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Bafilomycin A_1 Inhibits the Targeting of Lysosomal Acid Hydrolases in Cultured Hepatocytes

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SUMMARY: Effects of bafilomycin A_1, an inhibitor of vacuolar H^+-ATPase, on the synthesis and processing of cathepsin D and cathepsin H were investigated in primary cultured rat hepatocytes. Pulse-chase experiments showed that after being synthesized as procathepsin D and procathepsin H the precursors were converted into mature forms in the control cells as the chase time elapsed. However, in the presence of 5×10^{-7} M of bafilomycin A_1, both precursors were largely secreted into the medium and no mature forms were found within the cells. Thus bafilomycin A_1 mimics lysosomotropic amines with regard to perturbation of the targeting of lysosomal acid hydrolases. In contrast, bafilomycin A_1 was found not to inhibit processings of proalbumin and procomplement component 3, which are thought to occur at the acidic trans-Golgi, implying that the proteolytic event of the proproteins is not sensitive to an increase of intra-Golgi pH. The results suggest that bafilomycin A_1 is useful as a pH-perturbant to study the role of acidity in living cells.

There is increasingly accumulating evidence that acidic compartments are involved in a variety of cellular events such as degradation of macromolecules, targeting of lysosomal acid hydrolases, polarized exocytosis, receptor-mediated endocytosis, limited proteolysis and virus and toxin entry into the host

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Abbreviations: C3, the third component of complement; MEM, Eagle's minimum essential medium; SDS, sodium dodecyl sulfate; v-ATPase, vacuolar H^+-ATPase.

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cells (1, 2). Acidotropic amines (lysosomotropic amines) have been widely used to study the acidic compartments in living cells and have shed light on many physiologically important functions played by them (1, 2). These amines cause an increase in pH of acidic compartments by consuming proton, which is pumped into the lumens by vacuolar H^+-ATPases (v-ATPase) located on the membranes and further evoke osmotic imbalance across the membranes, culminating in osmotic swelling of acidic compartments (3).

Bafilomycin A_1, a macrolide antibiotic isolated from Streptomyces sp., is a potent inhibitor of v-ATPases (4, 5). Bafilomycin A_1 relatively specifically inhibits v-ATPase on chromaffin granules, lysosomes and the Golgi in vitro(5, 6), raising a possibility that bafilomycin A_1 can be used as a pH-perturbant by directly inhibiting v-ATPase in vivo in cultured cells. In this paper we describe the effects of bafilomycin A_1 on separate acidotropic amine-sensitive proteolytic events in cultured hepatocytes: processing of precursors of lysosomal enzymes (cathepsin D and cathepsin H) and processing of precursors of plasma proteins (serum albumin and the third component of complement).

MATERIALS AND METHODS

Materials: [^{35}S]Methionine (>800 Ci/mmol) and En^{3}Hance were purchased from Dupont-New England Nuclear (MA, Boston). Bafilomycin A_1 is a gift of Dr. Altendorf. 5×10^{-4} M stock solution in dimethylsulfoxide was stored at -20°C. Rabbit antisera against cathepsin D (7), cathepsin H (8) and serum albumin (9) were raised in rabbits as described previously. The antiserum against the third component of complement (C3) of rat was obtained from Cappel Laboratories (West Chester, PA).

Labeling of Hepatocytes: Hepatocytes were isolated from Fischer rats (body weight 250-350g) and cultured (2×10^6 cells/60 mm dish) for 24 h before use in Eagle's minimum essential medium (MEM) supplemented with 5 % new born calf serum as described previously (10). Pulse-chase experiments were performed as described previously (11, 12). Hepatocytes were preincubated in methionine-free MEM with or without bafilomycin A_1, labeled with 100 μCi of [^{35}S]methionine for 30 min and chased upto 5h. Bafilomycin A_1 was present at a final concentration of 5×10^{-7} M

throughout pulse-chase experiments. In control culture dimethylsulfoxide was added at a final concentration of 0.1 %. At the indicated chase-time the medium was removed, cells were washed and lysed in 0.5 ml of lysis buffer (phosphate buffered saline (pH 7.3) containing 1.0 % Triton X-100, 0.5 % sodium deoxycholate, 0.05 % sodium dodecyl sulfate (SDS) as described previously (12).

