

The Role of Intraorganellar Ca^{2+} in Late Endosome–Lysosome Heterotypic Fusion and in the Reformation of Lysosomes from Hybrid Organelles

Paul R. Pryor,^{*‡} Barbara M. Mullock,^{*‡} Nicholas A. Bright,^{*‡} Sally R. Gray,^{*‡} and J. Paul Luzio^{*‡}

^{*}Department of Clinical Biochemistry and [‡]Wellcome Trust Centre for the Study of Molecular Mechanisms in Disease, Cambridge Institute for Medical Research, University of Cambridge, Addenbrooke's Hospital, Cambridge, CB2 2XY, United Kingdom

Abstract. We have investigated the requirement for Ca^{2+} in the fusion and content mixing of rat hepatocyte late endosomes and lysosomes in a cell-free system. Fusion to form hybrid organelles was inhibited by 1,2-bis(2-aminophenoxy) ethane- N,N,N',N' -tetraacetic acid (BAPTA), but not by EGTA, and this inhibition was reversed by adding additional Ca^{2+} . Fusion was also inhibited by methyl ester of EGTA (EGTA-AM), a membrane permeable, hydrolyzable ester of EGTA, and pretreatment of organelles with EGTA-AM showed that the chelation of luminal Ca^{2+} reduced the amount of fusion. The requirement for Ca^{2+} for fusion was a later event than the requirement for a rab protein since the system became resistant to inhibition by GDP

dissociation inhibitor at earlier times than it became resistant to BAPTA. We have developed a cell-free assay to study the reformation of lysosomes from late endosome–lysosome hybrid organelles that were isolated from the rat liver. The recovery of electron dense lysosomes was shown to require ATP and was inhibited by bafilomycin and EGTA-AM. The data support a model in which endocytosed Ca^{2+} plays a role in the fusion of late endosomes and lysosomes, the reformation of lysosomes, and the dynamic equilibrium of organelles in the late endocytic pathway.

Key words: calcium • calmodulin • endosome • lysosome • membrane fusion

Introduction

In mammalian cells, the organelles of the late endocytic pathway have been shown to interact with each other and to be in dynamic equilibrium both in vivo and in vitro. Thus, content mixing and/or exchange of membrane proteins has been observed between late endosomes (Aniento et al., 1993), lysosomes (Storrie and Desjardins, 1996; Bakker et al., 1997; Ward et al., 1997), and between late endosomes and lysosomes (Mullock et al., 1998). It is now generally accepted that delivery of endocytosed macromolecules to lysosomes occurs by content mixing between late endosomes and lysosomes as a result of kiss-and-run events (Storrie and Desjardins, 1996) and/or direct fusion between the two organelles (Mullock et al., 1998). In our own experiments to discover the mechanisms of delivery to lysosomes, we have used a cell-free content mixing assay and demonstrated that late endosomes can fuse with lysosomes to form hybrid organelles of intermediate den-

sity (Mullock et al., 1998). Fusion was shown to be ATP-, cytosol-, and temperature-dependent, required the presence of *N*-ethyl maleimide-sensitive factor (NSF)¹ and soluble NSF attachment proteins (SNAPs), and was inhibited by GDP dissociation inhibitor (GDI). These characteristics implied the action of the common cytosolic fusion machinery used at many other sites on the secretory and endocytic pathways (Rothman, 1994). The site-specific components of the fusion machinery, e.g., SNAP receptors (SNAREs) and rab protein(s), required for late endosome–lysosome fusion have not yet been established.

In contrast to the paucity of information about specific molecules required for membrane traffic on the pathways to the lysosome in mammalian cells, many gene products necessary for membrane traffic to the yeast vacuole, which is the yeast equivalent of the lysosome (Klionsky et al., 1990), have been identified. Much of this information has

Address correspondence to Paul Luzio, Cambridge Institute for Medical Research, University of Cambridge, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2XY, UK. Tel.: 44-1223-336780. Fax: 44-1223-762630. E-mail: jpl10@cam.ac.uk

Paul R. Pryor and Barbara M. Mullock contributed equally to this work.

¹*Abbreviations used in this paper:* Av-ASF, avidin-asialofetuin; BAPTA, 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid; bpIgA, biotinylated polymeric IgA; EGTA-AM, methyl ester of EGTA; GDI, GDP dissociation inhibitor; GMP-PNP, 5'-guanylylimidodiphosphate; NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; STKM, STM containing 25 mM KCl.

come from genetic screens in *Saccharomyces cerevisiae* (e.g., the isolation of *vps* [vacuolar protein sorting]) mutants (Robinson et al., 1988; Raymond et al., 1992). In addition, the development of a cell-free homotypic vacuolar fusion assay has led to the identification of SNAREs and other factors required for vacuolar fusion and a growing understanding of the complex molecular events involved in this process (Nichols et al., 1997; Ungermann et al., 1999). While it has been suggested that SNAREs may be membrane fusion catalysts based on data from a liposome fusion assay (Weber et al., 1998), the study of yeast homotypic vacuole fusion has revealed that Ca^{2+} release from the vacuole is required in a postdocking phase of fusion and that the effects of released Ca^{2+} are mediated via calmodulin (Peters and Mayer, 1998). In these experiments, vacuole fusion was inhibited efficiently by 1,2-bis(2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), but very little by EGTA, a chelator with a similar dissociation constant for Ca^{2+} but an on rate that is much slower than BAPTA. More recently, *in vitro* homotypic fusion of mammalian early endosomes also has been shown to be inhibited by BAPTA and by a membrane permeable, hydrolyzable methyl ester of EGTA (EGTA-AM), but not by EGTA itself (Holroyd et al., 1999). We have reexamined the requirements for heterotypic fusion between rat hepatocyte late endosomes and lysosomes, which was previously shown to be unaffected by 5 mM EGTA (Mullock et al., 1998). We show that fusion is inhibited by BAPTA and by EGTA-AM, and that the Ca^{2+} -mediated event is likely to involve calmodulin and occurs at a later stage than the rab requirement for docking and fusion.

Since the fusion of late endosomes with lysosomes to form hybrid structures results in consumption of the starting organelles, we have proposed that a recovery system involving membrane retrieval and a lysosomal content condensation process must occur *in vivo* (Bright et al., 1997; Mullock et al., 1998). In the present study, we have developed a cell-free system to investigate the reformation of lysosomes from hybrid organelles isolated from the rat liver. We show that some reformation occurs in an ATP-dependent, cytosol-independent manner and can be inhibited by bafilomycin and EGTA-AM but not BAPTA. We propose a model in which endocytosed Ca^{2+} is required both for the fusion of late endosomes and lysosomes and for the condensation process, which must occur for electron dense lysosomes to reform from the resultant hybrid organelles.

