"Synchronized" Endocytosis and Intracellular Sorting in Alveolar Macrophages: The Early Sorting Endosome Is a Transient Organelle

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Abstract. Incubation of alveolar macrophages in hypo-osmotic K⁺-containing buffers results in persistent cell swelling and an inability to undergo regulatory volume decrease. We demonstrate that cells incubated in hypo-K⁺ show an inhibition of endocytosis without any observed alteration in recycling. The inhibition of endocytosis affected all forms of membrane internalization, receptor and fluid phase. Both increased cell volume and the inhibition of endocytosis could be released upon return of cells to iso-Na⁺ buffers. The ability to synchronize the endocytic apparatus allowed us to examine hypotheses regarding the origin and maturation of endocytic vesicles. Incubation in hypo-K⁺ buffers had no effect on the delivery of ligands to degradative compartments or on the return of previously internalized receptors to the cell surface. Thus, membrane recycling and movement of internalized components to lysosomes occurred in the absence of continued membrane influx. We also demonstrate that fluorescent lipids, that had been incorporated into early endosomes, returned to the cell surface upon exposure of cells to hypo-K⁺ buffers. These results indicate that the early sorting endosome is a transient structure, whose existence depends upon continued membrane internalization. Our data supports the hypothesis that the transfer of material to lysosomes can best be explained by the continuous maturation of endosomes.

Molecules internalized by endocytosis traverse the cell through a network consisting of an amorphous mixture of discrete vesicles and anastomosing tubules. While the boundaries between different parts of the endocytic pathway are imprecise, there is a consensus that the network consists of early and late sorting compartments of which the lysosome is the terminal organelle in the pathway. Elements of the early endocytic apparatus exhibit compositional and biochemical differences such as gradients of pH, proteolytic, esterolytic and fusogenic activities (Mayor et al., 1993; Aniento et al., 1993; Hopkins et al., 1990; Stoorvogel et al., 1991; Ward et al., 1990a,b; Roederer et al., 1990). Different theories of endosomal development have been proposed to account for the generation of these changes in activities (Helenius et al., 1983; Van Deurs et al., 1989). The early sorting endosome could act as a reservoir, transferring material to later compartments by means of a series of shuttle vesicles (Gruenberg et al., 1989; Griffiths and Gruenberg, 1991) similar to the vesicle transfer process observed in the Golgi Apparatus (Rothman and Orci, 1992). Aniento et al. (1993) have recently presented data suggesting a shuttle mechanism in which large vesicles transfer volume between early and late endosome. These large vesicles appear to bud from long lived early sorting endosomes.

An alternative hypothesis is that the early endosome could, by a process of maturation, be converted into a late endosome or lysosome (Murphy, 1991). The maturation process involves the removal of some components and the addition of others. Mechanistic consideration of the maturation hypothesis suggests two scenarios. The first is that maturation occurs by a continuous process in which components are continually added or removed. The second is by a discontinuous process where at some critical size the early endosome buds off, exhibits an abrupt change in its fusion properties and in a concerted fashion gains or loses components.

Existing studies however, have not lead to a consensus acceptance between either model. Dunn and Maxfield (1989) as well as our laboratory (Ward et al., 1990a) presented data indicating that newly internalized vesicles can fuse with a pre-existing or early sorting compartment. With time the ability of newly internalized vesicles to fuse with prelabeled vesicles is lost (Salzman and Maxfield, 1988; Ward et al., 1990a); yet, this sorting compartment was still considered to be an early endosome. Stoorvogel et al. (1991) using electron microscopy and subcellular fractionation, suggested that newly internalized vesicles were capable of fusing with pre-existing vesicles up until the point that the pre-existing vesicles were converted into lysosomes.
It is hard to directly compare these studies since different methodologies were used and there appear to be significant differences between cell types. It may well be that there is plasticity in the endocytic pathway between cell types. In considering a set of experiments that would distinguish between the various models we realized that there is a problem in using either a kinetic approach or one method which examines compositional differences between endocytic vesicles. The endocytic apparatus is normally asynchronous. Within a given cell, as well as in a population of cells, movement of molecules through the endocytic pathway may be determined by the "state" of the vesicles. Newly internalized vesicles require additional components to either transfer their contents on to or mature to become late endosomes. "Older" endosomes may already contain most of the components required to transfer their contents to late endosomes. Even with the shortest pulse times, internalized ligands may be introduced into vesicles of different sizes and in different stages of maturation. This situation is formally analogous to attempting to study early DNA synthesis in a non-synchronized population of cells using a radiolabeled nucleoside: a pulse of [3H]thymidine would label both new and old replication forks. This problem can be resolved by synchronizing cell mitosis and thus specifically labeling just early or late S phase. If the endocytic apparatus can be synchronized, then ligands introduced at specific times may be localized in specific classes of vesicles.

