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Effects of inhibitors of the vacuolar proton pump on hepatic heterophagy and autophagy

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Abstract

Bafilomycin A_1 (BAF) and concanamycin A (ConcA) are selective inhibitors of the H^+-ATPases of the vacuolar system. We have examined the effects of these inhibitors on different steps in endocytic pathways in rat hepatocytes, using [^{125}I]tyramine-cellobiose-labeled asialoorosomucoid ([^{125}I]TC-AOM) and [^{125}I]tyramine-cellobiose-labeled bovine serum albumin ([^{125}I]TC-BSA) as probes for respectively receptor-mediated endocytosis and pinocytosis (here defined as fluid phase endocytosis). The effects of BAF and ConcA were in principle identical, although ConcA was more effective than BAF. The main findings were as follows. (1) BAF/ConcA reduced the rate of uptake of both [^{125}I]TC-AOM and [^{125}I]TC-BSA. The reduced uptake of [^{125}I]TC-AOM was partly due to a redistribution of the asialoglycoprotein receptors (ASGPR) such that the number of surface receptors was reduced $\approx$40% without a change in the total number of receptors. (2) BAF/ConcA at the same time increased retroendocytosis (recycling) of both probes. The increased recycling of the ligand ([^{125}I]TC-AOM) is partly a consequence of the enhanced pH in endosomes, which prevents dissociation of ligand. (3) It was furthermore found that the ligand remained bound to the receptor in presence of BAF/ConcA and that the total amount of ligand molecules internalized in BAF/ConcA-treated cells was only slightly in excess of the total number of receptors. These data indicate that reduced pH in endosomes is the prime cause of receptor inactivation and release of ligand in early endosomes. (4) Subcellular fractionation experiments showed that [^{125}I]TC-AOM remained in early endosomes, well separated from lysosomes in sucrose gradients. The fluid phase marker, [^{125}I]TC-BSA, on the other hand, seemed to reach a later endosome in the BAF/ConcA-treated cells. This organelle coincided with lysosomes in the gradient, but hypotonic medium was found to selectively release a lysosomal enzyme (β-acetylglucosaminidase), indicating that even [^{125}I]TC-BSA remained in a prelysosomal compartment in the BAF/ConcA-treated cells. (5) Electron microscopy using horseradish peroxidase (HRP) as a fluid phase marker verified that BAF/ConcA inhibited transfer of material from late endosomes ('multivesicular bodies'). (6) BAF/ConcA led to accumulation of lactate dehydrogenase (LDH) in autophagic vacuoles, but although the drugs partly inhibited fusion between autophagosomes and lysosomes a number of autolysosomes was formed in the presence of BAF/ConcA. This

Abbreviations: AOM, asialoorosomucoid; AOM-Au10, AOM-gold particles (10 nm); ASGPR, asialoglycoprotein receptors; BAF, bafilomycin A; ConcA, concanamycin A; DAB, diaminobenzidine; HRP, horseradish peroxidase; [^{125}I]TC-AOM, [^{125}I]tyramine-cellobiose-asialoorosomucoid; [^{125}I]TC-BSA, [^{125}I]tyramine-cellobiose-bovine serum albumin; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline TGN, trans-Golgi network; TGN, *trans*-Golgi network

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observation explains the reduced buoyant density of lysosomes (revealed in sucrose density gradients). In conclusion, BAF/ConcA inhibit transfer of endocytosed material from late endosomes to lysosomes, but do not at the same time prevent fusion between autophagosomes and lysosomes.

1. Introduction

The endocytic and the biosynthetic-secretory pathways of eucaryotic cells have been called 'the vacuolar system', because they consist of a series of communicating vesicles. Membrane traffic between the compartments of the vacuolar system occurs by budding and membrane fusion processes that are governed by a number of specific proteins including small GTP-binding proteins [1,2]. Several of the organelles in the vacuolar system, including *trans*-Golgi, lysosomes, and endosomes, are acidified due to a so-called vacuolar type proton pump [3–5]. Besides creating an optimal milieu for acid hydrolases and for dissociating ligands from receptors, the proton gradient may also be important for membrane trafficking in both the exocytic and the endocytic pathways. A number of transport steps between compartments of the vacuolar system has been shown to be affected by agents that neutralize the acidic organelles [3]. Yeast mutants that are defective in their vacuolar ATPase exhibit defects in sorting of the carboxypeptidase Y from the *trans*-Golgi network to the yeast vacuole. This is consonant with the observation that inhibition of the yeast vacuolar ATPase by bafilomycin A_1 (BAF) results in missorting of the vacuolar hydrolases carboxypeptidase Y and proteinase A [6,7]. Inadequate acidification of the TGN in presence of concanamycin A (ConcA) may also result in a delay in the secretion of proteins following the constitutive secretory pathway in HepG2 cells [8].

The endocytic process is particularly susceptible to acidotropic agents. An endocytosed marker will encounter a gradually increasing acidity along the endocytic pathway. Ligands and solutes taken up by endocytosis are first seen in small 'primary endosomes' which carry their cargo to 'early endosomes' [9]. The early endosomes serve as a sorting station: Endocytosed membrane proteins and fluid markers may be brought to the terminal lysosomes (via late endosomes), to the TGN, to the opposite side of the (epithelial/polarized) cell, back to the plasma membrane (retroendocytosis), and in certain specialized cells such as oocytes to storage granules [9].

Acidotropic agents and/or specific inhibitors of the vacuolar proton pump (BAF, ConcA) have been shown to interfere both with retroendocytosis [10,11], transport to lysosomes [12–16], and transport to the TGN [17]. It was suggested several years ago that chloroquine, ammonium chloride and monensin inhibited degradation of endocytosed probes indirectly, by inhibiting the transport from endosomes to lysosomes, rather than inhibiting degradation in the lysosomes [18–20]. Furthermore, it was shown that high concentrations of chloroquine inhibit the recycling of the asialoglycoprotein receptor (ASGPR) from endosomes back to the plasma membrane. As a result, the number of ASGPRs at the cell surface was reduced by 75% [19]. Similar effects of lysosomotropic amines have been observed in studies of the ASGPR in HepG2 cells [11,21], the transferrin and the mannose receptor in macrophages [22,23] and the LDL receptor in macrophages [24].

The lysosomotropic amines induce a tremendous swelling of lysosomes [25–28]. The membrane properties of these organelles may therefore change and any inhibitory effects on membrane traffic may not necessarily be due to increased endosomal/lysosomal pH. BAF and ConcA are rather specific inhibitors of the vacuolar proton pump and would not be expected to induce gross changes in the membrane properties of vacuoles in the endocytic pathway. In the present investigation we have used these inhibitors to determine whether acidification of endosomes is a prerequisite for retroendocytosis and for transport of endocytosed probes from endosomes to lysosomes in isolated rat hepatocytes. To this end we studied the effects of BAF and ConcA on the uptake and intracellular transport of [^{125}I]tyramine-cellobiose-labeled asialoorosomucoid ([^{125}I]TC-AOM) and [^{125}I]tyramine-cellobiose-labeled bovine serum albumin ([^{125}I]TC-BSA) in isolated rat hepatocytes. [^{125}I]TC-AOM is internalized by clathrin-dependent receptor-mediated endocytosis [29–31] whereas [^{125}I]TC-BSA is a fluid phase marker [32,33]. It

would presumably be necessary to use a fluid phase marker to follow transport from endosomes to lysosomes since an enhanced endosomal pH may prevent dissociation of [^{125}I]TC-AOM from the ASGPR.

