Acidification of Endocytic Vesicles by an ATP-dependent Proton Pump

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ABSTRACT One of the early events in the pathway of receptor-mediated endocytosis is the acidification of the newly formed endocytic vesicle. To examine the mechanism of acidification, we used fluorescein-labeled α_2-macroglobulin (F-α_2M) as a probe for endocytic vesicle pH. Changes in pH were determined from the change in fluorescein fluorescence at 490-nm excitation as measured with a microscope spectrofluorometer. After endocytosis of F-α_2M, mouse fibroblast cells were permeabilized by brief exposure to the detergent digitonin. Treatment with the ionophore monensin or the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) caused a rapid increase in the pH of the endocytic vesicle. Upon removal of the ionophore, the endocytic vesicle rapidly acidified only when MgATP or MgGTP was added. Neither ADP nor the nonhydrolyzable analog, adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP) could support acidification. The ATP-dependent acidification did not require a specific cation or anion in the external media. Acidification was insensitive to vanadate and amiloride but was inhibited by Zn^{2+} and the anion transport inhibitor diisothiocyanostilbene disulfonic acid (DIDS).

We also examined the acidification of lysosomes with the permeabilized cell system, using fluorescein isothiocyanate dextran as probe. DIDS inhibited the ATP-dependent reacidification of lysosomes, although at a lower concentration than that for inhibition of endocytic vesicle reacidification. These results demonstrate that endocytic vesicles contain an ATP-dependent acidification mechanism that shares similar characteristics with the previously described lysosomal proton pump.

Many different peptide hormones, serum proteins, bacteria toxins, and enveloped viruses bind to receptors on cell surfaces and are internalized by the process of receptor-mediated endocytosis (reviewed in references 1 and 2). For many ligands this pathway has been shown to involve clustering of occupied receptors over clathrin-coated pits and entry into the cell through endocytic vesicles. It has been demonstrated by fluorescent double labeling studies that the serum protease inhibitor α_2-macroglobulin (α_2M) is internalized within the same endocytic vesicle as diphtheria toxin (3), epidermal growth factor (4), insulin (4), low density lipoprotein (5), and thyroid hormone (6). These results suggest that ligands share a common entry point in the pathway of endocytosis.

The final destination for many of these ligands is lysosomes, where they are degraded. However, it has been demonstrated that a sorting process can take place that enables the receptor to escape degradation and return to the cell surface where it is reused. This has been shown to occur for several receptors, including the α_2M receptor (7), asialoglycoprotein receptor (8–10), insulin receptor (11), low density lipoprotein receptor (12), and the mannose receptor (13). For this differential processing to occur, it is evident that the ligand must dissociate from its receptor, allowing the unoccupied receptor to recycle to the cell surface.

We have previously demonstrated that endocytic vesicles containing α_2M rapidly acidify to a pH of 5.0 ± 0.2 (14). Acidification of nonlysosomal endocytic vesicles has also been shown to occur for the internalization of transferrin by human erythroleukemia cells (15) and asialo-orosomucoid by human hepatoma cells (Tycko, B., C. H. Keith, and F. R. Maxfield, manuscript submitted for publication). Since many ligands dissociate from their receptors at acidic pH (16–20), we have suggested that acidification of endocytic vesicles is responsible for the uncoupling of ligands from their receptors (14, 20, 21). This mechanism is supported by studies that have shown that weak bases and ionophores that can dissipate the endocytic vesicle pH gradient (20) interfere with receptor recycling (7, 12, 13, 18, 22). Recent evidence has demonstrated that dissociation of the ligand-receptor complex can occur within endocytic vesicles and is mediated by changes in the intravesicular pH (23).

The mechanism by which the endocytic vesicle creates an

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acidic environment has yet to be elucidated. The ability of the endocytic vesicles to reacidify after the ionophore monensin was removed from the medium (20), indicated that the vesicles contain a mechanism for re-establishing the pH gradient across their membranes. In this study, we used a digitonin permeabilized cell system to examine the acidification of endocytic vesicles. Our results indicate that acidification of endocytic vesicles is due to an ATP-dependent proton pump located in the endocytic vesicle membrane.