Materials and Methods

Reagents

Unless otherwise stated, reagents were purchased from Sigma Chemical Co. Avidin-asialofetuin (Av-ASF) and ^{125}I -labeled-biotinylated polymeric IgA (bpIgA) were as previously described (Mullock et al., 1994). Affinity-purified goat antiavidin (egg white) was obtained from Calbiochem. Amine-terminated magnetic beads (Biomag beads) were purchased from Metachem Diagnostics and conjugated to rabbit anti-goat IgG (Dako) according to the manufacturer's instructions. The calmodulin antagonists W-7 and W-5 were aliquoted and kept at -20°C as 25-mM stocks in DMSO. Calmidazolium chloride (Calbiochem) was kept at 4°C as a 14.5-mM stock in DMSO. EGTA-AM (Calbiochem) was aliquoted and kept at -20°C as a 0.5-M stock in DMSO neutralized with NaOH to pH 7.

A plasmid containing NH_2 -terminal His-tagged bovine rab GDI cDNA (gift of Drs. H. Davidson and D. McDonald, from our department) was expressed in *Escherichia coli* BL21(DE3), and the recombinant GDI was purified according to Ullrich et al. (1995). Polyclonal rabbit anti-goat Ig antibodies conjugated to 8 nm colloidal gold were purchased from Sigma Chemical Co.

Content Mixing Assay

The content mixing assay was carried out as previously described (Mullock et al., 1998). In brief, late endosomes were prepared from the liver of a rat that received ~ 10 nmol of Av-ASF intravenously 6 min before killing; lysosomes were prepared from a rat that received ~ 50 pmol of ^{125}I -labeled bpIgA 30 min before killing. Organelle fractions, prepared by density gradient centrifugation, were resuspended in pig brain cytosol, which was prepared by extracting the tissue with 0.25 M sucrose containing 10 mM N-*TES*, 1 mM Mg^{2+} , and 25 mM KCl, pH 7.4 (STKM). Duplicate samples containing late endosomes and lysosomes in 0.2 ml of brain cytosol were routinely incubated for 10 min at 37°C with 1 mM ATP, 1 mM GTP, an ATP-regenerating mixture of phosphocreatine and creatine kinase (Mullock et al., 1994), and 60 $\mu\text{g}/\text{ml}$ biocytin to block any formation of avidin-bpIgA outside a membrane-bounded compartment. The total incubation volume was 240 μl . Potentially inhibitory compounds were added to the content mixing reaction tubes for 10 min on ice before the addition of ATP, GTP, and the ATP-regenerating system and subsequent incubation at 37°C . After incubation, dilution in the presence of protease inhibitors (Roche Molecular Biochemicals), lysis, and immunoprecipitation of the biotin-avidin conjugate, the total immunoprecipitable radioactivity in the samples was measured.

For experiments in which organelles were pretreated with potentially inhibitory compounds and reisolated for content mixing assays, the organelles were initially resuspended in STM (STKM without KCl) and incubated with the compound for 15 min on ice before reisolation, resuspension in cytosol, and use in the content mixing assay. In some experiments, hybrid organelles resulting from late endosome-lysosome fusion in the cell-free content mixing assay were isolated by ultracentrifugation on 20% Nycodenz/20% Ficoll step gradients as previously described (Mullock et al., 1998). Ca^{2+} concentrations in the content mixing assays, in the presence of 5 mM BAPTA, were calculated using the Freeware computer program BAD4 (version 4.0) developed by Brooks and Storey (1992). The pH of the content mixing assay, in the presence of cytosol, was measured as ~ 7 and calculated Ca^{2+} concentrations were adjusted accordingly.

Unless otherwise stated, all of the results from the content mixing assays are expressed as percentages of total immunoprecipitable counts formed within late endosome-lysosome hybrid organelles after incubation for 10 min at 37°C in the presence of cytosol, an ATP-regenerating system, 1 mM ATP, and 1 mM GTP.

Preparation and Characterization of Late Endosome-Lysosome Hybrids from Rat Liver

Hybrid-enriched fractions from the rat liver were prepared using a slight modification of the method used previously (Mullock et al., 1998). The endocytic pathway was loaded with ^{125}I -labeled bpIgA by intravenous injection 30 min before killing the rat. A postmitochondrial supernatant was prepared from the liver by centrifugation for 10 min at 1,100 *g* (removing nuclei and the bulk of large mitochondria; Branch et al., 1987), and 11 ml was loaded over a step gradient composed of 4 ml of 45% Nycodenz, 12 ml of 20% Nycodenz, and 12 ml of 20% Ficoll. The material found at the Nycodenz/Ficoll interface after centrifugation for 1 h, at 206,000 *g* in a vertical rotor, contained the hybrids. Fractions of 1 ml were collected, and the three most highly labeled fractions from the Nycodenz/Ficoll interface were pooled and diluted with an equal volume of STKM or, when appropriate, with pig brain cytosol. The pooled hybrid-enriched fraction was assayed for protein, *N*-acetyl- β -glucosaminidase, glucose 6-phosphatase, succinate dehydrogenase, and 5'-nucleotidase as previously described (Branch et al., 1987; Mullock et al., 1994).

The following criteria were used to show that the hybrid-enriched fraction, which was prepared directly from the rat liver contained hybrid organelles with the same characteristics as those formed in cell-free content mixing assays between late endosomes and lysosomes: (1) the same density after isopycnic centrifugation on Ficoll and Nycodenz gradients; (2) immunoelectron microscopic characterization showing the presence of cation-independent mannose 6-phosphate receptor and cathepsin L; and (3) the ability to recover immunoprecipitable avidin-biotin conjugates

from the organelles after intravenously injecting into the same rat ^{125}I -labeled bpIgA followed by Av-ASF. When the time gap between injections was increased from 9 to 16 min, the radioactivity in lysosomal fractions immunoprecipitated by antiavidin antibodies increased, whereas that in the hybrid-enriched fractions (and endosomes) decreased, which is consistent with the reformation of lysosomes from hybrids.

Lysosome Reformation Assay

Samples of the hybrid-enriched fraction (0.7 ml) in STKM were usually incubated at 37°C with ATP and an ATP-regenerating system as for the fusion reaction (Mullock et al., 1994). At the end of the reaction time, normally 10 min, the incubation mixtures were chilled in ice and loaded over 4.2-ml linear 12–30% Nycodenz gradients with 0.3 ml 45% Nycodenz cushions. Centrifugation for 20 min in a Beckman VTi90 vertical rotor at 585,000 g was followed by the collection of five drop fractions (~ 0.1 ml). Fractions were counted and refractive indices were measured to establish boundaries for lysosomes (refractive index, 1.3785 and above) and hybrids (refractive indices, 1.3784–1.3695). The fractions were stored at -20°C before determination of *N*-acetyl- β -glucosaminidase on a 25- μl sample from each fraction according to Maguire et al. (1983). Radioactive counts and *N*-acetyl- β -glucosaminidase levels, which were measured after fractionation of incubation mixtures that were kept on ice throughout, were treated as blanks and subtracted from the matching incubated mixtures.

Electron Microscopy

Hybrid-enriched fractions for electron microscopy were prepared from rat livers after loading the endocytic pathway with Av-ASF by i.v. injection 15 min before killing the rat. For electron microscopy of reformed lysosomes, fivefold larger samples of the hybrid-enriched fraction (3.5 ml) were incubated under the usual conditions for reformation. After chilling, the samples were loaded onto the 45% Nycodenz/20% Nycodenz/20% Ficoll gradients used to prepare hybrids, centrifuged, and fractionated. The starting hybrid-enriched fractions and the reformed lysosomes (taken from gradient fractions with a refractive index >1.3785) were separately pooled, diluted threefold with STM-containing protease inhibitors and centrifuged for 15 min at 104,000 g before preparation for either conventional transmission electron microscopy or ultrastructural immunocytochemistry according to Griffiths (1993) as described previously (Mullock et al., 1998).

For quantitation of the number of electron dense lysosomes in samples from density gradients, random sections were cut through conventionally treated organelle pellets. Electron dense lysosomes were defined approximately as spherical structures, 0.2–0.5 μm diam, with an amorphous electron dense matrix (Holtzmann, 1989), and >200 were scored in random sections of each pellet. The numbers of lysosomes in a hybrid-enriched fraction pellet or reformed lysosome pellet were calculated by calculating the area functions of lysosomes in sections and deriving the volume fractions of lysosomes in pellets according to the principle of Delesse (Griffiths, 1993). The number of lysosomes present in each fraction was calculated from the derived volume of lysosomes. In calculating the yields of reformed lysosomes by electron microscopy the following assumptions were made: 169×10^6 cells/g wet weight rat liver (Hommes et al., 1970); 270 lysosomes/hepatocyte (Sewell et al., 1986); that the number of hybrid organelles per cell is $\sim 5\%$ of the number of lysosomes (based on the *N*-acetyl- β -glucosaminidase recovery data in this study). Lysosomes were reformed from the hybrid-enriched fraction prepared from 3.8 g wet weight of rat liver.

Results

Using our established cell-free content mixing assay (Mullock et al., 1998) to measure the fusion of late endosomes and lysosomes, we examined the effect of adding the Ca^{2+} -chelating agent BAPTA directly to the assay. In contrast to EGTA, which we have shown previously to have no effect on the content mixing assay (Mullock et al., 1998), BAPTA inhibited content mixing with an IC_{50} of ~ 2 mM (Fig. 1 a). Pretreatment of the organelles with BAPTA had no effect, which is consistent with it being membrane impermeant (Fig. 1 b). The addition of increasing concentrations of CaCl_2 in the presence of 5 mM BAPTA added

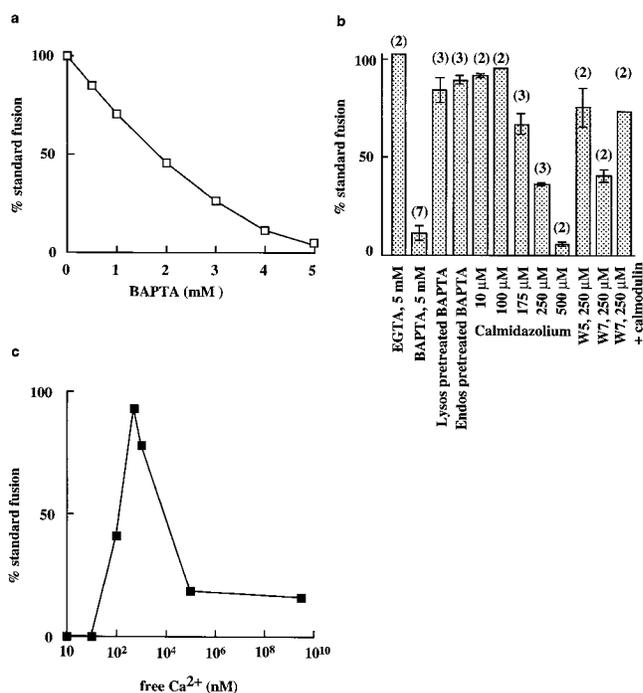


Figure 1. Inhibition of late endosome–lysosome content mixing by BAPTA. (a) Dose–response curve of inhibition. BAPTA at the concentrations stated was present throughout a standard incubation (i.e., for 10 min at 37°C in the presence of cytosol), an ATP-regenerating system, 1 mM ATP, and 1 mM GTP. The results are expressed as the percentage of the total immunoprecipitable counts that had formed within late endosome–lysosome hybrid organelles. Data are from a representative experiment. (b) Effects on content mixing of EGTA, pretreatment of organelles with BAPTA, or calmodulin antagonists. Content mixing was not inhibited by 5 mM EGTA in contrast to the effect of 5 mM BAPTA. Pretreatment of lysosomes (lysos) or late endosomes (endos) with 5 mM BAPTA was for 15 min on ice. The calmodulin antagonists (calmidazolium at concentrations stated or W5 and W7 each at 250 μM) without or with calmodulin (60 U) were present throughout standard incubations. For the calmodulin antagonists, the results are expressed as the percentage of the total immunoprecipitable counts that were formed within late endosome–lysosome hybrid organelles in the presence of DMSO. The numbers above the bars indicate the number of experiments. Data are presented as mean \pm SEM or range. (c) Reversal of BAPTA inhibition of content mixing by addition of CaCl_2 . The standard content mixing assay was carried out in the presence of 5 mM BAPTA together with different concentrations of CaCl_2 , resulting in the calculated free Ca^{2+} concentrations shown (for method of calculation see Materials and Methods).

to the assay without preincubation, resulted in the restoration of content mixing (Fig. 1 c) such that at a calculated free $[\text{Ca}^{2+}]$ of 0.5 μM , no difference from the standard assay was observed. Further addition of CaCl_2 , resulting in a higher free $[\text{Ca}^{2+}]$, inhibited content mixing. These data are all consistent with observations in the cell-free yeast vacuole fusion assay that led to the hypothesis that intravacuolar Ca^{2+} was released after vacuolar docking and SNARE assembly, and it was required for fusion via a calmodulin-mediated process.

Mobilization of Endosomal Ca^{2+} Is Required for Late Endosome–Lysosome Fusion

To investigate the requirement for luminal Ca^{2+} in content mixing, we examined the effects of EGTA-AM. In contrast to the lack of inhibition of content mixing by EGTA, EGTA-AM completely blocked fusion with an IC_{50} of ~ 0.2 mM (Fig. 2 a). Pretreatment of the late endosomes, but not lysosomes, with 1 mM or 2.5 mM EGTA-AM caused inhibition of content mixing (Fig. 2 b). Under these conditions adding back Ca^{2+} to the content mixing assay reversed the inhibition observed (data not shown). Pretreatment of either organelle with 5 mM EGTA-AM resulted in an inhibition of content mixing, but this inhibition was not reversed when Ca^{2+} was added back to the content mixing assay, suggesting irreversible damage (data not shown). The data presented are consistent either with the major source of Ca^{2+} required for fusion being that released from the lumen of the late endosome, or it coming from both organelles, with the lack of effect of low concentrations of EGTA-AM on lysosomes being a consequence of them containing a higher concentration of Ca^{2+} . The differences in the dose–response curves for the effects of EGTA-AM that were seen in Fig. 2 (a and b) are probably because of a leakage of EGTA-AM out of the pretreated organelles (Fig. 2 b) during reisolation and before warming to 37°C when hydrolysis of the ester occurs.

For the yeast cell-free homotypic vacuole fusion, Ca^{2+} released from the vacuole lumen functions by binding to calmodulin (Peters and Mayer, 1998). In our assay, we found that two structurally different antagonists of calmodulin, calmidazolium, and W-7, blocked late endosome–lysosome fusion (Fig. 1 b) at concentrations consistent with effects seen on the other calmodulin-dependent events in mammalian cells (Watanabe et al., 1999). When used at the same concentration, the calmodulin antagonist W-7 was more effective than W-5, which has a weaker affinity for calmodulin. The effect of W-7 was reversed by the presence in the content mixing assay of calmodulin (Fig. 1 b).

The GDI-sensitive Step in Fusion Occurs before the BAPTA-sensitive Step

We have previously shown that the addition of rab GDI inhibits late endosome–lysosome fusion, presumably by removal of an essential rab from the fusing membranes (Mullock et al., 1998). The putative rab required for fusion has not been positively identified. To determine at which stage of fusion Ca^{2+} acts compared to the stage at which the rab is required, we determined when the content-mixing assay would become resistant to appropriate inhibitors, i.e., we applied the approach used by Peters and Mayer (1998) with yeast vacuole fusion. We observed that over a time course of 15 min, inhibition of content mixing was greater when BAPTA was added to the assay at 5 min than when GDI was added at this time point although both inhibited equally if present from the beginning of the incubations (Fig. 3). This implies that by 5 min, $>50\%$ of the GDI-sensitive event(s) had already occurred but none of the BAPTA-sensitive events had. In this experiment, the effects of totally stopping the content mixing assay at different time points were observed simply by placing

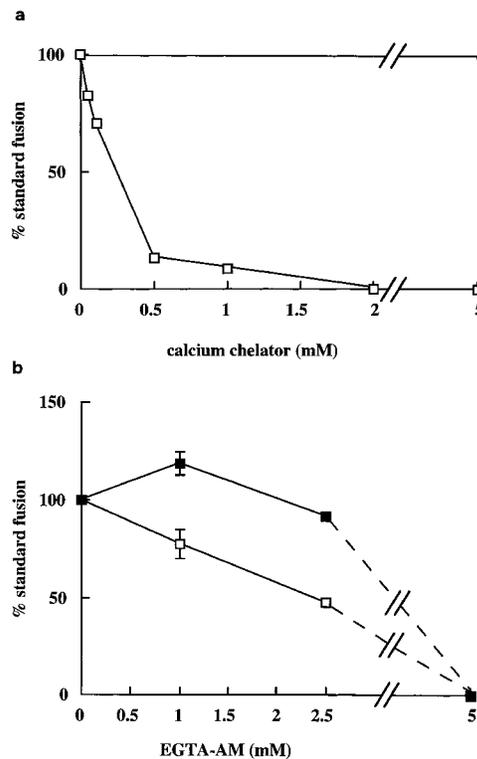


Figure 2. Inhibition of late endosome–lysosome content mixing by EGTA-AM. (a) Dose–response curve of inhibition with EGTA-AM (□) or EGTA (●) present at the concentrations stated throughout a standard incubation. For treatment with EGTA-AM, results are expressed as the percentage of the total immunoprecipitable counts that had formed within the late endosome–lysosome hybrid organelles in the presence of DMSO. (b) Effects of organelle pretreatment with EGTA-AM on content mixing in a standard incubation. (□) Late endosomes; and (■) lysosomes. Data are presented as mean \pm SEM of three experiments. The solid line depicts the range of EGTA-AM concentration where inhibition can be reversed by the addition of CaCl_2 . At 5 mM EGTA-AM, addition of CaCl_2 does not reverse inhibition.

tubes on ice for the remainder of the assay period. As previously noted (Mullock et al., 1998), a clear lag occurred in the first few minutes of incubation at 37°C , during which no measurable content mixing took place. The observed displacement of the BAPTA inhibition curve from that seen when inhibiting with GDI (Fig. 3) is consistent with data from the yeast vacuole fusion assay, implying that the GDI-sensitive event(s) occurs earlier than that which is sensitive to BAPTA. In contrast, the observed displacement of the BAPTA inhibition curve, from the time course obtained when the content mixing assay was simply stopped by placing tubes on ice (Fig. 3), was not seen in the yeast vacuole fusion assay. The displacement is easily explained, if subsequent to the Ca^{2+} requirement, a further, presently unknown, event is required for fusion.

Lysosomes Can Be Reformed from Hybrid Organelles in a Cell-free System

One of the processes thought to occur in the reformation

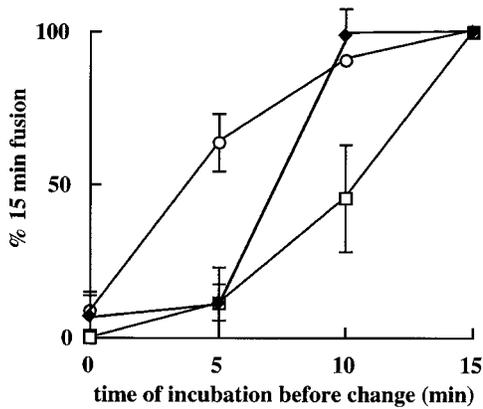


Figure 3. Luminal Ca^{2+} is required in a late phase of late endosome-lysosome fusion. BAPTA (5 mM; \blacklozenge) and GDI (20 μM ; \circ) were added to the ongoing content mixing assay at different times after the start and the effect of these inhibitors on the rest of the incubation period was monitored. First, as controls, a content mixing assay was carried out under standard conditions except that the incubation time at 37°C was 15 min, resulting in the 100% value by definition. Second, content mixing assays were carried out under standard conditions except that after defined times at 37°C , the assays were stopped by placing the incubation tubes in ice (\square). Data are presented as mean \pm SEM of three experiments.

of lysosomes is the recondensation of content such that lysosomes appear electron dense by comparison with hybrid organelles or endosomes (Bright et al., 1997; Mullock et al., 1998) and can be separated from hybrid organelles and endosomes by density gradient centrifugation. We decided to establish a cell-free system to study whether luminal Ca^{2+} also plays a role in the recondensation and reformation of lysosomes from late endosome-lysosome hybrid organelles. A hybrid organelle-enriched fraction was isolated from the rat liver using isopycnic centrifugation on a step gradient and was characterized as described in Materials and Methods. Using ^{125}I -labeled bpIgA endocytosed for 15 min *in vivo* as a marker of the endocytic pathway, the hybrid organelle-enriched fraction was found to contain $4.7 \pm 0.2\%$ (18 separate experiments) of the radiolabel in the homogenate. In a representative experiment where 4.1% of the homogenate radiolabel was in the hybrid organelle-enriched fraction, it was also found to contain 2.2% homogenate protein, 4.1% *N*-acetyl- β -glucosaminidase, 0.5% succinate dehydrogenase, 7.6% glucose 6-phosphatase, and 5.1% 5'-nucleotidase. Just as in the case of the rat liver late endosome fraction isolated by isopycnic centrifugation on a Ficoll gradient (Mullock et al., 1994), the major organelle contaminant in the hybrid-enriched fraction is the ER.

When rat liver hybrid organelle-enriched fractions containing ^{125}I -labeled bpIgA were incubated at 37°C for 10 min and analyzed by centrifugation on a 12–30% continuous Nycodenz gradient (Fig. 4), it was found that some of the radioactivity had moved to a lysosomal position on the gradient (defined previously by centrifuging purified rat liver lysosomes). Endogenous *N*-acetyl- β -glucosaminidase had also moved from the hybrid position to the lysosomal position on the gradient (Fig. 4). In contrast, there was no

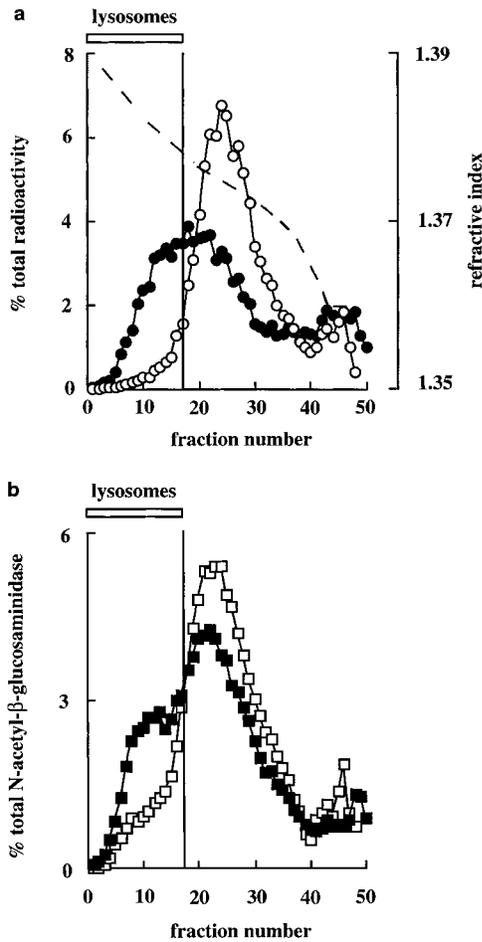


Figure 4. The reformation of lysosomes from hybrid organelles. A hybrid organelle-enriched fraction from rat liver containing endocytosed ^{125}I -labeled bpIgA was resuspended in STKM and incubated for 10 min at 37°C (closed symbols), or kept on ice (open symbols) in the presence of an ATP-regenerating system and 1 mM ATP. The incubation mixture (0.7 ml) was loaded onto a 4.2-ml 12–30% Nycodenz gradient prepared on top of a cushion of 0.3 ml 45% Nycodenz. After centrifugation, followed by fractionation, the distributions of radioactivity (a) and *N*-acetyl- β -glucosaminidase (b) were measured. A representative experiment is shown. The lysosomal region of the gradient is indicated by the bars to the left of the vertical lines. The broken line in a shows the refractive index of gradient fractions after centrifugation.

movement of glucose 6-phosphatase to this position (data not shown). To confirm that the movement of radiolabel and *N*-acetyl- β -glucosaminidase was due to the reformation of lysosomes, electron microscopy was carried out. A hybrid-enriched organelle fraction from a rat liver in which the endocytic pathway had been loaded with Av-SF *in vivo* was examined by immunoelectron microscopy. The fraction contained many organelles with the characteristic size and morphology of hybrids, as previously defined (Mullock et al., 1998), and these were decorated with gold-labeled antiavidin (Fig. 5). When this hybrid enriched fraction was kept at 4°C and then analyzed by centrifugation on a 12–30% continuous Ficoll gradient, no membrane-bound organelles could be recovered from

