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Isolation and Characterization of Endosome Subpopulation in Chinese Hamster Ovarian Cells

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=ABSTRACT=

Endosomes lower their internal pH by an ATP-driven proton pump, which is critical to dissociation of many receptor-ligand complexes, the first step in the intracellular sorting of internalized receptors and ligands. Endosomes are known to exhibit a great range of pH values that can vary between 5.0 and 7.0 within a single cell although the factors that regulate endosomal pH remain uncertain.

To evaluate the morphological and topological differences of endosomes in the different stages, confocal microscopy was used. The early endosomes labeled with fluorescein isothiocyanate- dextran for 10 min at 37℃ were identifiable at the peripheral and tubulo-vesicular endosome compartment. In contrast, the late endosomes formed by 10 min pulse and 20 min trace were located deeper in the cytoplasm and showed more vesicular features than early endosomes.

For the purpose of determining whether ATP-dependent acidification was heterogeneous and whether the differences in acidification were attributed to differences in the activity of Na^+-K^+-ATPase and/or Cl^- channel, endocytic compartments were fractionated into subpopulation using percoll gradient and measured ATP-dependent acidification. While all fractions exhibited ATP-dependent acidification activity, both the initial rate of acidification and extent of proton translocation were lower in early endosomes and gradually increased in late endosomes. Phosphorylation by PKA and ATP enhanced ATP-dependent acidification in both early and late endosomes, but there was no difference in the degree of enhancement by phosphorylation between two subpopulations. When ATP-dependent acidification was determined in the presence or absence of vanadate (Na_3VO_4) or ouabain, only early endosomes exhibited the vanadate or ouabain dependent stimulation of acidification activity, suggesting the inhibition of Na^+-K^+-ATPase.

Therefore, it seems probable that the inhibition of early endosome acidification by Na^+-K^+-ATPase observed *in vitro* at least in part plays a physiological role in controlling the acidification of early endosomes *in vivo*.

Key Words: Early and late endosome, Vacuolar H^+-ATPase, Confocal microscopy, FITC fluorescence, Na^+-K^+-ATPase

INTRODUCTION

Endosomes are a heterogenous class of organelles that play a central role in maintaining the orderly traffic and processing of receptors and ligands internalized during endocytosis (Helenius et al, 1983; Iris et al, 1992; Brown et al, 1983; Geuze et al, 1984). It is now well established for endosome to have an acidic internal pH, which is critical to

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dissociation of many receptor-ligand complexes, the first step in the intracellular sorting of internalized receptors and ligands (Mellman et al, 1986). Dissociation typically results in the recycling of free receptors back to the cell surface and the delivery of discharged ligands to lysosomes for degradation. However, for receptor-ligand complexes, which fail to dissociate at low pH, endosomes can also mediate other transport events. For example, transferrin returns back to the plasma membrane bound to its receptor (Iris et al, 1992; Klausner et al, 1983), macrophage-lymphocyte Fc receptor is selectively targeted to lysosome without discharging its ligand (Dautry-Varsat et al, 1983; Mellman & Pluter, 1984), and ricin is routed to the trans-Golgi network or cytoplasm (Bruno et al, 1993).

While endosomes, like lysosomes, are known to lower their internal pH by an ATP-driven proton pump (Merion et al, 1983; Galloway et al, 1983; Yamashiro et al, 1984), the mechanisms that regulate acidification in either organelles are unclear. Lysosomes are typically the most acidic organelle in mammalian cells and generally maintain their internal pH at 4.0~5.0 (Ohkuma et al, 1982). Endosomes exhibit a great range of pH values that can vary between 5.0 and 6.5 within a single cell (Yamashiro et al, 1984; Murphy et al, 1984; Shi et al, 1991). Given the role played by endosomal pH in regulating endocytic membrane traffic, it is likely that the observed variations in pH are functionally significant. In intact cells, materials internalized during endocytosis encounter endosomes of increasingly acidic pH en route to lysosomes (Merion et al, 1983; Murphy et al, 1984). This gradient in pH reflects the existence of distinct subpopulations of endosomes that differ not only in their capacity for acidification but also in composition and function (Schmid et al, 1988).

Although the factors that regulate endosomal pH remain unknown, several hypotheses could explain the mechanisms by which the rate of endosome acidification and the steady-state endosome pH could be regulated. The rate of acidification depends upon the number and activity of proton pumps, the movement of counterions, the membrane potential, and the passive proton conductance (Mellman et al, 1986; Xie et al, 1983; Van Dyke, 1988; Fuchs et al, 1989). There is evidence that acidification of early endosomes is regulated by a positive interior membrane potential generated by an ouabain-sensitive $3Na^+$-$2K^+$ pump in cultured fibroblasts (Fuchs et al, 1989; Cain et al, 1989), but not in rat liver endocytic vesicles (Anbari et al, 1994). In endosomes from rabbit kidney, a chloride channel has been identified which is activated by protein kinase A-dependent phosphorylation (Storrie, 1988). In contrast the rate of acidification, the steady-state endosome pH would depend more upon the number/activity of proton pumps and the proton conductance than upon the permeability of counterions. There has been no direct experimental evidence to demonstrate the existence of heterogeneity in the activity of proton pumps or the passive proton conductance in endocytic vesicles. Because fusion events between early endosomes and more acidic intracellular vesicles occur it is possible that the number of proton pumps might vary considerably among endosomes. It is more difficult to devise a scheme by which passive proton conductance could be regulated, particularly because regulated proton channels have not been demonstrated in biological membranes.