Results obtained using BAF/ConcA indicate that the individual steps in endocytosis may depend differently on the vacuolar proton pump in different cells. The key roles of hepatocytic endocytosis and autophagy in turnover of physiologically important macromolecules warrant a study of the specific effects of inactivation of the proton pump on endocytic and autophagic steps in hepatocytes.

2. Materials and methods

2.1. Biochemicals

Collagenase was obtained from Sigma Chemical Co. (St. Louis, MO). Na^{125}I was from the Radiochemical Centre (Amersham, United Kingdom). All other chemicals were of analytical grade. As markers for clathrin-dependent endocytosis and fluid phase endocytosis were used asialoorosomucoid (AOM) and bovine serum albumin (BSA), respectively. The probes were labeled with [^{125}I]tyramine cellobiose ([^{125}I]TC) as described earlier [34].

2.2. Preparation of gold–AOM complexes

Colloidal gold (10 nm diameter) was made as described by Slot and Geuze [35]. Gold–AOM complexes (AOM–Au10) were prepared as follows. The pH of the solutions of gold and AOM were adjusted to 7.0 by means of NaOH [36] in order to optimize the adsorption of AOM to the gold particles [37]. The minimal concentration of AOM needed to stabilize the gold-particles was found by adding 30 μl of AOM solution of increasing concentration to a series of tubes containing 200 μl gold solution. After 2 min 20 μl 10% NaCl was added, and the solution was shaken and left for 5 min. Electrolyte-induced aggregation of gold particles was seen as a change in color from pink to blue. The procedure showed that 3.5 μg AOM per ml of 10 nm gold-particle solution was an optimal concentration. The gold–protein conjugates were separated from non-absorbed protein and clusters of gold by centrifugation (60 min at 25 000 × g in a Beckman Model J2-21 centrifuge at 4°C). The sediment was resuspended in PBS.

2.3. Cell preparation and incubations

Hepatocytes were prepared from 18h starved male Wistar rats (250–300 g) by collagenase perfusion [38]. The cells were incubated as suspensions (4-ml aliquots with shaking at 37°C, usually 50–75 mg wet weight/ml) in suspension buffer [38] fortified with pyruvate (20 mM) and Mg^{2+} (2 mM). To remove [^{125}I]TC-BSA possibly associated with the cell, the cells were routinely treated with 0.3% pronase at 0°C for 1 h. This treatment efficiently removed remnants of [^{125}I]TC-BSA, and the remaining radiolabeled probes were therefore associated with intracellular structures. No would, on the other hand, remain bound to the plasma membrane since the cells were washed in Ca^{2+}-free solution [39]. For determination of cell surface bound and internalized [^{125}I]TC-AOM the cells were washed by centrifugation in suspension buffer thrice. Cell surface bound ligand was measured as radioactivity released by 10 mM EGTA. Ligand remaining in the cells following EGTA treatment was defined as internalized ligand.

2.4. Enzyme assays

β-Acetylglucosaminidase was determined according to Barrett [40].

2.5. Determinations of radioactivity

Radioactivities were measured in a Kontron gamma counter. Degradation of [^{125}I]TC-AOM and [^{125}I]TC-BSA was followed by measuring radioactivity soluble in 10% (w/v) trichloroacetic acid (acid-soluble radioactivity). Bovine serum albumin (0.5%) was added as a carrier.

2.6. Subcellular fractionation [33]

Cell suspensions (in 0.25 M sucrose/10 mM Hepes/1 mM EDTA, pH 7.3, referred to as homogenization buffer) were homogenized by five strokes in a Dounce homogenizer (tight-fitting pestle). The homogenates were fractionated by isopycnic centrifuga-

tion in sucrose gradients. A postnuclear fraction was prepared by centrifuging a 5-ml sample of the homogenate at $4000 \times g$ for 2 min, the resulting nuclear fraction (N-fractions) was resuspended in 5 ml homogenization buffer and centrifuged again at $4000 \times g$ for 2 min. In some experiments the postnuclear fraction was centrifuged at $48\,000 \times g$ for 60 min to obtain an MLP-fraction (a combined mitochondrial, light mitochondrial and particulate fraction). Differential centrifugation was carried out in a Sorvall RC-2B centrifuge using a SS-34 rotor and 13 ml centrifuge tubes. Homogenization and centrifugations were carried out at 0–4°C. In isopycnic centrifugation experiments 4 ml aliquots of an MLP-fraction or a postnuclear fraction were initially layered on top of the gradients. The centrifuge tubes (38 ml) were centrifuged at $85\,000 \times g$ in a Beckman SW 28 rotor at 4°C for 7 h. Following centrifugation the gradients were divided into 18×2-ml fractions by upward displacement using Maxidens as displacement fluid. The densities of the fractions were calculated from the refractive indices.

2.7. Electron microscopy

Hepatocytes were prepared for electron microscopy as follows: After internalization of different markers the cells were washed in suspension buffer [38] containing 10 mM EGTA, and fixed in 2.0% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 30 min at room temperature. The cells were then washed in 0.1 M PBS, postfixed for 60 min with 2% OsO_4 solution containing 1.5% potassium ferricyanide, and then stained with 1.5% uranyl acetate for 30 min. After dehydration in ethanol series, the cells were embedded in Epon, sectioned and stained with 0.2% Pb-citrate.

In order to label lysosomes we injected 700–800 μl AOM–Au10 into rats 24 h prior to sacrifice. To visualize the uptake of a fluid phase marker we incubated cells with 5 mg/ml horseradish peroxidase (Type VI, Sigma) for 1 h at 37°C. The cells were then washed thrice in ice-cold 0.1 M phosphate buffer and fixed in 2.0% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Subsequently they were reacted with diaminobenzidine (DAB, 2.0 mg/ml) for 15 min, followed by a 45 min incubation with DAB and 0.02% H_2O_2. The reaction was stopped by washing with cacodylate buffer and the cells were postfixed in 1.5% OsO_4 and processed for Epon embedding without further staining. Sections of 100–200 nm were cut on a LKB microtome and examined at 80 kV in a Phillips CM100 microscope.

2.8. Measurement of autophagic sequestration of lactate dehydrogenase (LDH)

Autophagic sequestration of LDH was measured as described previously [41], using the percentage of total cellular LDH present in cell corpses as the basic measurement parameter. Cell corpses were prepared by electrodisrupting suspensions of hepatocytes [42]. The corpses, containing intact cell organelles, were separated from the cytosol by centrifugation through metrizamide/sucrose density cushions [42].