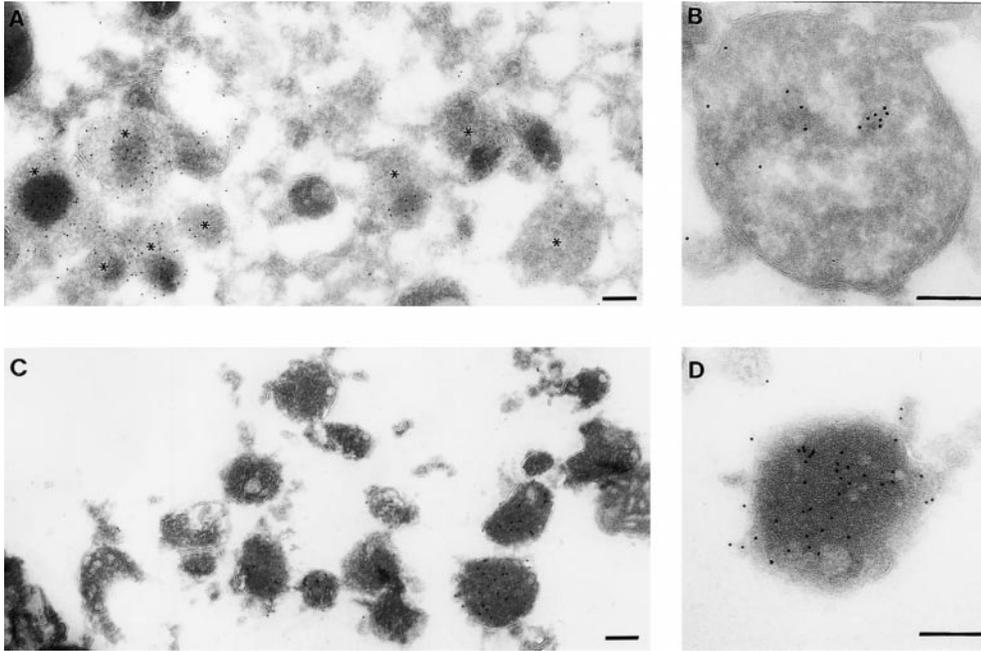


Figure 5. Electron microscopic analysis of the reformation of lysosomes from hybrid organelles. (A) Immunoelectron micrograph of a representative field of a hybrid organelle-enriched fraction from rat liver showing the presence of endocytosed Av-ASF as detected with anti-avidin (8 nm gold). Asterisks identify eight examples of organelles with the characteristics of hybrids. (B) Immunoelectron micrograph of a representative hybrid organelle. (C) Immunoelectron micrograph of a representative field of electron dense lysosomes, reformed from the hybrid organelle-enriched fraction after 3 min of incubation in STKM at 37°C in the presence of an ATP-regenerating system and 1 mM ATP and subse-

quent isolation on a 12–30% Nycodenz gradient. Note the presence of the endocytosed marker in several of the electron dense lysosomes. (D) Immunoelectron micrograph of a representative reformed lysosome. Bars, 200 nm.

the lysosomal region on the gradient. In contrast, when the hybrid-enriched fraction was incubated in the presence of ATP for 3 min at 37°C and then centrifuged, an organelle fraction was recovered from the lysosomal region on the gradient. Immunoelectron microscopy revealed the presence of many classical electron dense lysosomes decorated with gold-labeled anti-avidin (Fig. 5). Quantitation of morphologically defined lysosomes that were present in conventionally fixed samples of the hybrid-enriched fraction and the reformed lysosome fraction showed that the lysosomes were formed *de novo* in the assay and were not simply lysosomes that were previously trapped in the hybrid fraction (data not shown). The calculated efficiency of reformation assessed by electron microscopy was ~20% after 3 min of incubation at 37°C (see Materials and Methods), which is consistent with the biochemical data (Fig. 6). No further reformation of lysosomes, as assessed by electron microscopy, was observed by increasing the incubation time to 40 min.

Further study of the time course of reformation of lysosomes from hybrids in the cell-free system also revealed that either by measuring the movement of ¹²⁵I-labeled bpIgA or endogenous *N*-acetyl glucosaminidase, essentially all of the reformation was complete in 10 min with no further reformation observed after 40 min at 37°C (Fig. 6). Discrepancies in the relative amounts of radioactivity and *N*-acetyl glucosaminidase transferred from the hybrid gradient fractions to the lysosome gradient fractions in the time course experiment may reflect the origin of the ¹²⁵I-labeled bpIgA in individual hybrids since even after 15 min uptake *in vivo*, some ¹²⁵I-labeled bpIgA may already have recycled from lysosomes to hybrids, but some will have come directly from late endosomes. At present, we do not know whether the hybrid content that is derived

from the lysosome fusion partner is preferentially recondensed during lysosome reformation.

Reformation of Lysosomes Is ATP-dependent and Inhibited by Bafilomycin and EGTA-AM

The cell-free reformation of dense core lysosomes was cytosol-independent but required the presence of ATP (Fig. 7). The ATP dependence was also observed when hybrid organelles, which were prepared in a cell-free content mixing assay, were used as the starting material for lysosome reformation (data not shown). Endocytic transport to the lysosomes has been reported to be dependent on a bafilomycin-sensitive vacuolar proton pumping ATPase in cultured cells (van Weert et al., 1995). Therefore, we examined the effect of bafilomycin on lysosome reformation in the cell-free system and found that it was a potent inhibitor (Fig. 7), implying a role for ATP-dependent acidification in recondensation and lysosome reformation. Finally, to study the role of Ca²⁺ in the reformation of dense core lysosomes, the effects of BAPTA and EGTA-AM on the cell-free reformation assay were examined. Whereas BAPTA had no effect on the reformation, the membrane permeable, hydrolyzable chelating agent EGTA-AM completely abolished reformation, suggesting that intracellular Ca²⁺, as well as acidification, is required for recondensation and lysosome reformation.