Understanding the basis for the structural and functional heterogeneity of endosomes is critical for understanding the control of membrane traffic on the endocytic pathway. In this study endocytic compartments were fractionated into subpopulation using percoll gradient. The goal of this experiment was to determine whether ATP-dependent acidification was heterogeneous and whether the differences in acidification were attributed to differences in the activity of Na^+-K^+-ATPase and/or Cl^- channel.

METHODS

Cells and Cell Culture

Chinese hamster ovarian (CHO) cells, obtained from the American Type Culture Collection (ATCC), were maintained by serial passages in 75 cm^2 tissue culture flasks (Corning). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co.) supplemented with 5% fetal calf serum (Gibco Lab, Grand Island, NY), penicillin (100 U/ml), and streptomycin (100 g/ml) and were incubated at 37°C in 5% CO_2 in a humidified air atmosphere. At confluency (1.5×10^7 cells per flask), the cells were trypsinized and plated on 15 cm tissue culture dishes. Cells were routinely deprived of serum for 24 hr before experiments.

Endosome Labeling

Endosomes were labeled with pH-sensitive endocytic tracers, fluorescein isothiocyanate (FITC)-conjugated dextran (FITC-dextran, M.W.: 70,000, Sigma). FITC-dextran was dialyzed for 48 hr against three changes of 100 volumes each of phosphate buffered saline (PBS). Labeling with FITC-dextran (5 ~ 10 mg/ml) was performed in complete medium at 37°C for 15 min to get total endosomes, for 5 min to label early endosomes selectively, and 5 min followed by a 10-min chase in FITC-dextran-free medium to label late endosomes selectively (Schmid et al, 1988). After the labeling period, the monolayers were washed several times with ice cold PBS and harvested with a rubber scraper.

Isolation of Enriched Endosome Fractions

FITC-dextran-labeled cells were washed three times by centrifugation (350 × g, 5 min at 4°C) and resuspended in cold PBS. The final pellet was suspended in cold TEA buffer (0.25 M sucrose; 10 mM triethanolamine; 10 mM NaOAc; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride, pH 7.4) and disrupted with a Dounce homogenizer. The homogenate was centrifuged at 1,200 × g for 10 min to generate a postnuclear supernatant. Crude membrane fraction obtained by centrifuging postnuclear supernatant at 40,000 × g for 15 min (17,000 rpm; JA-17 rotor, Beckman) could be used freshly for the experiments of averaged data. For fractionation, the pellet was resuspended with 1 ml of homogenization buffer and then applied to a 1.05 g/ml self-performing percoll gradient (30 ml) and centrifuged at 40,000 × g for 45 min (Storrie, 1988). Fractions (1.2 ml) collected from the top of the tube, were analyzed for the fluorescene of FITC-dextran.

Cell-Free Assay of ATP-Dependent Acidification

ATP-dependent acidification of isolated endosomes was determined using FITC-dextran-containing endosomes in suspension. Endosomes were equilibrated for 2 ~ 4 hr at 0°C in iso-osmotic buffer, conditions that completely dissipated any preexisting pH gradients. Endosomes representing 50 g of protein were placed in a acrylic cuvette at 37°C and FITC fluorescence intensity was determined with a spectrofluorometer (Spex CM3000) with excitation and emission wavelengths set at 495 nm and 515 nm, respectively. Acidification was initiated by the addition of 1 mM ATP (stock 100 mM ATP titrated to pH 7.4) and followed with time as a decrease in FITC fluorescence intensity. After endosomal pH reached a steady-state level, the pH gradient was collapsed by addition of 5 μM nigericin. Absolute endosomal pH was calculated from the fluorescence vs pH relation and the endosomal FITC-dextran signal determined by addition of the anti-FITC antibody to quench external concentration of 1 mM and added during the 2- to 4-hr equilibration step at 0°C.

Enzyme Assays

The marker enzymes for lysosome (β-galactosidase) and for plasma membrane (alkaline phosphatase) were assayed according to Pool et al using the fluorogenic substrates 4-methylumbelliferyl-

thymidine 5'-phosphate (Pool et al, 1983). Protein was assayed using the Bradford assay (Bradford, 1976).

Subcellular Localization of Endosomes

For the studies designed to identify the location and morphology of endosomes, confocal microscopy was used. CHO cells were cultured on the coverslips which had been washed with acetone and ethanol, and autoclaved. After labeling endosomes with FITC-dextran by pulse and chase method, coverslips were washed 5 times with the ice-cold PBS and fixed for 30 mins with 2.5% (vol/vol), glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). Coverslips were then rinsed with PBS and mounted on clean slide glass with help of one drop of Aqua-poly mount (Polyscience) and examined under the confocal microscope.

RESULTS

ATP-Dependent Acidification of Whole Population of CHO Endosomes

Recent studies using organelles labelled with endocytic tracers conjugated to pH-sensitive probe FITC showed that endosomes, like many other elements of the vacuolar apparatus, lower their internal pH in vitro via the activity of an N-ethylmaleimide (NEM)-sensitive ATPase (Galloway et al, 1983; Schmid et al, 1988; Anbari et al, 1994). In order to characterize the mechanism of acidification in CHO cells, ATP-dependent acidification was measured in crude membrane fraction at first (Fig. 1).

Addition of 1 mM ATP caused a rapid quenching of FITC fluorescence reflecting intravesicular acidification. Acidification of endosomes in response to external ATP was blocked by removal of permeable ions or by 1 mM NEM. In the absence of Cl^-, K isethionate alone induced submaximal acidification, which was stimulated by the K^+ ionophore valinomycin by permitting the rapid efflux of internal

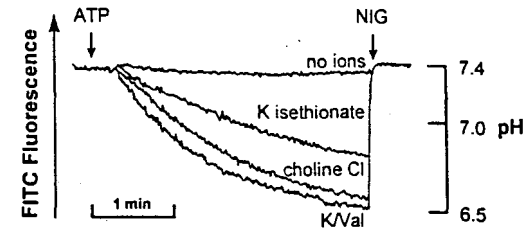

Fig. 1. Effects of various ions on the endosomal ATP-dependent acidification. Endosomes were loaded and suspended with the solution of 300 mM mannitol and 20 mM Hepes/Tris buffer (no ions); 100 mM mannitol, 100 mM K isethionate and 20 mM Hepes/Tris buffer (K isethionate); 100 mM mannitol, 100 mM K isethionate, 5 μM valinomycin and 20 mM Hepes/Tris buffer (K/val); 100 mM mannitol, 100 mM choline cholide and 20 mM Hepes/Tris buffer (choline Cl).

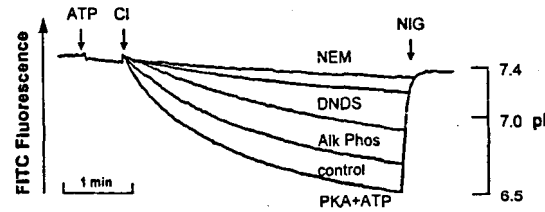

Fig. 2. The effect of phosphorylation on ATP-dependent acidification. ATP (1 mM), choline cholide (40 mM) and nigericin (5 μM) were added where indicated. Endosomes were pretreated with 100 Uml^{-1} alkaline phosphatase (Alk phos), 10 Uml^{-1} PKA +0.5 mM ATP (PKA+ATP) or 0.5 mM ATP alone in the absence (control) or the presence of 0.5 mM NEM (NEM) or 0.1 mM DNDS (DNDS). The reaction was started with the addition of 1 mM ATP.

K^+. Chloride in the absence of permeable cations gave strong acidification, although less than that of K^+/valinomycin. Thus, ATP-driven endosome acidification is electrogenic and, like acidification of isolated lysosome, and coated vesicle, it is dependent on the membrane permeability to Cl^-.

To investigate the regulatory role of Cl^- conductance on the endosomal acidification, ATP-dependent acidification under condition in which Cl^- was the major permeable ion was measured in Fig. 2. Phos-

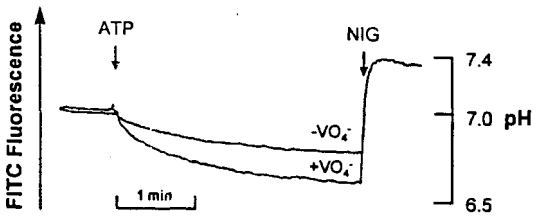

Fig. 3. Inhibition of endosome acidification by Na^+-K^+-ATPase activity. CHO endosomes were equilibrated with Na^+/K^+ buffer (145 mM NaCl; 5 mM KCl; 10 mM tetramethylammonium Hepes, pH 7.4; 5 mM $MgCl_2$). Sodium orthovanadate ($+VO_4^-$) was induced at a final concentration of 1 mM in parallel samples just before the final centrifugation.