3. Results

3.1. Effects of BAF and ConcA on uptake and degradation of [^{125}I]TC-AOM

Suspensions of hepatocytes were incubated in the presence or absence of BAF (in concentrations ranging from 1 to 4 μM) or ConcA (in concentrations ranging from 1 nM to 1 μM). Fig. 1 shows cell-associated radioactivities in cells incubated with [^{125}I]TC-AOM in the presence and absence of BAF (Fig. 1A) or ConcA (Fig. 1B). Degradation, as indicated by the formation of acid-soluble radioactivity, was nearly blocked by 1 μM BAF or by 10 nM ConcA (not shown). The two drugs also markedly reduced rate of uptake of [^{125}I]TC-AOM. Following incubation for 90 min at 1 μM BAF or 5 nM ConcA uptake was reduced to about 50% of control values. BAF (but not ConcA) was evidently metabolized by the hepatocytes as indicated by the different dose-response curves obtained following 30 and 90 min of incubation (Fig. 1A). BAF at 1 μM inhibited uptake of [^{125}I]TC-AOM maximally after 30 min. The cells then recovered from the drug treatment and after 90 min the uptake (in presence of 1 μM BAF) was reduced about 50% as compared to 80% in presence of 4 μM BAF.

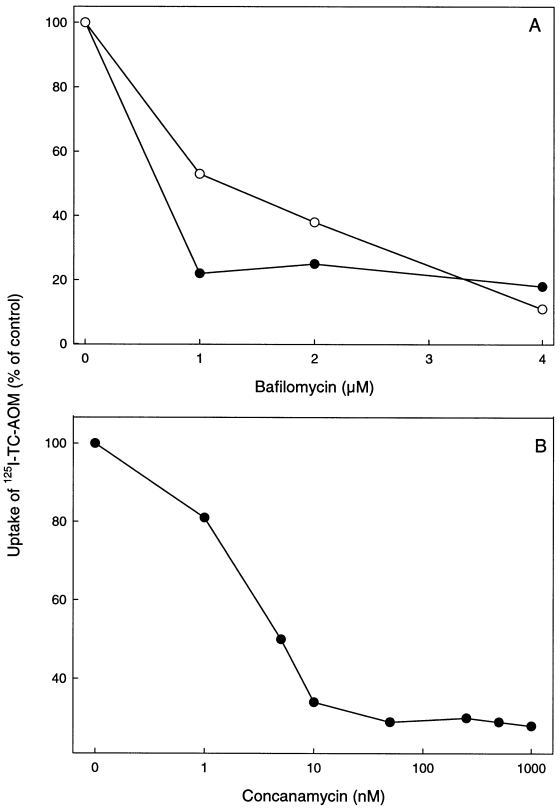

Fig. 1. ConcA (A) and BAF (B) inhibit uptake of [^{125}I]TC-AOM in hepatocytes. Suspensions of hepatocytes were incubated at 37°C with the indicated concentrations of BAF (A) or ConcA (B). After 15 min [^{125}I]TC-AOM (10 nM) was added and the incubation continued for 30 min (●) or 90 min (○). Samples of cells were then removed and washed thrice in ice-cold 10% sucrose. The results show cell-associated [^{125}I]TC-AOM presented as percent of control values. Representative data from three different experiments are shown.

3.2. BAF and ConcA reduce the amounts of surface ASGPR and enhance recycling of [^{125}I]TC-AOM

Reduced net uptake of ligand bound to ASGPR could be due to a reduced number of surface receptors, a reduced rate of formation of endosomes (coated vesicles), an enhanced recycling of ligand in association with the recycling receptor, or a combination of these effects.

Fig. 2 shows Scatchard plots of ligand binding to hepatocytes at 0°C. These data show that the number of active surface binding sites is reduced with about 40% following preincubation of the cells with BAF (1 μM) or ConcA (200 nM). To determine whether the reduced binding were due to inactivation or redistribution of ASGPR, binding of [^{125}I]TC-AOM was measured in cells permeabilized with digitonin according to the method described by Weigel and coworkers [43]. We did not observe any significant differences in the number of binding sites between control cells and BAF/ConcA treated cells (data not shown). Therefore, the reduced number of surface receptors is due to a redistribution of the ASGPR.

Fig. 3 shows the ratio between internalized and surface-bound ligand in cells incubated in presence and absence of BAF at increasing times at 37°C. The control cells internalize ligand twice as rapidly initially than the BAF-treated cells. Furthermore, whereas control cells continue to accumulate ligand the internalization of ligand levels off in the treated cells, evidently because the ligand recycles with the receptor. The initial rate of internalization of ligand in presence of BAF (before recycling brings ligand back to the cell surface) is, however, about 50% of control values, indicating that the drug inhibits the formation of endosomes (coated vesicles).

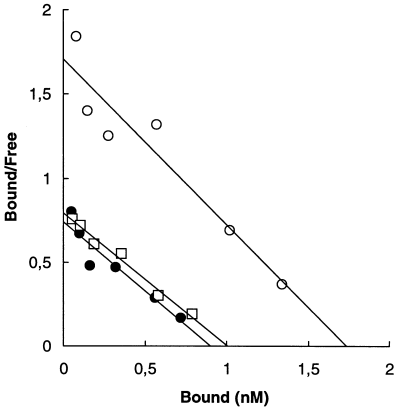

Fig. 2. Effects of BAF or ConcA on equilibrium binding of [^{125}I]TC-AOM. Suspensions of hepatocytes were preincubated without (○) or with ConcA (100 nM, □) or BAF (1 μM, ●) for 15 min at 37°C. The cells were then incubated at 4°C in presence of different concentrations of [^{125}I]TC-AOM. Cell-associated radioactivities were determined after 2 h of incubation. Data are represented as Scatchard plots. Similar results were obtained in five different experiments. The number of receptors per cell were in these experiments 243 000 ± 44 000 and 151 000 ± 30 000 for control cells and BAF-treated cells, respectively. Association equilibrium constants (K_a) were (13.0 ± 3.7) × 10^7 M^{-1} and (11.2 ± 2.2) × 10^7 M^{-1} for control cells and BAF/ConcA-treated cells.

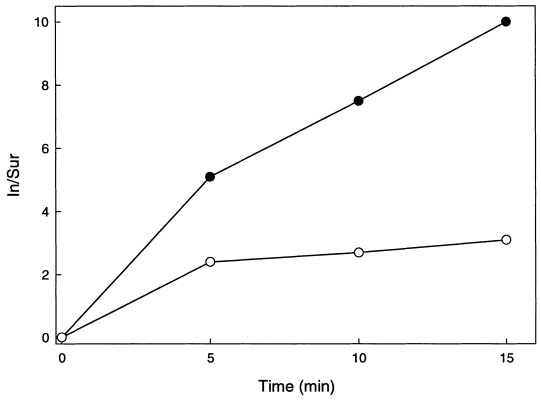

Fig. 3. Effects of BAF on the In/Sur ratio for internalization of [^{125}I]TC-AOM. Suspensions of hepatocytes (3×10^6/ml) were incubated at 37°C in presence (○) and absence (●) of BAF for 15 min, prior to the addition of [^{125}I]TC-AOM (100 nM). Samples of cells were removed at the indicated time points and internalized and cell-surface bound ligands were assayed as described in Section 2. The amount of internalized ligand divided by cell-surface bound ligand is plotted against time of incubation. The data presented are representative of two experiments.

To determine whether recycling of ligand were influenced by BAF or ConcA the cells were allowed to take up [^{125}I]TC-AOM for 15 min or 60 min at 37°C. The cells were then washed in the presence of 10 mM EGTA and subsequently incubated at 37°C with or without EGTA (10 mM) in presence and absence of BAF or ConcA. The data presented in Fig. 4 show that the treated cells release twice as much ligand in presence of EGTA than the controls after a 15 min pulse with labeled ligand. After a 60-min pulse the BAF/ConcA-treated cells released nearly the same proportion of ligand as after the 15-min pulse. The proportion released by the control cells, on the other hand, was only 50% of that released after the 15-min pulse with [^{125}I]TC-AOM. This result indicates that more ligand remains associated with the receptors in early endosomes (from which recycling takes place) in presence of the drugs.

If BAF or ConcA completely block ligand dissociation in the endosomes, then the maximal uptake of ligand should equal the total number of receptors in the cell. To see whether uptake of ligand matches receptor number in presence of BAF or ConcA we incubated cells in presence of saturating concentrations of labeled ligand and at the same time determined receptor number in digitonin-permeabilized cell samples from the same cell suspension by means of Scatchard plots. Fig. 5 shows the results of a representative experiment in which BAF-treated and control hepatocytes were incubated at 37°C in the presence of 100 nM [^{125}I]TC-AOM. The concentration of ligand was far in excess of the K_d for binding to the ASGPR (about 5 nM) and the receptors should therefore be saturated during the experiment. The total number of receptors in the cells used in the experiments was for control cells and BAF-treated cells, respectively 5.4×10^5 and 4.3×10^5 receptors/cell (as determined by Scatchard analysis). The corresponding numbers of surface receptors were, in comparison, 1.8×10^5 and 0.9×10^5. The data presented in Fig. 5 suggest that the BAF-treated cells took up 5.8×10^5 ligand molecules during 2 h of incubation. The corresponding number in control cells was 4.9×10^6. These results indicate that the amount of ligand internalized is only slightly in excess of the total number of receptors in the BAF-treated cells. It should be noticed, however, that a proportion (about 20%) of the ASGPR may not be involved in endocytosis [44].

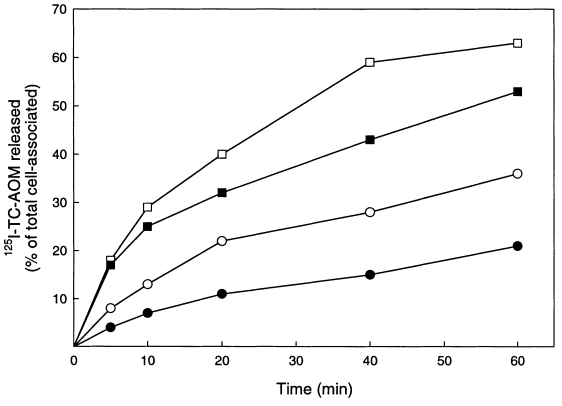

Fig. 4. Retroendocytosis of [^{125}I]TC-AOM in the presence and absence of BAF. Hepatocytes were first incubated at 37°C for 15 min (open symbols) or 60 min (closed symbols) with 10 nM [^{125}I]TC-AOM. The cells were subsequently washed in ice-cold medium containing 10 mM EGTA to remove cell-surface bound ligand and then incubated again at 37°C with (squares) or without BAF (circles) in the presence of 10 mM EGTA. Samples were removed at the indicated time points, and radioactivity released in the medium was measured and expressed as percentage of total radioactivity initially in the cells. Principally identical results were obtained in four different experiments. More than 95% of the released radioactivities were acid precipitable.

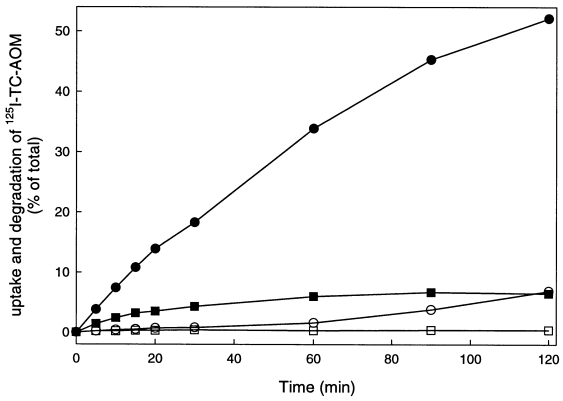

Fig. 5. Endocytic uptake and degradation of [^{125}I]TC-AOM in presence and absence BAF. Suspensions of hepatocytes (3×10^6/ml) were incubated at 37°C with saturating concentrations of ligand (50 nM) in presence (squares) and absence (circles) of BAF. Samples were removed at the indicated time points and cell-associated radioactivities measured. Acid-soluble (open symbols) and acid-precipitable (filled symbols) radioactivities are presented as percentage of total amount of acid precipitable radioactivity added at the start of the experiment. The results presented are representative of ten different experiments.

3.3. *Effect of BAF and ConcA on the intracellular transport of [^{125}I]TC-AOM and [^{125}I]TC-BSA from endosomes to lysosomes*

The reduced degradation of [^{125}I]TC-AOM in presence of BAF or ConcA could either be due to an inhibition of transport of ligand to lysosomes or an inhibition of degradation in lysosomes. To distinguish between these possibilities we performed subcellular fractionation of cells that had taken up [^{125}I]TC-AOM in presence and absence of BAF/ConcA. The result of a typical experiment is shown in Fig. 6. In this experiment the cells were incubated with [^{125}I]TC-AOM for 1h in the presence and absence of BAF. In the BAF-treated cells no labeled ligand coincides with lysosomes (identified by the marker enzyme β-acetylglucosaminidase (Fig. 6B) whereas in control cells acid-soluble radioactivity appears in the lysosomal region of the gradient. These results thus demonstrate that BAF (and ConcA, not shown) prevents transport of [^{125}I]TC-AOM from endosomes to lysosomes. Instead, the ligand is available for recycling from the early endosomes. The results shown in Fig. 6 furthermore demonstrate that [^{125}I]TC-AOM accumulates in endosomes of lower density (peak at 1.12 g/ml) in BAF-treated cells than in control cells. The reason for this is probably that the ligand only reaches early endosomes (the compartment from which it is recycled) in BAF-treated cells whereas in control cells it is distributed (after a 60-min chase) between late endosomes and lysosomes. It has been shown earlier that early and late endosomes have different densities in sucrose gradients [45]. Fig. 6B also reveals that lysosomes are shifted to a lower density by BAF. The peak of radioactivity (at 1.12 g/ml) is, however, well separated from the peak of the lysosomal marker enzyme (at 1.16 g/ml) in these cells.