Discussion

The present experiments demonstrate that, just as for yeast cell-free homotypic vacuole fusion (Peters and Mayer, 1998), cell-free heterotypic fusion of mammalian late endosomes and lysosomes requires Ca²⁺, probably

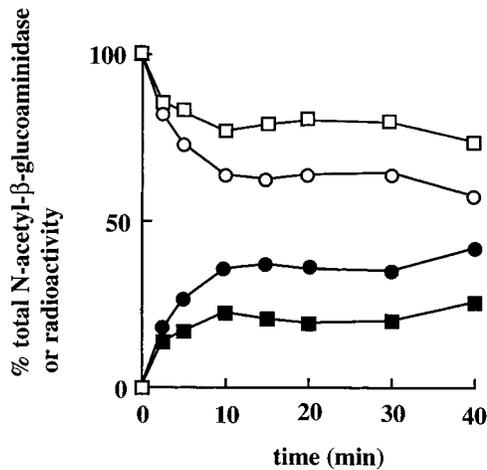


Figure 6. Time course of the reformation of lysosomes from hybrid organelles. A hybrid organelle-enriched fraction, from the rat liver containing endocytosed ^{125}I -labeled bpIgA, was incubated in STKM for different times at 37°C in the presence of an ATP-regenerating system and 1 mM ATP. The incubation mixtures were loaded onto 12–30% Nycodenz gradients and, after centrifugation and fractionation, the amounts of radioactivity (● and ○) and *N*-acetyl- β -glucosaminidase (■ and □) in the fractions containing hybrids (open symbols) and lysosomes (solid symbols) were measured. The results are expressed after subtracting the amounts of radioactivity (7% of total on gradient) and *N*-acetyl- β -glucosaminidase (16% of total on gradient) found at the lysosomal position after centrifugation of hybrid organelles kept on ice.

mediating its effects via calmodulin. The Ca^{2+} is derived from the organelle lumen and is required at a late step in fusion after the requirement for a rab protein.

While the observation that BAPTA inhibits late endosome-lysosome membrane fusion with an IC_{50} of ~ 2 mM, although EGTA has no effect even at 5 mM, is at first sight surprising, it is not without precedent in vertebrate membrane fusion systems. Thus, cell-free nuclear vesicle fusion during nuclear envelope assembly was shown to be inhibited by 5 mM BAPTA but unaffected by 12 mM EGTA (Sullivan et al., 1993). This effect was explained by the fact that at physiological pH, BAPTA exchanges Ca^{2+} ~ 100 times faster than EGTA, reflecting faster rates of association and dissociation. Therefore, facilitated diffusion can cause dispersal of cytosolic $[\text{Ca}^{2+}]$ gradients fast enough to suppress the biological response to mobilized Ca^{2+} . Sullivan et al. (1993) also speculated that Ca^{2+} mobilization could be a general feature of fusion in the secretory and endocytic pathways, although at that time convincing evidence for this being the case existed only for various steps of the secretory pathway.

Cytosolic Ca^{2+} concentration is tightly regulated with resting levels usually estimated at ~ 0.1 μM (Campbell, 1983). Endocytosis can provide a significant contribution to cellular calcium since uptake can occur from the extracellular fluid or, in the case of cultured cells, the incubation medium containing millimolar concentrations of this ion (Gerasimenko et al., 1998). Indeed, after 20 min of endocytosis by cultured fibroblasts in an incubation medium

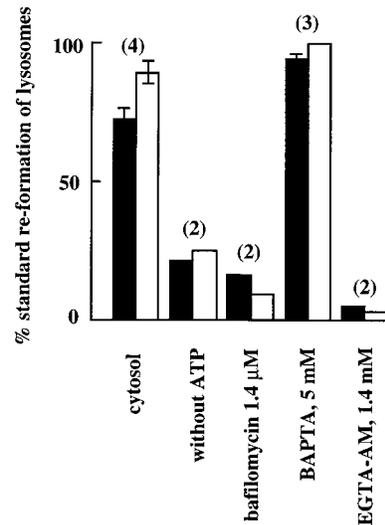


Figure 7. Requirements for the reformation of lysosomes from hybrid organelles. Lysosome reformation was calculated from the recovery of ^{125}I -labeled bpIgA (solid bars) and *N*-acetyl- β -glucosaminidase (open bars) at the lysosomal position on a 12–30% Nycodenz gradient, after incubation as indicated. Results are expressed as percentages of lysosome reformation obtained on the same day under standard conditions, i.e., with a hybrid organelle enriched fraction isolated from rat liver incubated in STKM for 10 min at 37°C in the presence of an ATP-regenerating system and 1 mM ATP. Cytosol, bafilomycin A1 (1.4 μM), BAPTA (5 mM), and EGTA-AM (1.4 mM) when added, were present throughout a standard reformation assay. In the cases of treatment with bafilomycin A1 and EGTA-AM, the results are expressed as the percentage of lysosome reformation obtained on the same day under standard conditions in the presence of DMSO. The numbers above the bars indicate the number of experiments. Data are presented as mean \pm SEM (range errors on pairs too small to show).

containing 2 mM Ca^{2+} , the endosomal free Ca^{2+} concentration was estimated at ~ 3 μM (Gerasimenko et al., 1998). There is also evidence that lysosomes contain a mobilizable pool of Ca^{2+} (Haller et al., 1996), and that both endosomal (Hilden and Madias, 1989) and lysosomal (Lemons and Thoene, 1991) membranes contain Ca^{2+} transport systems. Furthermore, a rise in cytosolic Ca^{2+} has been shown to be necessary for lysosome-lysosome fusion (Bakker et al., 1997), lysosome-phagosome fusion (Jaconi et al., 1990), and lysosome-plasma membrane fusion (Rodriguez et al., 1997). While earlier cell-free studies suggested that Ca^{2+} is not required for cytosol- and ATP-dependent fusion between early endosomes (Diaz et al., 1988; Wessling-Resnick and Braell, 1990; Mayorga et al., 1994), they did not use chelators with fast rates of association. Recently, Holroyd et al. (1999) have provided convincing evidence that BAPTA and EGTA-AM inhibit the cell-free fusion of early endosomes derived from both PC-12 and BHK cells. Taken together with our present experiments on heterotypic late endosome-lysosome fusion, these data are consistent with endocytosed Ca^{2+} being released from the lumen of endocytic organelles and used to mediate membrane fusion events at several stages on the endocytic pathway.