Immunoprecipitation and Electrophoresis: Immunoprecipitation of cathepsin D and cathepsin H was carried out as described previously (12). Albumin and C3 were immunoprecipitated as described previously (11). The immunoprecipitates were analyzed in SDS-polyacrylamide gel electrophoresis (7.5 % gel for C3; 12.5 % gel for cathepsin D and H) or in gel isoelectric focusing (albumin), followed by fluorography (10).

RESULTS

Cathepsin D and cathepsin H are synthesized as preproenzymes (13-15). The presequences are cleaved off cotranslationally on the rough endoplasmic reticulum, while prosequences are believed to be cleaved at post-Golgi compartments (prelysosomal compartments and/or lysosomes) during the passage of lysosomal enzymes (16, 17). Figs. 1 and 2 show effects of bafilomycin A_1 on the synthesis, processing and transport of cathepsin D and H, respectively. In control cells a 45 KDa procathepsin D (Fig. 1, lane 1) and a 41 KDa procathepsin H (Fig. 2, lane 1) were only forms detected in the cells after the pulse-time. As the chase time passed, the proforms were converted into a mature 43 KDa cathepsin D (Fig. 1A, lanes 3-4), and a 28 KDa cathepsin H (Fig. 2A, lanes 2-4), respectively. Cathepsin D was not detected in the medium throughout the experiment (Fig. 1A, lanes 5-7) and only a minor portion of newly synthesized procathepsin H was secreted into the medium (Fig. 2A, lanes 5-7) as reported previously (12). However, no mature enzymes appeared in the bafilomycin-treated cell even at 5 h-chase period(Figs. 1B and 2B, lane 4). Furthermore significant amounts of proenzymes were found to be released into the medium (Figs. 1B and 2B, lanes 5-7). These effects of bafilomycin on the delivery of lysosomal enzymes are reminiscent of those of acidotropic amines such as

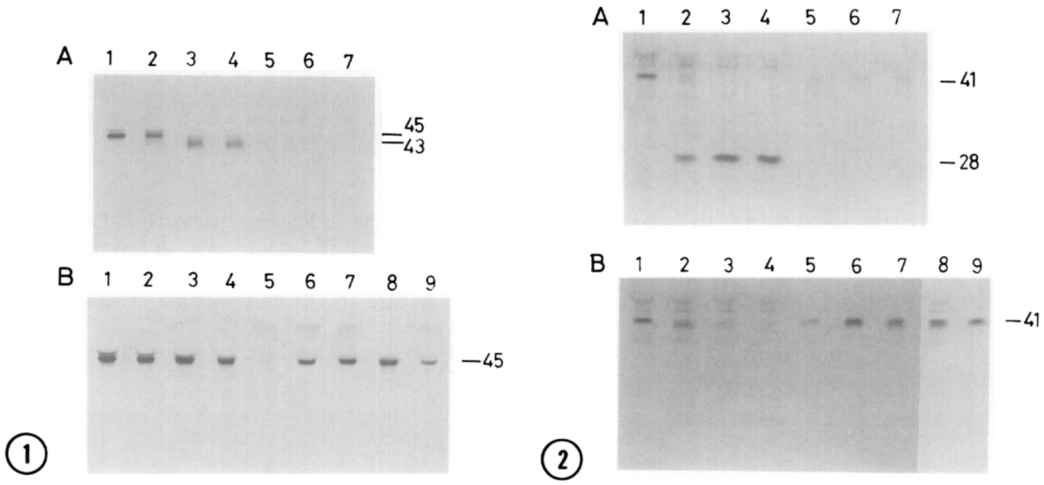
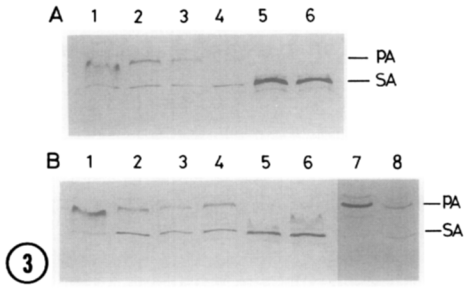


Figure 1. Effects of bafiomycin A_1 on the processing of cathepsin D. Rat hepatocytes were preincubated at 37°C for 30 min in the absence (A) or presence (B) of bafilomycin A_1 (lanes 1-7) at 5×10^{-7} M and methylamine (lanes 8 and 9) at 10 mM. The cells were labeled with [^{35}S]methionine for 30 min and chased in the complete MEM. At indicated times, cell lysates (lanes 1-4, 8) and media (lanes 5-7, 9) were prepared and used for immunoprecipitation of cathepsin D. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography as described under "Materials and Methods". Lane 1, pulse; lanes 2 and 5, 1-h chase; lanes 3 and 6, 3-h chase; lanes 4 and 7-9, 5-h chase. The Numbers at the right side of the gel indicate the masses (in kilodalton) of procathepsin D and cathepsin D from the top of the gel.

Figure 2. Effect of bafilomycin A_1 on the processing of cathepsin H. Hepatocytes were preincubated at 37°C for 30 min in the absence (A) or presence (B) of bafilomycin A_1 (lanes 1-7) at 5×10^{-7} M and methylamine (lanes 8, 9) at 10 mM. The cells were labeled with [^{35}S]methionine for 30 min and chased in the complete MEM. At indicated times, cell lysates (lanes 1-4, 8) and media (lanes 5-7, 9) were prepared and used for immunoprecipitation of cathepsin H. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography. Lane 1, pulse; lanes 2, 5, 8 and 9, 1-h chase; lanes 3 and 6, 3-h chase; lanes 4 and 7, 5-h chase. The Numbers at the right side of the gel indicate the masses (in kilodalton) of procathepsin H and cathepsin H from the top of gel.

chloroquine, ammonium chloride and methylamine (18-20). In fact both procathepsin D and procathepsin H were secreted from the methylamine-treated hepatocytes into the medium (Figs. 1B and 2B, lane 9; 21).

Serum albumin and C3 are synthesized as preproalbumin and preproC3, respectively. After cleavage of presequences on the rough endoplsmic reticulum, both proproteins migrate to the Golgi, where the profoms are believed to undergo a cleavage at



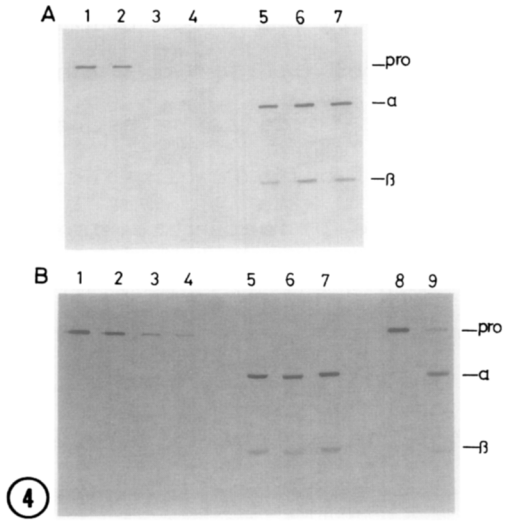


Figure 3. Effects of bafilomycin A_1 on the proteolytic conversion of proalbumin. Hepatocytes were labeled and chased without (A) or with (B) bafilomycin A_1 (lanes 1-6) and methylamine (lanes 7, 8) as described in the legend to Fig 1. After cathepsin D had been immunprecipitated, the remaining cell lysates (lanes 1-4, 7) and the media (lanes 5, 6, 8) were subjected to the immunoprecipitation of albumin. The immunoprecipitates were analyzed by isoelectric gel focusing, followed by fluorography. Lane 1, pulse; lane 2, 1-h chase; lanes 3 and 5, 3-h chase; lanes 4, 6, 7, 8, 5-h chase. PA and SA denote proalbumin and serum albumin, respectively. The pH gradient (8-5) was formed from the top to bottom of the gel.

Figure 4. Effects of bafilomycin A_1 on the proteolytic processing of proC3. Hepatocytes were labeled and chased without (A) or with (B) bafilomycin A_1(lanes 1-7) and methylamine (lanes 8-9) as described in the legend to Fig. 2. After cathepsin H had been immunoprecipitated, the remaining cell lysates (lanes 1-4, 8) and media (lanes 5-7, 9) were subjected to immunoprecipitation of C3. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography. Lane 1, pulse; lanes 2, 5, 8 and 9, 1-h chase; lanes 3 and 6, 3-h chase; lanes 4 and 7, 5-h chase. Pro, α and β denote proC3, α and β subunits of mature C3, respectively.

the dibasic residues Arg-Arg (22-25). Since a basic hexapeptide is removed from proalbumin upon cleavage, serum albumin has a pI value lower than that of proalbumin, allowing us to separate the precursor and product from each other on isoelectric focusing gel. In the case of processing of proC3, a single chain precursor molecule is cleaved to become a mature C3 consisting of α and β subunits linked by disulfide bond. No proforms appeared in the medium throughout the experiment (Fig. 3A, lanes 5, 6 and Fig. 4A, lanes 5-7), indicating that the proteolytic conversion

of proalbumin and proC3 become completed before discharge from the control cells. As shown in Figs. 3B and 4B, only mature forms were found to be secreted into the medium even in the presence of bafilomycin, indicating that bafilomycin does not affect the proteolytic processing of two plasma protein precursors. Contrast to bafilomycin, proalbumin and proC3 were secreted into the medium from methylamine-treated cells (Fig. 3B, lane 8 and Fig. 4B, lane 9), confirming that acidotropic amines inhibit the proteolytic processing occurring at the Golgi (26-29).

DISCUSSION

After being synthesized on the rough endoplasmic reticulum, lysosomal enzymes and plasma proteins move to the Golgi and undergo a variety of posttranslational modifications (16, 17). The sorting for targeting to different destinations is thought to occur at the trans Golgi. Acidity is crucial in the sorting event of lysosomal enzymes. Acidotropic amines, which cause an increase of intraprelysosomal and intralysosomal pH, are known to inhibit the targeting of lysosomal enzymes, resulting in the release of the enzymes (18-21). Bafilomycin mimics acidotropic amines. We have demonstrated that procathepsin D and procathepsin H are released into the medium in the presence of bafilomycin (Figs 1B and 2B). Thus it appears highly likely that bafilomycin elevates intraprelysosomal and intralysosomal pH by directly inhibiting v-ATPase in vivo in cultured hepatocytes, thereby causing the missorting of lysosomal enzymes.

The trans Golgi cisterna and Golgi-associated vesicles are acidic as demonstrated by using pH-sensitive cytochemical probes (30, 31). Acidotropic amines cause not only the swelling of the trans Golgi (32, 33), but also the inhibition of processing of proalbumin and proC3 in cultured hepatocytes (25-

29),suggesting that these processings occur at acidic trans compartments within the Golgi. However, due to the inherent limitations of acidotropic amines, it is difficult to ascertain whether the inhibition of proteolysis by the amines is ascribed to an increase in luminal pH or osmotic swelling of the Golgi, which might reduce a contact between the soluble proproteins and the membrane-bound processing enzyme(s). Since bafilomycin never exerts an inhibitory effect on the limited proteolysis occurring in the Golgi (Figs. 3B and 4B) under the condition in which the targeting of lysosomal enzymes is almost abrogated, we consider it unlikely that the processing enzyme(s) at the Golgi is sensitive to a raise in pH. Recently Umata et al. has shown that bafilomycin A_1 blocks the acidotropic amine sensitive-endocytotic pathway of diphteria toxin in Vero cells (34). Bafilomycin is also reported to affect the degradation of internalized epidermal growth factor in A431 cells (35), the maturation of autophagic vacuoles in H35 cells (36) and the secretion of prolactin in GH3 cells (37). Thus, it appears likely that bafilomycin is able to affect various acidic compartments in cultured cells, though the possibility that bafilomycin somehow fails to gain access to the Golgi in vivo in cultured cells can not be ruled out at present.

Taken together these findings reveal that bafilomycin A_1 can be used as a pH-perturbant for the investigation of physiological role of acidity of subcellular organelles instead of hitherto widely used acidotropic amines, which inherently have a side effect to cause osmotic swelling of acidic compartments.

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REFERENCES

1) de Duve, C. (1983) Eur. J. Biochem. 137, 391-397
2) Mellman, I, Fucks, R , and Helenius A. (1986) Ann. Rev. Biochem. 55, 663-700
3) Poole, B., Ohkuma, S. (1981) J. Cell Biol. 90, 665-669
4) Werner, G., Hagenmajer, H., Drautz, H., Baumbartner, A., and Zahner, H. (1984) J. Antibiot. (Tokyo) 37, 110-117
5) Bawman, E. J., Siebers, A., and Attendorf, K. (1988) Proc. Natl. Acad. Sci. USA 85, 7972-7976
6) Moriyama, Y., and Nelson, N.(1989) J. Biol. Chem. 264, 18445-18450
7) Yamamoto, K., Katsuda, N., Himeno, M., and Kato, K. (1979) Eur. J. Biochem. 95, 459-467
8) Nishimura, Y., and Kato, K. (1987) Biochem. Biophys. Res. Commun. 146, 159-164
9) Ikehara, Y., Oda, K., and Kato, K. (1977) J. Biochem.(Tokyo) 81, 1293-1297
10) Oda, K., Misumi, Y., and Ikehara, Y. (1983) Eur. J. Biochem. 135, 209-216
11) Oda, K., Ogata, S., Koriyama, Y., Yamada, E., Mifune, K., and Ikehara, Y. (1988) J. Biol. Chem. 263, 12576-12583
12) Oda, K. and Nishimura, Y. (1989) Biochem. Biophys. Res. Commun. 163, 220-225
13) Erickson, A. H., and Blobel, G. (1979) J. Biol. Chem. 154, 11771-11774
14) Faust, P., Kornfeld, S., and Chirgwin, J. M. (1985) Proc. Natl. Acad. Sci. USA 82, 4910-4914
15) Takio, K., Towatari, T., Katsunuma, N., Teller, D. C., and Titani, K. (1983) Proc. Natl. Acad. Sci. USA 80, 3666-3670
16) von Figura, K., and Hasilik, A. (1986) Annu. Rev. Biochem. 55, 167-193
17) Kornfeld, S. (1987) FASEB J. 462-468
18) Gonzalez-Noriega, A., Grubb, J. H., Talkad, V., and Sly W. S. (1980) J. Cell Biol. 85, 839-852
19) Hasilik, A., and Neufeld, E. F. (1980) J. Biol. Chem. 255, 4937-4945
20) Rosenfeld, M. G., Kreibich, G., Popov, D., Kato, K., and Sabatini, D. D. (1982) J. Cell Biol. 93, 135-143
21) Nishimura, Y., and Kato, K. (1988) Arch. Biochem. Biophys. 262, 159-170
22) Ikehara, Y., Oda, K., and Kato, K. (1976) Biochem. Biophys. Res. Commun. 72, 319-326
23) Oda, K.. Ikehara, Y., and Kato, K. (1978) Biochim. Biophys. Acta 536, 97-105
24) Redman, C. M., Banerjee, D., Manning, C., Haung, C. Y., and Green, K. (1978) J. Cell Biol. 77, 400-416
25) Oda, K., Miki, K., Hirose, S., Takami, N., Misumi, Y., and Ikehara, Y. (1986) J. Biochem. (Tokyo) 100, 1669-1675
26) Oda, K., and Ikehara, Y. (1985) Eur. J. Biochem. 152, 605-609
27) Oda, K., Koriyama, Y., Yamada, E., and Ikehara, Y. (1986) Biochem. J. 240, 739-745
28) Oda, K., Hirose, S., Takami, N., Misumi, Y, Takatsuki, A., and Ikehara, Y. (1987) FEBS Lett. 214, 135-138
29) Hotin, G., and Strauss, A. W. (1986) Biochem., Biophys. Res. Commun. 136, 603-609
30) Anderson, R. G. W., and Pathak, R. K. (1985) Cell 40, 635-643
31) Schwartz, A., Strous, G. J.A. M., Slot, J. W., and Geuze, H. J. (1985) EMBO J. 4, 899-904
32) Strous, G. J., Du Maine, A., Zijderhand-Bleekemolen, J. E., Slot, J. W., and Schwartz, A. L. (1985) J. Cell Biol. 101, 531-539

33) Koriyama, Y., Oda, K., and Yamada, E. (1989) Biomedical Research 10, 341-451
34) Umata, T., Moriyama, Y., Futai, M., and Mekada, E. (1990) J. Biol. Chem. 265, 21940-21945
35) Yoshimori, T., Yamamoto, A., Moriyama, Y., Futai, M., and Tashiro, Y. (1990) Cell Struct. Funct. 15, 447 (Abstr.)
36) Yamamoto, A., Yoshimori, T., Masaki, R., Moriyama, Y., Futai, M., and Tashiro, Y. (1990) Cell Struct. Funct. 15, 446 (Abstr.)
37) Henomatsu, N., Yoshimori, T., Moriyama, Y., Futai, M., and Tashiro, Y. (1990) Cell Struct. Funct. 15, 447 (Abstr.)