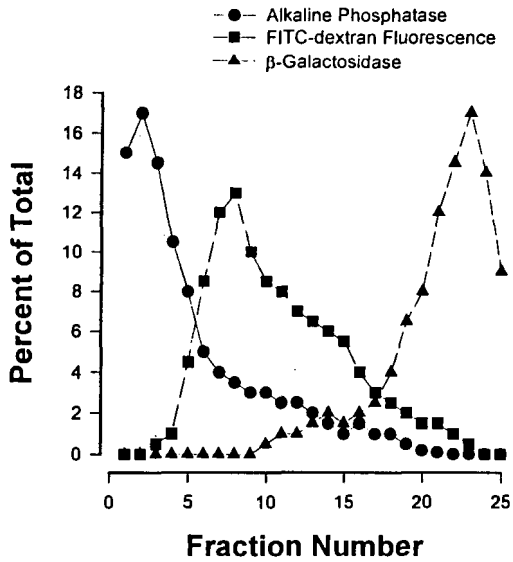

Fig. 4. Internalization of FITC-dextran in percoll density gradient fractionation. CHO cells were incubated with PBS solution containing FITC-dextran at 37°C for 15 min. Postnuclear supernatant was then prepared and subjected to density gradient separation. Fractions (1.2 ml) were collected and activities were determined.

phorylation of endosomes by PKA and ATP enhanced ATP-dependent acidification. Treatment with alkaline phophatase caused a remarkable decrease in acidification. Acidification was strongly inhibited by inhibition of the proton pump (NEM) or Cl^- conductance (DNDS). Thus, Cl^- is an effective counterion for control of ATP-dependent acidification and that PKA acts on a stilbene-sensitive Cl^- conductance. Na^+-K^+-ATPase might also act to regulate endosomal acidification by changing transmembrane potential.

To test this possibility, the effect of Na^+-K^+-ATPase inhibitor on the ATP-dependent acidification was examined in endosomes equilibrated in an iso-osmotic buffers containing 145 mM Na^+ and 5 mM K^+, ionic conditions that favor the activity of Na^+-K^+-ATPase (Fig. 3). Preincubation of endosomes with 1 mM sodium orthovanadate (Na_3VO_4), a selective Na^+-K^+-ATPase inhibitor increased the degree of ATP-dependent acidification presumably by reversing inhibition effect of acidification by Na^+-K^+-ATPase. This observation supports the possibility of the involvement of Na^+-K^+-ATPase in regulating endosomal acidification.

Subcellular Fractionation of CHO Endosomes

Endosomes are a heterogenous compartment which has several different subpopulations probably according to the different stages in the maturation of their pathway (Murphy, 1991). To understand the structural and functional heterogeneity of endosomes, fractionation of postnuclear supernatants was done by self-forming percoll gradients (1.105 g/ml). CHO endosomes were uniformly labeled by internalization of fluid phase marker for a single 15 min pulse. Fig. 4. shows the separation profile of crude membrane fraction on percoll gradient. FITC-dextran-labeled endosomes detected by FITC fluorescence, distributed as a broad band with a peak at fraction number 8. This endosome fraction was well separated from the high density lysosome fraction containing the activity of marker enzyme, β-galactosidase, and from the low density plasma membrane containing alkaline phosphatase activity.

Because the peak of fractionation profile after a single 15 min pulse was too broad to separate into a distinct subpopulation, CHO cells were fractionated after labeling under conditions that kinetically

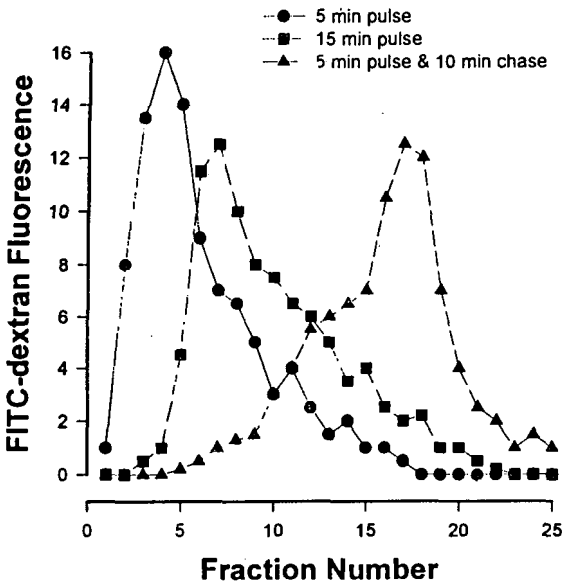

Fig. 5. Resolution of early and late endosomes by percoll density gradient fractionation. CHO monolayers were incubated with PBS solution containing FITC-dextran at 37°C for 5 min to label early endosomes or for 5 min followed by a 10 min chase in FITC-dextran-free medium to label late endosomes.

