

# Lysosomes Behave as $\text{Ca}^{2+}$ -regulated Exocytic Vesicles in Fibroblasts and Epithelial Cells

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**Abstract.** Lysosomes are considered to be a terminal degradative compartment of the endocytic pathway, into which transport is mostly unidirectional. However, specialized secretory vesicles regulated by  $\text{Ca}^{2+}$ , such as neutrophil azurophil granules, mast cell-specific granules, and cytotoxic lymphocyte lytic granules, share characteristics with lysosomes that may reflect a common biogenesis. In addition, the involvement of  $\text{Ca}^{2+}$  transients in the invasion mechanism of the parasite *Trypanosoma cruzi*, which occurs by fusion of lysosomes with the plasma membrane, suggested that lysosome exocytosis might be a generalized process present in most cell types.

Here we demonstrate that elevation in the intracellular free  $\text{Ca}^{2+}$  concentration of normal rat kidney (NRK) fibroblasts induces fusion of lysosomes with the plasma membrane. This was verified by measuring the release of the lysosomal enzyme  $\beta$ -hexosaminidase, the

appearance on the plasma membrane of the lysosomal glycoprotein Igp120, the release of fluid-phase tracers previously loaded into lysosomes, and the release of the lysosomally processed form of cathepsin D. Exposure to the  $\text{Ca}^{2+}$  ionophore ionomycin or addition of  $\text{Ca}^{2+}$ -containing buffers to streptolysin O-permeabilized cells induced exocytosis of  $\sim 10\%$  of the total lysosomes of NRK cells. The process was also detected in other cell types such as epithelial cells and myoblasts. Lysosomal exocytosis was found to require micromolar levels of  $\text{Ca}^{2+}$  and to be temperature and ATP dependent, similar to  $\text{Ca}^{2+}$ -regulated secretory mechanisms in specialized cells.

These findings highlight a novel role for lysosomes in cellular membrane traffic and suggest that fusion of lysosomes with the plasma membrane may be an ubiquitous form of  $\text{Ca}^{2+}$ -regulated exocytosis.

**L**YSOSOMES are acidic organelles delimited by a single membrane, containing a characteristic set of acid hydrolases (Novikoff, 1961). They receive newly synthesized lysosomal enzymes from the biosynthetic pathway and are responsible for the degradation of internalized material from the endocytic and autophagic pathways. Therefore, lysosomes are generally considered to be a terminal degradative compartment, the final destination of unidirectionally transported soluble macromolecules (DeDuve, 1963; Kornfeld and Mellman, 1989).

Extracellular release of lysosomal contents, however, has been described in several cell types, such as hepatocytes (LeSage et al., 1993), activated platelets (Silverstein and Febbraio, 1992), pancreatic acinar cells (Hirano et al., 1991), macrophages (Tapper and Sundler, 1990), and osteoclasts (Baron et al., 1990). In neutrophils, fusion of lysosomes with incompletely formed phagosomes also results in extracellular leakage of their contents (Pryzwansky et al., 1979). Based on the presence of hydrolytic enzymes and

lysosomal membrane glycoproteins, a common biogenesis has been proposed between lysosomes and specialized  $\text{Ca}^{2+}$ -regulated secretory vesicles, such as azurophil granules in neutrophils (Borregaard et al., 1993), specific granules in mast cells (Jamur et al., 1986), and lytic granules in cytotoxic T lymphocytes (Burkhardt et al., 1990; Peters et al., 1991).

Despite these similarities, conventional lysosomes of nonspecialized cells have not been generally described as organelles that fuse with the plasma membrane upon stimulation. Recent studies of cell infection with the protozoan parasite *Trypanosoma cruzi* have, however, provided evidence for the existence of a lysosomal exocytic pathway in fibroblasts and epithelial cells. During *T. cruzi* invasion, lysosomes are recruited to the parasite entry site and gradually fuse with the plasma membrane (Tardieux et al., 1992; Rodríguez et al., 1996). Intracellular free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ )<sup>1</sup> transients, triggered in host cells by a

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1. *Abbreviations used in this paper:*  $[\text{Ca}^{2+}]_i$ , intracellular free  $\text{Ca}^{2+}$  concentration; LDH, lactate dehydrogenase; Igp, lysosomal membrane glycoprotein; NRK, normal rat kidney; SLO, streptolysin O; TSF, trypanosome-soluble fraction.

trypanosome-soluble factor (Burleigh and Andrews, 1995), cause reversible disassembly of the cortical actin cytoskeleton of normal rat kidney (NRK) fibroblasts (Rodríguez et al., 1995), similar to what has been described in regulated exocytosis. The *T. cruzi*-triggered  $[Ca^{2+}]_i$  transients appear to be required for the lysosome-mediated invasion process, since buffering or depletion of host cell intracellular  $Ca^{2+}$  blocks parasite entry (Tardieux et al., 1994; Rodríguez et al., 1995).

Taken together, the observations described above suggested that increases in  $[Ca^{2+}]_i$  might be sufficient to trigger lysosomal exocytosis in most cell types, in a similar fashion to the well-characterized process of  $Ca^{2+}$ -regulated exocytosis in specialized secretory cells. In this work we provide evidence in favor of this hypothesis, demonstrating that conventional lysosomes can be induced to fuse with the plasma membrane in a  $Ca^{2+}$ , temperature- and ATP-dependent fashion.

## Materials and Methods

### Materials

BSA, bombesin, and gold chloride were obtained from Sigma Chemical Co. (St. Louis, MO); ionomycin and colchicine were from Calbiochem-Novabiochem Corp. (La Jolla, CA). Lucifer yellow and FITC-transferrin (human) were from Molecular Probes, Inc. (Eugene, OR). Reduced streptolysin O was from Murex Diagnostics (Dartford, UK), and hexokinase was from Boehringer Mannheim Biochemicals (Indianapolis, IN).  $^3H$ -dextran (mol wt 70,000) was obtained from Amersham Corp. (Arlington Heights, IL). Human diferric  $^{125}I$ -transferrin was obtained from DuPont (Wilmington, DE). Purified rabbit antibodies against cathepsin D were from Biosdesign Intl. (Kennebunk, ME). Trypanosome-soluble fraction (TSF) was prepared from the infective stages of *T. cruzi* as described in Rodríguez et al. (1995).

### Cell Culture

All cells were grown at 37°C with 5% CO<sub>2</sub>. Cultures of primary human fibroblasts (NIGMS; Coriell Institute for Medical Research, Camden, NJ), NRK, J774, IMR-90, L<sub>6</sub>E<sub>9</sub>, and LLC-MK<sub>2</sub> cell lines were grown in DME containing 10% FBS. CHO cells were grown in  $\alpha$ -MEM with 5% FBS. Confluent monolayers containing  $6 \times 10^4$  cells per cm<sup>2</sup> were used for all experiments.

### Ionomycin Treatment

Confluent monolayers of NRK cells in 60-mm culture dishes were washed with PBS and incubated with 0.5 ml of either PBS or 10  $\mu$ M ionomycin in PBS for the indicated times. The incubation buffer was collected and centrifuged at 11,000 *g* for 5 min before performing  $\beta$ -hexosaminidase,  $^3H$ -dextran, lucifer yellow, or cathepsin D detection assays. Total cell extracts were obtained by incubation of culture dishes with 0.5 ml of PBS 1% NP-40 (NP-40), followed by a 5-min centrifugation of the extract at 11,000 *g*.

### *N*-Acetyl- $\beta$ -D-Glucosaminidase ( $\beta$ -Hexosaminidase) Activity Assay

For each sample, 350  $\mu$ l of the incubation buffer was incubated for 15 min at 37°C with 50  $\mu$ l of 6 mM 4-methyl-umbelliferyl-*N*-acetyl- $\beta$ -D-glucosaminide in sodium citrate-phosphate buffer, pH 4.5. The reaction was stopped by addition of 100  $\mu$ l of 2 M Na<sub>2</sub>CO<sub>3</sub> and 1.1 M glycine, and the fluorescence was measured in an F-2000 spectrofluorometer (Hitachi Instruments, Inc., Danbury, CT) at excitation 365 nm/emission 450 nm. To determine the total cellular content of  $\beta$ -hexosaminidase, cell extracts were diluted 1:10 and 350  $\mu$ l was used for enzyme detection. In the TSF experiment, background levels of  $\beta$ -hexosaminidase in TSF (<0.2% of the total content of control NRK cells) were subtracted from each sample.

### Lactate Dehydrogenase Activity Assay

For each point, 100  $\mu$ l of the incubation buffer or 1:10 dilutions of NP-40 extracts prepared as described above were incubated with 900  $\mu$ l of reaction buffer (0.23 mM NADH, 1 mM sodium pyruvate, 0.1% Triton X-100, 0.2 M Tris-HCl, pH 7.3). Decrease in absorbance at 340 nm was measured in the spectrophotometer for either 2 min (permeabilized cells) or 5 min (nonpermeabilized cells).

### Lucifer Yellow and $^3H$ -Dextran Loading of Lysosomes and Detection

Cells were incubated for 4 h at 37°C in DME containing 10 mg/ml lucifer yellow or for 1 h with 100  $\mu$ Ci/ml  $^3H$ -dextran, washed extensively with PBS, and chased for another 2 h in DME 10% FCS before performing the ionomycin or permeabilization treatments. The incubation buffer was collected and centrifuged for 5 min at 11,000 *g*. Lucifer yellow was detected in each sample of supernatant by measuring the fluorescence at excitation 428 nm/emission 540 nm;  $^3H$ -dextran was measured in a scintillation counter.

### Staining for Surface Igp120

After the different treatments, NRK cells were incubated at 4°C for 30 min with culture supernatant from a mouse hybridoma line (Ly1C6) producing antibodies to rat Igp120 (kindly provided by I. Mellman, Yale University School of Medicine, New Haven, CT). Cells were then fixed with 2% paraformaldehyde for 15 min at 4°C, washed in PBS, and incubated with rhodamine-conjugated anti-mouse IgG antibodies (Boehringer Mannheim Biochemicals) for 30 min at room temperature.

### Flow Cytometry

Confluent NRK cells were trypsinized and washed twice with PBS before incubation with PBS or 10  $\mu$ M ionomycin in PBS for 5 min at 37°C. 10<sup>6</sup> cells for each assay were centrifuged and resuspended in Ly1C6 supernatant (mAb to rat Igp120) or FITC-transferrin (0.5 mg/ml) at 4°C for 30 min. Cells were washed in PBS and fixed in 2% paraformaldehyde for 15 min. Anti-Igp-treated cells were further incubated with FITC-conjugated anti-mouse antibodies (Boehringer Mannheim Biochemicals) for 30 min at room temperature. Finally, cells were washed, resuspended in 0.5 ml PBS, and analyzed on a FACS<sup>®</sup> Vantage Flow Cytometer (Becton Dickinson & Co., Mountain View, CA). Cells were illuminated at 488 nm and emission was detected at 525 nm. Forward angle scatter, right angle scatter, and fluorescence intensity were recorded from 10,000 cells whose forward angle scatter fell above a threshold used to distinguish intact from damaged cells.

### BSA-Gold Labeling of Lysosomes and EM

Lysosomes were labeled by the internalization of BSA adsorbed to 5 nm gold particles, prepared according to published procedures (Slot and Geuze, 1985). Cells were incubated with BSA-gold (OD 4.0 at 255 nm) for 3 h at 37°C, washed, and chased for 2 h in DME 10% FBS before addition of 10  $\mu$ M ionomycin for 2.5 and 10 min at 37°C. For resin embedding, cells were fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate (pH 7.4), post-fixed in 1% aqueous osmium tetroxide, en bloc stained in 1% uranyl acetate in 50 mM sodium maleate (pH 5.2), dehydrated in methanol, and embedded in Epon. Sections were contrasted with uranyl acetate and lead citrate. For immunocytochemistry, cells were fixed in 4% paraformaldehyde in 200 mM Hepes buffer, pH 7.2, scraped from the dish, pelleted, and embedded in 10% gelatin. Embedded pellets were infiltrated with 2.1 M sucrose in PBS and frozen by immersion in liquid nitrogen. Cryosectioning was performed using established methods, as described previously (Webster et al., 1994). Thawed sections were labeled with affinity-purified rabbit anti-Igp120-tail antibodies (Rodríguez et al., 1996) or mouse anti-rat lysosomal membrane glycoprotein (Igp120) mAbs (Ly1C6). The mAb was followed by application of a rabbit anti-mouse bridging antibody. Binding of antibodies was visualized using protein A-gold probes (purchased from Department of Cell Biology, University of Utrecht, The Netherlands).

### Streptolysin O Permeabilization

Confluent monolayers of NRK cells in 60-mm culture dishes were washed twice with 1 ml ice-cold buffer A (20 mM Hepes, 110 mM NaCl, 5.4 mM KCl, 0.9 mM Na<sub>2</sub>HPO<sub>4</sub>, 10 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, and 11 mM glucose,

pH 7.4) and incubated for 10 min at 4°C with streptolysin O (SLO) at 0.5 U/ml in buffer A. Cells were then washed once with 1 ml ice-cold buffer B (20 mM Hepes, 100 mM K-glutamate, 40 mM KCl, and 5 mM EGTA, pH 7.2) containing 2 mM MgATP and 5 mM free Mg<sup>2+</sup> (added as MgCl<sub>2</sub>). 0.5 ml of buffer B with MgATP and Mg<sup>2+</sup>, or buffer B with MgATP, Mg<sup>2+</sup>, and Ca<sup>2+</sup>/EGTA at the indicated concentrations, was added to the cells at 37°C for the indicated time periods. The concentrations of free Mg<sup>2+</sup> and Ca<sup>2+</sup> were maintained using a Ca<sup>2+</sup> or Mg<sup>2+</sup>/EGTA buffering system, calculated using the software developed by Foehr and Warchol (Ulm, Germany). Incubation buffer was collected for each sample and centrifuged for 5 min at 11,000 g before performing enzyme assays. Total extracts were obtained by incubation of culture dishes with 0.5 ml of PBS 1% NP-40, followed by a 5-min centrifugation of the extract at 11,000 g. In the ATP depletion experiment, cell permeabilization was performed in cells in suspension, which were previously trypsinized and washed before SLO permeabilization as described above. 10<sup>6</sup> cells were used for each assay. After permeabilization, cells were incubated with hexokinase (150 U/ml) and glucose 5 mM in buffer B with Mg<sup>2+</sup> for 15 min at 37°C, before performing the Ca<sup>2+</sup>-induced exocytosis assay.

### Detection of Cathepsin D

Confluent IMR-90 cells in 150-mm culture dishes were either treated with PBS or 10 μM ionomycin or permeabilized with SLO and incubated with a 0 or 1 μM Ca<sup>2+</sup> buffer as described above. The supernatants of these cells (3 ml) were collected after 5 min and concentrated with a Centricon-10 (Amicon Corp., Beverly, MA) to 50 μl (25 μg of total protein for PBS and ionomycin samples and 80 μg for permeabilized cell samples). The total extract was obtained by addition of 1 ml of lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.6, 1% NP-40) to the cells, and 15 μl (20 μg) was used for detection. 4× concentrated SDS-PAGE loading buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol) was added to the samples, which were heated to 95°C for 4 min before electrophoresis in a 10% SDS-polyacrylamide gel. Proteins were then transferred to Nytran filters by semidry electroblotting (Schleicher & Schuell, Keene, NH). Blots were probed with rabbit anti-cathepsin D antibodies (dilution 1:1,000 of a 12.5 mg/ml stock solution), followed by peroxidase-conjugated goat anti-rabbit immunoglobulin G and enhanced chemiluminescence detection (ECL; Amersham Intl., Buckinghamshire, UK).

### Transferrin Release Assay

<sup>125</sup>I-transferrin release from SLO-permeabilized cells was performed essentially as described by Galli et al. (1994). Confluent NRK cells plated at 2 × 10<sup>4</sup>/cm<sup>2</sup> 24 h before were trypsinized and washed in buffer K (20 mM Hepes, 128 mM NaCl, 3 mM KCl, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 2.7 mM CaCl<sub>2</sub>, and 11 mM glucose, pH 7.4, with NaOH) before incubation of 1.5 × 10<sup>5</sup> cells per μl in buffer K containing 20 μCi/ml human <sup>125</sup>I-transferrin for 1 h at 37°C. Cells were washed once with ice-cold buffer K and twice with buffer A, and permeabilization was performed as described above. Cells were resuspended in buffer B and divided into 100-μl aliquots containing 10<sup>6</sup> cells each for determining transferrin release. Supernatants were collected after incubation at 37°C for 20 min or the indicated times, and the amount of <sup>125</sup>I-transferrin was determined in a scintillation counter. In each experiment, samples in triplicate were pelleted at the beginning of the 37°C incubation (defined as time 0), the amount of <sup>125</sup>I-transferrin in the supernatant was determined, and this value was subtracted from each sample. Cell extracts to quantify the total amount of internalized <sup>125</sup>I-transferrin were obtained for each sample by incubation of pellets with 100 μl of PBS 1% NP-40.

## Results

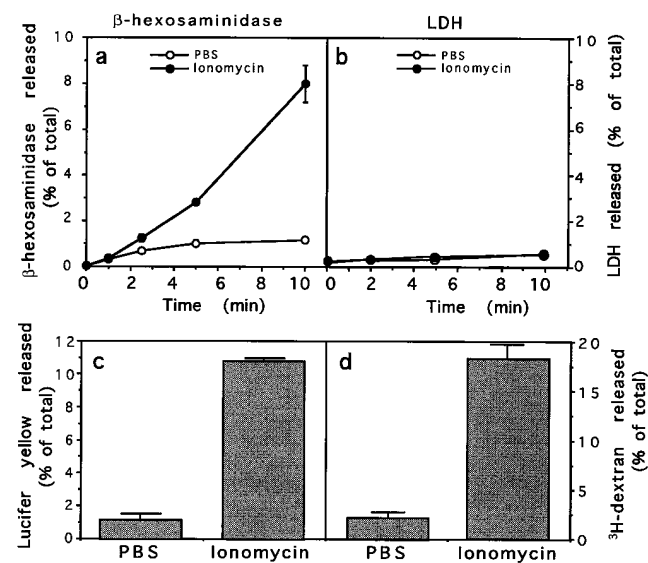
### Ionomycin Induces the Release of Lysosomal Contents into the Extracellular Medium

To test if an increase in [Ca<sup>2+</sup>]<sub>i</sub> would stimulate exocytosis of lysosomes, the calcium ionophore ionomycin was added to NRK fibroblasts in a Ca<sup>2+</sup>-containing buffer (PBS with 1 mM CaCl<sub>2</sub>). Cells were incubated at 37°C with 10 μM ionomycin, and release of the lysosomal enzyme β-hexosaminidase was measured in the incubation buffer. This enzyme is frequently used as a marker for lysosomes, since

90% of the total β-hexosaminidase in NRK cells is located in this compartment (Griffiths et al., 1990). A continuous release of β-hexosaminidase, typically reaching ~10% of the total enzyme content of the cells after 10 min, was specifically triggered by ionomycin (Fig. 1 a).

To verify if cell integrity was affected by the exposure to ionomycin, activity of the cytoplasmic enzyme lactate dehydrogenase (LDH) was measured in the incubation buffer of treated and untreated cells. Fig. 1 b shows that LDH levels in the incubation buffer were low in both conditions, indicating that the observed release of β-hexosaminidase induced by the ionophore was not due to cell lysis.

Lysosomes can be specifically labeled by incubation of cells with an endocytosis fluid-phase marker, followed by a chase of several hours (Adams et al., 1982). To confirm that the β-hexosaminidase release described above was indeed a consequence of lysosome exocytosis, similar experiments were performed after loading lysosomes with the soluble fluorescent tracer lucifer yellow or <sup>3</sup>H-dextran. Release into the supernatant of ~10% of the total amount of the tracers taken up by NRK cells was detected after 10-min exposure to 10 μM ionomycin (Fig. 1, c and d). This corresponds to an approximate increase of 6–10-fold over the levels observed during the same period in PBS-treated control cells.



**Figure 1.** Ionomycin induces exocytosis of β-hexosaminidase and lysosomal fluid-phase tracers but not LDH from intact NRK fibroblasts. (a and b) NRK cells were incubated at 37°C with either PBS (open circles) or 10 μM ionomycin in PBS (black circles). At the indicated time points, the incubation buffer was collected and assayed for β-hexosaminidase and LDH activity. The amount of enzyme released at each point is expressed as a percentage of the total content of enzyme in control cells. (c and d) Lysosomes of NRK cells were loaded with lucifer yellow or <sup>3</sup>H-dextran by fluid-phase endocytosis followed by a 2-h chase, before treatment with PBS or 10 μM ionomycin for 10 min. The incubation buffer was collected, lucifer yellow was detected by reading the fluorescence, and <sup>3</sup>H-dextran was detected by scintillation counting. The amount of lucifer yellow or <sup>3</sup>H-dextran released in each sample is expressed as a percentage of the total amount of tracer present in control cells. The data represent the average of triplicate determinations ± SD.

Another fluid-phase tracer, BSA-gold, was used for specific visualization of lysosomes by EM. NRK cell lysosomes were loaded with BSA-gold, and then treated with 10  $\mu\text{M}$  ionomycin for different periods of time. In PBS-treated control NRK cells, a population of vesicles containing BSA-gold and with the characteristic morphology and dimensions of lysosomes (Holtzman, 1989) was observed, mostly clustered in the perinuclear area (Fig. 2, *A* and *B*). In contrast, cells exposed to ionomycin showed a more dispersed distribution of gold-loaded vesicles, with accumulation in the proximity of the plasma membrane (Fig. 2, *C–F*). Images that strongly suggested exocytic events, with extracellular release of the gold complexes and other membranous lysosomal contents, were observed at both 2.5 and 10 min after exposure to ionomycin (Fig. 2, *D* and *F*). No similar images were detected in control cells (Fig. 2, *A* and *B*).

To confirm that the BSA-gold-loaded vesicles consisted of lysosomes, labeling with antibodies against the abundant lysosomal membrane glycoprotein lgp120 (Kornfeld and Mellman, 1989) was performed on cryosections. Specific lgp120 label was detected on the membrane of vesicles accumulated next to the plasma membrane in ionomycin-treated cells (Fig. 2, *G* and *H*). Anti-lgp120 labeling was also detected on the plasma membrane of ionomycin-treated cells (Fig. 2 *H*), an observation that was confirmed by immunofluorescence in live cells, as described below (Fig. 3, *a–d*).

#### ***Ionomycin Induces the Appearance of lgp120 on the Plasma Membrane***

Additional evidence for  $\text{Ca}^{2+}$ -dependent lysosomal fusion with the plasma membrane was provided by exposure of the luminal domain of lgp120 on the cell surface. After treating NRK cells with 10  $\mu\text{M}$  ionomycin, surface staining was performed in live cells at 4°C with an mAb recognizing the luminal domain of lgp120. A gradual increase in extracellularly exposed lgp120 was detected over time in ionomycin-treated cells, but not in control PBS-treated cells (Fig. 3, *a–c*). As a control, surface staining of ionomycin-treated cells with an mAb for the cytosolic protein kinesin was performed, showing no detectable fluorescence under the same conditions (not shown).

To quantify the percentage of the ionomycin-treated cell population that presented lgp120 on the plasma membrane, a similar experiment was performed with cells in suspension. NRK fibroblasts were trypsinized, washed, and incubated with 10  $\mu\text{M}$  ionomycin for 5 min before incubation at 4°C with the anti-lgp120 mAb, followed by fixation. Cells were analyzed for surface staining by flow cytometry. An increase in membrane staining for anti-lgp120 was observed in 95% of ionomycin-treated cells (Fig. 3 *d*).

#### ***Agonists That Mobilize $\text{Ca}^{2+}$ from Intracellular Stores Induce a Lower Level of Lysosomal Exocytosis***

The results described above indicated that ionomycin-mediated influx of  $\text{Ca}^{2+}$  from the extracellular medium induced fusion of lysosomes with the plasma membrane. To verify if  $\text{Ca}^{2+}$  mobilized from intracellular stores by receptor-mediated agonists had the same effect, release of  $\beta$ -hexosaminidase was measured in the supernatant of cells treated with several concentrations of the signaling peptide bombesin. As shown in Fig. 4 *a*, bombesin also induced lysosomal exocytosis in a dose-dependent way, but to a much lesser extent than ionomycin (a 1.5-fold maximum increase). When tested for induction of translocation to the plasma membrane of the lysosomal protein lgp120, bombesin was found to induce levels detectable by immunofluorescence (Fig. 4, *c* and *d*).

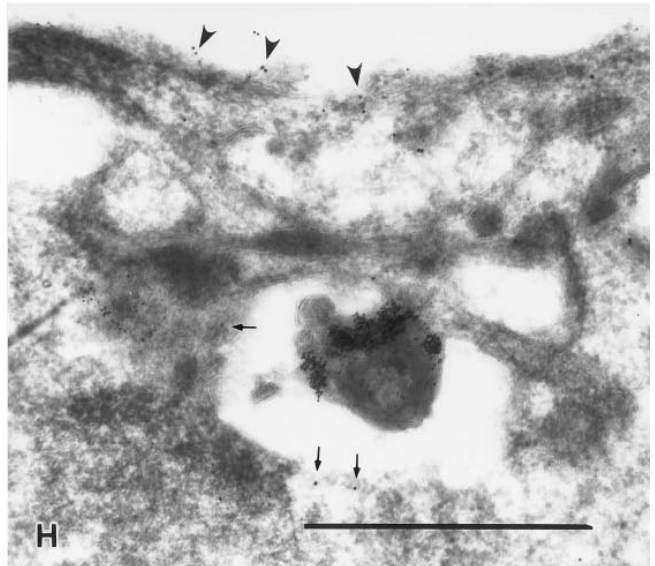
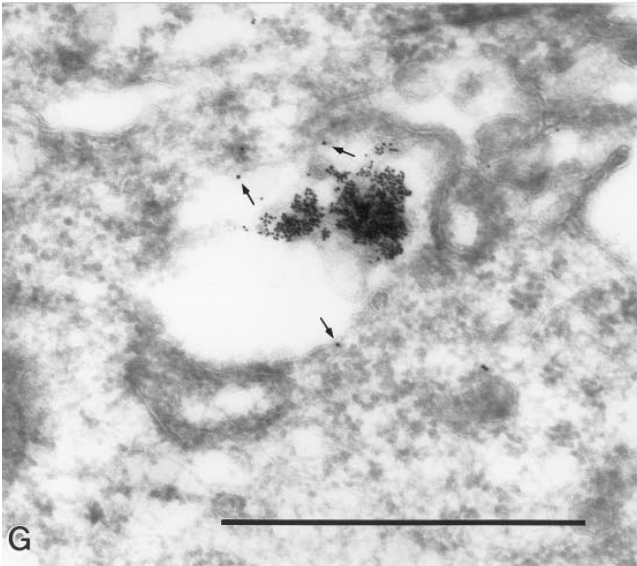
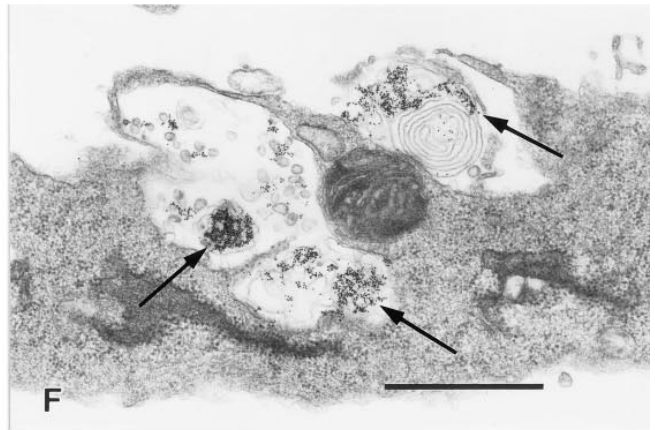
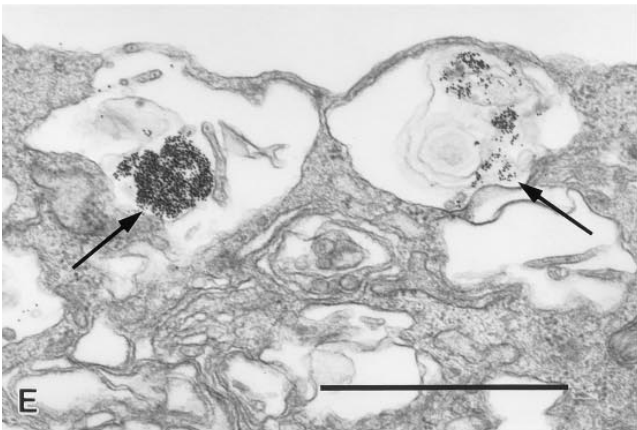
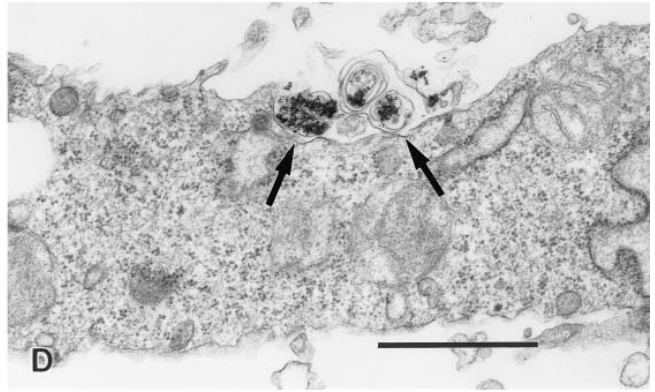
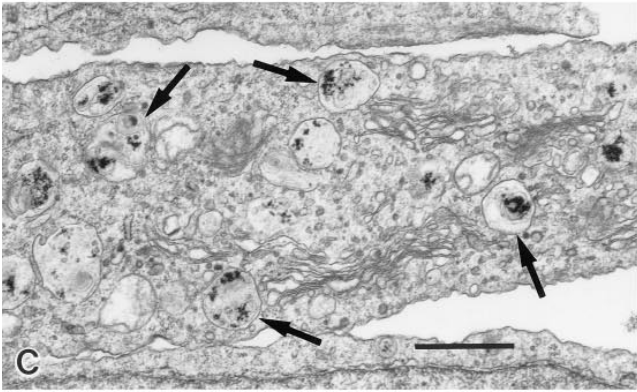
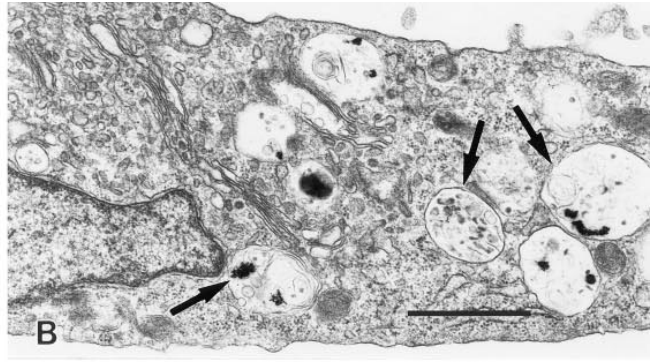
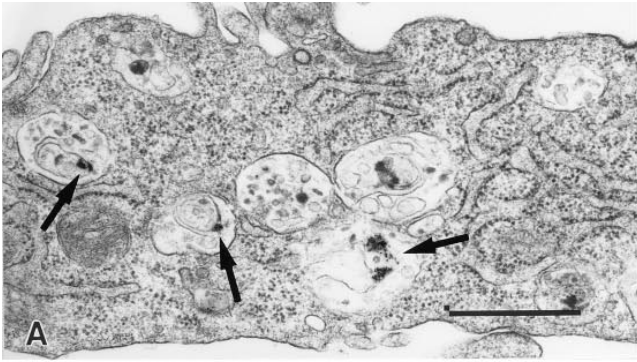
The protozoan parasite *Trypanosoma cruzi* induces fusion of lysosomes with the plasma membrane at its invasion site (Tardieux et al., 1992; Rodríguez et al., 1996). It also triggers  $\text{IP}_3$  formation and release of  $\text{Ca}^{2+}$  from intracellular stores in host cells, through a signaling factor present in soluble extracts of the parasites (Burleigh and Andrews, 1995; Burleigh et al., 1997). To verify if the trypanosome-soluble factor induced lysosome exocytosis, release of  $\beta$ -hexosaminidase was measured in the supernatant of cells treated with native or heat-inactivated TSF. As shown in Fig. 4 *b*, a low level of stimulated  $\beta$ -hexosaminidase exocytosis, of  $\sim 1.2$ -fold over the background levels, was induced in NRK cells by native TSF in a 5-min assay. Heat inactivation of TSF (95°C, 5 min) resulted in the loss of  $\text{Ca}^{2+}$ -agonist activity (not shown) and of the ability to induce lysosomal exocytosis (Fig. 4 *b*).

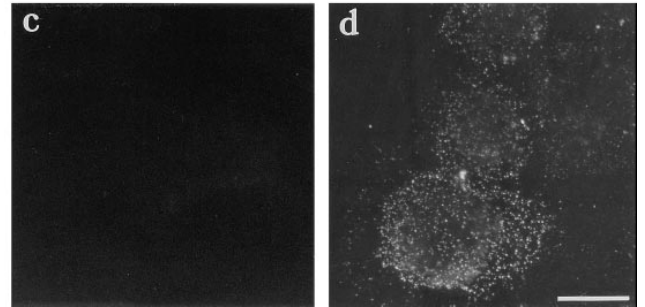
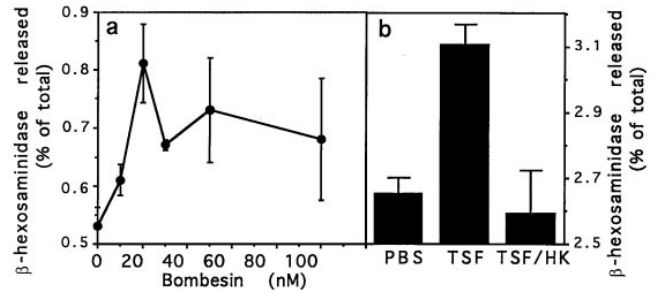
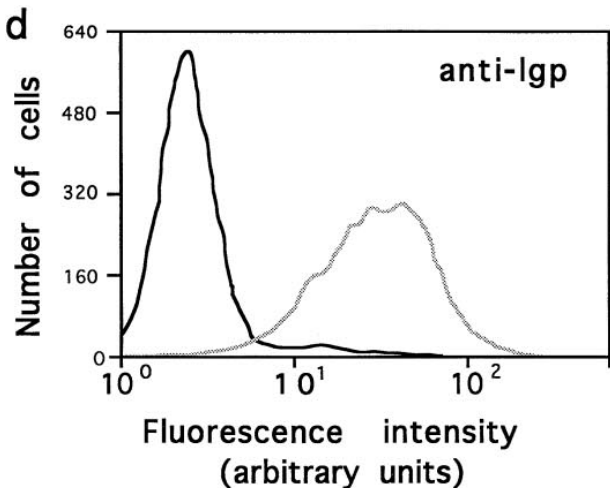
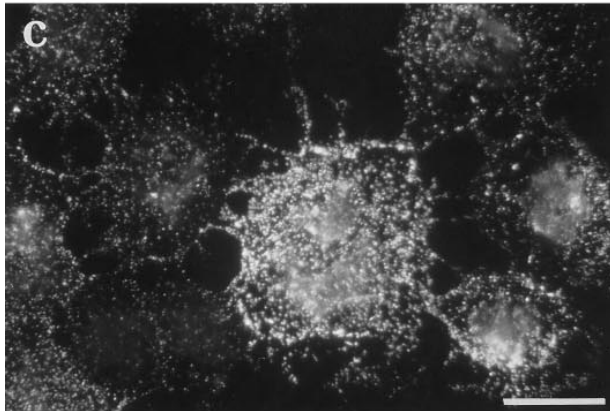
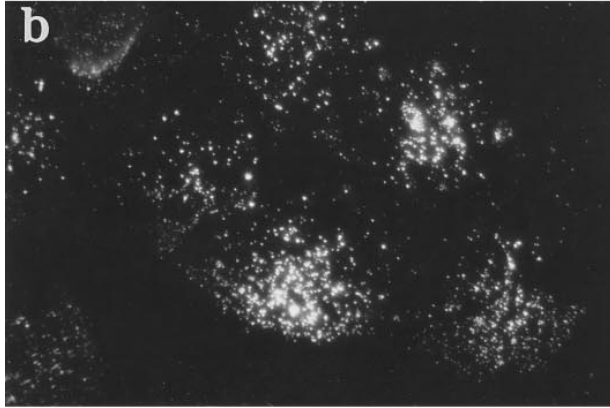
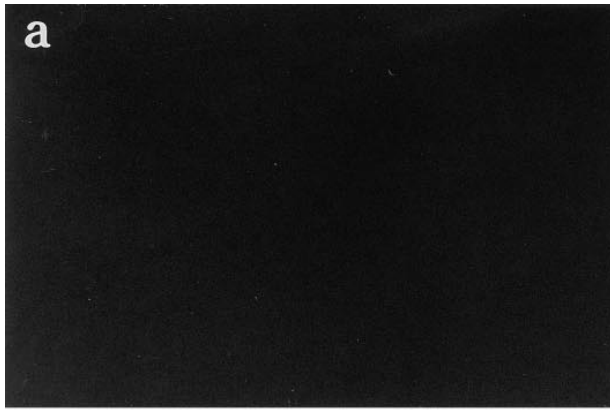
#### ***$\text{Ca}^{2+}$ Concentrations between 1 and 5 $\mu\text{M}$ Are Sufficient for Optimal Exocytosis of Lysosomes in Permeabilized Cells***

To further characterize the process of  $\text{Ca}^{2+}$ -dependent lysosome exocytosis, we next performed experiments in fibroblasts permeabilized with SLO (Miller and Moore, 1991), a system that allows precise control of the intracellular  $\text{Ca}^{2+}$  concentration.

Initially, to determine the efficiency of the permeabilization procedure under our experimental conditions, SLO-treated NRK cells were stained with propidium iodide, a fluorescent nuclear stain that does not penetrate intact cells. Virtually all cells were found to be permeabilized, when observed under a fluorescence microscope (not shown). The release of  $\beta$ -hexosaminidase was then measured in permeabilized and nonpermeabilized cells, in the absence or presence of 1 and 5  $\mu\text{M}$   $\text{Ca}^{2+}$ . As shown in Fig. 5 *a*, in a 5-min assay, an approximate fivefold increase in the re-

**Figure 2.** Exocytosis of BSA-gold-loaded lysosomes is triggered by  $[\text{Ca}^{2+}]_i$  elevation. NRK cells loaded with 5 nm BSA-gold complexes for 4 h followed by a chase of 2 h were incubated with PBS (*A* and *B*) or 10  $\mu\text{M}$  ionomycin (*C–H*) for 2.5 (*C* and *D*) or 10 min (*E–H*). Transmission EM sections show gold-loaded vesicles (arrows), observed in close proximity to the plasma membrane in ionomycin-treated cells (*C*, *E*, and *F*). Exocytosed BSA-gold was also detected after exposure to ionomycin (*D* and *F*). Labeling with antibodies against lgp120 (10 nm gold) was detected on BSA-gold-containing vesicles (*G* and *H*). Small arrows indicate anti-lgp120 labeling on lysosomes; arrowheads indicate anti-lgp120 labeling on the plasma membrane after ionomycin treatment. Bars, 1  $\mu\text{M}$ .





**Figure 4.**  $\text{Ca}^{2+}$  agonists induce a low level of  $\beta$ -hexosaminidase release and appearance of lgp120 in the plasma membrane. (a) NRK cells were incubated with different concentrations of bombesin for 5 min, and the incubation buffer was assayed for  $\beta$ -hexosaminidase activity. The amount of enzyme released at each point is expressed as a percentage of the total content of enzyme in control cells. The data represent the average of triplicate determinations  $\pm$  SD. (b) Same as a, except that cells were incubated for 5 min with PBS, TSF, or heat-inactivated TSF. (c) lgp120 surface staining 5 min after exposure to PBS; (d) lgp120 surface staining of NRK cells 5 min after exposure to 10  $\mu\text{M}$  bombesin. Bar, 5  $\mu\text{M}$ .

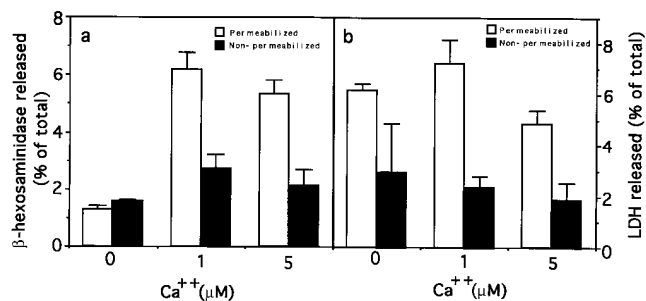
lease of  $\beta$ -hexosaminidase from permeabilized cells was triggered by addition of 1 or 5  $\mu\text{M}$   $\text{Ca}^{2+}$ .

The optimal concentration of SLO for the permeabilization of NRK cells was determined for each batch of toxin used and was found to vary between 0.2 and 0.5 U/ml, as determined by titrations using the  $\text{Ca}^{2+}$ -dependent  $\beta$ -hexosaminidase release assay described above. Concentrations of SLO lower or higher than the optimal one resulted in decreased  $\text{Ca}^{2+}$ -induced release of  $\beta$ -hexosaminidase (not shown).

Release of the cytosolic enzyme LDH from SLO-permeabilized cells was measured as a further control for permeabilization. Fig. 5 b shows that a significantly higher level of the enzyme was released when the cells were per-

**Figure 3.** Ionomycin induces appearance of lgp120 on the plasma membrane. NRK cells, either attached to coverslips (a–c) or in suspension (d), were incubated with PBS or 10  $\mu\text{M}$  ionomycin in PBS at 37°C, followed by immunofluorescent surface labeling with an mAb to a luminal domain of lgp 120. (a) lgp120 surface staining 10 min after exposure to PBS; (b) lgp120 surface staining 3 min after exposure to ionomycin; (c) lgp120 surface staining 10 min after exposure to ionomycin. (d) FACS<sup>®</sup> analysis of NRK cells in suspension treated for 5 min with PBS (black line) or 10  $\mu\text{M}$  ionomycin in PBS (gray line). Cells were incubated with anti-lgp120 mAbs for 30 min at 4°C before fixation. Bar, 5  $\mu\text{m}$ .





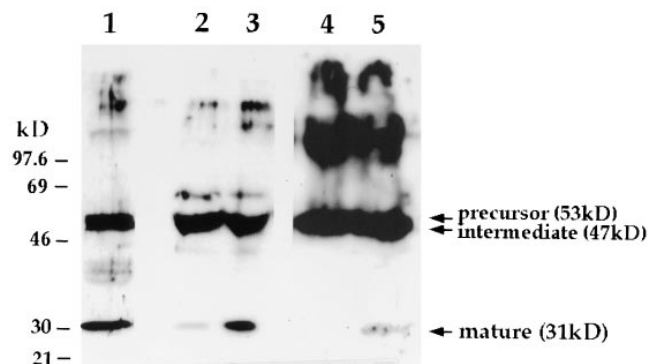
**Figure 5.** Release of  $\beta$ -hexosaminidase and LDH from intact and SLO-permeabilized NRK cells. Intact cells (*black columns*) and SLO-permeabilized cells (*white columns*), were incubated in permeabilization buffer containing either 0, 1, or 5  $\mu\text{M}$   $\text{Ca}^{2+}$  for 5 min at 37°C. Supernatants were assayed for (a)  $\beta$ -hexosaminidase and (b) LDH activity. The amount of enzyme released in each sample is expressed as a percentage of the total enzyme content of control cells. The data represent the average of triplicate determinations  $\pm$ SD.

meabilized (Fig. 5 b). The total amount of LDH released (5–7% of the total cellular content) reflects the relative loss of cytosolic components occurring during the 5-min period of the assay. Such minor cytosolic loss has been shown not to interfere significantly with secretory functions in previously characterized systems (Pimplikar et al., 1994).

To determine the optimal  $\text{Ca}^{2+}$  concentration inducing exocytosis of lysosomes in the permeabilized cell system, we performed the  $\beta$ -hexosaminidase, lucifer yellow, and <sup>3</sup>H-dextran release assays in buffers with  $\text{Ca}^{2+}$  concentrations ranging from 0–5  $\mu\text{M}$ . Optimal release of lysosomal markers, reaching 7–10-fold above the levels observed in the absence of  $\text{Ca}^{2+}$ , was found to require 1–5  $\mu\text{M}$   $\text{Ca}^{2+}$ , although exocytosis was already detectable at 0.1  $\mu\text{M}$   $\text{Ca}^{2+}$  (Fig. 6, a–c).