We present studies demonstrating that, in alveolar macrophages, endocytosis and the sorting apparatus can be synchronized. Our results suggest that the components of the early endocytic apparatus "disappear" in the absence of continued internalization. This observation suggests that the early endocytic apparatus comprises a steady state organelle and its existence results from continued membrane influx. Once internalized, ligands destined for the lysosome move there with similar kinetics independent of whether internalization continues or is inhibited. We provide further data indicating that when the endocytic block is released, internalized ligands are directed to the lysosome without any apparent lag. Similarly, the return of recycled components to the plasma membrane does not require continued internalization. These results are consistent with a maturation model of early endosome formation.

Materials and Methods

Cells
Rabbit alveolar macrophages were obtained by bronchial lavage (Myrvik et al., 1961) and maintained as described previously (Kaplan, 1980). In all experiments cells were placed in Hank's Minimal Essential Media (HMEM) (iso-Na+ 300 mOsm) at 37°C and incubated for 45 min before experimental manipulations (Kaplan and Keogh, 1982). Hypo-K+ buffers 1. Abbreviations used in this paper: aM-T, a-macroglobulin-trypsin complex; BODIPY-SM, boron dipyrromethene difluoride-sphingosine; DF-BSA, defatted-BSA; DF-HSA, defatted human serum albumin; DFO, deferoxamine mesylate; Fluor-Dex, Fluorescein-Dextran (10K); HMEM, Hank's minimal essential media; hypo-K+; hypo-osmotic K+ containing buffers (150 mOsm); iso-Na+, iso-osmotic Na+ containing buffers (300 mOsm); man-BSA, mannosylated-BSA; RVD, regulatory volume decrease; Tf(Fe)2, diferric transferrin; TR-Dex, Texas red-Dextran (10K).

Figure 1. Model of RVD in alveolar macrophages. See text for explanation.

(hypo-osmotic K+ containing buffers 150 mOsm) were prepared using Baenziger and Fiete (1982) K+ buffer (142 mM KCl, 3.6 mM CaCl2, 20 mM Hepes, 0.34 mM K2HPO4, 0.35 mM KH2PO4, and 0.81 mM MgSO4) diluted 1:1 with H2O to obtain 150 mOsm. All experiments were performed a minimum of three times.

Materials
α-Macroglobulin (αM) was isolated from either rabbit or human plasma.

Figure 2. Volume measurements of alveolar macrophages incubated in hypo-K+ buffers. Cells were incubated for the indicated times in hypo-K+ (○) buffers at 37°C. At the time denoted by the arrow cells were resuspended in iso-Na+ media (●). Cell volume was determined using a FACS®. Volumes were calibrated using latex beads of known size (9-4.4-μm diam) as standards.
in cells incubated in iso-Na+ and hypo-K+ buffers. Cells were incubated in either iso-Na+ (□) or hypo-K+ (○) buffers at 37°C for 30 min. One set of hypo-K+-treated cells was then incubated in iso-Na+ buffers for an additional 30 min at 37°C (△). Cells were washed once in the appropriate buffers and incubated with 1 x 10^-8 M αM • 125I-T for various times in those buffers. At each specified time samples were placed at 0°C, washed extensively, and then surface ligand removed using HBSS-Ca2+-Mg2+ + 5 mM EDTA, pH 6.0, or iso-K+-Ca2+-Mg2+ + 5 mM EDTA, pH 6.0. Cells were solubilized in 1% SDS and radioactivity in the cells as well as that "stripped" from the surface determined. The data are expressed as internalized αM • 125I-T versus the integral of surface ligand bound as per Opresko and Wiley (1987). (B) HRP uptake in alveolar macrophages incubated in iso-Na+ or hypo-K+ buffers. Cells were placed in either HMEM + 2 mg/ml BSA (●) or hypo-K+ + 2 mg/ml BSA (○) and incubated in these buffers at 37°C for 30-45 min. A final concentration of 1 mg/ml HRP was added to αM • 125I-T was prepared as previously described (Kaplan and Nielson, 1979). Mannosylated-BSA (man-BSA) was obtained from E. Y. Laboratories, Inc. (San Mateo, CA) and iodinated as described previously (Ward and Kaplan, 1990). Human transferrin (Tf) was obtained from Sigma Chem. Co. (St. Louis, MO) and iron saturated as described previously (Ward et al., 1982). Tf(Fe3)2 was iodinated using iodogen (Sigma Chem. Co.). HRP type IV was obtained from Sigma Chem. Co. Deferoxamine (DFO) (CIBA, Summit, New Jersey) was used at a concentration of 0.8 mM.

Methods

Binding of radioactive ligands to cells and discrimination between internalized and surface bound ligand was performed as described (Kaplan and Nielson, 1979; Kaplan, 1980). Internalization rates were calculated according to the methods of Opresko and Wiley (1987). The acid stripping procedures for 125I-TT were performed as described (Ward et al., 1990a) and uptake of HRP was assayed using dianisidine as described (Novak et al., 1988). Subcellular fractionation and Percoll gradients were performed as described previously (Ward et al., 1990a). TCA soluble radioactivity was determined as described (Ward et al., 1989). Protein determinations were performed as described by Lowry et al. (1951), using BSA (fraction V; Sigma Chem. Co.) as a standard.