defined either "early" or "late" endosomes. Early endosomes were labeled by incubation of FITC-dextran for 5 min at 37°C. A further incubation for 10 min in marker-free medium was used to chase internalized FITC-dextran into kinetically defined late endosomes. For comparison, all possible endosome populations were uniformly labeled by continuous incubation medium containing FITC-dextran for 15 min at 37°C. As shown in Fig. 5, all labeled endosome subpopulations were shifted toward the bottom of the tube and were well resolved from plasma membrane and from the bulk of microsomal proteins. However, the early and late endosomes labeled by FITC-dextran were not deflected equally. The 5 min FITC-labeled early endosomes were shifted less to the bottom than the 15 min FITC-labeled total endosomes (Fig. 5). After a 10 min chase into late endosomes, however, the peak of FITC-dextran fluorescence was shifted more to the bottom, higher density in a percoll gradient than the total endosome. These data demonstrate fluid-phase endocytic markers are first found in endosomes of relatively low density but are transferred to much higher density endosomes at later times. Thus, percoll gradient in combination with various pulse/chase labeling techniques can separate crude membrane fraction into early and late endosome subpopulations.

Localization of FITC-dextran in CHO cells

In order to identify the various endocytic compartments after internalization, cells grown on coverslips were examined with confocal microscope. For information of labeling kinetics endosomes were labeled with various intervals of pulse and chase of FITC-dextran: 5 min pulse (early endosome), 5 min pulse and 10 min chase (late endosome), 15 min pulse (total endosome) and 5 min pulse and 30 min chase (lysosome). With 5 min labeling, FITC fluorescence was localized just beneath the plasma membrane and features of early endosome judged from boundary of FITC-fluorescence were tubular-cisternal-vesicular (Fig. 6A). In contrast to these findings, 5 min pulse and 10 min chase shows FITC fluorescence localized deep in cytoplasm far from plasma membrane and shows typical vesicular features (Fig. 6C). And further chasing after 5 min pulse made the fluorescence locate deeper and near nucleus(data not shown). 15 min pulse without chase shows the fluorescence scattered in the whole area of the cytoplasm from plasma membrane to the nucleus (Fig. 6B).

These data showed that early and late endosomes have different locations in the cells and distinct morphology and that the labeling method used in this study made it possible to discriminate early and late endosomes.