### Elevated $[\text{Ca}^{2+}]_i$ Induces Secretion of the Lysosomally Processed Form of Cathepsin D

To confirm the lysosomal origin of the  $\text{Ca}^{2+}$ -triggered exocytic products, we examined the lysosomal protease cathepsin D released into the supernatant of cells with elevated  $[\text{Ca}^{2+}]_i$ . Cathepsin D is processed into a mature polypeptide of 31 kD only after being transported to lysosomes (Gieselmann et al., 1983). Elevation of  $[\text{Ca}^{2+}]_i$  in the hu-

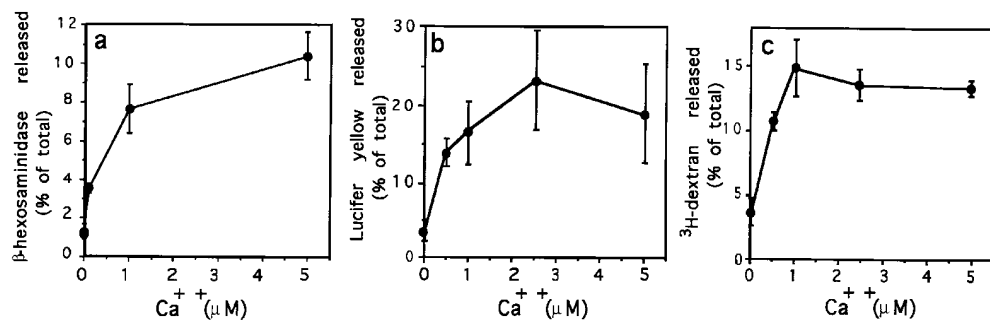


**Figure 7.** Elevation in  $[\text{Ca}^{2+}]_i$  induces secretion of the 31-kD lysosomally processed form of cathepsin D. Rabbit anti-cathepsin D antibodies were used to probe a Western blot containing a lysate and concentrated supernatants of IMR-90 human fibroblasts. (Lane 1) total lysate; (lane 2) concentrated supernatant of cells treated with PBS for 5 min; (lane 3) concentrated supernatant of cells treated with 10  $\mu\text{M}$  ionomycin for 5 min; (lanes 4 and 5) concentrated supernatants of SLO-permeabilized cells incubated for 5 min in buffers containing 0 or 1  $\mu\text{M}$   $\text{Ca}^{2+}$ , respectively. Different ECL exposures were performed for each treatment to allow visualization of the secreted mature 31-kD cathepsin D band.

man fibroblast cell line IMR-90, by treatment with ionomycin or by addition of 1  $\mu\text{M}$   $\text{Ca}^{2+}$  buffer to SLO-permeabilized cells, induced the appearance in the supernatant of the 31-kD mature form of cathepsin D (Fig. 7, lanes 3 and 5). A band of identical migration was also detected in whole cell extracts (Fig. 7, lane 1). The intermediate (47 kD) and precursor (53 kD) forms of the protein (Gieselmann et al., 1983) were detected in the supernatant at similar levels in each condition, probably reflecting the constitutive extracellular release of unprocessed cathepsin D that can occur before reuptake and targeting to lysosomes (von Figura and Hasilik, 1986; Kornfeld and Mellman, 1989). The higher molecular mass bands also appearing in Fig. 7 are due to nonspecific cross-reaction of the antibodies.

### Elevated $[\text{Ca}^{2+}]_i$ Has a Minor Stimulatory Effect on the Recycling of Transferrin Receptor-containing Endosomes

To verify if the elevation in  $[\text{Ca}^{2+}]_i$  triggering secretion of



**Figure 6.**  $\text{Ca}^{2+}$ -dependent release of  $\beta$ -hexosaminidase, lucifer yellow, and <sup>3</sup>H-dextran from permeabilized NRK cells. SLO-permeabilized cells were incubated for 5 min at 37°C in permeabilization buffer containing different  $\text{Ca}^{2+}$  concentrations. (a)  $\beta$ -hexosaminidase activity released. (b) Lucifer yellow released from previously loaded cells. (c) <sup>3</sup>H-Dextran released from previously loaded cells. Values are expressed as a percentage of the total content of either  $\beta$ -hexosaminidase, lucifer yellow, or <sup>3</sup>H-dextran in control cells. The data represent the average of triplicate determinations  $\pm$ SD.

lysosomes also affected the exocytosis of other elements of the endocytic pathway, we measured the release of previously internalized  $^{125}\text{I}$ -labeled transferrin using the same SLO-permeabilized cell system. As described previously (Galli et al., 1994), permeabilized cells released  $^{125}\text{I}$ -transferrin linearly for  $\sim 20$  min after permeabilization (Fig. 8 a). Confirming previous reports that describe a small stimulation in the exocytosis of transferrin receptor-containing vesicles upon treatment with ionophores or  $\text{Ca}^{2+}$ -mobilizing agonists (Buys et al., 1984; Wiley and Kaplan, 1984), there was a minor increase (1.5-fold) in the extracellular release of transferrin at increasing  $\text{Ca}^{2+}$  concentrations (Fig. 8 b). The release of apotransferrin into the medium after recycling to the cell surface allows the use of externally added, labeled transferrin to estimate the number of receptors on the surface at a given time. Using this method, we found that the number of receptors available for binding of FITC-transferrin on the surface of NRK cells was also slightly increased after exposure to  $10 \mu\text{M}$  ionomycin (Fig. 8 c).

### Characterization of Lysosome Exocytosis in Permeabilized Cells

The presence of  $\text{Ca}^{2+}$ -dependent lysosomal exocytosis was tested in different cell types. Cells were permeabilized and assayed for  $\beta$ -hexosaminidase release for 5 min at  $37^\circ\text{C}$ . To control for permeabilization efficiency in each cell type, LDH release was measured during the 5-min assay time. Levels of LDH release ranging from 10–20% confirmed that cells were efficiently permeabilized (not shown). In LLC-MK<sub>2</sub> and L<sub>6</sub>E<sub>9</sub> cells, a preincubation of 3 min at  $37^\circ\text{C}$  in the absence of  $\text{Ca}^{2+}$  was necessary to allow pore formation before addition of the  $1 \mu\text{M}$   $\text{Ca}^{2+}$  buffer. As shown in Table I,  $\text{Ca}^{2+}$ -dependent lysosomal exocytosis was detected in various cell types, including a primary culture of human fibroblasts as well as epithelial and myoblast cell lines. Enhancement in  $\beta$ -hexosaminidase release over background levels varied from 2–12-fold among the various cell types. The lowest  $\text{Ca}^{2+}$ -induced lysosome exocytosis levels in this group were observed with the macrophage cell line J774. Interestingly, the fusion of lysosomes with phagosomes in J774 and monocyte-derived macrophages was recently reported not to be regulated by  $\text{Ca}^{2+}$  (Zimmerli et al., 1996).

Table I.  $\text{Ca}^{2+}$ -dependent  $\beta$ -Hexosaminidase Secretion in Different Cell Types

	Species	Cell type	No $\text{Ca}^{2+}$	$1 \mu\text{M}$ $\text{Ca}^{2+}$
NRK	rat	fibroblast	$0.9 \pm 0.5^*$	$11.1 \pm 0.5$
Human fibroblasts	human	fibroblast	$0.4 \pm 0.09$	$4.4 \pm 0.6$
IMR-90	human	fibroblast	$1.1 \pm 0.2$	$4.7 \pm 1.6$
J774	mouse	monocyte	$2.4 \pm 0.1$	$4.8 \pm 0.1$
CHO	hamster	epithelial-fibroblastoid	$0.8 \pm 0.01$	$6.6 \pm 0.8$
LCC-MK <sub>2</sub>	monkey	epithelial	$0.9 \pm 0.4$	$5.0 \pm 1.5$
L <sub>6</sub> E <sub>9</sub>	rat	myoblast	$0.4 \pm 0.06$	$4.3 \pm 0.09$

\*The incubation buffer was assayed after a 5-min incubation, as described in Materials and Methods. Values shown correspond to the percentage of the total cellular content of  $\beta$ -hexosaminidase released  $\pm$  SD (average of triplicate determinations).

The kinetics of  $\text{Ca}^{2+}$ -induced lysosomal secretion in NRK cells, as measured by release of  $\beta$ -hexosaminidase, showed a rapid increase in the first 5 min that did not significantly change with longer incubation times (Fig. 9 a). Conversely, there was a continuous increase in the extracellular accumulation of LDH, due to cytoplasmic leakage from the permeabilized cells. As mentioned before, however, the degree of cytosol loss that occurred during the first 5 min after permeabilization is not sufficient to account for the sharp decrease in  $\beta$ -hexosaminidase secretion; as shown in Fig. 8 a, transferrin recycling, a process known to depend on cytosolic factors (Podbilewicz and Mellman., 1990; Galli et al., 1994), proceeds in a linear fashion for at least 20 min, under the same conditions.

To investigate the role of microtubules in the  $\text{Ca}^{2+}$ -regulated lysosome exocytosis, NRK cells were treated with  $10 \mu\text{M}$  colchicine for 1 h in the absence of serum. This is a condition that induces extensive microtubule depolymerization, as visualized by immunofluorescence with anti-tubulin antibodies (not shown). A small reduction of  $\sim 15\%$  in lysosomal exocytosis, reproducible in several experiments, was observed (Fig. 9 b).