MATERIALS AND METHODS

Materials: Monensin was obtained from Calbiochem Corp. (La Jolla, CA), sodium vanadate from Fisher Scientific Co. (Fairlawn, NJ), and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)[1] from Aldrich Chemical Co. (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO). MgATP (vanadate free), ADP, adenosine 5′-(β,γ-imido)triphosphate (AMP-PNP), NaGTP, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), digitonin (80% pure), 4,4′-diisothiocyano-2,2′disulfonic acid stilbene (DIDS), fluorescein isothiocyanate dextran (FITC-dextran, 40,000 mol wt), and methylamine were obtained from Sigma Chemical Co. Amiloride was a gift of Merck, Sharpe, and Dohme Research Laboratories (West Point, PA).

α_2M was prepared from whole human plasma as previously described (24), with a slight modification (25). The preparative procedure converts native α_2M to a form that binds to the fibroblast α_2M-protease receptor with high affinity (21). Fluorescein-labeled α_2M (F-α_2M) was prepared as previously described (4).

Cell Culture: BALB/c 3T3 mouse fibroblast cells were grown in Dulbecco-Vogt modified Eagle's medium (DME; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) containing 10% (vol/vol) calf serum at 37°C under a 95% air–5% CO_2 humidified atmosphere. Culture dishes were prepared for fluorescence microscopy by punching 13-mm holes in 35-mm culture dishes (Falcon Labware, Oxnard, CA) and attaching coverslips to the bottom surface using a mixture of hot paraffin (Tissue-Tek, Fisher) and petroleum jelly (Vaseline) (3:1, vol:vol) to form a watertight seal. Dishes were sterilized under ultraviolet irradiation. Cells were plated at a density of $\sim 10^5$ cells/dish and were used 2–4 d after plating.

Quantitative Fluorescence Microscopy: Fluorescence intensities were measured using a Leitz MPV Compact microscope spectrofluorometer mounted on a Diavert microscope. The microscope was equipped with a 75-W xenon lamp for epifluorescence illumination, a 63× (N.A. 1.3) objective, and with 490- and 450-nm narrow bandpass filters. Cellular fluorescence was observed with a DAGE/MTI 65 MKII SIT camera and fluorescence intensity simultaneously measured with a photomultiplier tube. The changes in the internal pH of endocytic vesicles and lysosomes were determined from the change in fluorescein intensity at 490-nm excitation. Techniques for intensity measurements and video intensification microscopy have been previously described (14, 20, 26).

BALB/c 3T3 cells were incubated with F-α_2M (150 μg/ml) for 12 min in DME at 37°C in 95% air–5% CO_2. To remove free F-α_2M, cells were rinsed four times with 2 ml of Medium 1 (low K^+) which contained 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 30 mM sucrose, and 20 mM HEPES, pH 7.4. The dish was then placed on a microscope stage which, once positioned, was sufficiently rigid to prevent dislocation when various substances were added by pipette or removed by suction. Substances were added in appropriate buffers at a pH of 7.4.

A group of two to six cells were centered in the measuring area as observed on the television monitor under bright-field illumination. An initial intensity measurement at 490-nm excitation (I_{init}) was taken with 0.1-s illumination. Digitonin in 1 ml of Medium 1 was pipetted into the dish for a final concentration between 30–80 μg/ml. The concentration of digitonin required for a set of parallel culture dishes was determined from the concentration that caused over 95% of the cells to become permeabilized as assessed by trypan blue uptake. In addition, the permeability of the experimental cells could be determined by addition of trypan blue at the termination of the experiment. After 1–2 min the digitonin was removed by suction and the cells rinsed three times (2 ml) with either Medium 1 or Medium 2 (low Na^+) that contained 150 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 30 mM sucrose, and 20 mM HEPES, pH 7.4.

The pH gradient of the endocytic vesicles was dissipated by adding one of the following ionophores; monensin (in Medium 1, final concentration 10 μM), FCCP (in Medium 2, final concentration 10 μM), or CCCP (in Medium 2, final concentration 10 μM). Cells were treated with the appropriate ionophore for 2 min, the ionophore removed, and the cells rinsed three times (2 ml) with the same medium. MgATP or other agents were then added. NaGTP, AMP-PNP, and ADP were added with equimolar amounts of $MgCl_2$. Inhibitors, when used, were added 1 min prior to addition of MgATP. After 5 min, methylamine (final concentration 20 mM) or monensin (final concentration 7.5 μM) was added to dissipate any pH gradient produced. The acidifications obtained with the different experimental conditions were compared to ATP controls done with dishes plated from the same culture ("parallel culture dishes").

Intensity measurements were normally taken (*a*) before addition of digitonin (I_{init}), (*b*) after removal of digitonin prior to addition of the ionophore, (*c*) after 2 min incubation with the ionophore, (*d*) prior to addition of ATP ($I_{ATP,0min}$), (*e*) 3 min after ATP addition ($I_{ATP,3min}$), (*f*) 5 min after ATP addition, and (*g*) 1 min after addition of methylamine. The loss of fluorescence intensity due to photobleaching over the time course of the experiment was <5% of the total intensity. A parameter related to the extent of reacidification, the relative fluorescence intensity (RFI, in percent), was determined from:

$$RFI = 100 \times (I_{ATP,0min} - I_{ATP,3min})/(I_{ATP,0min} - I_{init})$$

If one assumes that the pH prior to addition of ATP ($I_{ATP,0min}$) is 7.4, then the pH after addition of ATP can be determined by reference to a standard curve, such as the one previously obtained using the same instrument (20). A reacidification >50% corresponds to pH values below 6.5. Approximately 2–3 min were required between rinsing of the cells and the first intensity measurement. This should allow for the internalization of nearly all receptor-bound F-α_2M (27). Extracellular F-α_2M should not contribute to the RFI value, since all buffers were at pH 7.4.

To determine the ion dependence of reacidification, the following media were used: (*a*) K^+-free medium containing 155 mM NaCl, 1 mM $CaCl_2$, 30 mM sucrose, and 20 mM HEPES, pH 7.4; (*b*) Na^+-free medium containing 155 mM KCl, 1 mM $CaCl_2$, 30 mM sucrose, and 20 mM HEPES, pH 7.4; and (*c*) Sucrose medium, composed of 300 mM sucrose, 1 mM $CaCl_2$, 10 mM Tris-HEPES, pH 7.4.

FITC-dextran was used as an indicator of lysosomal pH (28, 29). BALB/c 3T3 cells were incubated overnight with FITC-dextran (2 mg/ml) at 37°C in DME containing 10% calf serum. Cells were rinsed with DME + calf serum and incubated for at least 30 additional minutes prior to use in experiments, following the procedures described for endocytic vesicles.

Computer Digitization of Fluorescence Images: Video images were obtained using a Dage/MTI 65 MKII SIT camera that is modified to allow for manual control of gain and black level and to linearize the intensity response curve. A Panasonic NV 8030 video tape recorder was used to record images. Images were digitized using a CAT-800 Image Digitizer housed in a Northstar 64K microcomputer. Digitized images were transmitted to a MINC 11/23 (Digital Equipment Corp., Marlboro, MA) for processing. Images from eight video frames were averaged and transmitted back to the CAT-800 to allow for video taping of a continuously displayed image. The averaged fluorescent image was then displayed on an Ikegami Model PM-121T high resolution television monitor and photographed. The fluorescence intensity of individual bright dots was determined as described by B. Tycko, C. H. Keith, and F. R. Maxfield (manuscript submitted for publication).

RESULTS

ATP-dependent Acidification

Fluorescein fluorescence is strongly pH dependent, and with excitation at 490 nM the fluorescence intensity increases sharply with increasing pH. Fluorescein labeled macromolecules have been widely used to examine the pH of other acidic intracellular compartments such as lysosomes by Ohkuma and Poole (28, 29) and pinosomes by Heiple and Taylor (26). We have previously used this characteristic of fluorescein to demonstrate that endocytic vesicles of BALB/c 3T3 mouse fibroblast cells have an acidic pH of 5.0 ± 0.2 (14).

To examine the mechanism by which endocytic vesicles become acidic, we have permeabilized cells with the detergent

[1] *Abbreviations used in this paper:* AMP-PNP, adenosine 5′-(β,γ-imido)triphosphate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone: DIDS, diisothiocyanostilbene disulfonic acid; F-α_2M, fluorescein-labeled α_2-macroglobulin; and FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

digitonin to permit the direct intracellular addition of substances such as Mg-ATP. By briefly exposing the cells to digitonin, and subsequently removing the detergent, we were able to selectively permeablize the plasma membrane leaving the endocytic vesicle relatively intact. Prolonged exposure to digitonin or high concentrations of the detergent (>100 μg/ml), caused the endocytic vesicles to become leaky and disrupted the pre-existing pH gradient. Treatment with digitonin has been previously used to observe ATP induced proton uptake into secretory granules of pancreatic β-cells in monolayer culture (30).

Mouse fibroblast cells were incubated with F-α_2M for 12 min and the fluorescence measured at 490-nM excitation with a microscope spectrofluorometer. It has been previously shown by electron microscopy, after a 15-min uptake of colloidal gold-α_2M and 5 min chase, that the ligand is predominantly in nonlysosomal compartments as determined by the absence of detectable acid phosphatase activity (14). Therefore, over the time course of the experiments the vast majority of F-α_2M is still in endocytic vesicles. This compartment has also been termed an endosome (1) or a receptosome (2).

After incubation with F-α_2M, free ligand was removed by repeated rinsing. The dish was placed on the microscope and a group of two to six cells were centered in the measuring area. Fig. 1 illustrates the results of a typical experiment. An initial fluorescence measurement was made (0 min), just prior to the addition of digitonin. The figure shows that brief treatment with digitonin did not permeabilize the endocytic vesicles to a great degree, since there was only a small rise in the fluorescence intensity.

Upon addition of the carboxylic ionophore monensin, there was a large and rapid increase in the fluorescence intensity, indicating that the pH gradient was being dissipated. Similar results were obtained with the protonophores FCCP (Fig. 2) and CCCP (data not shown). Treatment with all three ionophores caused a complete disruption of the proton gradient, raising the pH of the endocytic vesicles to approximately 7.4.

Previous work has shown that in intact cells, the effect of monensin was rapidly reversed when the ionophore was removed (20). In permeabilized cells, removal of the ionophore was not accompanied by acidification, (Fig. 1, open circles).

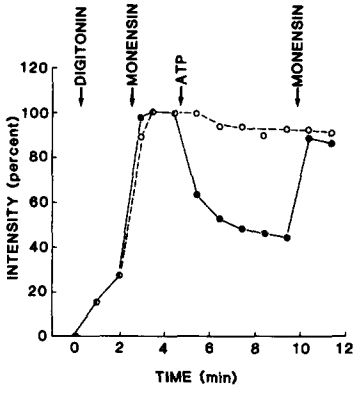

FIGURE 1 Time course of ATP-dependent acidification of endocytic vesicles. Cells incubated with F-α_2M were rinsed with Medium 1, placed on a microscope stage and their fluorescence intensity measured at 0 min, with 490 nm excitation. Cells were treated with digitonin (50 μg/ml) for 1 min, an intensity measurement taken, and the digitonin removed and the cells rinsed with Medium 1. At 2 min, a measurement was taken, monensin (10 μM final concentration) added, and measurements taken at 3 min and 3.5 min. After removal of monensin, either MgATP (closed circles, final concentration 3 mM) or Medium 1 (open circles) was added. Measurements were taken 1, 2, 3, 4, and 5 min after the addition of ATP or Medium 1. Monensin (7.5-μM final concentration) was added after 5 min to dissipate any pH gradient produced.

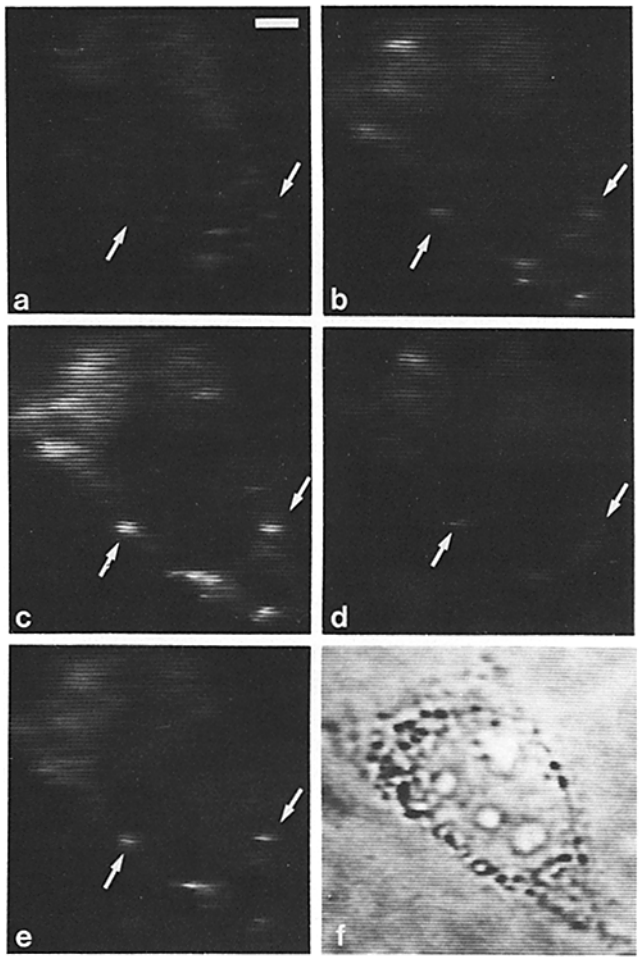

FIGURE 2 Fluorescence visualization of the ATP-dependent acidification of endocytic vesicles. Cells incubated with F-α_2M were rinsed with Medium 1 and placed on the microscope stage. Fluorescence images (*a–e*) of a single cell, with 490-nM excitation, were recorded on videotape. Images from eight frames were digitized and averaged. The processed image was videotaped, redisplayed and photographed from a high resolution monitor. The images are: (*a*) initial fluorescence; (*b*) after permeabilization and removal of digitonin; (*c*) after 2 min treatment and removal of FCCP (10 μM); (*d*) 3 min after the addition of 3 mM ATP; (*e*) 1 min after the addition of 20 mM methylamine; (*f*) the cell shown under bright-field illumination after treatment with digitonin. Bar, 4 μm. × 1,350.

However, with the addition of ATP there was a rapid decrease in the fluorescence intensity (Fig. 1, closed circles), indicating that a pH gradient was being established. The maximum acidification was reached after 3 min, with this change corresponding to a decrease of approximately 1 pH unit.

When monensin was added to the dish after 5 min of incubation with ATP, with fluorescence intensity rapidly increased. This result confirmed that ATP had caused a proton gradient to be re-established across the endocytic vesicle membrane. The pH gradient could also be dissipated by use of the weak base methylamine (Fig. 2). With cells in which no ATP was added, monensin did not cause a significant rise in the fluorescence intensity indicating that no significant pH gradient had been established.

The ATP-dependent acidification of the endocytic vesicles can be observed visually as demonstrated in Fig. 2. For each fluorescent image (Fig. 2, *a–e*), we digitized and averaged eight video frames and then photographed the resultant image.

Initially, the fluorescence was faint (Fig. 2 *a*), with dots visible in the later part of the experiment (arrows) barely detectable. After treatment with digitonin (Fig. 2 *b*), there was an increase in fluorescence and endocytic vesicles can now be seen. After treatment with FCCP (Fig. 2 *c*), there was a large increase in fluorescence and an increase in the number of dots visible. After incubation with ATP (Fig. 2 *d*), there was a marked decrease in the fluorescence, to a level similiar to the initial fluorescence. The fluorescence intensity of two bright dots (arrows) was determined by image digitization procedures and indicated that these vesicles had reacidified >65%. Upon treatment with methylamine (Fig. 2 *e*), the pH gradient produced by ATP was dissipated.

Nucleotide Specificity

Fig. 3 illustrates that the acidification produced by ATP is dose dependent and maximal at 3 mM. Throughout the course of this study, the ATP-dependent acidification was relatively constant within a set of parallel culture dishes. The error bars in Fig. 3 provide an estimate of the precision. There was however, a day to day variation in the maximal acidification obtained with 3 mM ATP. The relative fluorescence intensity (RFI) ranged from 50% to 100%, with these values corresponding to changes of approximately 1 to 2 pH units. It was therefore necessary to conduct ATP controls of each experimental condition, using dishes plated from the same culture. The acidification produced in cells in which no ATP was added was <10% for all experiments.

The majority of the remaining experiments of this study used the protonophore FCCP instead of monensin. The reason we switched ionophores, is that the carboxylic ionophore monensin exchanges protons for monovalent cations, with a 10-fold preference for Na^+ over K^+ (31). This will cause an alteration of the internal ionic composition of the endocytic vesicle and may affect the acidification.

Table I illustrates the nucleotide specificity of acidification. Reacidification could be partially supported by guanosine triphosphates, but not by ADP or the nonhydrolyzable analog AMP-PNP. ADP and AMP-PNP also prevented ATP-dependent acidification (Table II), indicating that these compounds interact with the ATPase site of the proton pump.

Electrolyte Requirements of ATP-dependent Acidification

Two different buffers were used in this study: Medium 1 (low K^+) and Medium 2 (low Na^+). Table III demonstrates that there is no significant difference between the extent of reacidification in the two buffers. In addition, media that contained only one type of monovalent cation, Na^+-free medium or K^+-free medium, and a sucrose buffer (containing 1 mM $CaCl_2$ and 10 mM Tris-HEPES) supported acidification. These results indicate that the ATP-dependent proton pump does not require a specific electrolyte in the external media. Since the internal ionic composition of the endocytic vesicle is unknown, it remains to be determined whether a specific ion is required for proton pump activity.

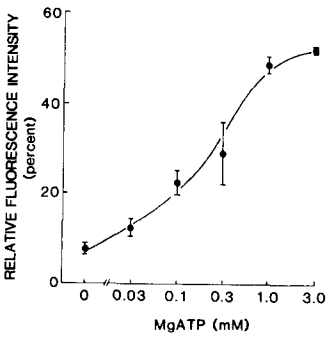

FIGURE 3 Dose response curve of ATP-dependent acidification. The procedure used in Fig. 1 was used for this experiment. The relative fluorescence intensity was calculated as described in Materials and Methods. Each point represents the mean of three determinations plus or minus the standard error.

TABLE I

Nucleotide Specificity for Acidification of Endocytic Vesicles

Nucleotide	Nucleotide-dependent acidification	ATP-dependent acidification
GTP (3 mM)	40	53
GTP (1 mM)	12	46
ADP (3 mM)	0	50
AMP-PNP (3 mM)	0	63

Cells incubated with F-α_2M were rinsed in medium 1 and the fluorescence of a group of two to six cells measured with 490-nM excitation. Cells were permeabilized with digitonin, rinsed with Medium 2, and treated with FCCP. After removal of FCCP, nucleotide was added along with an equimolar amount of $MgCl_2$ and the acidification determined after 3 min of incubation. The results are expressed in terms of the relative fluorescence intensity (percent) and are compared to the ATP-dependent acidification obtained in parallel culture dishes. The concentration of ATP was 3 mM, except in the case of GTP (1 mM), where 1 mM ATP was used for comparison. Each point represents the mean of at least three determinations.

TABLE II

Effect of Inhibitors on the ATP-dependent Acidification of Endocytic Vesicles

Inhibitor	ATP-dependent acidification	
	+Inhibitor	−Inhibitor
ADP (2.25 mM)	3	62
AMP-PNP (2.25 mM)	11	71
Amiloride (1 mM)	63	66
Vanadate (100 μM)	78	80
$ZnCl_2$ (1 mM)	1	62

Cells incubated with F-α_2M were rinsed in Medium 1 and permeabilized with digitonin. For experiments with ADP, AMP-PNP, and amiloride, FCCP was used to raise the endocytic vesicle pH, while monensin was employed for experiments with vanadate and $ZnCl_2$. The nucleotides, ADP and AMP-PNP, were incubated with cells for 5 min and then an equimolar concentration of ATP was added (2.25 mM final concentration). For experiments with amiloride, vanadate, and $ZnCl_2$, cells were preincubated with the inhibitor for 1 min, and then ATP (3 mM final concentration) plus inhibitor was added. The acidification is expressed as the relative fluorescence intensity (percent) and was calculated as described in Materials and Methods. Each point represents the mean of at least three determinations.

TABLE III

Electrolyte Requirement for the ATP-dependent Acidification of Endocytic Vesicles

Medium	ATP-dependent acidification
Medium 1 (low K^+)	76
Medium 2 (low Na^+)	98
K^+-free medium	99
Na^+-free medium	87
Sucrose medium	89

Cells were incubated with F-α_2M, rinsed in Medium 1, and treated with digitonin. The cells were then rinsed in Medium 2, treated with FCCP, and rinsed with the medium to be tested. ATP (3 mM final concentration) was added in the same medium. The acidification is expressed as the relative fluorescence intensity (percent) and was calculated as described in Materials and Methods. Each point represents the mean of at least three determinations.

Effects of Inhibitors of ATP-dependent Acidification

Table II demonstrates the effects of agents that have been reported to inhibit various ATP-dependent ion pumps. Vanadate, an inhibitor of the Na^+,K^+-ATPase, the Ca^{2+}-ATPase, and the K^+,H^+-ATPase (32–34), did not prevent acidification at concentrations up to 100 μM. The diuretic drug amiloride, which inhibits Na^+/H^+ exchange (35–38), did not prevent acidification. Zn^{2+} inhibited the ATP-dependent acidification and has been reported to inhibit the ATP-dependent acidification of lysosomes (39). DIDS, an inhibitor of anion transport in erythrocytes (40) and the lysosomal proton/phosphate pump (41, 42), partially inhibits the acidification of endocytic vesicles at a concentration of 100 μM, (Fig. 4).

Lysosomal ATP-dependent Proton Pump

Previous work by Schneider (41, 42) Ohkuma et al. (39) have demonstrated that hepatic lysosomes contain an ATP-dependent proton pump. We used our digitonin-permeabilized cell system to examine the acidification of intact lysosomes. FITC-dextran was used as the pH probe since it accumulates in lysosomes due to its resistance to lysosomal enzyme degradation (28). Fibroblast cells were incubated with FITC-dextran overnight and their fluorescence measured with the microscope spectrofluorometer. Cells were permeabilized with digitonin and treated with the protonophore FCCP. FCCP caused a rapid increase in fluorescein fluorescence. The protonophore dissipated the pH gradient to an average maximum of 74% as judged by the fluorescence increase obtained when 20 mM methylamine was added at the end of the experiment. The addition of 3 mM ATP caused a rapid decrease in the fluorescence intensity (data not shown).

Since DIDS partially inhibited the ATP-dependent acidification of endocytic vesicles, we wanted to determine if DIDS had a comparable effect on the acidification of lysosomes. As Fig. 4 demonstrates, the lysosomal ATP-dependent acidification is also inhibited by DIDS. This result is in excellent agreement with the work of Schneider (41, 42) who found maximal inhibition with 60 μM DIDS in intact lysosomes (41) and with 30 μM DIDS in lysosomal membrane vesicles (42). As shown here, the lysosomal acidification mechanism appears to be significantly more sensitive to the effects of DIDS than the endocytic vesicle acidification mechanism.

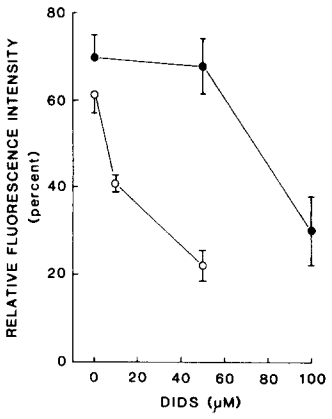

FIGURE 4 Inhibitory effect of diisothiocyanostilbene disulfonic acid on the ATP-dependent acidification of endocytic vesicles and lysosomes. A procedure similiar to that used in Fig. 2 was used for this experiment. Cells were incubated with either F-α_2M for 12 min or overnight with FITC-dextran. Each point for endocytic vesicles (closed circles) represents the mean of six determinations plus or minus the standard error. Each point for lysosomes (open circles) represents the mean of three to five determinations plus or minus the standard error.

DISCUSSION

One of the early events in the pathway of receptor-mediated endocytosis is the rapid acidification of the newly formed endocytic vesicle (14, 15, 20, 21). Evidence is accumulating which indicates that the creation of an acidic internal pH plays an important role in the intracellular routing of various ligands and receptors. Acidification appears to be involved with the penetration of viruses such as Semliki Forest into the cytosol (43). We have developed a permeabilized cell system to directly study the mechanism by which endocytic vesicles develop and maintain an acidic internal pH. This method allows us to examine the endocytic vesicle pH in situ with a minimum of manipulations. Since the endocytic pathway of α_2-macroglobulin has been extensively characterized, the pH measurements can be made at a time when F-α_2M is almost exclusively in endocytic vesicles (14). The results presented in this paper indicate that endocytic vesicles contain an ATP-dependent proton pump that can drop the pH within the vesicle below a value of 6.0.

There are several alternative mechanisms other than the presence of a proton pump which could account for the low pH within acidic organelles. For example, endocytic vesicles could be acidified at formation and then passively maintain the low pH. However, the observation that endocytic vesicles can quickly reacidify in intact cells (20) or in the presence of MgATP (Fig. 1) demonstrates that endocytic vesicles actively regulate their internal pH. Another alternative is that the proton gradient is established indirectly by creating a gradient in one ion (e.g., Na^+ or K^+) followed by a passive exchange with protons. This type of indirect acidification mechanism might involve the generation of a Na^+ gradient, established by the plasma membrane Na^+,K^+ ATPase, followed by Na^+/H^+ exchange resulting in acidification of the endocytic vesicle. This mechanism is unlikely, as the Na^+,K^+ ATPase inhibitor vanadate does not inhibit the ATP-dependent acidification. Furthermore, ATP-dependent acidification occurred equally well in media which had no external Na^+ or K^+, conditions which should completely alter the establishment of a Na^+/K^+ gradient. Acidification is also not inhibited by amiloride which inhibits Na^+/H^+ exchange (including a Na^+/H^+ antiport in Chinese hamster lung fibroblasts [38]).

All of our data are consistent with the presence of an ATP driven proton pump on the membrane of endocytic vesicles. There have been several recent reports describing ATP-dependent proton pumps in other organelles which are involved in the endocytic pathway. These organelles include lysosomes (39, 41, 42), pinosomes involved in fluid phase uptake of FITC-dextran (44), and clathrin-coated vesicles isolated from bovine brain (45, 56). The precise relationship between these proton pumps and the acidification mechanism for endocytic vesicles described in this paper remains to be defined by future work. The proton pumps of these different organelles may be identical and it is possible that pumps shuttle between various membranes of the cell. For example, the acid-secreting cells of the turtle bladder have an intracellular pool of vesicles containing proton pumps which can be exported to the luminal membrane upon stimulation with CO_2 (47).

From the limited data currently available, it appears that the endocytic vesicle proton pump shares similiar characteristics with other proton pumps located along the endocytic pathway. These proton pumps are apparently insensitive to vanadate, suggesting that they do not require the formation of a phosphorylated intermediate in contrast with many

cation-transporting ATPases (32, 34, 48). Acidification is independent of the external ionic environment, although it is unknown whether all of the pumps translocate phosphate as reported for the lysosomal proton pump (42). The ATP-dependent acidification of both lysosomes (39) and endocytic vesicles are inhibited by Zn^{2+}. As reported here for endocytic vesicles, the lysosomal (39, 42) and clathrin-coated vesicle (45) pumps can use GTP but not nonhydrolyzable ATP analogues to support acidification.

We find that there is, however, a quantitative difference between the sensitivity of lysosome and endocytic vesicle acidification to DIDS. This may be due to differences in the proton pumps themselves or to other differences in the membrane components of these organelles. The differences we observe could be accounted for by a difference in a DIDS sensitive site on the proton pump or possibly by the presence of additional DIDS-sensitive anion transporters in the endocytic vesicle membrane. Whatever the reason, this difference indicates that it may be possible to independently change the pH of endocytic vesicles and lysosomes within cells.

In this paper, we have described a method for studying the mechanism of endocytic vesicle acidification and have shown that the mechanism is similar, but not identical, to the acidification mechanism in secondary lysosomes. Many questions about the acidification mechanism remain. It is not known whether the pump is electrogenic or whether the intravesicular ionic composition plays any role. Also, we do not know if the pump can move freely to the membranes of other organelles in the endocytic pathway. The methods we have described in this paper should be useful for answering these questions and also for examining the factors that regulate the fusion of endocytic vesicles with other cellular organelles.

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