ATP-dependent Acidification of Early and Late Endosomes

It has been demonstrated that the two distinct

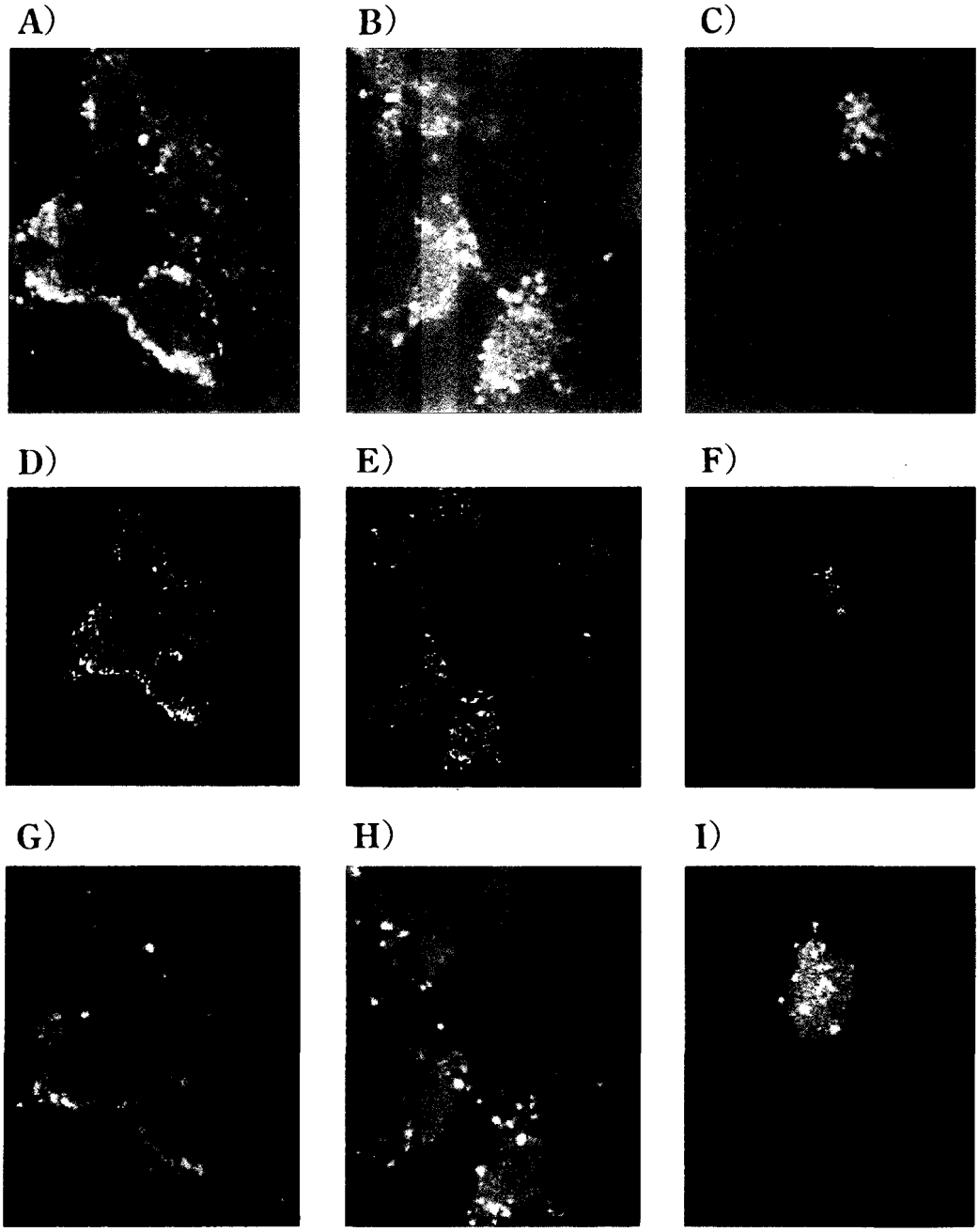

Fig. 6. Intracellular localization of internalized FITC-dextran in CHO cells. Cells were grown on the coverslips and loaded with FITC-dextran for 5 min-pulse (A, D, G), 15 min-pulse (B, E, H) or 10 min-chase after 5 min-pulse (C, F, I). Labeled coverslips were washed with ice-cold PBS, fixed and examined under the confocal microscopy. A-C; Epifluoresence images of FITC-dextran (orange) overlapped with phase contrast images in the same fields. D-F; Epifluorescence images of FITC-dextran (white) obtained from the middle section of cells in y axis. G-I; Three dimensional images of FITC fluorescence in the cells reconstructed with the 10-15 sections of y axis.

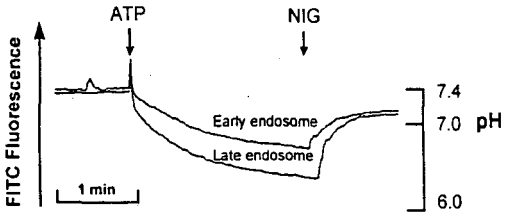

Fig. 7. ATP-dependent acidification of early and late endosomes. CHO cells were incubated at 37°C with the solution containing FITC-dextran for 5 min (to label early endosomes) or for 5 min followed by an 10 min chase in the absence of FITC-dextran (to label late endosomes). Microsomal fractions were prepared and equilibrated by incubation for 2 hr at room temperature in a buffer containing 100 mM mannitol, 100 mM NaCl, 2 mM $MgSO_4$ and 20 mM Hepes/Tris(pH 7.4). Acidification was measured after addition of 1 mM ATP.

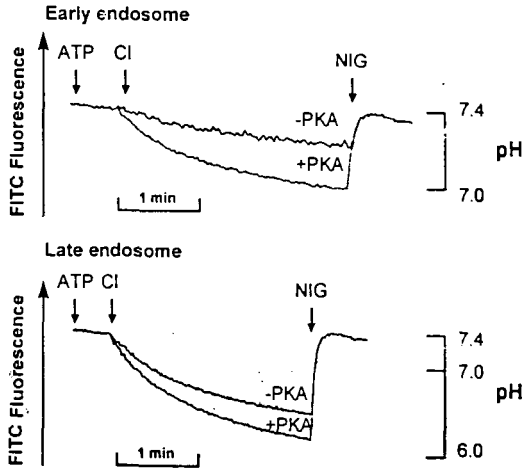

Fig. 8. Effect of phosphorylation on ATP-dependent acidification of early and late endosomes. ATP (1 mM) and choline chloride (40 mM) were added where indicated. Early (A) or late endosomes (B) were labeled with the same method and preincubated with 10 Uml^{-1} PKA and 0.5 mM ATP (+PKA) or 0.5 mM ATP alone (-PKA).

endosome subpopulations are associated with distinct sorting functions (Schmid, 1988); early endosomes are involved in receptor recycling and late endosomes are involved in the delivery of ligands to lysosomes. Since pH is known to be important for endosomal function and since in vivo studies have shown that internalized ligands encounter decreasing pH as they traverse the endocytic pathway, the acidification properties of early and late endosomes were studied first using FITC-dextran endocytic tracers in conjunction with established in vitro acidification assays (Galloway et al, 1983).

