Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 5315–5319, September 1983
Cell Biology

Defective acidification of endosomes in Chinese hamster ovary cell mutants "cross-resistant" to toxins and viruses

(lysosomes/diphtheria toxin/subcellular fractionation/endocytosis)

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Communicated by Stuart Kornfeld, May 18, 1983

ABSTRACT **Like many physiological ligands, several viruses and toxins enter mammalian cells through receptor-mediated endocytosis. Once internalized, the nucleic acids of several viruses and the toxic subunit of diphtheria toxin gain access to the cytosol of the host cell through an acidic intracellular compartment. In this report, we present evidence that one class of mutants of Chinese hamster ovary (CHO)-K1 cells, which is "cross-resistant" to *Pseudomonas* exotoxin A, diphtheria toxin, and several animal viruses, has a defect in acidification of the endosome. Cells were allowed to internalize fluorescein isothiocyanate-conjugated dextran before subcellular fractionation. Fluorescence measurements on subcellular fractions permitted measurement of the internal pH of the isolated endosomes and lysosomes. Our results show that (*i*) endosomes and lysosomes from CHO-K1 cells maintain an acidic pH, (*ii*) acidification of both endosomes and lysosomes is mediated by a Mg^{2+}/ATP-dependent process, (*iii*) GTP can satisfy the ATP requirement for acidification of lysosomes but not of endosomes, and (*iv*) at least one class of mutants that is cross-resistant to toxins and animal viruses has a defect in the ATP-dependent acidification of their endosomes. These studies provide biochemical and genetic evidence that the mechanisms of acidification of endosomes and lysosomes are distinct and that a defect in acidification of endosomes is one biochemical basis for cross-resistance to toxins and viruses.**

Receptor-mediated endocytosis is a specific cell-directed mechanism by which extracellular substances can enter the intracellular vesicular system (1, 2). Steps in the entry pathway include (*i*) binding to cell surface receptors; (*ii*) internalization within coated vesicles, (*iii*) distribution of ligand to appropriate intracellular compartments; and (*iv*) in many cases, return of the receptor to the cell surface. For some pathogenic viruses and toxins, step *iii* includes transfer of a portion of the ligand to the cytosol (3–8). Recently, it has become apparent that this transfer, which is critical for the biologic activity of these viruses and toxins, requires an acidic environment (7–13). Although lysosomes are known to be acidic (14, 15), recent evidence (16) that the endosome is the site at which ligands are first exposed to an acidic environment makes the endosome a suitable site for viral and toxin penetration of the cytoplasm of the host cell (17).

If viruses and diphtheria toxin (DT) penetrate cells through the same acidic compartment, or through different compartments acidified by the same mechanism, cells selected for resistance to one agent that simultaneously acquire resistance to the others are good candidates for mutants that have lost the ability to acidify this compartment. Moehring and Moehring (18) reported the isolation of such "cross-resistant" mutants from KB cells. These mutants were selected for resistance to DT but proved also to be resistant to a number of RNA viruses. More recently, Didsbury *et al.* (19) isolated cross-resistant mutants of Chinese hamster ovary (CHO)-K1 cells and characterized a class, designated DPV^r, that was selected on the basis of resistance to *Pseudomonas* exotoxin A (PT) and proved to be 10–100 times more resistant to DT than CHO-K1 cells and also resistant to infection by several viruses including Sindbis virus, Semliki Forest virus, and vesicular stomatitis virus (20). The resistance of this class of mutants to DT was overcome by exposing the mutant cells to low pH (20), as would be expected for mutants with a defect in acidification. Robbins *et al.* (21) recently reported the isolation and characterization of mutants of CHO cells with some similarities to the DPV^r mutants.

Merion and Sly (22) recently reported a two-step Percoll density gradient fractionation that resolved two prelysosomal endocytic compartments through which several physiological ligands pass enroute to lysosomes. In the present paper, we report the extension of these studies to CHO-K1 cells and the DPV^r mutants and the use of the method of Ohkuma and Poole (14) to measure the internal pH of isolated endocytic compartments loaded with fluorescein isothiocyanate-conjugated dextran (FITC-dextran). These studies show that the DPV^r mutants of CHO-K1 cells have a defect in ATP-dependent acidification of endosomes.

MATERIALS AND METHODS

Cells, Medium, and Culture Conditions. CHO-K1 cells were obtained from the American Type Culture Collection. RE.31 cells, resistant to DT, were isolated from CHO-K1 cells mutagenized with ethylmethane sulfonate, as described (23). RPE.28 and RPE.44 cells, resistant to PT, DT, and certain animal viruses were isolated from similarly mutagenized CHO-K1 cells by using PT (1.4 μg/ml) as a selective agent (19, 20).

All cells were cultured in 10-cm-diameter tissue culture dishes (Scientific Products, St. Louis, MO) in Eagle's minimal essential medium (EME medium) containing nonessential amino acids, penicillin, and streptomycin, plus 10% heat-inactivated fetal bovine serum, in 5% CO_2/95% air at 37°C. Cells were used 24–48 hr after reaching confluency. Cells for studies of the kinetics of acidification in intact cells were grown on 1 × 2 cm glass plates and used 24–48 hr after reaching confluency.

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Abbreviations: DT, diphtheria toxin; CHO, Chinese hamster ovary; PT, *Pseudomonas* exotoxin A; DPV^r, (cells) resistant to both DT and PT; FITC, fluorescein isothiocyanate; EME medium, Eagle's minimal essential medium; HBSS, Hanks' balanced saline solution.

Uptake of FITC-Dextran and Subcellular Fractionation. For subcellular fractionation studies, FITC-dextran (M_r, 70,000; Sigma) was incubated at 15 mg/ml with cells in EME medium at 37°C for various periods of time. Cells were then washed and fractionated by the two-step Percoll density gradient procedure. The amount of cell-associated FITC-dextran/mg of cell protein after a 15-min incubation was the same for all cell types. Fractionation was conducted using the self-forming gradient material Percoll (Pharmacia) as described (22, 24). After incubation with FITC-dextran, cells were washed 10 times at 4°C in 0.25 M sucrose/1 mM K_2EDTA and adjusted to pH 7.3 with KOH prior to fractionation. Lysosomal enzyme activities were determined fluorometrically on subcellular fractions (25) using substrates purchased from Sigma. All assays were carried out in the presence of 0.1% Triton X-100 and terminated by the addition of 1 ml of 50 mM glycine/NaOH, pH 10.5/5 mM EDTA.

Lactoperoxidase-catalyzed iodination of cell surface proteins was carried out as described (22, 24). Cell viability after labeling was determined to be greater than 95% by trypan blue exclusion.

Measurement of pH in Subcellular Fractions. A standard curve relating the ratio of fluorescence intensities at 550 nm after excitation at 495 and 450 nm was constructed according to the method of Ohkuma and Poole (14) using FITC-dextran (5 μg/ml) in 100 mM sodium phosphate or sodium acetate buffers at various pH values. The pH within isolated vesicles containing FITC-dextran was determined by relating the measured ratio of fluorescence intensities to the standard curve described above. Blank values were determined from fractions from an identical Percoll gradient to which no postnuclear supernatant had been applied. Autofluorescence was determined from Percoll density gradient fractions to which a postnuclear supernatant, prepared from cells not exposed to FITC-dextran, was applied. Extravesicular fluorescence was determined by monitoring the instantaneous change in fluorescence after addition of 50 mM sodium acetate (pH 6.0). All fluorescence measurements were made using a SPF 500 Aminco ratio mode spectrofluorometer.

Measurement of the Kinetics of Acidification of Endocytic Vesicles in Intact Cells. FITC-dextran was prepared (26) using dextran of mean M_r 40,000 and fluorescein isothiocyanate isomer 1 (Sigma). Cells on glass plates were washed several times with Hanks' balanced saline solution (HBSS)/15 mM Hepes, pH 7.4, and incubated in HBSS/Hepes with FITC-dextran at 240 mg/ml for 5 min at 37°C. The cells were then washed extensively with cold isotonic saline (pH 7.2) for 5–10 min. The glass plate was mounted in a Lucite holder and placed in a standard fluorescence cuvette containing medium thermostated at 37°C. The intravesicular pH was determined at intervals as described by Ohkuma and Poole (14).

RESULTS

Four different cell lines were examined. The normal control line was CHO-K1, the parental cells from which the mutant cell lines were isolated. Two independently isolated cross-resistant lines were used, RPE.28 and RPE.44. Both cell lines were selected for resistance to PT and found to be resistant to DT and several viruses. A control for isolated resistance to DT was provided by RE.31 (23), a cell line selected for DT resistance that retained normal sensitivity to viruses.

Two-Step Percoll Density Gradient Fractionation of CHO Cells. The two-step Percoll density gradient fractionation was used previously for isolation of endosomes and lysosomes from human (22) and murine (24) fibroblasts. The distribution of plasma membrane marker, lysosomal enzymes, and internalized FITC-dextran in the first Percoll gradient is shown in Fig. 1. For both parental CHO-K1 and RPE.28 mutant cells, lysosomal enzyme activities distribute within three peaks. The activity on top of the gradient (fractions 1–4) sediments with soluble enzymate markers (22, 24, 27). The small amount of activity at a density of 1.040 g/ml (fractions 9–14) may represent lysosomal enzyme activity associated with GERL-derived structures, endoplasmic reticulum, Golgi, or endosomes (22, 27). The majority of lysosomal enzyme activity is localized at a peak of modal density 1.07 g/ml (fractions 21–24), an area of the gradient shown previously to contain typical secondary lysosomes (22, 27). To label plasma membranes specifically, cells were iodinated at 4°C using soluble lactoperoxidase immediately prior to fractionation. Radioactivity that entered the gradient was localized at a modal density of 1.04 g/ml (fractions 9–12). Fractionation of RPE.44 and RE.31 cells gave similar results (data not shown). The kinetics of internalization of FITC-dextran was examined using the first Percoll gradient on cells fractionated after 5 and 15 min of internalization (Fig. 1). In both CHO-K1 and RPE.28 cells (after 5 min), fluorescence was localized at a density of 1.04 g/

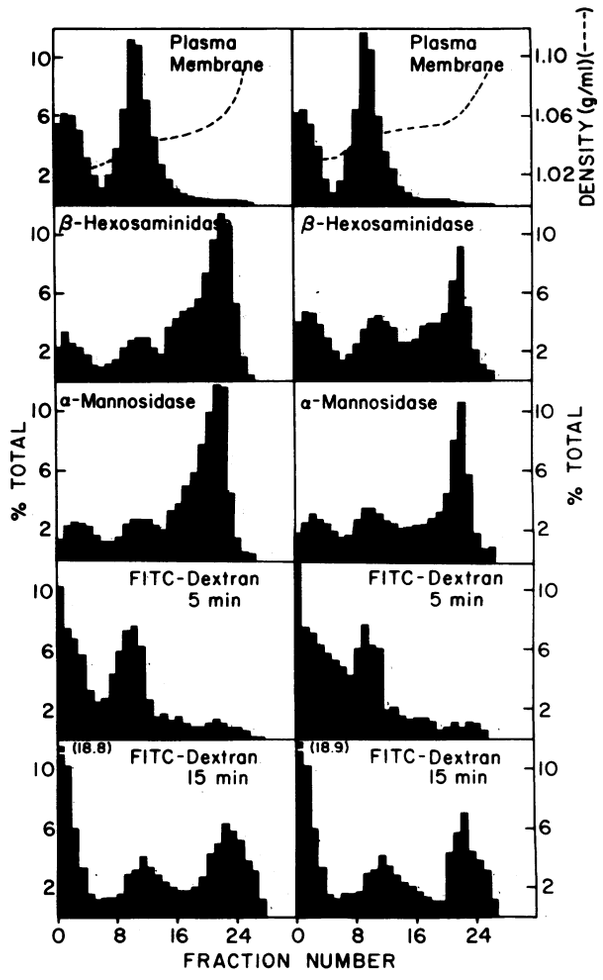

FIG. 1. First Percoll density gradient fractionation. CHO-K1 (*Left*) and cross-resistant mutant RPE.28 (*Right*) cells were iodinated at 4°C with soluble lactoperoxidase and homogenized, and the postnuclear supernatant was fractionated on a Percoll gradient. Fractions were collected by upward flow displacement and plasma membrane, β-hexosaminidase, and α-mannosidase activities were determined. In some cases, prior to fractionation, cells were incubated with FITC-dextran (15 mg/ml) for either 5 or 15 min at 37°C, and fluorescence of the gradient fractions was determined. Cell-associated fluorescence after a 15-min incubation was 2.5 times that found after a 5-min incubation.

ml (fractions 9–14). After internalization for 15 min, an additional peak of fluorescence appeared at 1.07 g/ml (fractions 21–24), where secondary lysosomes are localized (22). Similar results were obtained with RPE.44 and RE.31 cells (data not shown). Thus, in the CHO-K1 cells and the mutants derived from them, newly internalized FITC-dextran is first localized in a prelysosomal compartment and subsequently appears in secondary lysosomes.

To resolve the prelysosomal compartment(s) from the plasma membrane, fractions 9–14 from the first Percoll density gradient were pooled and fractionated on a second Percoll density gradient (Fig. 2). For both CHO-K1 and RPE.28 cells, the plasma membrane marker is unimodal in distribution at 1.037 g/ml (fractions 3–8). The small amount of lysosomal enzyme activity applied to this gradient (<10% of total activity) is distributed within a broad area between 1.037 and 1.041 g/ml (fractions 3–17), as well as in a discrete peak at 1.045 g/ml (fractions 15–27). Similar results were obtained for RPE.44 and RE.31 cells (data not shown). The distribution of newly internalized FITC-dextran in the second Percoll gradient, after 5 min of loading of CHO-K1 and RPE.28 cells, is also presented in Fig. 2. Fluorescence is distributed in peaks at densities of 1.040 (fractions 9–14) and 1.043 (fractions 20–24) g/ml. These two fluorescent peaks, which were separated from secondary lysosomes in the first gradient, were discrete from the plasma membrane marker in the second gradient. Similar results were obtained with cells incubated with FITC-dextran for 15 min prior to fractionation. Vesicles of similar densities have been shown (22) to be transport intermediates in the transfer of ligands from the cell surface to lysosomes and are referred to as light endosomes (Fig. 2, fractions 9–14) and heavy endosomes (Fig. 2, fractions 20–23).

Acidification of Endosomes and Lysosomes Isolated from CHO-K1 Cells. CHO-K1 cells were incubated with EME medium containing FITC-dextran (15 mg/ml) for 15 min at 37°C and fractionated; lysosomes were isolated from the first gradient and the two endosome peaks were isolated from the second gradient. Fractions were then buffered with 50 mM Hepes/KOH, pH 7.0, and the intravesicular pH was measured using the A_{495}/A_{450} ratio and the standard curve. The internal pH of both light and heavy endosomes was 5.9 ± 0.2 and that of lysosomes was 5.5 ± 0.2. Next, we examined the effect of ATP on the internal pH of isolated endosomes and lysosomes. Fractions were buffered with 50 mM Hepes/KOH, pH 7.0, in the presence (Fig. 3*A*) and in the absence (Fig. 3*B*) of 5 mM $MgCl_2$ and the pH was monitored for 10 min, after which 1 mM K_2ATP was added, and the pH was monitored for an additional 5 min. After the addition of ATP in the presence of Mg^{2+}, the pH of the heavy endosomes decreased 0.5 unit (Fig. 3*A*). ATP did not produce this decrease in pH in the absence of added Mg^{2+} (Fig. 3*B*). