Biochemical Pharmacology, Vol. 31, No. 4, pp. 593–595, 1982.
Printed in Great Britain.

0006-2952/82/040593-03 $03.00/0

Evidence for the selective inhibition of fusion between endocytic vesicles and lysosomes by benzyl alcohol

(*Received* 6 *April* 1981; *accepted* 25 *August* 1981)

Two main problems in endocytosis are (1) the mechanism of fusion between endocytic vesicles and lysosomes, and (2) whether all endocytic vesicles fuse with lysosomes, or just certain types of vesicles. Analysis of these problems is made difficult by the circumstance that no common chemical agent is really a selective inhibitor of either proteolysis within the lysosomes or fusion between endocytic vesicles and lysosomes.

Leupeptin acts primarily by inhibiting cathepsin B and related proteases [1]. Prolonged treatment of cells with leupeptin leads to a reduced rate of fusion between endocytic vesicles and lysosomes [2], probably because of the accumulation of undegraded protein in the lysosomes. Chloroquine or ammonia accumulate in the lysosomes [3], causing a reduction in the rate of fusion [4, 5], while the rate of proteolysis within the lysosomes may not be seriously impaired. Coincidentally, the equilibrium density of the lysosomes in a sucrose gradient becomes considerably lower [4]. Chloroquine also inhibits some systems of receptor-mediated endocytosis by inhibiting the recycling of the receptors to the cell surface [6–8].

Concanavalin A does act on the rate of fusion between lysosomes and endocytic vesicles [9, 10], but it has to be added before the ligand [10]. It may interfere with the binding of ligand [10, 11] and it may cause 'capping' in some cell types.

In this paper, we suggest the use of benzyl alcohol as a selective inhibitor of the fusion between lysosomes and endocytic vesicles. In concentrations of about 50 mM, benzyl alcohol has been shown to have well-defined effects on model membrane systems [12, 13]. In high concentrations, it may induce fusion between cells [14].

Materials and methods

Cells and incubations. Hepatocytes were prepared from rat liver by the collagenase perfusion method [15]. Non-parenchymal cells were removed by differential centrifuging [16]. They were incubated in a minimal salt medium containing bovine serum albumin [16] in a shaking water bath. Viability of the cells was checked by the trypan blue exclusion method.

Ligands. Orosomucoid was obtained from Calbiochem AG, Lucerne, Switzerland. It was desialylated by incubation with neuraminidase [17] and labelled with ^{125}I by the sodium hypochlorite oxidation method [17, 18] or conjugated with [^{14}C]sucrose by chemical coupling with 2,4,6-trichlorotriazine [2, 19]. The advantage of the latter method is that the sucrose label does not leave the lysosomes, so that after fractionating the cells, a quantitative measure of the rate of transfer of labelled protein into the lysosomes may be obtained. In contrast, the label from iodine-labelled proteins leaves the cells very quickly [20].

Determination of cell-associated ligand. The hepatocytes were separated from the medium by placing a 250 μl aliquot on top of 200 μl of dibutyl phthalate in a narrow test tube and centrifuging in a table centrifuge for 30 sec [16]. In these samples, the cell-associated radioactivity included surface-bound as well as internalized ligand. Before centrifugation through oil, surface-bound ligand may be removed by making an aliquot of the cell suspension 3 mM with respect to EGTA [17]. If the cells are incubated at 10°, the amount of internalized ligand is negligible if the incubation lasts for less than 30 min [17], so that the rate of binding to the cell surface may be studied. The rate of binding may be taken as a measure of the number of available receptors on the cell surface. [6].

Determination of degradation. At selected time points, 250 μl aliquots of the cell suspension were mixed with 250 μl of 2% (w/v) phosphotungstic acid in 4 N HCl. After centrifuging the samples, the extent of degradation in the cell suspension is determined as acid-soluble radioactivity. Only cell-associated asialo-orosomucoid is degraded [16]. Accordingly, the extent of degradation in the suspension varies with the percentage of cell-associated radioactivity as well as with the actual rate of degradation of cell-associated ligand. In order to measure the latter rate selectively, the cells may be exposed to labelled asialo-orosomucoid for 10 min, then the medium is changed, the cell suspension is divided into a suitable number of portions, and the compounds to be tested are added. In this paper, only degradation measurements obtained by this procedure are discussed.

Isopycnic centrifuging in a sucrose gradient. This method shows the intracellular distribution of the radioactive label. Post-nuclear supernatants were prepared after homogenization of the cells and centrifuged in a linear sucrose gradient as described previously [4, 16].

On fractionating hepatocytes that have been allowed to take [^{14}C]sucrose-labelled asialo-glycoproteins for 15 min or more, two peaks of radioactivity are found in the gradient: one 'endosomal' peak at d = 1.15 g/ml and one 'lysosomal' peak at d = 1.20 g/ml. The size of the lysosomal peak increases throughout the incubation as a result of transfer of material from the endosomal peak [2, 4, 20].

Determination of ATP levels. A 50 μl aliquot of the cell suspension was mixed with 1 ml of ice-cold 0.2 M perchloric acid. After centrifugation, an aliquot of the supernatant was neutralized, and the concentration of ATP was determined using a highly purified luciferase preparation from LKB-Wallac (Bromma, Sweden) in a luminometer from the same company. When the pure luciferase is used, the decrease in the light level is negligible over a period of several minutes. The variability between samples taken at the same time from the same cell suspension is 5–10%. These measurements were used as a control on the condition of the cells.

Results and discussion

Effects of benzyl alcohol on the uptake of asialo-glycoproteins in isolated hepatocytes. Figure 1 shows the uptake of asialo-orosomucoid into cells that had been pre-incubated with benzyl alcohol for 30 min. The viability of the cells was better than 75% throughout the experiment. Forty mM benzyl alcohol reduced the viability of the cells to 65% by the end of the pre-incubation (the results at this concentration were not included in the figure). In cells that were incubated with 10 mM benzyl alcohol, the ATP levels declined to 90% of controls during a one-hour incubation, suggesting that toxic effects of benzyl alcohol were not a problem.

A relatively low concentration of asialo-orosomucoid was used in the experiment shown in Fig. 1. In control cells, the uptake of the ligand was complete by the time the first samples were taken after 5 min. In these cells, the amount of cell-associated radioactivity decreased after 10 min because radioactive degradation products left the

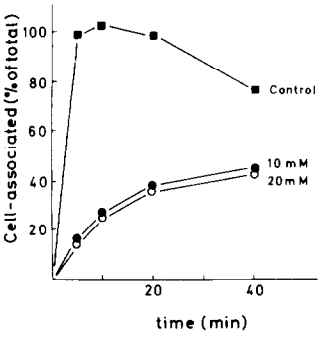

Fig. 1. Effect of benzyl alcohol on uptake of asialo-orosomucoid by isolated rat hepatocytes. To the cell suspensions (8×10^6 cells/ml) benzyl alcohol (●, 10 mM: ○ 20 mM; ■, no addition) was added and the cells were incubated at 37°. After 30 min, 0.25 nM ^{125}I-asialo-orosomucoid was added to each flask.

cells. Both concentrations of benzyl alcohol reduced the rate of uptake to less than 15% of that in the control cells.

In order to examine further the inhibition of asialo-orosomucoid uptake by benzyl alcohol, hepatocytes were incubated with the drug for 5–30 min at 37°, then the temperature was lowered to 10°, and the binding of asialo-orosomucoid to receptors on the cell surface [17] was studied (Fig. 2). In hepatocytes to which benzyl alcohol was added immediately before labelled asialo-orosomucoid at 10°, binding was only slightly impaired compared to untreated cells, showing that the presence of benzyl alcohol hardly affected the properties of the receptor itself. Accordingly, the progressive reduction in binding that was observed with increasing length of the pre-incubation with benzyl alcohol at 37° reflects a decrease in the number of receptors. This effect is very similar to that of chloroquine [6].

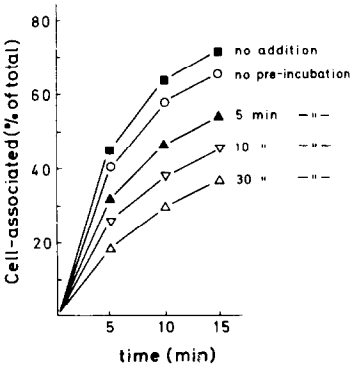

Fig. 2. Effect of various times of pre-incubation with benzyl alcohol on the binding of asialo-orosomucoid to suspended hepatocytes at 10°. The cell suspensions were incubated at 37°. To three of the flasks, benzyl alcohol (10 mM) was added, so that the time of incubation with benzyl alcohol would be 30 min (△), 10 min (▽) or 5 min (▲) when the flasks were removed from the water-bath and put on ice. To a fourth flask (○), benzyl alcohol (10 mM) was added after it had been chilled. The fifth flask (■) received no addition. After 5 min on ice, 0.2 nM ^{125}I-asialo-orosomucoid was added to all of the flasks, which were placed on a shaking water bath at 10°. Under the conditions of this experiment, the amount of internalized asialo-glycoprotein is negligible [14].

Effect of benzyl alcohol on the degradation of ^{125}I-asialo-orosomucoid. The effect of benzyl alcohol on degradation apart from its effect on uptake is shown in Fig. 3. In these experiments, extracellular labelled asialo-orosomucoid was removed by washing before the inhibitor was added, so that its effect is only on the intracellular handling of endocytosed protein. The effect of benzyl alcohol (Fig. 3A) is comparable to that of other powerful inhibitors of protein degradation, such as chloroquine [4] or leupeptin [21]. A curve showing the effect of 10 mM benzylamine is also included in Fig. 3A. As expected, this compound is a more powerful inhibitor than benzyl alcohol on a molar basis, because benzylamine, possessing an aliphatic amino group, may be expected to accumulate in the lysosomes, just like other aliphatic amines [22], and cause an increase in intralysosomal pH [3].

The effect of adding benzyl alcohol and leupeptin simultaneously to the cells is shown in Fig. 3B. If the incubation with leupeptin lasts for less than about one hour, it affects intralysosomal degradation only [2]. Addition of 20 μM leupeptin reduces the extent of degradation after 20–40 min by about two-thirds. This relative reduction is the same whether leupeptin acts in the benzyl alcohol-treated cells or in untreated cells. This result is compatible with the suggestion that leupeptin and benzyl alcohol act on different stages of the degradation process.

In an effort to elucidate the mechanism of inhibition of degradation, hepatocytes were allowed to endocytose [^{14}C]sucrose-labelled asialo-orosomucoid, then benzyl alcohol was added to a portion of the cell suspension, and intracellular processing was allowed to proceed. The sucrose label does not escape from the lysosomes [2, 19] allowing an estimation of the rate of transfer from the endosomal peak (at d = 1.15 g/ml in the gradient) to the lysosomal peak (d = 1.20 g/ml).

The plasma membrane marker enzyme, 5′-nucleotidase, showed a shift towards higher density in the benzyl alcohol-treated cells (Fig. 4B). The lysosomes of benzyl alcohol-treated cells were, in contrast, recovered at the same density as the control cells, indicating that benzyl alcohol does not accumulate in the lysosomes to the extent that chloroquine or ammonia do; these compounds induce

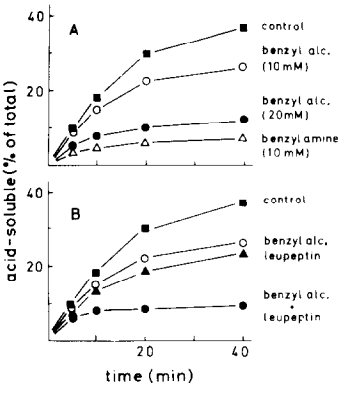

Fig. 3. Comparison of the effects of benzyl alcohol and various other inhibitors. The cell suspension (8×10^6 cells/ml) was made 0.25 nM with respect to ^{125}I-asialo-orosomucoid and incubated at 37° for 10 min. The suspension was put on ice, and extracellular labelled protein was removed by a change of medium. The suspension was divided into six portions, to which were added, respectively, 10 mM (○) or 20 mM (●) benzyl alcohol, 10 mM benzylamine (△), 20 μM leupeptin (▲), 10 mM benzyl alcohol *and* 20 μM leupeptin (●) or no addition (■). All of the points in panel A and panel B are from the same experiment.

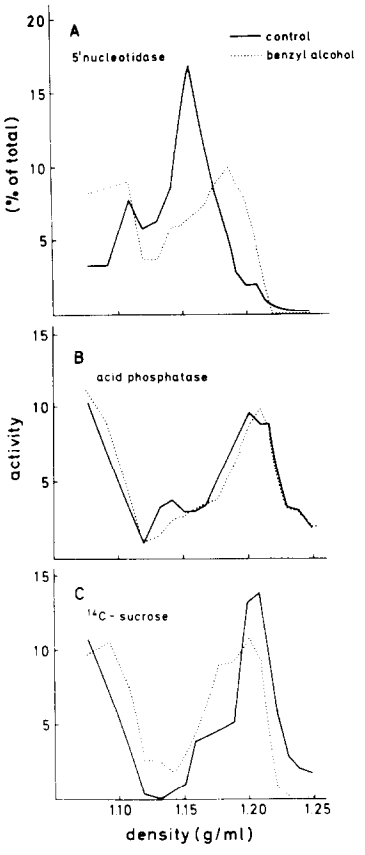

Fig. 4. Effect of benzyl alcohol on the intracellular distribution of asialo-orosomucoid. The cell suspension (8×10^6 cells/ml) was made 115 nM with respect to [^{14}C]sucrose-asialo-orosomucoid and incubated for 30 min at 37°. Extracellular asialo-orosomucoid was removed by two changes of medium, and the suspension was divided into two portions, one of which was made 10 mM with respect to benzyl alcohol. The incubation was continued for 2 hr at 37°, when the cells were put on ice and fractionated by isopycnic centrifugation in a sucrose gradient [3, 13]. Panel A: distributions of 5'-nucleotidase (marker enzyme for the plasma membrane); B: acid phosphatase (marker enzyme for the lysosomes); C: radioactivity from [^{14}C]sucrose. Dotted line: Benzyl alcohol-treated cells; continuous line: control cells.

a migration of the lysosomal marker enzymes towards much lower densities on fractionation in a sucrose gradient, reflecting the presence of a high concentration of osmotically active particles in the lysosomes of cells that have been treated with chloroquine or ammonia [3].

After 2 hr of incubation, the distributions of acid phosphatase and radioactivity were very similar in the control cells, indicating a substantially complete transfer of the endocytosed material into the lysosomes. In the benzyl alcohol-treated cells, much of the radioactivity in the gradient remained at a lower density than the lysosomes. Thus, the rate of transfer of labelled asialo-orosomucoid was much slower in the treated cells.

In the present report, we have shown that benzyl alcohol in concentrations of 10–20 mM reduces the uptake of asialo-glycoproteins into rat hepatocytes in suspension. Several other local anaesthetics also have this effect (H. Tolleshaug, unpublished observations), along with chloroquine, which has been shown to interfere with receptor recycling in several systems [4–6]. The local anaesthetics and chloroquine, which are amines, accumulate in the lysosomes, as shown by the reduced equilibrium density of lysosomes from treated cells in a sucrose density gradient. Intralysosomal pH is increased [3] and the rate of endosome–lysosome fusion is reduced ([4], H. Tolleshaug, unpublished). In contrast to the amines, benzyl alcohol does not reduce the equilibrium density of lysosomes in a sucrose density gradient. The simplest explanation for this is that benzyl alcohol is not present in the lysosomes in high concentrations. Benzyl alcohol lowers the rate of degradation of endocytosed asialo-glycoproteins. From the change in intracellular distribution of the radioactive label on addition of benzyl alcohol, it may be concluded that benzyl alcohol affects degradation by reducing the rate of transfer of endocytosed protein from the endosomes to the lysosomes. The binding properties of the receptor were substantially unchanged in the presence of benzyl alcohol. Accordingly, the simplest explanation of the reduced degradation is that benzyl alcohol reduces the rate of endosome–lysosome fusion. Further work is required in order to establish whether benzyl alcohol acts primarily on the endosomes or on the lysosomes.

Institute for Nutrition Research HELGE TOLLESHAUG
University of Oslo TROND BERG
P.O. Box 1046
Blindern
Oslo 3, Norway

REFERENCES

1. W. A. Dunn, J. H. LaBadie and N. N. Aronson, *J. biol. Chem.* **254**, 4191 (1979).
2. H. Tolleshaug and T. Berg, *Expl Cell Res.* **134**, 207 (1981).
3. B. Poole and S. Ohkuma, *Proc. natn. Acad. Sci. U.S.A.* **75**, 3327 (1978).
4. T. Berg and H. Tolleshaug, *Biochem. Pharmac.* **29**, 917 (1980).
5. A. H. Gordon, P. D'Darcy Hart and M. R. Young, *Nature, Lond.* **286**, 79 (1980).
6. H. Tolleshaug and T. Berg, *Biochem. Pharmac.* **28**, 2919 (1979).
7. C. Tietze, P. Schlesinger and P. Stahl, *Biochem. biophys. Res. Commun.* **93**, 1 (1980).
8. A. Gonzalez-Noriega, J. H. Grubb, V. Talkad and W.-S. Sly, *J. Cell Biol.* **85**, 839 (1980).
9. P. J. Edelson and Z. A. Cohn, *J. exp. Med.* **140**, 1364 (1974).
10. H. Tolleshaug, M. Abdelnour and T. Berg, *Biochem. J.* **190**, 697 (1980).
11. M. E. Costlow and P. E. Gallagher, *Biochim. biophys. Acta* **587**, 192 (1979).
12. G. L. Turner and E. Oldfield, *Nature, Lond.* **277**, 669 (1979).
13. L. Ebihara, J. E. Hall, R. C. MacDonald, T. J. McIntosh and S. A. Simon, *Biophys. J.* **28**, 185 (1979).
14. O. F. Ahkong, G. M. Bortham, A. W. Woodward and J. A. Lucy, *Biochem. J.* **192**, 829 (1980).
15. P. O. Seglen, in *Methods in Cell Biology* (Ed. D. M. Prescott), Chap. 13, p. 29. Academic Press, New York (1976).
16. H. Tolleshaug, T. Berg, M. Nilsson and K. R. Norum, *Biochim. biophys. Acta* **499**, 73 (1977).
17. H. Tolleshaug, *Int. J. Biochem.* **13**, 45 (1981).
18. M. R. Redshaw and S. S. Lynch, *J. Endocr.* **60**, 527 (1974).
19. R. C. Pittman, S. R. Green, A. D. Attie and D. Steinberg, *J. biol. Chem.* **254**, 6876 (1979).
20. H. Tolleshaug, T. Berg, W. Frølich and K. R. Norum, *Biochim. biophys. Acta* **585**, 71 (1979).
21. T. Berg, T. Ose, L. Ose and H. Tolleshaug, *Int. J. Biochem.* **13**, 253 (1981).
22. P. O. Seglen, B. Grinde and A. E. Solheim, *Eur. J. Biochem.* **95**, 215 (1979).