Inhibition of asialoglycoprotein endocytosis and degradation in rat hepatocytes by protein phosphatase inhibitors

Ingunn HOLEN,* Paul B. GORDON,* Per E. STRØMHAUG,* Trond O. BERG,* Monica FENGSRUD,* Andreas BRECH,† Norbert ROOS,† Trond BERG‡ and Per O. SEGLEN*§

*Department of Tissue Culture, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, 0310 Oslo, Norway, †Electron Microscopical Unit for Biological Sciences, Department of Biology, University of Oslo, Oslo, Norway, and ‡Unit for Molecular Cell Biology, Department of Biology, University of Oslo, Oslo, Norway

In isolated rat hepatocytes, a radiolabelled tyramine-cellobiose conjugate of asialo-orosomucoid, ^{125}I -TC-AOM, was rapidly taken up by receptor-mediated endocytosis and proteolytically degraded in the lysosomes, where radioactive degradation products accumulated. Okadaic acid and other protein phosphatase inhibitors (microcystin-LR, calyculin A) strongly reduced the fraction of asialoglycoprotein (ASGP) receptors localized to the cell surface, and correspondingly inhibited the uptake of ^{125}I -TC-AOM. In addition, the inhibitors suppressed ^{125}I -TC-AOM degradation strongly (90 % at 150 nM) and potently (half-maximal effect at 20 nM okadaic acid), indicating an involvement of protein phosphorylation, and of a protein phosphatase of type 2A, in the regulation of intracellular endocytic flux. The effects of okadaic acid on ^{125}I -TC-AOM accumulation, as well as on degradation, could be eliminated by the protein kinase inhibitor genistein. Okadaic acid prevented the transfer of ^{125}I -TC-AOM to a non-recycling endocytic compartment, causing its retention in a recycling compartment from which about one-third of the endocytosed ^{125}I -TC-AOM could be returned to the cell surface and detached from its receptor in the presence of EGTA. ASGP receptors recycled extensively both in the presence and absence of okadaic acid, as indicated by a sustained uptake of ^{125}I -TC-AOM. Sucrose density gradient analysis and sedimentation studies indicated that okadaic acid caused accumulation of ^{125}I -TC-AOM in light endosomes (1.11 g/ml),

preventing its transfer to dense endosomes (1.14 g/ml) and lysosomes (1.18 g/ml). The lysosomes could be identified in density gradients by their contents of lysosomal marker enzymes and acid-soluble radioactivity, and by their sensitivity towards the lysosome-disrupting agent glycyl-L-phenylalanine-2-naphthylamide. By using endocytosed AOM-gold particles as an ultrastructural endocytic marker, it could be shown that the light endosomes accumulating ASGP in the presence of okadaic acid had the morphological appearance of small endocytic vesicles/tubules and multivesicular endosomes. Whereas in control cells 4 % of the AOM-gold was in small vesicles/tubules, 55 % in multivesicular endosomes and 41 % in lysosomes, the corresponding figures for okadaic acid-treated cells were 17 %, 73 % and 11 %. Our results thus indicate that protein phosphatase inhibitors have two effects on ASGP endocytosis: (1) an early inhibition of ligand uptake, due to a reduction in the fraction of ASGP receptors at the cell surface, and (2) an inhibition of ASGP transfer from a recycling compartment consisting of light, small endocytic vesicles and multivesicular endosomes, to a non-recycling compartment consisting of dense multivesicular endosomes. This late step may represent the detachment of multivesicular endosomes from a continuous tubulovesicular network, a process apparently subject to regulation by a genistein-sensitive protein kinase and a protein phosphatase of type 2A.

INTRODUCTION

The endocytic pathway is subject to regulation by protein phosphorylation at several steps. Surface receptors can be phosphorylated both at serine/threonine [1] and tyrosine groups [2], resulting in inhibition or stimulation respectively of endocytosis. Endosomes contain several protein kinase activities [3,4] that may be involved in signal transduction [5] as well as in the control of endosomal function [6].

To study the functional significance of protein phosphorylation in intact cells, okadaic acid and related protein phosphatase inhibitors have been found to be useful. Okadaic acid inhibits protein phosphatases 1 and 2A [7], and has been shown to induce a general hyperphosphorylation of hepatocytic phosphoproteins [8]. Hepatocytic autophagy is potently inhibited by okadaic acid [9a], possibly as a consequence of okadaic acid-induced disruption of the cytokeleton intermediate filament network [9b],

both effects probably being mediated by Ca^{2+} /calmodulin-dependent protein kinase II (CaMK-II) or a closely related enzyme [10]. In other cell types, okadaic acid has been found to either stimulate [11] or inhibit fluid-phase endocytosis [12], and to prevent fusion between endosomes in a cell-free system [13].

The present investigation was undertaken to see if okadaic acid might interfere with receptor-mediated endocytosis. We have used isolated rat hepatocytes as an experimental system, and ^{125}I -tyramine cellobiose-conjugated asialo-orosomucoid (^{125}I -TC-AOM) as an endocytic probe. This probe has the advantage that acid-soluble degradation products remain inside the vacuoles in which they are generated and thus can be used to distinguish between proteolytic and non-proteolytic compartments [14]. To further characterize the various endocytic compartments, we have employed sedimentation analysis, sucrose density gradient fractionation and electron microscopy. The results indicate that okadaic acid inhibits both the uptake

Abbreviations used: ^{125}I -TC-AOM, ^{125}I -tyramine-cellobiose-asialo-orosomucoid; ACP, acid phosphatase; β -AGA, *N*-acetyl- β -D-glucosaminidase; ASGP, asialoglycoprotein; CaMK-II, Ca^{2+} /calmodulin-dependent protein kinase II; GPN, glycyl-L-phenylalanine-2-naphthylamide; PP1, protein phosphatase type 1; PP2A, protein phosphatase type 2A; TCA, trichloroacetic acid.

§ To whom correspondence should be addressed.

and degradation of ^{125}I -TC-AOM, causing its retention in a recycling compartment consisting of small endocytic vacuoles/tubules and multivesicular endosomes.

MATERIALS AND METHODS

Cell preparation and incubation

Hepatocytes were isolated from 18 h-starved male Wistar rats (250–300 g) by two-step collagenase perfusion [15]. The cells were washed and suspended in suspension buffer [15] containing 15 mM pyruvate and extra Mg^{2+} (to 2 mM). Samples (2 ml) of cell suspension (15–20 mg of cellular wet weight) were incubated at 37 °C in 5-cm-diam. albumin-coated plastic Petri dishes. For dose-response measurements the cells were preincubated for 30 min at 37 °C with various concentrations of okadaic acid before addition of ^{125}I -TC-AOM, at approx. 50000 c.p.m./sample (10 nM); the cells were then incubated for an additional 2 h at 37 °C. For time-course studies and isopycnic centrifugations the cells were preincubated with 30 nM okadaic acid for 15 min and 30 min respectively, before addition of ^{125}I -TC-AOM. After incubation the samples were cooled to 0 °C, transferred to 15-ml plastic tubes and washed once with perfusion buffer containing 10 mM EGTA (final pH 7.5) to remove receptor-bound ^{125}I -TC-AOM from the cell surface; the cells were then washed either twice with 10% sucrose (for sedimentation and gradient studies) or once with perfusion buffer [15] (for uptake and degradation measurements).

Measurements of uptake and degradation of ^{125}I -TC-AOM

After washing, the cell pellets were counted in an LKB gamma-counter to measure the cellular uptake of ^{125}I -TC-AOM, i.e. the amount of cell-associated radioactivity, expressed as a percentage of the total acid-insoluble radioactivity added initially (since the ^{125}I -TC-AOM preparation contained 10–20% acid-soluble radioactivity, all cellular radioactivity values are related to acid-insoluble rather than to total initial radioactivity). An aliquot (1 ml) of 10% trichloroacetic acid (TCA) was then added to each pellet, the suspension was kept on ice for a minimum of 15 min, and thereafter centrifuged for 15 min at 3700 g. The supernatant, containing the acid-soluble degradation products of ^{125}I -TC-AOM, was transferred to a separate tube and counted. The amount of ^{125}I -TC-AOM degraded was calculated as a percentage of the total acid-insoluble radioactivity added to each sample initially.

Asialoglycoprotein (ASGP) receptor studies

Binding of ^{125}I -TC-AOM to cell-surface receptors was measured after incubating the cells for 90 min at 0 °C with ^{125}I -TC-AOM. The cells were then washed twice in washing buffer with or without 10 mM EGTA to determine unspecific (Ca^{2+} -independent) and total binding respectively. Specific binding (on average, 96% of total) was calculated as the difference between total and unspecific binding, and the number of surface ASGP receptors per cell determined by Scatchard analysis as previously described [16].

Electrodisruption and preparation of cell corpses

After washing in 10% unbuffered, electrolyte-free sucrose, the samples were resuspended in 1 ml of 10% sucrose and disrupted by a single high-voltage pulse (2 kV/cm) [17]. A 200- μl aliquot of the disrupted sample was transferred to a separate tube for measurement of total ^{125}I -TC-AOM uptake, and a 300- μl aliquot was layered on top of a 4-ml density cushion of metrizamide-

sucrose (2.2% sucrose, 8% metrizamide, 50 mM potassium phosphate, 1 mM dithiothreitol, 1 mM EDTA, pH 7.5; density 1.05 g/ml). Sedimentation through the cushion was performed at 0 °C at a high ($2.9 \times 10^6 \text{ g} \cdot \text{min}$) centrifugation force, except in one experiment performed to illustrate the effect of okadaic acid on structure-associated sedimentability, where a low centrifugation force ($1.1 \times 10^5 \text{ g} \cdot \text{min}$) was employed. The sediments (cohesive or dissociated cell corpses) were counted in the gamma counter, and their content of endocytosed ^{125}I -TC-AOM calculated as a percentage of the total acid-insoluble radioactivity added to each sample initially.

Cell fractionation and isopycnic sucrose density gradient centrifugation

All fractionation and gradient work was performed at 0 °C. Cell samples were electrodisrupted, and a disruptate volume of 4.5 ml was layered on top of a 25-ml metrizamide-sucrose cushion and centrifuged at a high centrifugation force ($2.9 \times 10^6 \text{ g} \cdot \text{min}$). Pellets were carefully resuspended in 3 ml of buffered sucrose (0.25 M sucrose, 10 mM Hepes, 1 mM EDTA, pH 7.3), followed by four strokes in a Dounce homogenizer with a tight-fitting pestle. The homogenate was thereafter centrifuged at 3000 g·min to obtain a nuclear pellet and a postnuclear supernatant. The nuclear pellet was washed once in 2 ml of buffered sucrose, and the two postnuclear supernatants were combined. A 1.5-ml sample of postnuclear supernatant or large-granule fraction was layered on top of a linear sucrose density gradient. The gradient was made by layering 5.5 ml of 63% (w/v) buffered sucrose underneath 5.5 ml of 15% (w/v) buffered sucrose (both sucrose solutions containing 1 mM EDTA and 10 mM Hepes, pH 7.3) and mixing with a Biocomp Gradient Master (Nycomed Pharma, Oslo, Norway) to obtain densities ranging from 1.04 g/ml in the top fraction to 1.23 g/ml in the bottom fraction. The gradients were centrifuged at 4 °C in a Beckman L8-70M Ultracentrifuge with an SW40/9E 2800 rotor for 1 h 45 min at 40000 rev./min ($3 \times 10^7 \text{ g} \cdot \text{min}$). Following centrifugation the gradients were fractionated into approx. 20 fractions by upward displacement using Maxidenz as displacement fluid. The densities of the fractions were calculated from the refractive indices. Acid phosphatase (ACP) or β -acetylglucosaminidase (β -AGA) in the fractions was determined spectrophotometrically in a Technicon RA-1000 autoanalyser and expressed as a percentage of the total amount of enzyme in the cell. Total radioactivity, acid-soluble and acid-insoluble radioactivity in each fraction were measured in a gamma counter as described above, except that albumin was added to a final concentration of 0.1% (w/v) before acid precipitation (0.4 ml of gradient fraction, 0.1 ml of 1% albumin and 0.5 ml of 20% TCA). Radioactivities in the fractions were expressed as percentages of the total cell-associated radioactivity at that time point.

Disruption of lysosomes by glycyl-L-phenylalanine-2-naphthylamide (GPN)

GPN, freshly dissolved in DMSO at 75 mM, was diluted 100-fold with buffered sucrose and mixed with half its volume of electrodisrupted hepatocytes to a final concentration of 0.5 mM GPN and 0.67% DMSO. The disruptate was incubated with GPN for 5 min at 37 °C, cooled to 0 °C, and cell corpse isolation and subcellular fractionation were performed by standard procedures.

Measurement of ^{125}I -TC-AOM recycling

To measure the release of endocytosed ^{125}I -TC-AOM from ligand-recycling compartments, hepatocytes were first incubated

for 5 or 40 min at 37 °C with ^{125}I -TC-AOM, then rapidly cooled by adding ice-cold buffer and washed once at 0 °C with buffer containing 10 mM EGTA to remove surface-bound ligand. The cells were subsequently incubated at 37 °C in the presence or absence of 2.5 mM EGTA for up to 30 min. In the presence of the chelator, any AOM that recycled to the cell surface would be released into the medium and could be measured and expressed as a percentage of the cell-associated radioactivity in the absence of EGTA.

Electron microscopy

Colloidal gold particles (5 and 10 nm diam.) were prepared according to Slot and Geuze [18] and coated with AOM by adsorption according to established procedures [19,20]. Gold sol (240 ml) was complexed with AOM (1.5 mg for 10-nm gold; 0.75 mg for 5-nm gold) by mixing at room temperature, and the gold-protein complexes recovered by centrifugation at 45000 *g* for 45 min. To prelabel hepatocytic lysosomes, 1 ml of 10-nm AOM-gold in colloidal suspension (absorbance at 520 nm after 100-fold dilution = 2.42) was injected intravenously into rats 24 h before cell isolation. To label the endosomes (and lysosomes) *in vitro*, 2 ml of hepatocytes in suspension (30 mg wet wt. cells) were incubated at 37 °C with 200 μl of 5-nm AOM-gold (absorbance at 520 nm after 100-fold dilution = 0.245) for 5 or 120 min. The cells were then washed twice in ice-cold 0.1 M phosphate-buffered saline (PBS; pH 7.4) and fixed with 4.0% formaldehyde and 0.1% glutaraldehyde in 0.1 M PBS. Pellets were post-fixed for 60 min with 2% OsO_4 containing 1.5% potassium ferrocyanide, followed by staining with 1.5% uranyl acetate (all solutions in distilled water). After serial dehydration in ethanol and propylene oxide, specimens were embedded in Epon, sectioned and post-stained with 0.2% lead citrate. Sections were examined in a JEOL 100CX electron microscope at 80 kV.

Enzyme and radioactivity measurements

ACP activity [21] was determined spectrophotometrically in a Technicon RA-1000 autoanalyser, using a kit for automated analysis from Boehringer Mannheim. β -AGA (hexosaminidase) activity was determined according to Barrett [22]. Radioactivities were measured in an LKB Wallac gamma counter (1261 multigamma).

Reagents

^{125}I -TC-AOM was synthesized as previously described [14]. Okadaic acid and calyculin A were purchased from Moana Bioproducts Inc., Hawaii, U.S.A.; microcystin-LR and genistein from Gibco BRL, Gathersburg, MD, U.S.A.; Nycodenz, Maxident and Metrizamide from Nycomed A/S, Oslo, Norway. Kits for automated analysis of ACP were obtained from Boehringer (Mannheim, Germany). All other biochemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. or other suppliers of analytical grade chemicals.

RESULTS

Inhibition of hepatocytic ASGP endocytosis by protein phosphatase inhibitors

^{125}I -TC-AOM is endocytosed by way of the ASGP receptor, and eventually reaches the lysosomes, where radiolabelled degradation products accumulate [14]. With this probe, overall endocytosis could, therefore, be followed as the continuous accumulation of radioactivity. After 3 h, about 80% of the ^{125}I -

Figure 1 Effect of okadaic acid on cellular accumulation of ^{125}I -TC-AOM

(a) Hepatocytes were preincubated for 30 min at 37 °C in the absence (○) or presence (●) of 100 nM okadaic acid. ^{125}I -TC-AOM (100 nM) was then added, and the incubation continued for the length of time indicated. The samples were then cooled to 0 °C, washed, and the amount of cell-associated radioactivity (given as a percentage of the total acid-insoluble radioactivity added) was determined. Each value is the mean $\pm$ S.E.M. of three experiments. (b) Hepatocytes were preincubated for 30 min at 37 °C with okadaic acid (○), calyculin A (●) or microcystin-LR (△) at the concentration indicated, 100 nM ^{125}I -TC-AOM was added, and the cells were incubated at 37 °C for another 2 h. The amount of cell-associated radioactivity is given as a percentage of the untreated control value. The data were obtained from a single experiment.

TC-AOM added had been endocytosed (Figure 1a). The protein phosphatase inhibitor okadaic acid inhibited ^{125}I -TC-AOM accumulation strongly (Figure 1a), with a half-maximal effect around 20 nM, i.e. okadaic acid was about an order of magnitude more potent than calyculin A or microcystin-LR (Figure 1b). None of the inhibitors had any detectable effect on general cellular appearance within their effective concentration ranges. At higher concentrations (e.g. okadaic acid above 100 nM) some surface blebbing was observed.

Alterations in ASGP receptor distribution after okadaic acid treatment

To see if the reduced accumulation of ^{125}I -TC-AOM in the presence of protein phosphatase inhibitors might, at least in part, be due to a reduced uptake, the effect of okadaic acid pretreatment on binding of ^{125}I -TC-AOM to cell-surface ASGP receptors at 0 °C was studied. Within 15 min of okadaic acid treatment at 37 °C, the number of surface ASGP receptors, as measured by binding of ^{125}I -TC-AOM (20 nM) at 0 °C, was reduced by about one-half at 40 nM okadaic acid (Figure 2a), with a half-maximal effect of okadaic acid at about 20 nM (Figure 2b). Scatchard plot analysis of binding data obtained after incubating the cells at 0 °C with concentrations of ligand ranging from 1 nM to 60 nM showed that okadaic acid pretreatment did not affect receptor affinity (the equilibrium dissociation constants were 2.7×10^{-8} M and 2.9×10^{-8} M for control cells and okadaic acid-pretreated cells, respectively). The number of surface receptors was, on the other hand, reduced from 2.6×10^5 /cell to 1.2×10^5 /cell at 40 nM okadaic acid. Inhibition of ^{125}I -TC-AOM uptake by okadaic acid paralleled the reduction in receptor number in this experiment (results not shown), suggesting that the drug reduced ASGP uptake in hepatocytes by a reduction in the number of ASGP receptors available at the cell surface.

Inhibition of ASGP degradation by protein phosphatase inhibitors

Isolated rat hepatocytes degrade ASGP rapidly [23,24]; under the present conditions about one-half of the ^{125}I -TC-AOM initially added to the medium was degraded within 3 h (Figure

Figure 2 Effect of okadaic acid on the number of ^{125}I -TC-AOM-binding cell-surface receptors

(a) Hepatocyte suspensions (2×10^6 cells/ml) were incubated with 40 nM okadaic acid at 37 °C. At the indicated times cell samples were removed and incubated for 90 min at 0 °C with 20 nM ^{125}I -TC-AOM. Specific receptor binding was determined as the difference between samples incubated with and without 10 mM EGTA. Control cells (before okadaic acid addition) bound 502 fmol of ligand/ 10^6 cells. (b) Hepatocytes were incubated with the indicated concentrations of okadaic acid for 15 min at 37 °C; the specific binding of ^{125}I -TC-AOM (20 nM) was then determined by incubation at 0 °C for 90 min. Control cells (no okadaic acid added) bound 435 fmol of ligand/ 10^6 cells.

Figure 3 Effect of okadaic acid on the degradation of endocytosed ^{125}I -TC-AOM

(a) Hepatocytes were preincubated for 30 min at 37 °C in the absence (○) or presence (●) of 30 nM okadaic acid, a tracer amount of ^{125}I -TC-AOM was added, and the incubation was continued for the length of time indicated. The samples were then cooled to 0 °C, washed, precipitated with 10% ice-cold TCA, and the amount of acid-soluble radioactivity was measured and expressed as a percentage of the total acid-insoluble radioactivity initially added. Each value is the mean $\pm$ S.E.M. of seven or eight experiments. (b) Hepatocytes were preincubated for 30 min at 37 °C with okadaic acid (○), calyculin A (●) or microcystin-LR (△) at the concentration indicated, a tracer amount of ^{125}I -TC-AOM was added, and the cells were incubated at 37 °C for another 2 h. At the end of the incubation the cells were washed and precipitated with TCA, and the relative amount of acid-soluble radioactivity was measured and expressed as a percentage of the corresponding control (included in each experiment). Each value is the mean of seven experiments with okadaic acid; for calyculin A and microcystin-LR the data were obtained from a single experiment.

3a). The degradation of ^{125}I -TC-AOM was strongly suppressed by okadaic acid (Figure 3a), half-maximal inhibition being observed around 20 nM (Figure 3b). Calyculin A and microcystin-LR were likewise effective inhibitors of ASGP degradation, albeit not as potent as okadaic acid (Figure 3b).

Figure 4 Antagonistic effect of genistein on the inhibition of ^{125}I -TC-AOM uptake and degradation by protein phosphatase inhibitors

Hepatocytes were incubated with ^{125}I -TC-AOM for 3 h at 37 °C in the absence (○) or presence of okadaic acid, 60 nM (●); calyculin A, 100 nM (△); or microcystin-LR, 100 nM (▲), at the concentration of genistein indicated. The cells were then washed and precipitated with 10% TCA, and the total amount of cell-associated radioactivity (a) as well as the acid-soluble, i.e. degraded, fraction of ^{125}I -TC-AOM (b) was measured and expressed as a percentage of the total acid-insoluble radioactivity added initially. Each point represents the mean of two experiments (triplicate samples in each experiment).

Okadaic acid-antagonistic effect of a protein kinase inhibitor, genistein

Since okadaic acid is a protein phosphatase inhibitor, its effect might conceivably be antagonized by inhibition of the protein kinase(s) responsible for suppression of ^{125}I -TC-AOM endocytosis. Genistein, an isoflavone that inhibits tyrosine- as well as serine/threonine-protein kinases [25,26], had very little effect on endocytosis on its own, but reversed the effects of okadaic acid and microcystin-LR both on the cellular accumulation (Figure 4a) and on the degradation (Figure 4b) of ^{125}I -TC-AOM. Genistein did not antagonize the effect of calyculin A, a paradox that warrants further investigation.

Separation of ^{125}I -TC-AOM-containing endocytic vacuoles by isopycnic sucrose density gradient centrifugation

Figure 5 shows the time-dependent accumulation of endocytosed ^{125}I -TC-AOM in endocytic vacuoles from postnuclear supernatants of homogenized cell corpses, separated on sucrose density gradients. At the early time points (Figures 5a and 5b), nearly all of the radioactivity was acid-insoluble, distributed between a major, light peak at 1.10–1.11 g/ml and a minor, denser peak at 1.14 g/ml. These two peaks were also observed in hepatocytes homogenized without prior electrodisruption, i.e. they would seem not to be a result of the electrical treatment. Beyond 10 min, the amount of acid-insoluble radioactivity in the 1.14-peak increased relative to the 1.11-peak, consistent with a transfer of ^{125}I -TC-AOM from light to dense endosomes. Eventually (around 30 min), acid-soluble radioactivity began to appear, both in a very dense peak at 1.18–1.19 g/ml and in the 1.14–1.16 g/ml region (Figures 5c and 5d). Subsequent accumulation of acid-soluble radioactivity was predominantly in the denser of these two peaks, coinciding at all times with the distribution of lysosomal marker enzymes like β -AGA (Figure 5) and ACP (results not shown). Treatment of electrodisrupted cells with GPN, a lysosome-disrupting cathepsin C substrate [27], strongly reduced the β -AGA peak and largely eliminated the acid-soluble radioactivity (Figure 5f). Similar results (not shown) were obtained with L-methionine-O-methyl ester, a lysosome-

Figure 5 Time-dependent alterations in density distribution and sedimentability of endocytic vacuoles

Hepatocytes were incubated at 37 °C with ^{125}I -TC-AOM for 5 (a), 15 (b), 30 (c), 45 (d) or 60 min (e and f). The cells were then electrodisrupted, and the disruptate centrifuged through a metrizamide-sucrose density cushion at a high centrifugation force ($2.9 \times 10^5 \text{ g} \cdot \text{min}$). In (f), the disruptate was first incubated for 5 min at 37 °C in the presence of 0.5 mM GPN. The sediments were homogenized, and postnuclear supernatants fractionated by isopycnic sucrose density gradient centrifugation. A sample from each fraction was precipitated with TCA, and acid-soluble (○) and acid-insoluble (●) radioactivity measured separately; in addition, the fractions were assayed for β -acetylglucosaminidase (β -AGA, Δ). The values per fraction are expressed as percentages of the total cell-associated radioactivity or β -AGA, respectively.

disrupting esterase substrate [28]. The acid-insoluble radioactivity at 1.18–1.19 g/ml was, likewise, eliminated by GPN, whereas the acid-insoluble radioactivity at 1.14 g/ml was unaffected (Figure 5f). The peak at 1.18–1.19 g/ml would thus seem to represent lysosomes, containing degraded as well as some undegraded ^{125}I -TC-AOM. The peak at 1.11 g/ml would appear to be purely endosomal (light endosomes), whereas the region around 1.14–1.16 g/ml may represent a mixture of dense endosomes and light lysosomes.

Altered subcellular distribution of endocytosed ^{125}I -TC-AOM after okadaic acid treatment

Okadaic acid has previously been shown to cause cytoskeletal alterations resulting in a structural disintegration of electro-disrupted cells [10], thereby reducing the tendency of organelles such as lysosomes and autophagosomes to sediment as part of the general cell structure [9a]. Figure 6 indicates that this also applies to endosomes: whereas 80% of the endocytosed ^{125}I -TC-AOM from control hepatocytes sedimented with the cell corpses at a low centrifugation force ($1.1 \times 10^5 \text{ g} \cdot \text{min}$), only about 10% was sedimented at this centrifugation force after treatment with maximally effective concentrations of okadaic acid.

To assess the effect of okadaic acid on organelle distributions

Figure 6 Effect of okadaic acid on the cell structure-dependent sedimentability of endocytic vacuoles

Hepatocytes were preincubated for 30 min at 37 °C with okadaic acid at the concentration indicated. A tracer dose of ^{125}I -TC-AOM was then added, and the incubation continued for another 2.5 h. The cells were electrodisrupted, and a sample of the disruptate centrifuged through a metrizamide-sucrose density cushion at a low centrifugation force ($1.1 \times 10^5 \text{ g} \cdot \text{min}$). The radioactivity in the resulting pellet was measured and expressed as a percentage of the total cell-associated radioactivity measured in the disruptate. Each value is the mean $\pm$ S.E.M. of three experiments.

Figure 7 Effect of okadaic acid on the density distribution of endocytic vacuoles during ^{125}I -TC-AOM endocytosis

Hepatocytes were preincubated for 30 min at 37 °C without (a, c) or with (b, d) 30 nM okadaic acid, then given ^{125}I -TC-AOM and incubated further under the same conditions for 15 (a, b) or 120 min (c, d). The cells were then electrodisrupted, and the disruptate centrifuged through a metrizamide-sucrose density cushion at a high centrifugation force ($2.9 \times 10^5 \text{ g} \cdot \text{min}$). The sediments were homogenized, and postnuclear supernatants fractionated by isopycnic sucrose density gradient centrifugation. Acid-insoluble radioactivity (●), acid-soluble radioactivity (○) and acid phosphatase activity (Δ) were measured in each fraction and expressed as a percentage of the total cell-associated radioactivity or acid phosphatase activity, respectively.

Figure 8 Sedimentability of endosome fractions from untreated and okadaic acid-treated hepatocytes

Hepatocytes were preincubated for 30 min at 37 °C in the presence or absence of 30 nM okadaic acid, then given ^{125}I -TC-AOM and incubated for another 5 or 30 min under the same conditions. The cells were then electrodisrupted, the disruptates were homogenized, and postnuclear supernatants prepared directly. (a) Postnuclear supernatants were centrifuged through sucrose-metrizamide density cushions at the centrifugation force indicated (up to $2.9 \times 10^6 \text{ g} \cdot \text{min}$). The amount of radioactivity sedimented at various times was measured and calculated as percentage of the amount sedimented at $2.9 \times 10^6 \text{ g} \cdot \text{min}$. For clarity, the data are presented as a percentage unsedimented ^{125}I -TC-AOM at the centrifugation force used, relative to the maximum amount sedimentable. (○) Control cells at 5 min; (△) control cells at 30 min; (●) okadaic acid-treated cells at 5 min; (▲) okadaic acid-treated cells at 30 min. (b) Postnuclear supernatants were fractionated on sucrose density gradients as in Figure 6. Three pooled low-density-gradient fractions (1.09–1.11 g/ml; called '1.11') or high-density gradient fractions (1.13–1.16 g/ml, called '1.14') were diluted to the same density (1.05 g/ml) and re-centrifuged through sucrose-metrizamide density cushions at the centrifugation force indicated. (○) Control cells, '1.11', 5 min; (△) control cells, '1.11', 30 min; (□) control cells, '1.14', 30 min; (●) okadaic acid-treated cells, '1.11', 5 min; (▲) okadaic acid-treated cells, '1.11', 30 min. Each point in (a) represents the mean $\pm$ range of two experiments; the data in (b) are from a single experiment.

Figure 9 Effect of okadaic acid on recycling of endocytosed ^{125}I -TC-AOM

Hepatocytes preincubated for 30 min at 37 °C without or with 30 nM okadaic acid were incubated for 5 min or 40 min with ^{125}I -TC-AOM, rapidly cooled, washed once (0 °C) with 10 mM EGTA and reincubated at 37 °C in the presence or absence of 2.5 mM EGTA for the length of time indicated. The cells were separated from the medium by centrifugation, and the amount of ^{125}I -TC-AOM released into the medium was measured and expressed as a percentage of the total cell-associated radioactivity. (○) Control cells, 5 min ^{125}I -TC-AOM, + EGTA; (△) control cells, 40 min ^{125}I -TC-AOM, + EGTA; (●) okadaic acid-treated cells, 5 min ^{125}I -TC-AOM, + EGTA; (▲) okadaic acid-treated cells, 40 min ^{125}I -TC-AOM, + EGTA; (□) control cells, 5 min, - EGTA; (▽) control cells, 40 min, - EGTA. Each point represents the mean $\pm$ S.E.M. of three experiments.

independently of this alteration in general cell structure, the initial sedimentation was done at a centrifugation force sufficiently high ($2.9 \times 10^6 \text{ g} \cdot \text{min}$) to sediment all endosomes. The resulting pellets were homogenized, and postnuclear fractions analysed on sucrose density gradients (Figure 7). At 15 min, nearly all of the endocytosed ^{125}I -TC-AOM was acid-insoluble, most of it being found in light endosomes banding at 1.11 g/ml both in control (Figure 7a) and okadaic acid-treated cells (Figure 7b). However, the minor peak of dense endosomes at 1.14 g/ml seen in the control cells was largely missing in the okadaic acid-treated cells.

At 120 min, control cells contained acid-insoluble radioactivity at 1.14 and 1.18 g/ml, and acid-soluble radioactivity at 1.18 g/ml (Figure 7c). The latter coincided with the major peak of the lysosomal enzyme ACP, suggesting that this peak represented the lysosomes where a partial degradation of ^{125}I -TC-AOM had taken place. In okadaic acid-treated cells, most of the acid-insoluble radioactivity at 120 min was found at 1.11 g/ml (Figure 7d). The fractions at 1.14 and 1.18 g/ml were substantially reduced, along with the acid-soluble radioactivity at 1.18 g/ml, indicating that okadaic acid inhibited the transfer of ^{125}I -TC-AOM from light endosomes to dense endosomes and lysosomes.

Sedimentability of endocytic vacuoles following okadaic acid treatment

To characterize further the endosomes accumulating in the presence of okadaic acid, a sedimentation analysis was performed on the whole vacuole population (postnuclear fraction), and on material recovered from various density gradient regions. ^{125}I -TC-AOM-containing vacuoles from control cells sedimented faster after 30 min of endocytosis than after 5 min, suggesting that the dense (1.14 g/ml) endosomes in which ^{125}I -TC-AOM appeared at 30 min were more sedimentable, i.e. presumably larger, than the light (1.11 g/ml) endosomes (Figure 8a). Vacuoles from okadaic acid-treated cells, on the other hand, exhibited the same low sedimentation rate at both time points (Figure 8a), supporting the assumption that the light endosomes, shown to accumulate in the presence of the drug, were, on average, of smaller size than in control cells. Sedimentation analysis of gradient fractions (Figure 8b) confirmed the slow sedimentation of light endosomes (pooled 1.09–1.11 g/ml fractions) from either control cells (5 min) or okadaic acid-treated cells (at 5 and 30 min) as well as the rapid sedimentation of dense endosomes (pooled 1.13–1.16 g/ml fractions) from control cells at 30 min.

Ligand recycling after okadaic acid treatment

Endocytosed ASGP has been shown to recycle extensively from early endosomes [29]. During endosomal maturation the ligand is increasingly dissociated from its receptor, and gradually less ligand will, therefore, recycle [30]. The extent of ligand recycling at different time points of ^{125}I -TC-AOM endocytosis might, therefore, provide some information about progression along the endocytic pathway, and about the step affected by okadaic acid.

After removal of cell-surface-bound ^{125}I -TC-AOM by cold-washing with EGTA (which removed $95.7 \pm 1.2\%$ of the ^{125}I -TC-AOM bound at 4 °C, mean $\pm$ S.E.M. of 12 analyses), the hepatocytes were incubated at 37 °C in EGTA-containing medium (to detach ligand from its receptor upon return to the cell surface). In control hepatocytes, an appreciable fraction of the ^{125}I -TC-AOM taken up during 5 min of endocytosis was released from the cells in the presence of EGTA (Figure 9). In the absence of EGTA the release was negligible. If the uptake of ^{125}I -TC-AOM was allowed to proceed for 40 min, the fraction of

Figure 10 Ultrastructural appearance of endocytic vacuoles labelled with AOM-gold particles

Isolated hepatocytes, in which electron-lucent (a) and electron-dense (b) hepatocytic lysosomes had been prelabelled by an intravenous injection of 10-nm AOM-gold (large arrowheads) 24 h before cell isolation, were incubated with 5-nm AOM-gold (small arrowheads) for up to 2 h at 37 °C. In control cells, the endocytosed 5-nm AOM-gold labelled multivesicular endosomes (c) as well as lysosomes (e), co-localizing with 10-nm AOM-gold in the latter (asterisk). In okadaic acid-treated cells, 5-nm and 10-nm AOM-gold were found mostly in separate vacuoles (d), indicating that the endocytic flux to lysosomes had been interrupted. Bars, 0.1 μ m.