CHO cells were incubated at 37°C in the presence of FITC-dextran for 5 min to label early endosomes, or for 5 min followed by 10 min in the absence of FITC-dextran to label late endosomes. Crude microsomal fraction prepared before seperation by percoll gradient was used for comparison. The labeled endosomes were equilibrated in KCl buffer and ATP-dependent acidification was assayed by the quenching of FITC fluorescence (Fig. 7). All fractions exhibited acidification activity, indicating that both early and late endosomes contain an ATP-dependent proton pump. However, both the initial rate of acidification and extent of proton translocation were lower in early endosomes and gradually increased in late endosomes. To investigate whether phosphorylation by PKA has different effect on the ATP-dependent acidification of early and late endosomes, the fractionated endosomes were subject to both PKA and ATP (Fig. 8). Phosphorylation by PKA and ATP enhanced ATP-dependent acidification in both early and late endosomes. However, there was no difference in the degree of enhancement by phosphorylation between two subpopulations.

The possible physiological importance of Na^+-K^+-ATPase mediated inhibition of acidification activity would be supported by the fact that it occurs only in the subpopulation of early endosomes, which generally are thought to have a less acidic pH than late endosomes. To test this possibility, ATP-dependent acidification was determined in the presence or absence of Na_3VO_4. As shown in Fig. 9, only early endosomes exhibited the vanadate dependent stimulation of acidification activity, characteristic of the vanadate-mediated inhibition of Na^+-K^+-ATPase.

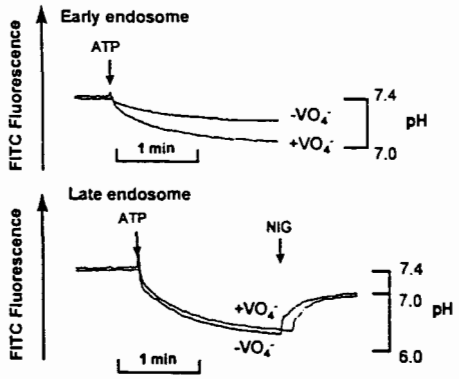

Fig. 9. Effect of Na_3VO_4 on ATP-dependent acidification of early and late endosome. Early (A) or late endosomes (B) were equilibrated in Na^+/K^+ buffer same as that used in Fig. 3. Sodium orthovanadate was induced at a final concentration of 1 mM in parallel samples just before the final centrifugation.

Thus, it seems probable, that the inhibition of early endosomes acidification by Na^+-K^+-ATPase observed *in vitro* at least in part plays a physiological role in controlling the acidification of early endosomes *in vivo*.

DISCUSSION

To determine whether ATP-dependent acidification was heterogenous and whether the degrees in acidification were attributed to differences in the activity of Na^+-K^+-ATPase and/or Cl^- channel, endocytic compartment was fractionated into subpopulations using percoll gradient and ATP-dependent acidification of each subpopulation was measured in CHO cells.

The fluid-phase marker FITC-dextran (79 kD) was used to label endocytic vesicles in these studies. FITC-dextran fluorescence is sensitive to vesicle volume and pH, which has made possible the measurement of osmotic water, solute and ion-coupled proton permeabilities, and ATP-dependent acidification in endocytic vesicles (Verkman et al, 1988; Shi & Verkman, 1989). FITC has good optical properties for use as an endocytic marker including high quantum yield (0.9) and molar absorbance (8×10^4 $M^{-1}cm^{-1}$), and blue/green excitation and emission wavelengths. Fluorophore brightness is particularly important because of the small size of endocytic vesicles: there were only 120 molecules of FITC in a vesicle of 150 nm diameter labeled in vivo with FITC. Another advantage of FITC is the availability of the anti-FITC antibody, an impermeant, and very efficient quencher of extravesicular FITC that is bound nonspecifically to the external vesicle surface or to the positively charged poly-*l*-lysine (Shi et al, 1991). Disadvantages of FITC-dextran are a moderately high rate of photobleaching and the inability to measure pH by an excitation ratio method because of the relatively poor signal-to-background at a 450 nm excitation wavelength. Photobleaching was minimized by removal of solution oxygen and use of low illumination intensity. Pyranine and BCECF (2',7'-bis (2-carboxyethyl)-5(6)-carboxy-fluorescein)-labeled dextran are potentially useful fluid-phase markers for a ratiometric measurement of pH in individual endocytic vesicles in the pH range 6.5-8.0 (Straubinger et al, 1990).

Endosomes represent an important intracellular site from which internalized plasma membrane, receptors, and extracellular macromolecules are selectively transported to one of several possible destinations (Helenius et al, 1983; Geuze et al, 1984; Mellman et al, 1986). Morphological studies had suggested that the endosomes involved in these sorting processes were both structurally and functionally heterogeneous. Internalized receptor-ligand complexes are first delivered to electrolucent vesicles and tubules near the periphery of the cell (Geuze et al, 1984; Wall & Hubbard, 1984; Marsh et al, 1986). Receptor recycling is thought to occur from the tubular extensions (Geuze et al, 1984), while dissociated ligands are carried into the perinuclear region of the cell where they are found in vesicles and multivesicular bodies before delivery to lysosomes. Heterogeneity has also been indicated by previous cell fractionation studies using rat liver (Wall & Hubbard, 1984) and BHK-21 cells (Marsh

et al, 1986).