We next investigated whether  $\beta$ -hexosaminidase release from SLO-permeabilized NRK cells exhibited the properties expected for an exocytic process. The release was found to be temperature dependent; when cells previously shifted for 5 min to  $37^\circ\text{C}$  to allow SLO pore formation were returned to ice,  $\text{Ca}^{2+}$ -dependent  $\beta$ -hexosaminidase

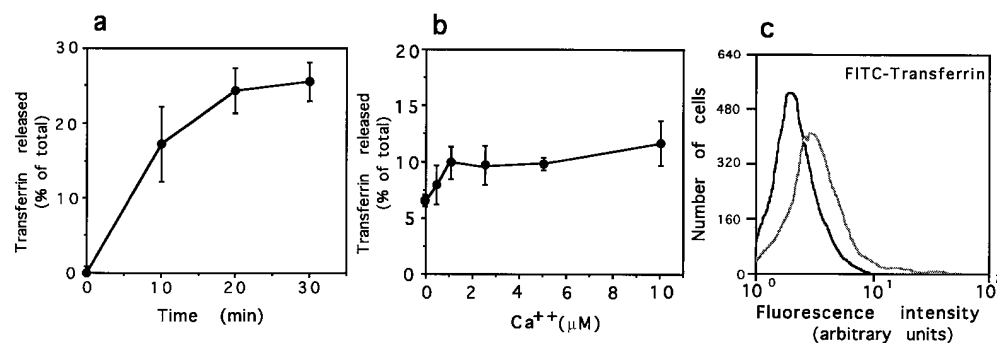
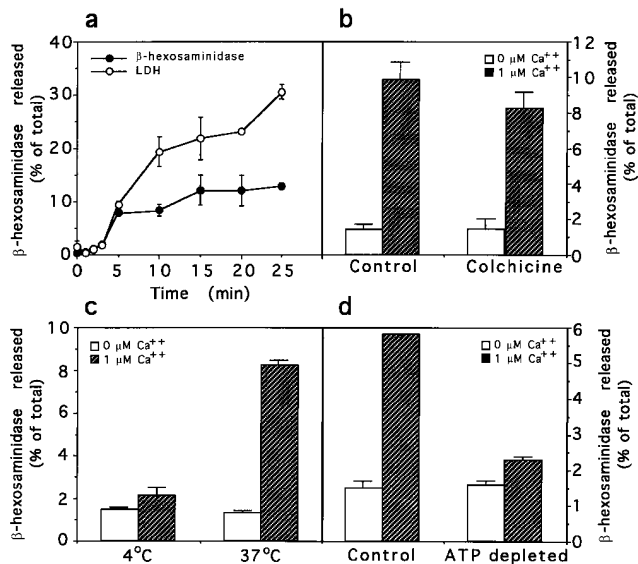


Figure 8. Effect of elevated  $[\text{Ca}^{2+}]_i$  on exocytosis of transferrin receptor-containing endosomes. (a) Kinetics of release of preinternalized  $^{125}\text{I}$ -transferrin from SLO-permeabilized NRK cells in the absence of  $\text{Ca}^{2+}$ . (b) Release of preinternalized  $^{125}\text{I}$ -transferrin from SLO-permeabilized NRK cells at different  $\text{Ca}^{2+}$  concentrations. Values are expressed as a percentage of the total  $^{125}\text{I}$ -

transferrin present in each sample (cell associated plus released). The data represent the average of triplicate determinations  $\pm$  SD. (c) FACS<sup>®</sup> analysis of NRK cells in suspension treated for 5 min with PBS (black line) or  $10 \text{ mM}$  ionomycin in PBS (gray line). Cells were incubated with FITC-transferrin for 30 min at  $4^\circ\text{C}$  before fixation.





**Figure 9.** Characterization of  $\text{Ca}^{2+}$ -induced lysosome exocytosis in permeabilized cells. Release of  $\beta$ -hexosaminidase from SLO-permeabilized NRK cells was measured after addition of 0 or 1  $\mu\text{M}$   $\text{Ca}^{2+}$  buffers. (a) Kinetics of release of  $\beta$ -hexosaminidase (black circles) and LDH (white circles) from NRK cells after addition of 1  $\mu\text{M}$   $\text{Ca}^{2+}$  at 37°C. (b) Sensitivity of  $\beta$ -hexosaminidase release to microtubule depolymerization. Cells were treated with 10  $\mu\text{M}$  colchicine for 1 h before addition of the  $\text{Ca}^{2+}$  buffers for 5 min at 37°C. (c) Temperature dependence of  $\beta$ -hexosaminidase release. Cells were incubated for 5 min at 37°C to allow SLO pore formation, and then the secretion assay was performed at 4° or 37°C for 5 min. (d) Sensitivity of  $\beta$ -hexosaminidase release to ATP depletion. After permeabilization, cells were incubated in the presence of 2 mM MgATP or an ATP-depleting system (5 mM glucose, 150 U/ml hexokinase) for 15 min at 37°C, before performing the 5-min secretion assay. In all experiments, the amount of enzyme released is expressed as a percentage of the total enzyme content of control cells. The data represent the average of triplicate determinations  $\pm$ SD.

release was almost totally blocked (Fig. 9 c).  $\beta$ -Hexosaminidase release was also found to be dependent on the presence of MgATP. Cells were first permeabilized and then exposed or not to an ATP-depleting system (hexokinase 150 U/ml, glucose 5 mM) for 15 min at 37°C. A significant inhibition of  $\text{Ca}^{2+}$ -triggered  $\beta$ -hexosaminidase secretion was observed in ATP-depleted cells (Fig. 9 d).

## Discussion

In this work we demonstrate that increase in  $[\text{Ca}^{2+}]_i$  triggers exocytosis of lysosomes, in NRK fibroblasts and other cell types not considered “professional”  $\text{Ca}^{2+}$ -regulated secretory cells. To confirm the lysosomal nature of the secretory vesicles in our experiments, we used four different approaches, taking advantage of molecular markers and functional characteristics of lysosomes.

First, we measured the release into the extracellular medium of the lysosomal enzyme  $\beta$ -hexosaminidase. About 90% of the total  $\beta$ -hexosaminidase of NRK cells is located in dense lysosomes (Griffiths et al., 1990). Therefore, the release of  $\sim$ 10% of the total cellular content of this en-

zyme into the medium upon increase in  $[\text{Ca}^{2+}]_i$  strongly suggested the occurrence of lysosomal exocytosis. This was observed in both ionomycin-treated and in SLO-permeabilized cells, in the presence of  $\text{Ca}^{2+}$ -containing buffers.

Second, we examined the extracellular release of tracers previously loaded into lysosomes by fluid-phase endocytosis. A chase of 2 h after the uptake period was used to ensure delivery of the endocytic tracer to lysosomes (Adams et al., 1982; Rodríguez et al., 1996). The release into the medium of lucifer yellow or  $^3\text{H}$ -dextran previously loaded into lysosomes was measured after elevating the  $[\text{Ca}^{2+}]_i$  with ionomycin or by exposing permeabilized cells to  $\text{Ca}^{2+}$ -containing buffers. The results were essentially identical to those obtained for  $\beta$ -hexosaminidase secretion, with maximum release values for fluid-phase tracers reaching between 10 and 20% of the total cellular content. Another fluid-phase tracer, BSA-gold, was used to visualize the distribution of lysosomes by EM after elevation in  $[\text{Ca}^{2+}]_i$ . In contrast to normal cells, after  $[\text{Ca}^{2+}]_i$  elevation, a large number of BSA-gold-containing vesicles, with the size and morphological characteristics of lysosomes, was observed closely associated with the plasma membrane. In several instances, lysosomal contents were detected in direct contact with the extracellular medium, strongly suggesting the occurrence of exocytosis.

Third, we monitored the appearance on the plasma membrane of a luminal epitope from the lysosomal integral membrane glycoprotein lgp120. This protein is highly enriched in lysosomes and is not detectable on the surface of NRK cells under normal conditions (Harter and Mellman, 1992). The appearance of lgp120 on the surface of the large majority of ionomycin-treated NRK cells, as demonstrated by flow cytometry, indicates that lysosomal exocytosis occurred homogeneously in the majority of cells of the population.

Fourth, we studied the extracellular release of the protease cathepsin D, frequently used as a marker for lysosomes. This enzyme is synthesized in the ER as a 53-kD precursor, which is subsequently cleaved into a 47-kD intermediate form, and then to an active 31-kD mature polypeptide. The last processing event that generates the 31-kD mature form occurs after transport to dense lysosomes (Gieselmann et al., 1983). We observed that after elevation of  $[\text{Ca}^{2+}]_i$  in both ionomycin-treated and SLO-permeabilized cells, a 31-kD band specifically recognized by anti-cathepsin D antibodies appeared in the extracellular medium.

The results obtained with these four different approaches, combining the use of ionomycin and of SLO-permeabilized cells, indicate that elevation in  $[\text{Ca}^{2+}]_i$  triggers exocytosis of lysosomes in fibroblasts. We cannot, however, rule out the participation of late endosomes, since in some cell types these organelles, in addition to containing hydrolases and lgp120 (Griffiths et al., 1988), appear to retain soluble tracers even after long chase periods (Rabinowitz et al., 1992).

Parallel experiments measuring the recycling of transferrin receptor-containing endosomes to the plasma membrane showed that lysosomes are the main vesicle population that undergoes exocytosis upon  $[\text{Ca}^{2+}]_i$  elevation. A small stimulation in transferrin release and in the number of transferrin receptors on the cell surface was observed

upon elevation of  $[Ca^{2+}]_i$ , confirming previous findings (Buys et al., 1984; Wiley and Kaplan, 1984). However, the increase in exocytosis triggered by  $Ca^{2+}$  in NRK cells was several fold higher for lysosomes than for early endosomes, under the same conditions. Furthermore, transferrin receptor-containing vesicles fused efficiently with the plasma membrane in the absence of  $Ca^{2+}$ , while lysosomes did not. Constitutive transport from the TGN to the plasma membrane is also known to occur at similar rates in the absence or presence of  $Ca^{2+}$  up to 1  $\mu M$  (Miller and Moore, 1991).

