-bearing vesicles with their address compartment.

The rapid onset of the stimulation of the secretion of lysozyme after the addition of 4β-PMA, as shown in Figure 3, is consistent with an involvement of the signalling cascade, commencing with protein kinase C. This suggestion is supported by the observation that both 4α- and 4β-PMA and R-59022, but not the 4α- isomer of 4β-PMA, enhance the secretion of lysozyme.

It is known that 4β-PMA affects the exocytosis of soluble proteins and the subcellular distribution of membrane proteins. For example, 4β-PMA stimulates the secretion of specific-granule contents, including lysozyme, in neutrophils (Barrowman et al., 1987), of milk protein in epithelial cells (Turner et al., 1992), or of noradrenaline in PC12 cells (Nishiizaki et al., 1992). Among a variety of proteins which are translocated to the plasma membrane as a result of treatment of cells with 4β-PMA there is also the mannose-6-phosphate/insulin-like growth factor II receptor (Braulke et al., 1990). This effect has been recognized in human fibroblasts and shown not to be accompanied by a stimulation of the secretion of mannose-6-phosphate-containing ligands. To find out if this holds also for procathepsin D, we examined its secretion in human fibroblasts, which had been treated with 5 × 10⁻⁷ M 4β-PMA. In the absence of NH₄Cl the targeting was not inhibited by 4β-PMA, but in its presence it was (A. Hasilik, unpublished work).

In reports from our and other laboratories (Hasilik et al., 1984; Akin and Kinkade, 1986; Strömberg et al., 1986) it has been suggested that in promyelocytes the packaging of myeloperoxidase does not depend on the mannose 6-phosphate pathway, because it is not inhibited by NH₄Cl. Our current study shows that in the presence of 4β-PMA myeloperoxidase is released into the medium (C. Uhänder, and A. Hasilik, unpublished work). Based on our present findings we suggest that, in differentiating leucocytes, transport of enzymes into granulolike organelles and fusion of the latter with lysosomes may contribute to the lysosomal targeting.

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