A simple explanation of the lack of transfer of ligand to lysosomes in the BAF/ConcA-treated cells is that the ligand remains to a large extent bound to the receptor (and therefore is not available for trans-

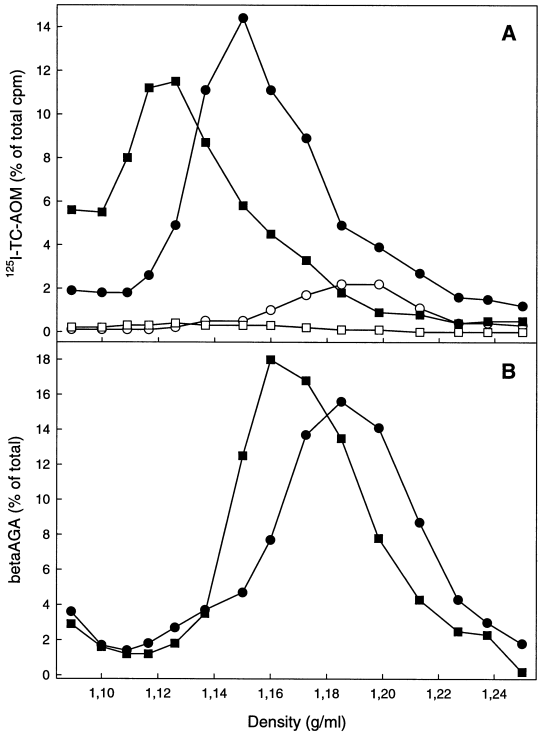

Fig. 6. BAF inhibits transport of [^{125}I]TC-AOM from endosomes to lysosomes. Suspensions of hepatocytes (3×10^6 cells/ml) were preincubated for 15 min with (squares) or without (circles) 4 μM BAF and were subsequently incubated for 1 h in the presence of [^{125}I]TC-AOM (10 nM). Postnuclear fractions were applied onto sucrose density gradients which were centrifuged for 7 h at $85\,000\times g$. Acid-soluble (open symbols) and acid-precipitable (filled symbols) radioactivities (A) as well as β-acetylglucosaminidase (B) were measured in the fractions and are presented as percentage of total recovered activity in the gradients.

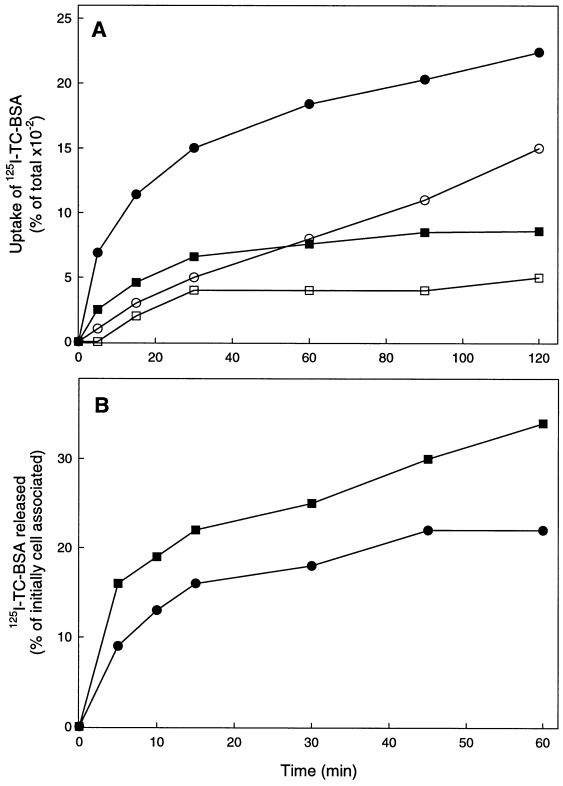

Fig. 7. BAF inhibits uptake and degradation but enhances recycling of [^{125}I]TC-BSA. (A) Suspensions of hepatocytes (3×10^6 cells/ml) were preincubated for 15 min with (squares) or without (circles) 4 μM BAF and were subsequently incubated in the presence of [^{125}I]TC-BSA (10 nM). Acid-soluble (open symbols) and acid-precipitable (filled symbols) radioactivities were measured in samples of cells removed at the indicated times, and are presented as percentage of total added acid-precipitable radioactivity. (B) Cells were given a 15-min pulse with [^{125}I]TC-BSA (10 nM) and were subsequently washed and incubated in presence and absence of BAF (4 μM). Cells were centrifuged at the indicated times at $1000\times g$ for 10 min. The released radioactivity was measured and is presented as percentage of radioactivity associated with the cells at the start of the re-incubation.

port to later endocytic compartments). It is, however, also conceivable that the drugs interfere with the formation of transport vesicles. To determine whether the drugs interfere with the formation of late endosomes and/or their transport to lysosomes we used [^{125}I]TC-BSA as a fluid phase marker of the endocytic vesicles. It has earlier been shown that this probe behaves as a reliable marker for fluid phase endocytosis in hepatocytes [32,33]. Fig. 7 shows that BAF strongly inhibits the uptake and also enhances the recycling of [^{125}I]TC-BSA. These results suggest that the drugs inhibit transfer of marker to later endocytic compartments and thereby make more marker available for recycling from the early endosomes. On the other hand, considerably less [^{125}I]TC-BSA than [^{125}I]TC-AOM was recycled in presence of BAF. This finding probably means that [^{125}I]TC-BSA reaches a later endocytic compartment than [^{125}I]TC-AOM in presence of BAF. The latter notion was underscored by subcellular fractionation experiments which showed that [^{125}I]TC-BSA reached denser endosomes (peak at 1.16 g/ml) than endosomes containing [^{125}I]TC-AOM (peak at 1.12 g/ml) (Fig. 8A). The interpretation of this result was, however, hampered by the fact the [^{125}I]TC-BSA containing endosomes sedimented at the same density as lysosomes (β-acetylglucosaminidase distribution shown in Fig. 8A). The effect of BAF on the buoyant density of lysosomes was, by the way, reversible, as indicated in Fig. 8B. These data show that peak activity of β-acetylglucosaminidase changes from 1.16 g/ml in cells pretreated with BAF for 1 h to 1.20 g/ml in cells subsequently incubated in absence of BAF for an additional hour.