Fluorescent Studies

Boron dipyrromethene difluoride-sphingosine (BODIPY-SM) (Molecular Probes, Eugene, Oregon) was diluted into defatted human serum albumin (DF-HSA) and back-exchanges performed as described by Martin and Pagano (1994). Quantification of the BODIPY-SM was performed using butanol extraction as described previously (Pagano et al., 1991). Cells were visualized using a Nikon inverted fluorescent microscope with a Zeiss 100× oil immersion objective. Images (512 × 512) were acquired using a Photometrics cooled CCD camera and a Macintosh workstation running OncorImage 3-D cytometry software. A multi-dye filter set was used in which excitation filters of 485 nm (fluorescein/BODIPY-SM) and 575 nm (Texas red) were selected from a computer-controlled filter wheel in conjunction with a multi-wavelength emitter and dichroic filter set (525 and 640 nm; XF56 set from Omega Optical). Out-of-focus blur was removed by adjacent plane Fast Fourier deconvolution using the inverse of the modulation transfer function of the microscope. Adjacent planes were taken at 0.5-μm intervals under control of the OncorImage software package. Prior to deconvolution, all images were corrected for background and flatfield. Images were then scaled to 256 levels of gray before output to a film recorder. Texas red dextran (TR-Dex) (10K) and fluorescein dextran (Fluor-Dex) (10K) were obtained from Molecular Probes (Eugene, Oregon).

Volume Measurements

Cells were placed in either iso-Na+ or hypo-K+ buffers at a concentration of 1 x 10⁷/ml. At specified times cell volumes were determined using FACS® with latex beads of known volume as standards. The parameters used to estimate volume were as per Salzman et al. (1990) using forward light scatter and a linear relationship between the light scatter and the actual volume. This procedure was verified by Coulter Counter volume measurements.

Results

Incubation of Macrophages in Hypo-K+ Solutions Inhibits Membrane Internalization

Previously we demonstrated that exposure of alveolar macrophages to hypo-osmotic Na+ containing solutions in-cells at 37°C and at various times cells were placed at 0°C, washed with HBSS-Ca2+-Mg2+ + 5.0 mM EDTA, pH 6.0, twice and then washed 10 times with HBSS to remove surface bound HRP. Cells were then solubilized in 0.1% Triton X-100 and the amount of HRP determined as described in the Materials and Methods. Each time point represents the average of four plates.
hibits endocytosis without inhibiting membrane recycling (Novak et al., 1988b; Buys et al., 1989). Cells exposed to hypo-osmotic Na⁺ solutions initially swell, and in a time and temperature-dependent manner regain their normal volume. This process, termed regulatory volume decrease (RVD) results from the stretch-activated opening of separate K⁺ and Cl⁻ channels (Grinstein et al., 1982). Since the intracellular concentration of K⁺ is higher than the extracellular concentration, there is a net loss of intracellular K⁺ (Fig. 1). To maintain electroneutrality the loss of K⁺ induces a loss of Cl⁻ and a concomitant osmotic loss of H₂O. Because of the ability of cells to regulate their volume the inhibition of endocytosis is transient, as cells regain their normal volume, endocytic activity also returns to normal (Novak et al., 1988a). Incubation of cells in hypo-osmotic medium in which K⁺ is the major cation prevents RVD. The cation and anion channels open in response to cell swelling, however, since the extracellular concentration of K⁺ is higher than the intracellular concentration, K⁺ as well as Cl⁻ and H₂O enter the cell. Cell volume increases and cells are unable to volume regulate (Fig. 2). The inability to regulate volume results in a sustained inhibition of receptor-mediated endocytosis (Fig. 3 A). Hypo-K⁺ treated cells internalize α-macroglobulin–protease complexes at a rate (Kₑ = 0.018) that is ~5% of that of control cells (Kₑ = 0.444; Fig. 3 A). The inhibition of endocytosis is not restricted to receptors, but extends to all forms of membrane internalization. As demonstrated in Fig. 3 B there is also a marked inhibition of fluid phase pinocytosis.

![Figure 4](image_url)  
**Figure 4.** Surface binding of αM·125I-T in alveolar macrophages incubated in hypo-K⁺ buffers. Cells were incubated for the indicated times in iso-Na⁺ (●) or hypo-K⁺ (○) buffers at 37°C. The cells were placed at 0°C, washed, and then incubated with 1 × 10⁻⁹ M αM·125I-T for 60 min. Cells were washed extensively, solubilized in 1% SDS and radioactivity determined using a Packard Autogamma 5780 counter. Cell protein was determined and the data is presented as specific binding.

Phagocytosis of either complement coated Zymosan or IgG-coated red blood cells is also inhibited following incubation of cells in hypo-K⁺ buffer (data not shown).

Incubation of hypo-K⁺ cells in iso-osmotic Na⁺ media results in a rapid decrease in cell volume (Fig. 2) and a recovery of endocytic activity (Fig. 3 A). The rate of endocytosis for cells which have recovered from hypo-K⁺ treatment (Kₑ = 0.463) was similar to that of cells maintained in iso-Na⁺ buffers (Kₑ = 0.444). The recovery of endocytosis in hypo-K⁺ incubated cells placed in iso-Na⁺ media has been measured with three different ligands (Tf[Fe]₂, αM·T and man-BSA), and in all cases occurs without any detectable lag (data not shown).

**Incubation in Hypo-K⁺ Does Not Inhibit Membrane Recycling**

While incubation of alveolar macrophages in hypo-K⁺ solutions inhibits endocytosis there is no apparent inhibition of membrane recycling. Hypo-K⁺-incubated cells demonstrate a persistent increase in the surface number of constitutively recycling αM·T receptors (Fig. 4) as well as for

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**Figure 5.** Recycling of 125I-Tf in cells incubated in iso-Na⁺ or hypo-K⁺ buffers. Cells were pulsed with 125I-Tf(Fe)₂ in HMEM + 2 mg/ml BSA for 2 min at 37°C. Cells were then placed at 0°C, washed, and surface ligand removed using the acid stripping procedures described in Materials and Methods. Cells were then placed back at 37°C in iso-Na⁺ or hypo-K⁺ buffers containing 0.8 mM deferoxamine and 1 mg/ml Tt[Fe]₂ for various times. Cells were placed at 0°C, media removed, and the cells acid stripped as described in Materials and Methods to remove 125I-Tf which has recycled to the plasma membrane. Cells were then solubilized in 1% SDS and the radioactivity in the media, acid strips, and cell associated determined as well as protein determinations performed. The open triangles represent cells which were pre-incubated in hypo-K⁺ buffer prior to the pulse of 125I-Tf(Fe)₂. The data are expressed as the percent of 125I-Tf released.
Hypo-K⁺ Solutions Do Not Prevent the Movement of Ligands to Late Endosomes or Lysosomes

We next examined whether hypo-K⁺ treatment affected the movement of ligands from early endocytic vesicles to late endosomes and lysosomes. Cells were incubated with αM·¹²⁵I-T for 2–3 min, washed extensively, and then incubated in either iso-Na⁺ or hypo-K⁺ buffers at 37°C. At specified times ligand degradation was measured using TCA precipitation (Ward et al., 1989). Although the rate of degradation was slightly decreased in hypo-K⁺-treated cells, the time at which degradation was initiated was similar to cells incubated in iso-Na⁺ buffers (Fig. 6A), indicating that movement of ligand to degradative compartments was not inhibited in hypo-K⁺ buffers.

To further evaluate the effect of hypo-K⁺ treatment on ligand movement within the endocytic apparatus we examined the distribution of internalized ligand by subcellular fractionation. Alveolar macrophages were incubated with αM·¹²⁵I-T for 2 min and then incubated either in iso-Na⁺ or hypo-K⁺ media. At specified times cells were homogenized and subcellular fractionation was performed as described in Materials and Methods. The data are expressed as the percent which colocalized with the lysosomal enzyme marker β-hexosaminidase. The maximum observed to be coincident with lysosomal marker in iso-Na⁺ was expressed as 100% and all other time points were then calculated using the maximum as the denominator.

other recycling receptors (Buys et al., 1989). This increase in surface receptors is due to a sustained inhibition of endocytosis in the face of continued recycling. Upon incubation of hypo-K⁺-treated cells in iso-Na⁺ media the recovery in endocytic activity was concurrent with a decrease in surface receptor number to the original value and often to slightly lower values, suggesting an overshoot phenomenon. The magnitude of the increase in surface receptors suggest that the majority of internal receptors have been exteriorized. To study the rate and extent of receptor recycling we examined the behavior of internalized Tf, since this ligand stays associated with its receptor as it traverses the endosomal recycling path. Cells pre-loaded with ¹²⁵I-Tf were exposed to iso-Na⁺ or hypo-K⁺ buffers and the loss of cell-associated Tf measured. The loss of radioactivity from cells incubated in either iso-Na⁺ or hypo-K⁺ buffer appeared biphase (Fig. 5). The majority of radioactivity (80%) was released with a t½ of 2–4 min, the remainder with a longer half-life. All of the radioactivity that was released from cells was released in a trichloroacetic acid precipitable form, that is consistent with recycling rather than degradation. Little detectable ¹²⁵I-Tf was retained within cells indicating that the majority of internal receptor/ligand complexes were exteriorized to the cell surface. These data suggest that the recycling pathway is unaffected by incubation of alveolar macrophages in hypo-K⁺ buffers. We observed, in a variety of cultured cell types, that while endocytosis was reduced upon exposure to hypo-K⁺, membrane recycling was also affected. Alveolar macrophages, however, seem to be unique in that hypo-K⁺ solutions have no effect on recycling.

Figure 6. (A) Movement of αM·¹²⁵I-T to degradative compartments in iso-Na⁺ or hypo-K⁺ buffers as determined by release of TCA soluble radioactivity. Cells were incubated in HMEM 2 mg/ml BSA with 1 × 10⁻⁸ M αM·¹²⁵I-T for 2 min. Cells were placed at 0°C, washed extensively, and surface ligand removed using EDTA containing solutions. Cells were then placed at 37°C in either iso-Na⁺ or hypo-K⁺ containing buffers. At specified times samples were removed and TCA precipitations performed as described in Materials and Methods. The data are expressed as percent of TCA soluble. (B) Movement of αM·¹²⁵I-T to the lysosome in iso-Na⁺ or hypo-K⁺ buffers as determined by Percoll gradients. Cells were incubated in HMEM + 2 mg/ml BSA with 1 × 10⁻⁵ M αM·¹²⁵I-T for 3 min. Cells were placed at 0°C, washed extensively, and surface ligand removed using EDTA containing solutions. Cells were then placed back at 37°C in either iso-Na⁺ or hypo-K⁺ containing buffers. At specified times samples were removed and TCA precipitations performed as described in Materials and Methods. The data are expressed as percent which colocalized with the lysosomal enzyme marker β-hexosaminidase. The maximum observed to be coincident with lysosomal marker in iso-Na⁺ was expressed as 100% and all other time points were then calculated using the maximum as the denominator.
mogenized, the post nuclear supernatant top loaded onto a Percoll gradient and the amount of radioactivity co-sedimenting with the lysosomal marker B-hexosaminidase was determined. The time of appearance of ligand in lysosomes was similar in iso-Na⁺- or hypo-K⁺-treated cells, however, there appeared to be a 30% reduction in the amount of ligand transferred to lysosomes in hypo-K⁺-treated cells (Fig. 6 B).

We have assumed that radioactivity which had the same density as lysosomes reflected transfer of molecules to lysosomes. This assumption may be wrong; the change in radioactive distribution on Percoll may simply reflect a change in buoyant density and not movement to lysosomes. To ascertain the appearance of molecules in the lysosome we used fluorescent microscopy. Cells were incubated at 37°C with fluorescein-dextran for 60 min followed by an additional 60 min “chase” period to label lysosomes. The cells were then incubated with TR-Dextran for 10 min. (Fig. 7 A) and then chased for extended times in either iso-Na⁺ or hypo-K⁺ buffers. Initial coincidence of the dyes occurred within 10 min of the chase for both iso-Na⁺- and hypo-K⁺-treated cells (data not shown) and increased with time (iso-Na⁺, Fig. 7 B and hypo-K⁺, Fig. 7 C). These results demonstrate that ligand was transferred to lysosomes in hypo-K⁺ treated cells.

Effect of Hypo-K⁺ Solutions on Internalization and Recycling of Lipids

The above data indicate the existence of the early endocytic apparatus, which is defined by the presence of recycling receptors and Tf, requires continued membrane internalization, and in the absence of internalization the early endocytic apparatus disappears. A critical caveat to that interpretation is that recycling receptors and fluid phase ligands may be transported but that a framework endosome remains. Due to a lack of unique early endosomal markers we may simply be unable to detect this organelle. To investigate this possibility we used the fluorescent sphingomyelin derivative (BODIPY-SM); available data indicates that it is a bulk flow membrane marker (Martin and Pagano, 1994).

Cells were incubated at 0°C with BODIPY-SM and then transferred to either solutions containing hypo-K⁺ or iso-Na⁺ at 37°C. At specified times the cells were placed at 0°C, back-exchanged to remove surface bound probe, lipids were butanol extracted and BODIPY-SM quantified (Koval and Pagano, 1989). Cells loaded with BODIPY-SM at 0°C and then incubated in hypo-K⁺ buffer at 37°C resulted in essentially a complete loss of fluorescence, whereas incubation of cells in iso-Na⁺ containing solutions

Figure 7. Movement of fluid-phase internalized fluorescent ligands to lysosome in iso-Na⁺ or hypo-K⁺ buffers. Cells were plated on coverslips at 37°C, 45 min in HMEM. All samples were incubated with 1 mg/ml fluorescein-dextran at 37°C for 60 min. Cells were washed extensively and then incubated for an additional 60 min at 37°C. Cells were then pulsed for 10 min at 37°C with 5 mg/ml TR-dextran (A). Cells were placed at 0°C, washed extensively, and then placed back at 37°C in either iso-Na⁺ (B) or hypo-K⁺ (C) buffers for 30 minutes. Cells were viewed at both 520 nm (fluorescein) and 670 nm (TR) using BDS-Image 3D microscopy as described in Materials and Methods.
at 37°C resulted in a progressive increase in cell associated fluorescence which was insensitive to back-exchange (Fig. 8). This result demonstrates that hypo-K⁺ solutions inhibit internalization of bulk membrane lipids. Another group of cells were loaded with dye at 0°C and then incubated at 37°C in iso-Na⁺ for 15 min to label early endosomes. The media was removed and the cells incubated in either iso-Na⁺ or hypo-K⁺ solutions. At selected times the cells were placed at 0°C, back-exchanged and cell associated fluorescence assayed. Cells incubated in hypo-K⁺ buffer lost cell-associated fluorescence which was insensitive to back-exchange (Fig. 8). The kinetics of fluorescence loss roughly matched the rate of Tf recycling from hypo-K⁺-treated cells. This loss of fluorescence was not irrevocable. The addition of iso-Na⁺ buffers to hypo-K⁺-treated cells (which were not back-exchanged) resulted in the appearance of fluorescence that became resistant to subsequent back-exchange (Fig. 8, arrow). Thin layer chromatography of dye extracted from cells that had been incubated for up to 30 min revealed no evidence of metabolism (data not shown). These results suggest that, upon exposure to hypo-K⁺ solutions, early endocytic structures containing lipids are recycled to the cell surface.

The above quantitative observations were verified by examining BODIPY-SM-loaded cells with fluorescence microscopy. Cells incubated with dye at 0°C demonstrated a bright ring-like fluorescence (Fig. 9 A) which could be removed by back-exchange (data not shown). Incubation of dye-loaded cells at 37°C resulted in a time dependent appearance of vesicular fluorescence which resisted back-exchange (Fig. 9, B and C). A rapid loss of intracellular fluorescence occurred when BODIPY-SM-loaded cells were transferred at 37°C to hypo-K⁺ solutions (Fig. 9 D). These observations suggest that inhibition of endocytosis induces the disappearance of some or all of the early endocytic apparatus. Occasionally we observed the appearance of a few internal fluorescent vesicles which were retained for extended periods of time in hypo-K⁺-treated cells (data not shown). These vesicles appeared larger than most of the vesicles observed and may be late endosomal or lysosomal in nature. They represent, however, only a small percentage of the originally internalized fluorescence.

**Examination of Vesicle Behavior Using Synchronized Endocytosis**

Our studies indicate that the hypo-K⁺ block on endocytosis resulted in the disappearance of components in the early endocytic pathway, and that when the hypo-K⁺ block was released endocytosis was reinitiated without a measurable lag. These observations suggest that the properties of the endocytic pathway can be examined by pulsing the system with a “synchronized” set of vesicles. These vesicles are defined as a set of vesicles internalized into cells which have no pre-existing early endosome with which to fuse, and no subsequent internalized vesicles with which to fuse. To determine if this was feasible we assessed the effect of repeated hypo-K⁺ exposure on membrane recycling. Cells were incubated in hypo-K⁺, the endocytic block released by incubation in iso-Na⁺, and then the cells were re-exposed to hypo-K⁺. Measurement of surface receptor number revealed that following recovery of receptor number, a second addition of hypo-K⁺ had no effect on recycling. Surface binding increased at a rate and magnitude that was similar to the initial hypo-K⁺ exposure (Fig. 10).

To determine if the release on the inhibition of endocytosis results in the reformation of the endocytic apparatus we examined two parameters, ligand hydrolysis, which reflects the movement of ligand to degradation competent compartments assumed to be late endosomes, and the movement of ligand to lysosomes. Following exposure to αM-¹²⁵I-T, the generation of mono-iodotyrosine reflects the degradation and presence of ligand in late endosomal compartments and the transfer of material from early to late endosomes. The initiation of ligand hydrolysis was similar in all cells (Fig. 11 A). The movement of ligand to lysosomes was also measured by subcellular fractionation. Cells were incubated in either iso-Na⁺ or hypo-K⁺ buffers at 37°C for 30 min before incubation with αM-¹²⁵I-T in iso-Na⁺ buffers. After 4 min of internalization, cells were then returned to iso-Na⁺ or hypo-K⁺ buffers at 37°C for additional times. Subcellular fractionation using Percoll gradients demonstrated that ligand was introduced into lysosomes at approximately the same time whether the cells
were incubated in iso-Na\(^+\) or hypo-K\(^+\) buffers. With continued incubation in hypo-K\(^+\), the rate of ligand entry in lysosomes decreased somewhat but still continued (Fig. 11B). These results suggest that the intracellular movement of vesicles, either to the cell surface or to the lysosome, does not require the continued influx of newly internalized membrane.

**Discussion**

**Recycling Represents the Default Pathway for Internalized Membrane Proteins**

Incubation of alveolar macrophages in hypo-K\(^+\) solutions results in a marked inhibition of endocytosis without an
inhibition of either membrane recycling or transfer of previously internalized components to lysosomes. The mechanism underlying the hypo-K\(^+\) induced inhibition of endocytosis is not defined. One possibility is that the force required to internalize plasma membrane can not overcome the surface tension resulting from increased hydraulic pressure. In alveolar macrophages, the rate of recycling was essentially unchanged in hypo-K\(^+\) solutions as assessed by measuring the number of surface receptors, the recycling of internalized Tf, and by the loss of internalized BODIPY-SM. All the various measurements of recycling gave consistent results, a half time for recycling of 2-4 min, a value similar to that reported previously (Ward et al., 1989).

While specific structural information on membrane proteins affects the rate of internalization, molecules lacking internalization sequences will still get internalized (Felder et al., 1990; McGraw and Maxfield, 1990; Jing et al., 1990; Wiley, et al., 1991; Mayor et al., 1993; Naim and Roth, 1994). In the absence of specific sequence information, membrane proteins are internalized at a rate specified by bulk flow endocytosis. Once internalized specific sequences are required to affect lysosomal movement or late endosome retention (Herbst et al., 1994; French et al., 1994). Thus, the information for recycling is intrinsic to the endocytic apparatus and specific structural information is required to prevent recycling.

Our results indicate that on a physical level, recycling to the cell surface is a default pathway for the bulk of newly internalized membrane. In the absence of continued internalization there is a net movement of endosomal membrane to the cell surface. This conclusion is supported by other studies. In a recent study, Sukhorukov et al. (1993) demonstrated an increase in plasma membrane surface area after exposure of cultured cells to hypo-osmotic solutions. The net movement of internal membrane to the plasma membrane is consistent with morphological studies on the Drosophila temperature-sensitive mutation, Shibire (Kessel et al., 1989; Tsuruhara et al., 1990). This mutation in the dynamin protein results in a decrease in endocytosis at the restrictive temperature. Incubation of cultured cells or embryos exposed to HRP at the permissive temperature show the appearance of tubulo-vesicles. Incubation of cells at the restrictive temperature show the disappearance of the tubulo-vesicular structures and an increase in plasma membrane surface area. While HRP only marked the contents of the vesicles rather than membrane, the increase in cell surface area suggests the disappearance of endocytic membrane and its redistribution to the cell surface. Suggestive confirmation of this observation has been reported by Mellman and colleagues (Bacon et al., 1994). They isolated a temperature sensitive mutant in Dictyostelium discoideum which showed an inhibition of endocytosis at the restrictive temperature. While the nature of the mutation is unknown, they observed that although endocytosis was inhibited at the restrictive temperature, movement of internalized molecules to the lysosome or to the cell surface appeared unaffected. These studies suggest that inhibition of internalization along with continued membrane recycling may result in depletion of the early endocytic apparatus.

**The Early Sorting Endosome Is A Transient Organelle**

In the absence of internalization the endocytic apparatus, or at least the early portion, disappears. Additionally, once the block on endocytosis is released movement to the lysosome is reinitiated without any obvious lag. These observations lead to the conclusion that the endocytic apparatus is a steady state organelle with no independent existence and results from continued membrane influx. The possibility exists that there is a remnant of the endocytic apparatus which is left behind. This endosomal remnant may act as the seed, or organizing unit to which incoming vesicles fuse. One of the drawbacks about using rabbit alveolar macrophages is that there are few defined immuno-markers for organelles; most antisera are made in rabbits and thus react poorly with rabbit antigens. To address the issue of endosomal remnants, we examined the behavior of BODIPY-SM. This lipid acts as a bulk membrane marker, and there is no evidence to suggest that it is either selectively

![Figure 10](https://example.com/figure10.png)

**Figure 10.** Effect of repeated incubation in hypo-K\(^+\) buffers on the surface number of recycling receptors. Cells were incubated in iso-Na\(^+\) (●) or hypo-K\(^+\) (○) buffers at 37°C for 30 min. One group of hypo-K\(^+\) cells was then incubated in iso-Na\(^+\) buffer for additional times (▲). Following recovery, cells were then re-exposed to hypo-K\(^+\) buffers (△). At the end of the various incubation times, cells were placed at 0°C, washed, and \(^{125}\)I-man BSA binding measured at 0°C for 60 min as described in Materials and Methods.
We demonstrated that proper intracellular ligand targeting was not affected by the absence of an early endocytic apparatus. This was determined by pulsing hypo-K⁺ treated cells with ligand, reinitiating the hypo-K⁺ block, and then examining the movement of ligand to both late endosomes and lysosomes. In alveolar macrophages late endosomes and lysosomes are defined using operational criteria. Ligand degradation occurs within 2–5 min of internalized or recycled. Quantitative studies suggest that, following 15 min of endocytosis, most of the BODIPY-SM is recycled to the cell surface upon exposure to hypo-K⁺ buffers. Observations using fluorescence microscopy confirm this result. Internalization of the fluorescent lipid gives rise to a punctate pattern of fluorescence and subsequent exposure to hypo-K⁺ results in the disappearance of the majority of intracellular fluorescence. Thus, if there is an endosomal remnant it is below our limits of resolution to detect by either fluorescent microscopy or quantitative analysis.

The disappearance of the early endosomal apparatus in the absence of continued membrane internalization suggests that, as an organelle, it does not have a unique existence. In this regard the endocytic apparatus is similar to the Golgi apparatus. In the absence of continued vesicle fusion from the endoplasmic reticulum the Golgi as an identifiable organelle disappears (Rothman and Orci, 1992). Further similarity between the two organelles comes from the observations that both are affected by brefeldin-A, addition of this drug results in changes both in organelle structure and in the distribution of organelle constituents (for review see Klausner et al., 1992). Brefeldin-A treatment results in the redistribution of Golgi contents to the endoplasmic reticulum. In the case of the endosomal apparatus brefeldin-A treatment results in a partial redistribution of endosomal markers to the cell surface.