125 I-TC-AOM subsequently released was considerably reduced, indicating transfer to a compartment from which the ligand could not recycle as easily.

In cells which had been preincubated with okadaic acid at 37 °C, however, 125 I-TC-AOM release was equally extensive after short and long loading periods (Figure 9). This would suggest that okadaic acid caused the endocytosed 125 I-TC-AOM to be maintained in a recycling endocytic compartment, presumably by inhibiting its transfer to a non-recycling compartment.

Effect of okadaic acid on ultrastructural elements involved in ASGP endocytosis

To investigate further the nature of the endosomes accumulating under okadaic acid, an electron microscopic study of cells endocytosing AOM-conjugated gold particles was undertaken. An attempt was made to prelabel hepatocytic lysosomes with 10-nm AOM-gold by intravenous injection on the day before the experiment. 10-nm gold particles could be found both in electron-lucent (Figure 10a) and electron-dense (Figure 10b) vacuoles, probably representing active and passive lysosomes respectively [31,32]. The electron-lucent lysosomes were, on average, larger

(1124 ± 94 nm diam., mean $\pm$ S.E.M. of 14 vacuole profiles) than the dense lysosomes (442 ± 33 nm diam., mean $\pm$ S.E.M. of nine vacuole profiles). However, in addition to the 10-nm-gold-positive vacuole profiles, there was an equivalent number of 10-nm-gold-negative vacuole profiles with an obvious lysosomal morphology (large, electron-lucent vacuoles containing remnants of degraded material, sometimes of a recognizable autophagic origin) which were also classified as lysosomes. Previous studies have similarly indicated that only about one-half of the lysosomes become labelled after an overnight exposure of cells to gold particles [33].

Early endosomes were labelled by allowing isolated hepatocytes to endocytose 5-nm AOM-gold for 5 min, whereas later endocytic vacuoles were labelled by incubation with 5-nm AOM-gold for 120 min. After 5 min of endocytosis in control hepatocytes, about one-half of the 5-nm AOM-gold was seen to reside in peripheral endocytic vesicles and tubules, often interconnected. These vesicles/tubules were of relatively small size (72 ± 7 nm diam., mean $\pm$ S.E.M. of 13 vesicle profiles). The other half of the AOM-gold was in multivesicular endosomes, which had a larger size (284 ± 39 nm diam., mean $\pm$ S.E.M. of nine vacuole profiles). No endocytosed 5-nm AOM-gold was seen in lysosomes at this time. In okadaic acid-treated cells, the

Table 1 Effect of okadaic acid on distribution of endocytosed AOM–gold among endocytic vacuoles

Isolated hepatocytes were incubated with 5-nm AOM–gold for 2 h at 37 °C in the presence or absence of 30 nM okadaic acid, and sections prepared by conventional electron microscopy. Lysosomes were identified by their morphology or by their content of 10-nm AOM–gold, injected intravenously 24 h before cell isolation; other vacuoles were classified on the basis of morphology. For all vacuoles that contained 5-nm AOM–gold, the number of 5-nm gold particles per vacuole profile was counted and expressed as a percentage of the total number of 5-nm gold particles within the respective cell profile. Each value is the mean $\pm$ S.E.M. of the number of cell profiles given in parentheses. Data from two different experiments have been combined. Control cells contained 740 ± 49 5-nm gold particles/cell profile (mean $\pm$ S.E.M. of 21 cell profiles); okadaic acid-treated cells contained 128 ± 19 particles/cell profile (mean $\pm$ S.E.M. of 20 cell profiles).

	Relative content of endocytosed 5-nm AOM–gold particles per vacuole profile type (as % of all gold particles per cell profile)	
	Control	Okadaic acid
Endocytic vesicles/tubules	3.9 ± 0.5 (21)	16.6 ± 2.3 (20) ^a
Multivesicular endosomes	54.9 ± 3.7 (21)	72.8 ± 4.5 (20) ^a
Lysosomes	41.2 ± 5.0 (21)	10.6 ± 2.8 (20) ^a

^a $P < 0.001$ versus control according to the *t*-test.

picture at 5 min was similar, but the number of gold-labelled endosomes was too small to allow a reliable estimate of the distribution between endocytic vesicles/tubules and multivesicular endosomes.

After 2 h of endocytosis, about one-half of the 5-nm AOM–gold endocytosed by control hepatocytes was found in multivesicular endosomes (Figure 10c), as indicated by a morphometric analysis (Table 1); most of these would presumably have a density of 1.14 g/ml (cf. the gradient analysis). They were larger than the multivesicular endosomes labelled at 5 min (428 ± 31 nm diam., mean $\pm$ S.E.M. of 18 vacuole profiles; $P < 0.01$ versus 5-min value), in accordance with the sedimentation analysis. However, a large fraction (41 %) of the endocytosed AOM–gold was now also found in lysosomes, identified morphologically or by their content of 10-nm AOM–gold (Figure 10e). Autophagosomes were abundant in control cells, which were incubated under amino acid-free conditions, known to induce maximal autophagic activity [34].

In okadaic acid-treated hepatocytes, the average cellular content of 5-nm AOM–gold after 2 h of endocytosis was 80 % lower than in control cells (cf. legend to Table 1). Independent experiments showed that AOM and AOM–gold were taken up with comparable kinetics, and that okadaic acid inhibited the uptake of both ligands similarly (results not shown). The morphometric analysis revealed that okadaic acid caused a significant retention of 5-nm AOM–gold in endocytic vesicles/tubules as well as in multivesicular endosomes (Figure 10d), both presumably being of low density (1.11 g/ml; cf. the gradient analysis). The fraction of 5-nm AOM–gold that had reached the lysosomes was strongly reduced relative to control cells (Table 1). The difference in sedimentability between endosomes from control and okadaic acid-treated cells (Figure 8) was probably mainly due to the higher proportion of tubulovesicular elements in the latter, since the multivesicular endosomes from okadaic acid-treated cells were not small enough (353 ± 30 nm diam., mean $\pm$ S.E.M. of 12 vacuole profiles) to account for the difference. In addition to reducing the number of gold particles per lysosome profile, okadaic acid reduced the number of lysosomes staining positively for 5-nm AOM–gold, from 9.2 ± 0.8

($n = 21$) to 1.9 ± 0.5 ($n = 20$) lysosome profiles per cell profile ($P < 0.001$). Very few autophagosomes were present after 2 h of okadaic acid treatment, consistent with the strong inhibitory effect of okadaic acid on hepatocytic autophagy [9a,10].

DISCUSSION

Okadaic acid, an inhibitor of serine/threonine protein phosphatases of type 1 (PP1) and type 2A (PP2A) [7], has been shown to either stimulate [11] or inhibit fluid-phase endocytosis [12], but to our knowledge its effect on receptor-mediated endocytosis has not previously been investigated. In the present experiments, okadaic acid and other protein phosphatase inhibitors were found to inhibit the accumulation as well as the degradation of endocytosed ^{125}I -TC–AOM in isolated hepatocytes. Okadaic acid was more potent than calyculin A or microcystin-LR, with a half-maximal effect already at 20 nM, indicating an involvement of PP2A [7]. One likely target for the effect of okadaic acid would be the ASGP receptors on the cell surface, the number of which was reduced to one-half by okadaic acid. Since okadaic acid causes a general hyperphosphorylation of hepatocytic proteins [8], it is possible that it could promote the phosphorylation of ASGP receptors, thereby shifting the equilibrium between intracellular receptors and surface receptors, the latter being largely unphosphorylated at serine and threonine residues [1]. That part of the receptor phosphorylation/dephosphorylation which is associated with ordinary receptor recycling [1] is probably not affected by okadaic acid, since the drug did not inhibit the receptor-mediated [29] recycling of ^{125}I -TC–AOM. However, a subpopulation of the ASGP receptors is sequestered, in a phosphorylated state, in an intracellular storage compartment [35]. The equilibrium between this compartment and the cell surface could well be disturbed by okadaic acid, which might suppress receptor dephosphorylation and hence recruitment from the storage compartment to the cell surface.

In addition to the effect of okadaic acid on ^{125}I -TC–AOM uptake, the density gradient data suggested that it also inhibited a later step in the endocytic pathway. Since okadaic acid caused retention of ^{125}I -TC–AOM in a recycling compartment, this later point of inhibition would seem to lie between recycling and non-recycling endosomes. This coincides with the localization of an important biological control point subject to inhibition by amino acids [36] and stimulation by insulin [37]. The fact that only one-third of the endocytosed ^{125}I -TC–AOM could be released by EGTA, even in the presence of okadaic acid, might implicate its residence in a compartment where most of the ligand had dissociated from the ASGP receptor. Furthermore, it could be calculated (by assuming a ligand concentration of 100 nM and 120000 ASGP receptors per cell) that the continuous uptake of ^{125}I -TC–AOM seen in the presence of okadaic acid would require each receptor to be used approximately 40 times during 2 h, indicating extensive recycling through a compartment of uncoupling of receptor and ligand [30].

The density gradient analysis indicated that the inhibitory effect of okadaic acid at the later step in endocytosis entailed a retention of endocytosed ^{125}I -TC–AOM in light (1.11 g/ml) endosomes, which apparently corresponded to the recycling endocytic compartment. Ultrastructurally, these light endosomes would appear to include both small endocytic vesicles/tubules and larger multivesicular endosomes, with a preponderance of the latter among the vacuoles accumulating endocytosed AOM–gold in okadaic acid-treated cells. It is well known that endocytosed material, taken up in coated pits, appears rapidly in small, peripheral endocytic vesicles/tubules, and shortly

afterwards in multivesicular endosomes [38–40], both elements probably being parts of a continuous tubulovesicular network [41] that may represent the recycling compartment, or compartment of uncoupling of receptor and ligand (CURL) [30]. Multivesicular endosomes are frequently equipped with tubular extensions containing membrane-bound ASGP receptors that probably recycle back to the cell surface. Free ASGP is gradually concentrated in the receptor-free lumen of the multivesicular vacuoles, which eventually bud off from their tubular connections, detach and become a non-recycling compartment [30,42,43]. Apparently, okadaic acid inhibits the detachment of multivesicular endosomes from the continuous tubulovesicular network, and hence the formation of free, dense (1.14 g/ml) endosomes, or 'carrier vesicles' [43]. These are also multivesicular according to the morphological analysis, but apparently somewhat larger than the earlier, light multivesicular endosomes (430 versus 280 nm mean diam.).

In control cells, the endocytosed ^{125}I -TC-AOM was eventually transferred to lysosomes. Acid-soluble radioactivity, indicative of intralysosomal hydrolysis, could be detected, with no difference in kinetics, both in a major, dense lysosome peak at 1.18–1.19 g/ml, and in a minor, light lysosome peak at 1.14–1.16 g/ml, the latter usually being observed only as a shoulder to the major peak. In hepatocytes, light lysosomes have been shown to be actively engaged in autophagy as well as in endocytosis [31,32]. This lysosomal subpopulation may be equivalent to the mannose-6-phosphate receptor-rich 'prelysosomal compartment' observed in other cell types [43]. Although light lysosomes and dense endosomes overlapped in density, they could be distinguished by the use of the cathepsin C substrate GPN to destroy the lysosomes without affecting the endosomes [44]. In the present study, GPN removed essentially all of the acid-soluble radioactivity from the gradient along with most of the acid-insoluble radioactivity in the dense region around 1.18–1.19 g/ml, whereas acid-insoluble radioactivity at 1.14 g/ml was virtually unaffected. Both the GPN-sensitivity and the presence of acid-soluble ^{125}I -TC-AOM degradation products would thus seem to be relatively specific identifying criteria for hepatocytic lysosomes. In okadaic acid-treated cells, the transfer of ^{125}I -TC-AOM to lysosomes and the accumulation of acid-soluble degradation products was strongly inhibited, probably due to the retention of ^{125}I -TC-AOM in dense endosomes.

Okadaic acid and other protein phosphatase inhibitors are known to induce hyperphosphorylation of various cytoskeletal proteins [45,46], which in rat hepatocytes results in extensive disruption of the cytostructure [9a,10], probably mediated by CaMK-II [10]. Specifically, the cytoskeletal element disrupted by okadaic acid is the cytokeratin intermediate filament network [9b]. Okadaic acid has, furthermore, been shown to induce hyperphosphorylation and detachment of a protein suspected to be involved in the binding of endosomes to microtubules [47]. Thus, the okadaic acid-induced retention of ^{125}I -TC-AOM in light endosomes might be due to a loss of contact between the endosomal network and the cytoskeleton, and a consequent impairment of the vacuolar motility needed to detach the dense endosomes.

Several protein kinases have been shown to be present in endosomes [3,4], and to play a role in cell-free endosome fusion [13,48]. In the present experiments, the protein kinase inhibitor genistein had little effect on its own on ASGP degradation (in contrast to previous observations, cf. [2]), but antagonized the inhibitory effect of okadaic acid. This could indicate that a genistein-sensitive serine/threonine protein kinase [26] inhibits the detachment of dense endosomes from the tubulovesicular endocytic network. In such a complex pathway as endocytosis,

the participation of several different protein kinases with contrasting effects may be anticipated.

This work has been generously supported by the Norwegian Cancer Society and by the Research Council of Norway. We wish to thank Tor Gjen for providing the ^{125}I -TC-AOM. The skilful technical assistance of Mona Birkeland and Marianne Synnes is gratefully acknowledged.