To determine whether [^{125}I]TC-BSA really had reached lysosomes in presence of BAF or ConcA we exposed the MLP-fractions to hypotonic media. It has been shown that such treatment ruptures lysosomes while leaving endosomes intact [46]. Fig. 9 shows results obtained with MLP-fractions prepared from cells that had been incubated with or without BAF for 2 h with [^{125}I]TC-AOM or [^{125}I]TC-BSA. Exposure of MLP-fractions from BAF-treated cells to 10-mosmol sucrose solutions released more than 70% of the lysosomal enzyme β-acetylglucosaminidase, whereas negligible amounts of [^{125}I]TC-AOM or [^{125}I]TC-BSA were released. Hypotonic treatment of control cells released, as expected, more radioactivity, since some of the radioactive probes had reached the lysosomes. This simple experiment thus demonstrates clearly that neither the ligand of the ASGPR nor the fluid phase marker ([^{125}I]TC-BSA) reach the terminal lysosomes in presence of BAF or ConcA. Fig. 9 indicates that a higher proportion of [^{125}I]TC-AOM than [^{125}I]TC-BSA was released by hypotonic treatment. This is due to the fact that most of the added ligand ([^{125}I]TC-AOM) was internalized by the cells during a short interval (<10 min) whereas the fluid phase marker [^{125}I]TC-BSA

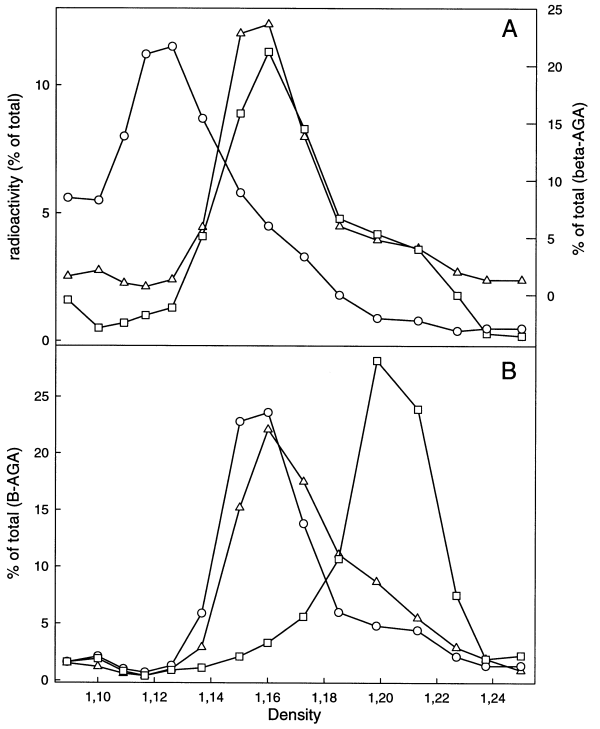

Fig. 8. [^{125}I]TC-BSA reaches denser endosomes than [^{125}I]TC-AOM in BAF-treated cells. (A) Suspensions of hepatocytes (3×10^6cells/ml) were preincubated for 15 min with 4 μM BAF and were subsequently incubated for 1 h in the presence of [^{125}I]TC-BSA (10 nM) (□) or [^{125}I]TC-AOM (10 nM) (○). Postnuclear fractions were applied to sucrose density gradients, which were centrifuged for 7 h at $85\,000\times g$. Radioactivities and β-acetylglucosaminidase (triangles) were measured in the fractions and are presented as percent of total recovered activity in the gradients. (B) Cells were first incubated for 1 h in the presence of BAF (4 μM) and were subsequently washed and reincubated in the presence or absence of 4 μM BAF for 1 h. Postnuclear fractions prepared from cells were removed at the end of the pulse (○) and at the end of the chase in presence (△) or absence (□) of BAF. Enzyme activities were measured in the fractions and are presented as percentage of total recovered activity in the gradients.

was taken up continuously during the incubation. Therefore, relatively more of the fluid phase marker was present in endosomes and not available for release by hypotonic treatment.

3.4. Electron microscopical studies of the intracellular distribution of HRP in cells treated with BAF

To further corroborate the results obtained with biochemical methods we performed electron microscopical studies on the distribution of the fluid phase marker horseradish peroxidase (HRP) and AOM-coated gold particles. To label lysosomes we injected rats with AOM–Au10 1 day before sacrifice. After 1 h of continuous endocytosis of HRP in control cells we found the marker distributed throughout the endocytic pathway, with a notable amount in lysosomes. Upon BAF treatment the amount of HRP

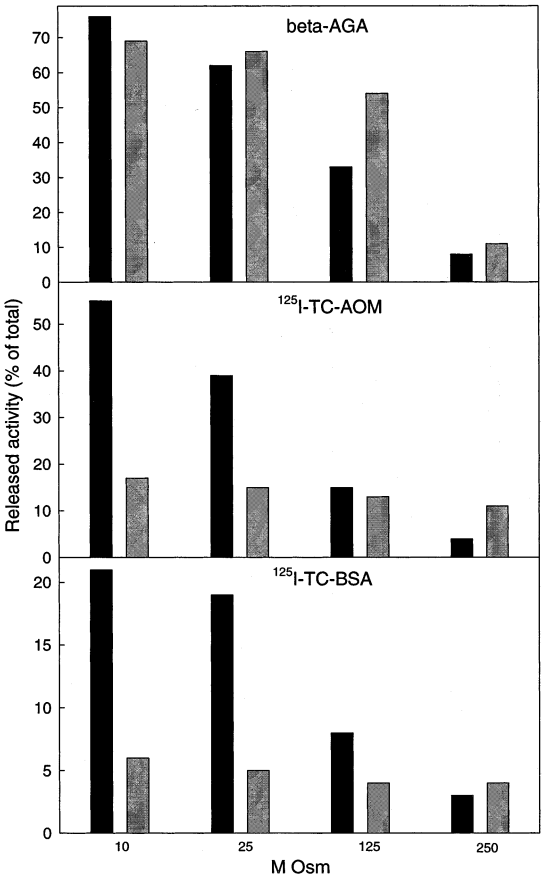

Fig. 9. Hypotonic medium releases β-acetylglucosaminidase but not [^{125}I]TC-AOM or [^{125}I]TC-BSA from BAF-treated cells. Suspensions of hepatocytes (3×10^6 cells/ml) were preincubated for 15 min with (gray bars) or without (black bars) 4 μM BAF and were subsequently incubated for 1 h in the presence of [^{125}I]TC-BSA (10 nM) or [^{125}I]TC-AOM (10 nM). MLP-fractions were prepared from postnuclear fractions (by centrifuging for 60 min at $50\,000\times g$) and incubated in sucrose solutions with the indicated osmolalities for 10 min at 0°C. The fractions were subsequently centrifuged at $50\,000\times g$ for 60 min, and β-acetylglucosaminidase (betaAGA) together with radioactivities were measured in supernatants and pellets. Released enzyme activities and radioactivities are presented as percentage of total activity in the MLP-pellets exposed to hypotonic or isotonic medium (0.25 M sucrose). Nearly identical results were obtained in four different experiments.