The data described demonstrate that endosomes consist of at least two distinct but kinetically related subpopulations. Internalized receptor-bound ligands and soluble fluid-phase markers must pass sequentially through each of these compartments before reaching lysosomes. Early endosomes, aside from being the first endosomal compartment labeled by endocytic tracers, are functionally distinguished from late endosomes in that they appear to be the primary site of recycling for a variety of internalized plasma membrane proteins. Late endosomes (8～60 min; $t_{1/2}$ = 25 min) also argues against passage of recycling membrane proteins through this compartment. The role of early endosomes in recycling is further supported by the observation that early endosomes share some protein components with the plasma membrane. Late endosomes, whose composition is completely distinct from the plasma membrane, would thus appear to be more directly involved in delivery of internalized material to lysosomes.

A functional distinction between early and late endosomes is indicated by their acidification properties. The receptor-ligand sorting functions of endosomes are dependent on their acidic internal pH (Mellman et al, 1986). Given that the sorting activities of early and late endosomes are physically distinct, it is of interest that their acidification properties are also distinct. Early endosome acidification *in vitro* occurs at a slower rate and to a lesser extent than for late endosomes, which is consistent with observations in intact cells. This indicates that early endosomes are generally less acidic than late endosomes and lysosomes (Merion et al, 1983; Murphy et al, 1984; Schmid et al, 1988). However, since these experiments could not demonstrate a precursor-product relationship between the two populations, their functional significance was not established. It has also been unclear whether endosome heterogeneity reflected the existence of distinct organelles defined by their own specific sets of resident proteins or simply the different stages of maturation of a single organelle. The development of procedures to isolate and resolve discrete endosome subpopulations has now produced some fundamental insights into the activities of these organelles involved in the sorting and regulation of intracellular membrane traffic.

Although all endocytic organelles appear to lower their internal pH via the activity of similar if not identical H^+-ATPases (Mellman et al, 1986), the means by which these characteristic pH values are maintained are unclear. Given the small and variable internal volumes of endosomes (in the range of 10～12 ml; Marsh et al, 1986) and, consequently, the small number of H^+ needed to achieve pH values between 5 and 6, it is unlikely that such careful regulation is mediated by controlling the number of H^+ pumps per vesicle. The present finding that the endosomal H^+ pumps, like its lysosomal counterpart (Ohkuma et al, 1982), is electrogenic suggests that acidification may be regulated by alterations affecting the ion permeability characteristics of early vs late endosomes. Such a mechanism would be expected to influence the net accumulation of intravesicular H^+ at equilibrium as well as the activity of the H^+ pump itself. However, it is not yet clear that different endosome subpopulations have distinct permeability properties.

The results of this study suggest the modulation of electrogenic ATP-driven H^+-transport by the activity of the vanadate-sensitive Na^+-K^+-ATPase; and, most interestingly, evidence for the Na^+-K^+ ATPase could only be found in early endosomes, the less acidic of the two endocytic organelles. The possible mechanism that the Na^+-K^+ ATPase might act to regulate acidification in early endosomes is presumed as follows. Conceivably, some molecules of Na^+-K^+-ATPase are internalized during endocytosis (Jesaitis & Yguerabide, 1986). The predicted orientation of the Na^+-K^+-ATPase in the endosomal membrane would result in the coupled transport of 3 Na^+ into the endosome for every 2 K^+ out, which would thus generate an interior-positive membrane potential opposing electrogenic ATP-driven H^+

transport. Concentrations of Na^+ needed to elicit this effect *in vitro* (>25 mM) are in the range of normal cytosolic Na^+ concentration (5～35 mM). Since the Na^+ effect was seen both in the presence of external Cl^- and gluconate, this mechanism of inhibition requires that the anion conductance of the endosomal membrane is limiting under these conditions, ie., for the Na^+-K^+-ATPase to generate an interior positive membrane potential in the presence of an external permeant anion, Na^+ transport must proceed faster than the rate of Cl^- influx (which would otherwise dissipate the forming potential). Na^+ transport must also proceed faster than net ATP-driven H^+ transport, since the Cl^- conductance is sufficient to collapse the potential produced by the H^+ pump alone.

Taken together, our results suggest that the inhibition of acidification by the Na^+-K^+-ATPase results from a net increase in interior-positive membrane potential due to Na^+ transport, which in turn slows the activity of the potential-sensitive H^+-ATPase. However, the fact that Na^+-K^+-ATPase mediated inhibition was observed only in early endosomes, regardless of mechanism, supports its likely physiological role in helping to maintain the internal pH of early endosomes at the slightly acidic levels associated with facilitating the selective dissociation and intracellular transport of cell surface receptors involved in rapid recycling.

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