The observation that the calmodulin antagonist calmidazolium and W-7 inhibit late endosome-lysosome fusion is consistent with experiments on yeast vacuole fusion where calmodulin was described as exerting an active, promoting effect on the postdocking events (Peters and Mayer, 1998). Subsequent work has shown that calmodulin is a component of a high molecular weight complex on the yeast vacuole that also contains protein phosphatase 1, an essential protein for vacuolar bilayer mixing (Peters et al., 1999). Previous use of calmodulin antagonists has suggested that this protein is important in endocytosis in mammalian cells, including having functions in endocytic uptake (Artalejo et al., 1996; Llorente et al., 1996), fusion of early endosomes (Colombo et al., 1997), recycling to the cell surface (De Figueiredo and Brown, 1995), delivery to the Golgi complex (Llorente et al., 1996) transcytosis (Apodaca et al., 1994; Hunziker, 1994; Llorente et al., 1996), and lysosomal degradation of endocytosed immune complexes (Hunziker, 1994). The last of these effects may well be explained by the ability of W-7 to inhibit fusion of late endosomes and lysosomes, thereby preventing the mixing of endocytosed ligands with active lysosomal hydrolases. Localization of intracellular calmodulin on both endosomal (Enrich et al., 1996) and lysosomal (Nielsen et al., 1987) membranes has been reported. What is not apparent from the present experiments is the nature of any downstream calmodulin effector required for heterotypic fusion of late endosomes and lysosomes, although clearly a wide variety of calmodulin-activated enzymes, transporters, cytoskeletal proteins, and other proteins are known (Klee et al., 1980; Niki et al., 1996; Peters et al., 1999). The requirement for a downstream effector of calmodulin would be consistent with the observed displacement of the time course of BAPTA inhibition of late endosome-lysosome fusion from the time course when stopping the reaction by placing the assay tubes on ice (Fig. 4). Intriguingly, the discovery of a calmodulin requirement for fusion of late endosomes and lysosomes helps resolve a previous anomaly. Chloroquine and primaquine had been observed to inhibit fusion, implying a role for luminal acidity, yet the vacuolar proton pumping ATPase inhibitor, bafilomycin, did not inhibit it. Chloroquine and primaquine have structural similarities to W-7 (Barry and Bernal, 1993), and there is evidence that such antimalarials, which are amphipathic amines that contain large hydrophobic regions and carry a positive charge at neutral pH, can act as calmodulin antagonists (for review see Weiss et al., 1982). This suggests that the effects of chloroquine and primaquine in inhibiting late endosome-lysosome fusion may in fact be due to their ability to inactivate calmodulin.

NSF-dependent membrane fusion events require the interaction of cognate SNAREs on the fusing membranes to provide at least one layer of specificity to the fusion process (Rothman, 1994; Advani et al., 1998). At present, there is no clear published evidence implicating specific mammalian SNAREs in late endosome-lysosome fusion. Several have been partially localized in late parts of the endocytic pathway (Advani et al., 1998) and the use of inhibitory antibodies to VAMP-7 in permeabilized cells has suggested that this v-SNARE is somewhere in the pathway of transport from early endosomes to lysosomes (Advani et al., 1999). In our present experiments, the evidence

indicating that calmodulin is a downstream effector of Ca^{2+} required for membrane fusion suggests that the release of Ca^{2+} from the endosome/lysosome lumen may occur after SNARE complex assembly, as suggested for the release of Ca^{2+} from the vacuole lumen (Peters and Mayer, 1998). However, in other systems, there is both evidence for (Chen et al., 1999) and against (Coorsen et al., 1998) a requirement for Ca^{2+} in SNARE complex assembly, and in the present experiments, we cannot rule out such a role. What is clear from our experiments is that BAPTA inhibition of late endosome-lysosome fusion occurs at a stage later than the requirement for a rab protein.

The formation of hybrid organelles after direct fusion of late endosomes with lysosomes implies that in vivo, a lysosome reformation process occurs to avoid consumption of the fusing organelles. Such a reformation process should involve a retrieval and/or digestion of those endosomal membrane components not found in lysosomes and recondensation of content to reform dense core lysosomes. To date we have only been able to reconstitute the latter reaction in a cell-free system although the inability to detect cation-independent mannose 6-phosphate receptor in the reformed dense core lysosomes (data not shown) could be due to proteolysis or a presently undetected membrane retrieval event. Our present reformation assay is cytosol-independent, but this does not rule out the presence of cytosolic components on the membrane of the hybrid organelles, i.e., that they are primed for reformation. Others have been able to show the recruitment of coat proteins to very late endocytic compartments that may contribute to a membrane retrieval process (Traub et al., 1996; Arneson et al., 1999). Since we have achieved much <100% reformation of lysosomes in a cytosol-free system, further modifications including appropriately activated cytosol may lead to a more efficient reformation system in which membrane retrieval can be demonstrated. Nevertheless, our present cell-free lysosome reformation assay does demonstrate the requirements for recondensation of lysosome content. ATP was found to be essential and the ability to inhibit reformation with the specific vacuolar H^+ pumping ATPase inhibitor bafilomycin A1 suggests that the requirement for ATP may be to support the maintenance of a proton gradient. It is not clear whether the function of such a proton gradient in lysosome reformation is due to an effect on luminal content condensation or on membrane retrieval by a process analogous to the formation of endocytic carrier vesicles earlier in the endocytic pathway (Clague et al., 1994). However, the present evidence that ATP is required for the generation of a dense organelle from the hybrids echoes earlier experiments in which isolated endosomes were shown to undergo an increase in density to that of lysosomes, and it was proposed that a low luminal pH induced aggregation of vesicle contents (Roederer et al., 1990). Bafilomycin A1 inhibition of lysosome reformation also provides an explanation of data showing that treatment of cultured hepatoma cells with bafilomycin A1 prevented transport of endocytosed HRP from endosomes to lysosomes (van Weert et al., 1995). The observation in the present experiments that EGTA-AM but not BAPTA inhibited lysosome reformation clearly suggests that luminal free Ca^{2+} in the hybrid organelle is required for recondensation. An interesting possibility is that endosomal

Ca²⁺ is not only required for fusion with lysosomes, but its presence in the newly formed hybrid also triggers the recondensation of lysosomal contents that were diluted into the hybrid lumen at the point of fusion. Thus, late endosome-lysosome fusion and lysosome reformation may be coupled by the requirement for luminal free Ca²⁺. However, we cannot rule out that the luminal Ca²⁺ required for reformation results from a specific transport process into the hybrid organelle.

Overall, our data are consistent with the mechanism of heterotypic fusion of late endosomes and lysosomes requiring release of luminal Ca²⁺ after tethering/docking to promote postdocking events. In addition, our data on the cell-free reformation of dense core lysosomes suggest that recondensation occurs by a very similar process to that observed in the formation of regulatory secretory granules where both luminal acidification and free Ca²⁺ are thought to be important in the aggregation process (Chanat and Huttner, 1991).

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