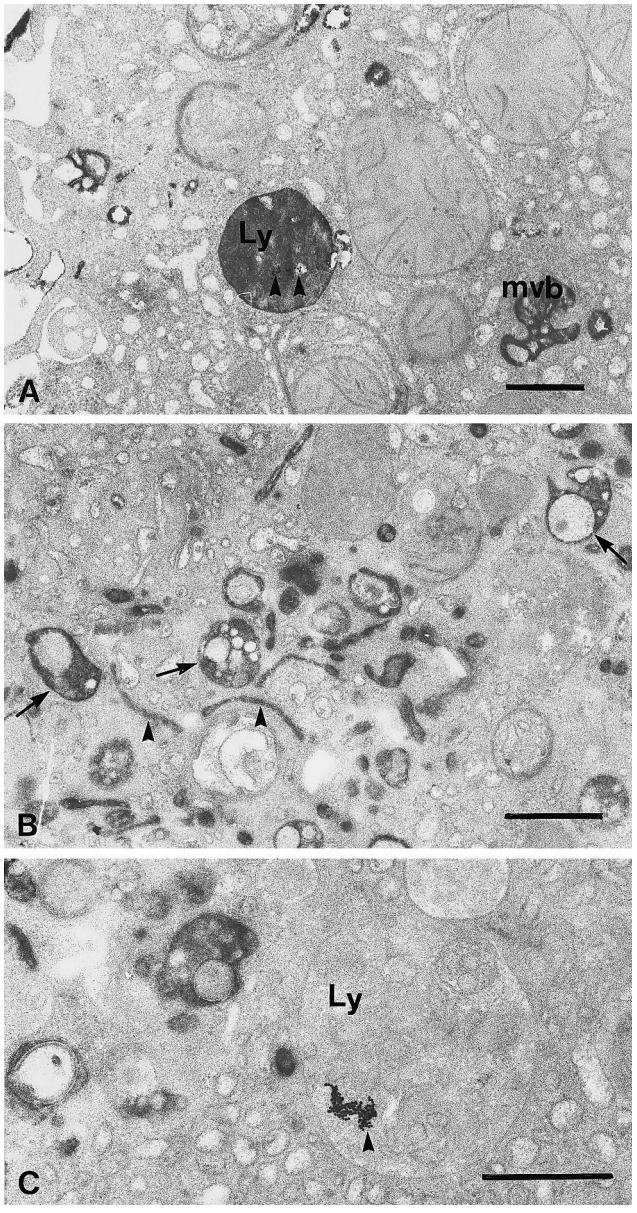

Fig. 10. Electron microscopical study of the intracellular transport of HRP. The intracellular distribution of HRP was studied in isolated rat hepatocytes, which had been preloaded (24 h before sacrifice) with AOM–Au10 in order to label lysosomes. In control hepatocytes (A) we observed HRP reaction product after 1 h continuous internalization in multivesicular structures (mvb) and colocalizing with AOM–Au10 (arrowheads) in lysosomes (Ly). Upon BAF treatment (B,C) HRP accumulated in tubulo-vesicular structures (arrows in B) and multivesicular endosomes (arrowheads in B), whereas lysosomes (Ly in C, arrowhead indicating AOM–Au10) were negative for HRP reaction product. Bars represent 500 nm in all figures.

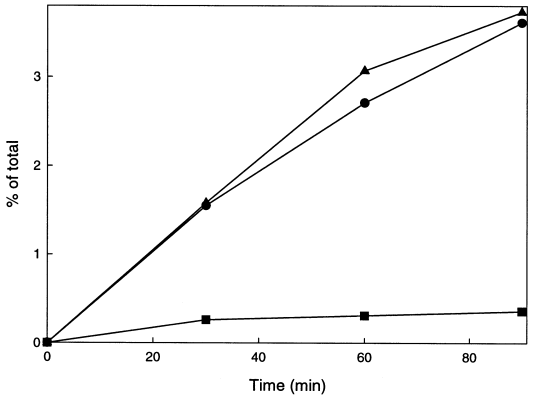

Fig. 11. Accumulation of autophaged LDH in the presence of BAF or leupeptin. Hepatocytes were incubated at 37°C in the presence (▲) or absence (■) of leupeptin (0.3 mM) or BAF (4 μM, ●). The amount of LDH in sedimentable cell corpses was measured at the indicated time points and is expressed relative to the total cellular LDH. The results are representative of five different experiments.

colocalizing with lysosomes was reduced considerably, and the marker was found mainly in tubulovesicular endosomes and in multivesicular endosomes (Fig. 10). Similar results were obtained with ConcA. We also investigated the effect of BAF/ConcA-treatment on the distribution of newly endocytosed AOM–Au15, which is endocytosed via the ASGPR-system. In this case we observed a greatly reduced number of internalized gold particles upon inhibitor treatment, especially in the case of ConcA-treatment. The gold-labeled probes were mainly found in peripheral, small endosomal structures (results not shown). In contrast to the fluid phase marker only small amounts of AOM–Au15 were seen in multivesicular endosomes. These observations are in excellent agreement with our biochemical results, and underline that the intracellular transport of receptor-mediated ligands and fluid phase markers is differentially affected by both inhibitors.

3.5. *Effects of BAF or ConcA on autophagic sequestration of lactate dehydrogenase*

BAF (1 μM) or ConcA (200 nM) inhibited degradation of autophagocytosed LDH to the same extent as leupeptin (Fig. 11). During 1 h of incubation about 3% of the cellular LDH was associated with autophagic vacuoles. This value is in good agreement with earlier data [47]. A morphometric quantitation

Table 1
Quantitation of the effect of BAF/ConcA treatment on the autophagosome volume in hepatocytes

Treatment	% Autophagosome volume	Number of micrographs
Control	3.70 ± 0.23	28
BAF	7.32 ± 0.27	29
ConcA	13.10 ± 0.58	28

Hepatocytes were prepared for electron microscopy as described in Section 2. The volume density of autophagosomes in the cytosol was estimated by point counting with a test lattice according to established techniques. Micrographs were taken at an initial magnification of 7600× and counting performed at a final magnification of 30 000×. Autophagosomes were morphologically recognized by their content of undegraded cellular material such as mitochondria, endosomes and ER membranes. Their volume was expressed as percentage of the cytoplasmic reference volume (mean ± S.E.M. of the indicated number of micrographs). Data from two different experiments have been combined. Differences between untreated and treated cells were statistically significant with $P < 0.05$ (according to Student's *t*-test).

of the cytoplasmic volume occupied by autophagic vacuoles indicated a 2-fold increase upon BAF-treatment, and a 6–7-fold increase upon ConcA treatment (see Table 1). This result shows that autophagic sequestration per se is not inhibited. To determine whether the drugs inhibit degradation of LDH in lysosomes or fusion between autophagosomes and lysosomes we combined subcellular fractionation and electron microscopy. In the biochemical approach LDH was used as a marker of autophagic vacuoles and β-acetylglucosaminidase as a lysosomal marker. To label lysosomes in the EM-studies AOM–Au10 was injected 24 h prior to the preparation of hepatocytes. Autophagic vacuoles were identified by their characteristic appearance.

The interpretation of the subcellular fractionation data was again impeded by the density shift of the lysosomes, induced by BAF/ConcA. Nevertheless, at concentrations of BAF/ConcA that did not completely inhibit LDH degradation (1 μM) the density distributions of LDH and β-acetylglucosaminidase showed peak activities at different densities (because β-acetylglucosaminidase was not density shifted) suggesting that the two markers were to some extent in different compartments, although some colocalization could not be ruled out (results not shown). The notion that BAF/ConcA inhibited fusion between lysosomes and autophagosomes was, however, not supported by results obtained by EM. In order to get a rough estimate of the fusion efficiency between autophagosomes and lysosomes upon BAF treatment, we simply counted the number of lysosomal gold aggregates localized in either lysosomes or autophagic vacuoles. We observed about 80% of all gold aggregates in autophagic vacuoles, strongly indicating that fusion between autophagosomes and lysosomes was not inhibited. Accordingly, the observed accumulation of undegraded material in these vesicles is probably caused by the inhibitory effect of BAF on acidification.

4. Discussion

The role of acidification of endosomes and lysosomes for the functional properties of these organelles has been the subject of a number of investigations [3]. In early studies neutralization of endosomes and lysosomes was achieved by means of acidotropic weak bases such as chloroquine and ammonia. Such agents may affect parameters other than pH that are essential for transport and processing in endocytic and autophagocytic pathways. They lead, for instance, to a tremendous osmotic swelling of acidic vesicles/lysosomes [25–28] and the membrane of the swollen organelle may act differently from the native membrane with regard to membrane traffic (budding/fusion reactions). BAF and ConcA act specifically on the vacuolar proton pump and should therefore presumably affect the pH of the endocytic vesicles/lysosomes without some of the side effects induced by acidotropic agents.

In the present investigation we have employed BAF and ConcA to study the role of the vacuolar proton pump for different transport steps in endocytosis and autophagy in rat hepatocytes. The main findings were as follows: BAF/ConcA (a) reduced rate of internalization of [^{125}I]TC-AOM and [^{125}I]TC-BSA, (b) increased rate of recycling of [^{125}I]TC-AOM and [^{125}I]TC-BSA, (c) reduced the number of surface ASGPR but did not affect the total number of ASGPR, (d) inhibited transport of both [^{125}I]TC-AOM and [^{125}I]TC-BSA from endosomes to lysosomes, and (e) enhanced accumulation of LDH in autophagic vacuoles, but did not signifi-

cantly inhibit fusion between autophagosomes and lysosomes. The autophagocytosed material instead accumulated in autolysosomes (because degradation was inhibited).

The reduced amount of surface ASGPRs in presence of BAF/ConcA seen in the present study is compatible with earlier reports [14,17,19,20] and explains partly the reduced rate of uptake of ligand. BAF leads to a redistribution of ASGPRs such that more receptors are in the early endosomes at the expense of the number of receptors at the cell surface. A possible reason for this may be that inactivation of the proton pump leads to increased amounts of membrane in the tubular extensions of the early endosomes. However, these receptors are still in a position to recycle to the cell surface. A further reduction of uptake is due to a reduced rate of internalization of receptor–ligand complexes, as indicated by measuring the ratio between the amounts of surface-bound and internalized ligand in BAF-treated and control cells. This result is in accordance with earlier reports [14]. Recycling of ligand (in association with receptor) was, on the other hand, enhanced in BAF-treated cells. A simple explanation of this effect is of course that reduced amounts of ligand dissociate from the receptor in early endosomes because of enhanced pH.

If the ligand does not dissociate from the receptor it would not be available for transfer/transport to lysosomes, and subcellular fractionation verified that no ligand entered lysosomes. However, the amount of ligand taken up in BAF-treated cells exceeded slightly the total amount of receptors in the cells. Moreover, a proportion of the ASGPR may be segregated from the endocytic pathway [44]. The low pH may therefore not be the only factor that controls receptor activity. The ASGPR is subject both to phosphorylation and acylation, and Weigel and coworkers have reported that such covalent modifications may control the activity of the ASGPR during each round through the sorting endosome [48,49]. Some release of ligand is conceivable in view of the extremely high concentrations of receptor–ligand complexes that may be present in this compartment. It is also possible that inactivation of receptor (by phosphorylation and acylation) may be dependent on a low endosomal pH.

Since transfer of [^{125}I]TC-AOM may be restricted in presence of BAF/ConcA we also studied intracellular transport of a fluid phase marker, [^{125}I]TC-BSA, which has been shown to be a useful probe for studying pinocytosis [32,33]. Using subcellular fractionation it was shown that the transfer of the fluid phase marker to lysosomes was also inhibited, and the use of hypotonic medium to selectively disrupt lysosomes clearly indicated that BAF/ConcA blocked transport of [^{125}I]TC-BSA to lysosomes. Since transport to lysosomes is inhibited the probe will accumulate in endosomes and this explains why more marker is released to the medium and why the net uptake of fluid phase marker is markedly reduced.

The effects of the drugs may be on maturation or transport of the endosomes or on the fusion between endosomes and lysosomes. Earlier studies have suggested that the specific effects of BAF on endocytic traffic may be cell-dependent. Thus, it has been shown that BAF in BHK-21 cells prevents transport from early to late endosomes, presumably by interfering with the formation of endocytic carrier vesicles [12], whereas in HepG2-cells and HEp-2 cells inactivation of the vacuolar proton pump seems primarily to block transport from late endosomes to lysosomes. Traffic through early endosomes and late endosomes is less affected [16,50]. The present data indicate that BAF/ConcA in hepatocytes interfere with the endocytic pathway by preventing fusion between late endosomes and lysosomes. A 'hybrid organelle' in which degradation of endocytosed material has been shown to take place [51] will therefore not be formed. The bulk of [^{125}I]TC-AOM in presence of BAF/ConcA remains in an early endosome from which recycling of receptor takes place because the ligand remains bound to the receptor. The fluid phase marker, [^{125}I]TC-BSA, on the other hand, is found in a denser endosome than the ligand in presence of BAF/ConcA, and by electron microscopy it could be shown that another fluid phase marker, HRP, also accumulates in a late (multivesicular) endosome. No HRP was seen in lysosomes. The mechanisms whereby endocytosed molecules are transported from early endosomes to lysosomes is not fully understood and the process may differ from cell to cell. The endocytic carrier vesicle that transports material from early to late endosomes in, e.g., BHK cells has not so far been described in liver cells.

Instead, endocytosed material is found in maturing multivesicular bodies that eventually deliver material to terminal lysosomes [52]. The different effects of BAF on the endocytic processes in BHK cells and hepatocytes may conceivably be due to a different layout of the endocytic pathways in the different cells.

While increased pH in endosomes and lysosomes in BAF/ConcA-treated cells prevents ligand dissociation in early endosomes and degradation in lysosomes, it is less clear why a proton gradient is necessary for membrane traffic in the 'vacuolar apparatus'. The present data indicate, in accordance with earlier reports [16,50], that a key step in the endocytic pathway with regard to pH-sensitivity is the communication between lysosomes and late endosomes. It is not entirely clear whether this step depends on fusion between lysosomes and late endosomes or whether the transport is mediated by small vesicles. At any event, it is likely that the internal pH of late endosomes affects structure(s) on the cytoplasmic face of the organelles. Such targets may be proteins involved in budding and fusion. Interestingly, it has been shown that association of an ADP-ribosylation factor to microsomes is dependent on luminal pH; BAF and the protonophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP) inhibit ATP-dependent association of ARF to the vesicle membrane [53]. The budding of carrier vesicles from the early endosome or the conformation of a receptor-like protein involved in budding/fusion may require a pH differential across the budding membrane. This notion is in agreement with the observation that the formation of an endosomal carrier vesicle (that mediate transport from early to late endosomes) is inhibited by BAF in BHK cells [54].

Acknowledgements

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