Increases in  $[Ca^{2+}]_i$  regulate many secretory events, such as neurotransmitter release (Smith and Augustine, 1988) and exocytosis of secretory granules (Holz et al., 1991). Intracellular constitutive fusion mechanisms, such as ER to Golgi transport (Beckers and Balch, 1989) or fusion of nuclear membrane vesicles (Sullivan et al., 1993), require basal levels of  $Ca^{2+}$  ( $\sim 100$  nM); regulated exocytosis fusion events in specialized secretory cells (Holz et al., 1991) and lysosome-phagosome fusion in neutrophils (Jaconi et al., 1990) require higher  $[Ca^{2+}]_i$ ,  $\sim 0.5$ – $5$   $\mu M$  in average. Significantly higher  $Ca^{2+}$  concentrations, reaching hundreds of micromolar in microseconds, are involved in triggering synaptic vesicle fusion at the neuron terminal (Heidelberger et al., 1994). Using the SLO-permeabilized system, which allows the equilibration of intracellular and extracellular  $Ca^{2+}$  concentrations (Ahnert-Hilger et al., 1989), we were able to define the optimal  $[Ca^{2+}]_i$  required to trigger lysosomal exocytosis in NRK cells as  $\sim 1$   $\mu M$ .

The similar  $[Ca^{2+}]_i$  levels necessary to trigger lysosome exocytosis in fibroblasts and regulated secretion in specialized cells add evidence in support of a common biogenesis between lysosomes and  $Ca^{2+}$ -regulated secretory granules (Borregaard et al., 1993; Jamur et al., 1986; Burkhardt et al., 1990; Peters et al., 1991). The temperature and MgATP dependence we observed for lysosomal exocytosis is also consistent with what is known for other regulated secretory systems. Kinetic studies of exocytosis in neuroendocrine cells revealed the existence of multiple  $Ca^{2+}$ -activated steps and have shown that secretion can be dissociated into MgATP-dependent and -independent stages, requiring distinct cytosolic factors (Bittner and Holz, 1992; Neher and Zucker, 1993; Hay and Martin, 1992; Chamberlain et al., 1995). Involvement of components of the NSF-SNAP-SNARE putative fusion machinery (Sollner et al., 1993) in several  $Ca^{2+}$ -dependent exocytic processes has been reported, but the precise events in which they participate are presently unclear (DeBello et al., 1995; Chamberlain et al., 1995; Bi et al., 1995).

The kinetics of  $Ca^{2+}$ -dependent lysosome exocytosis in SLO-permeabilized NRK cells showed no significant increase after the first 5 min. This does not appear to be due to depletion of essential cytosolic components, since recycling of transferrin receptor-containing vesicles, a process known to require cytosolic factors, proceeds in permeabilized cells at increasing rates for at least 20 min (Fig. 8 a, compare with Fig. 9 a). Furthermore, after a significantly longer period of permeabilization (15 min), the  $Ca^{2+}$ -induced  $\beta$ -hexosaminidase release measured in a 5-min assay was only reduced in  $\sim 40\%$  (Fig. 9 d, compare with 9 b). The fact that only 10–20% of the lysosome population (as defined by the total content of  $\beta$ -hexosaminidase or fluid-

phase tracers) appears to be capable of  $Ca^{2+}$ -triggered exocytosis might reflect heterogeneity in spatial distribution and/or functional characteristics. Similar results have been described for chromaffin cells, where the  $Ca^{2+}$ -triggered release of catecholamines is limited to 20–30% of the total cellular content (Dunn and Holz, 1983). Consistent with this interpretation, we observed only a very minor inhibition in lysosome secretion after microtubule depolymerization, suggesting that most of the lysosomes that undergo exocytosis are not transported for long distances, but are already located close to the plasma membrane before the  $Ca^{2+}$  stimulation. Microtubule-based movement was actually shown not to be required for exocytosis in several systems, being only critical for delivery of newly synthesized material to specific locations (Kelly, 1990).

A recent report, based on capacitance measurements, showed that elevation in  $[Ca^{2+}]_i$  triggers massive exocytosis in CHO cells and 3T3 fibroblasts, resulting in a surface area increase of 20–30% in 4 min (Coorsen et al., 1996). The  $Ca^{2+}$  concentration range described in that study to trigger exocytosis is the same we determined for lysosome exocytosis in NRK cells, from 0.1 to 4  $\mu M$ . Interestingly, the rate of exocytosis observed by those authors was found to increase strongly with  $[Ca^{2+}]_i$ , while the amount did not. This finding is consistent with our observation that only 10–20% of the lysosome population is capable of  $Ca^{2+}$ -triggered exocytosis, with no significant increase after 5 min. Our present study reinforces the suggestion that  $Ca^{2+}$ -regulated exocytosis is not restricted to specialized secretory cells, and it strongly suggests that lysosomes are the source of the large amount of exocytosed membrane detected in that previous study (Coorsen et al., 1996).

Another earlier observation consistent with our present results is the  $Ca^{2+}$ -dependent quantal release of acetylcholine loaded by endocytosis in CHO cells (Morimoto et al., 1995). Although the loading and chase periods used in that study were short ( $\sim 15$  min), it is conceivable that endocytosed acetylcholine reached lysosomes before exocytosis was triggered by  $Ca^{2+}$ . Recently,  $Ca^{2+}$ -regulated secretion of glycosaminoglycan chains was detected in CHO fibroblasts (Chavez et al., 1996). Although the  $Ca^{2+}$  concentration range for maximal secretion (1–5  $\mu M$ ) is the same as the one required for lysosomal exocytosis, the glycosaminoglycan-containing organelles described in that study were found to be less dense than lysosomes, and did not colocalize with IgG on immunofluorescence assays.

Our data indicate that optimal secretion of lysosomes in NRK cells occurs at  $Ca^{2+}$  concentrations of  $\sim 1$   $\mu M$ . Therefore, a more significant level of exocytosis was initially expected in our experiments with the agonists bombesin and TSF. These agents were previously shown to induce  $[Ca^{2+}]_i$  transients reaching low micromolar levels in NRK cells (Burleigh and Andrews, 1995). However, there is increasing evidence that  $IP_3$ -mediated mobilization of  $Ca^{2+}$  from intracellular stores can occur in a highly localized fashion, giving rise to a heterogeneous pattern of  $[Ca^{2+}]_i$  elevation in cells (Hahn et al., 1992; Paradiso et al., 1995). It is conceivable that in our agonist treatment experiments, only lysosomes in the immediate vicinity of areas with elevated  $Ca^{2+}$  were able to fuse with the plasma membrane. It is interesting to note, in this context, that the mobilization of lysosomes to the plasma membrane by the parasite *T. cruzi*

was recently shown to involve only lysosomes located at a distance of  $\sim 12 \mu\text{m}$  from the parasite invasion site (Rodríguez et al., 1996).

Regulated secretion of lysosomal components has been described previously mostly as part of specialized processes. In osteoclasts,  $\text{Ca}^{2+}$  regulation of lysosomal enzyme release was suggested (Davidson et al., 1994), and, in neutrophils, both secretion of the lysosome-related azurophil granules (Sengelov et al., 1993) and lysosome-phagosome fusion (Jaconi et al., 1990) are  $\text{Ca}^{2+}$ -dependent processes. Release of lysosomal contents from platelets and pancreatic acinar cells is triggered by specific agonists, suggesting a possible  $\text{Ca}^{2+}$ -regulated pathway (Febbraio and Silverstein, 1990; Hirano et al., 1991; Grondin and Beaudoin, 1996). Certain carcinoma cells present Igps in their surface (Saitoh et al., 1992; Garrigues et al., 1994) and secrete lysosomal enzymes (Sloane et al., 1986). A model of secretory lysosomes fusing directly with the plasma membrane was proposed for mediating this process in colon carcinoma cells (Saitoh et al., 1992). Therefore, it is conceivable that the  $\text{Ca}^{2+}$ -regulated lysosomal exocytosis we describe here corresponds to a ubiquitous mechanism that might be upregulated in certain pathological conditions.

A physiological function for a ubiquitous,  $\text{Ca}^{2+}$ -dependent exocytosis of lysosomes is unclear. One intriguing possibility, strongly suggested by our data, is that lysosomes are the intracellular organelles recently proposed to be responsible for membrane resealing in wounded cells (Bi et al., 1995; Miyake and McNeil, 1995). Membrane resealing was initially shown to require  $\text{Ca}^{2+}$ -regulated exocytosis, in 3T3 fibroblasts and sea urchin embryos (Steinhardt et al., 1994). More recently, the exocytic vesicles observed to fuse with the plasma membrane at sites of injury, in 3T3 fibroblasts and bovine endothelial cells, were proposed to belong to the endosomal/lysosomal compartment, based on their labeling with lipophilic fluorescent dyes (Miyake and McNeil, 1995). In light of our present results, it is conceivable that a localized  $[\text{Ca}^{2+}]_i$  elevation, caused by  $\text{Ca}^{2+}$  influx through damaged membranes, triggers the fusion of nearby lysosomes with the plasma membrane. Lysosome exocytosis may therefore be at least one of the mechanisms underlying the essential process of plasma membrane repair in all cells.

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