Studies have demonstrated self-fusion of early endosomes in vitro (Davey et al., 1985; Diaz et al., 1988; Gruenberg et al., 1989). Our data suggests that the early endosomal apparatus results primarily from homotypic fusion events. This is surmised from the observation that early endosomal events, membrane recycling, ligand degradation and ligand/receptor sorting, occurred in cells which, because of their previous exposure to hypo-K⁺, had no early endosomal apparatus. In the absence of pre-existing endosomes, the sorting endosome must result from self or homotypic membrane fusion. This conclusion is supported by studies which have examined the distribution of proteins on the cell surface or within endosomes (Casciola-Rosen and Hubbard, 1992; Schmid et al., 1988) by either compositional analysis or by impermeable labeling procedures. Although there are differences in the relative distribution of proteins present on the plasma membrane versus late endosomes, a unique class of proteins specific to the early endosome has not been detected.

**The Intracellular Targeting of Vesicles Does Not Require Pre-existing Early Endosomal Compartments**

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nalization, yet ligand does not reach lysosomes until 10–15 min. Initially, ligand degradation occurs in an endosomal fraction that can be differentiated from early endosomes due to the absence of internalized transferrin. Lysosomes can be differentiated from late endosomes on the basis of buoyant density and the presence of hexoseaminidase. The data demonstrate that even in the absence of a pre-existing early endosomal compartment internalized ligands can be found in late endosomes and lysosomes. These studies confirm the morphological observations in the temperature sensitive Drosophila mutation, Shibire (Tsuruhara et al., 1990). They synchronized endocytosis in oocytes using temperature shifts and then followed vitellogenin internalization and movement to yolk storage vesicles. They observed that vitellogenin was seen to move through a variety of vesicles and tubules and finally was deposited in yolk granules. Tsuruhara and colleagues suggest that this movement of ligand occurs through the maturation of vesicle.

Our studies on synchronized endocytosis indicate that degradation of internalized ligands can be initiated in endosomes within two minutes of internalization. If there is no pre-existing endocytic apparatus the question arises, Where do the proteases that initiate degradation initiate? Studies indicate that in some cell types proteases may enter endosomes from small vesicles which fuse with incoming endosomes (Guagliardi et al., 1990; Bowser and Murphy, 1990). An alternative hypothesis is that lysosomal enzymes are routed to the plasma membrane prior to moving to lysosomes. These lysosomal enzymes are internalized into endosomes and then moved to lysosomes, endosomal capture being part of the biosynthetic pathway of lysosomal enzymes (Braun et al., 1989). The ability to examine endosomal contents in a synchronized population of endosomes may provide a way to examine these two hypotheses.

Models of Endosome Formation

Our observations suggest a model of endosome development in which the early sorting compartment can result solely from homotypic fusion of newly internalized vesicles. These vesicles exhibit random motion and might either undergo self fusion or fusion with the cell surface. A consistent observation of in vitro and in vivo fusion systems is that early endosomes do not fuse with late endosomes or if they do is at a greatly reduced level. (Salzman and Maxfield, 1988; Ward et al., 1990a; Stoorvogel et al., 1991; Dunn and Maxfield, 1992). At some point the sorting endosome appears to lose the capacity to fuse with newly internalized vesicles. This event could occur if the proteins responsible for endosome fusion were restricted or clustered on membrane destined for recycling. Clustering of membrane components in sorting endosomes has in fact been demonstrated by a number of morphological studies (Geuze et al., 1983; Hopkins et al., 1994). As the distance from the cell surface increases the probability of fusion of newly internalized vesicles with the maturing endosome would decrease (Stoorvogel et al., 1991). At some point all of the pre-existing fusion machinery would have been recycled, and thus the maturing endosome would no longer be able to fuse with newly formed vesicles. This hypothesis could explain the generation of "carrier" vesicles of Gruenberg et al. (1989) and Aniento et al. (1993). This suggests that carrier vesicles are maturing endosomes (Dunn and Maxfield, 1992; Murphy, 1991; Stoorvogel et al., 1991).

We predict that recycling endosomes are capable of fusing with the plasma membrane, with other recycling endosomes and with early endosomes which still retain their fusion competency. In alveolar macrophages recycling occurs close to the cell surface and is extremely rapid. In other cell types recycling events may occur at locations distal to the original cell surface area, allowing for an opportunity for homotypic fusion between recycling vesicles. Such fusion events may result in the generation of tubulovesicular structures seen in the para-Golgi region in a number of cell types (Hopkins et al., 1990; Tooze and Hollinshead, 1991). Finally, depending upon the geometry of cells, such fusion events may also lead to fusion of recycling vesicles with the sorting endosome.

Summary

The ability to "synchronize" the endocytic apparatus and follow the movement of a single population of vesicles within cells provides a unique way to examine vesicle movement and the components involved. Our results indicate that in the absence of continued membrane internalization, processes such as membrane recycling and movement of internalized material to the late endosome and lysosome are little affected. The early sorting endosome, however, disappears indicating that this compartment is a transient organelle, predominantly derived from homotypic fusion of newly internalized vesicles. Our results are consistent with the maturation model of endocytosis.

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