REFERENCES

- Fallon, R. J. and Schwartz, A. L. (1988) *J. Biol. Chem.* **263**, 13159–13166
- Fallon, R. J., Danaher, M., Saylor, R. L. and Saxena, A. (1994) *J. Biol. Chem.* **269**, 11011–11017
- Kaplan, K. B., Swedlow, J. R., Varmus, H. E. and Morgan, D. O. (1992) *J. Cell Biol.* **118**, 321–333
- Rindress, D., Lei, X., Ahluwalia, J. P. S., Cameron, P. H., Fazel, A., Posner, B. I. and Bergeron, J. J. M. (1993) *J. Biol. Chem.* **268**, 5139–5147
- Faure, R., Baquiran, G., Bergeron, J. J. M. and Posner, B. I. (1992) *J. Biol. Chem.* **267**, 11215–11221
- Zen, K., Biwersi, J., Periasamy, N. and Verkman, A. S. (1992) *J. Cell Biol.* **119**, 99–110
- Cohen, P., Holmes, C. F. B. and Tsukitani, Y. (1990) *Trends Biochem. Sci.* **15**, 98–102
- Haystead, T. A. J., Sim, A. T. R., Carling, D., Honnor, R. C., Tsukitani, Y., Cohen, P. and Hardie, D. G. (1989) *Nature (London)* **337**, 78–81
- Holen, I., Gordon, P. B. and Seglen, P. O. (1993) *Eur. J. Biochem.* **215**, 113–122
- Blankson, H., Holen, I. and Seglen, P. O. (1995) *Exp. Cell Res.* **218**, 522–530
- Holen, I., Gordon, P. B. and Seglen, P. O. (1992) *Biochem. J.* **284**, 633–636
- Kreienbhl, P., Keller, H. and Niggli, V. (1992) *Blood* **80**, 2911–2919
- Lucocq, J., Warren, G. and Pryde, J. (1991) *J. Cell Sci.* **100**, 753–759
- Woodman, P. G., Mundy, D. I., Cohen, P. and Warren, G. (1992) *J. Cell Biol.* **116**, 331–338
- Berg, T., Kindberg, G. M., Ford, T. and Blomhoff, R. (1985) *Exp. Cell Res.* **161**, 285–296
- Seglen, P. O. (1976) *Methods Cell Biol.* **13**, 29–83
- Berg, T., Blomhoff, R., Naess, L., Tolleshaug, H. and Drevon, C. A. (1983) *Exp. Cell Res.* **148**, 319–330
- Gordon, P. B. and Seglen, P. O. (1982) *Exp. Cell Res.* **142**, 1–14
- Slot, J. W. and Geuze, H. J. (1985) *Eur. J. Cell Biol.* **38**, 87–93
- Horisberger, M. and Rosset, J. (1977) *J. Histochem. Cytochem.* **25**, 295–305
- Roth, J., Bendayan, M. and Orci, L. (1978) *J. Histochem. Cytochem.* **26**, 1074–1081
- Ames, B. N. (1966) *Methods Enzymol.* **8**, 115–118
- Barrett, A. L. (1972) in *Lysosomes: A Laboratory Handbook* (Dingle, J. T., ed.), pp. 46–135, North-Holland, Amsterdam
- Tolleshaug, H., Berg, T., Nilsson, M. and Norum, K. R. (1977) *Biochim. Biophys. Acta* **499**, 73–84
- Seglen, P. O., Gordon, P. B. and Poli, A. (1980) *Biochim. Biophys. Acta* **630**, 103–118
- Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. and Fukami, Y. (1987) *J. Biol. Chem.* **262**, 5592–5595
- Linossier, C., Pierre, M., Le Pecq, J.-B. and Pierre, J. (1990) *Biochem. Pharmacol.* **39**, 187–193
- Jadot, M., Colmant, C., Wattiaux-De Coninck, S. and Wattiaux, R. (1984) *Biochem. J.* **219**, 965–970
- Reeves, J. P., Decker, R. S., Crie, J. S. and Wildenthal, K. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4426–4429
- Townsend, R. R., Wall, D. A., Hubbard, A. L. and Lee, Y. C. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 466–470
- Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Lodish, H. F. and Schwartz, A. L. (1983) *Cell* **32**, 277–287
- Seglen, P. O. and Solheim, A. E. (1985) *Exp. Cell Res.* **157**, 550–555
- Kindberg, G. M., Refsnes, M., Christoffersen, T., Norum, K. R. and Berg, T. (1987) *J. Biol. Chem.* **262**, 7066–7071
- Lawrence, B. P. and Brown, W. J. (1992) *J. Cell Sci.* **102**, 515–526
- Kovcs, A. L., Grinde, B. and Seglen, P. O. (1981) *Exp. Cell Res.* **133**, 431–436
- Stoorvogel, W., Schwartz, A. L., Strous, G. J. and Fallon, R. J. (1991) *J. Biol. Chem.* **266**, 5438–5444
- Besterman, J. M., Airhart, J. A., Low, R. B. and Rannels, D. E. (1983) *J. Cell Biol.* **96**, 1586–1591
- Gibbs, E. M., Lienhard, G. E., Appleman, J. R., Lane, M. D. and Frost, S. C. (1986) *J. Biol. Chem.* **261**, 3944–3951
- Wall, D. A. and Hubbard, A. L. (1985) *J. Cell Biol.* **101**, 2104–2112

- 39 Limet, J. N., Quintart, J., Schneider, Y.-J. and Courtoy, P. J. (1985) *Eur. J. Biochem.* **146**, 539–548
- 40 Belcher, J. D., Hamilton, R. L., Brady, S. E., Hornick, C. A., Jaeckle, S., Schneider, W. J. and Havel, R. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6785–6789
- 41 Hopkins, C. R., Gibson, A., Shipman, M. and Miller, K. (1990) *Nature (London)* **346**, 335–339
- 42 Mueller, S. C. and Hubbard, A. L. (1986) *J. Cell Biol.* **102**, 932–942
- 43 Rabinowitz, S., Horstmann, H., Gordon, S. and Griffiths, G. (1992) *J. Cell Biol.* **116**, 95–112
- 44 Berg, T. O., Strømhaug, P. E., Løvdaal, T., Seglen, P. O. and Berg, T. (1994) *Biochem. J.* **300**, 229–235
- 45 Yatsunami, J., Fujiki, H., Suganuma, M., Yoshizawa, S., Eriksson, J. E., Olson, M. O. J. and Goldman, R. D. (1991) *Biochem. Biophys. Res. Commun.* **177**, 1165–1170
- 46 Ohta, T., Nishiwaki, R., Yatsunami, J., Komori, A., Suganuma, M. and Fujiki, H. (1992) *Carcinogenesis* **13**, 2443–2447
- 47 Rickard, J. E. and Kreis, T. E. (1991) *J. Biol. Chem.* **266**, 17597–17605
- 48 Tuomikoski, T., Felix, M.-A., Dorée, M. and Gruenberg, J. (1989) *Nature (London)* **342**, 942–945
-

Received 3 January 1995/19 May 1995; accepted 26 May 1995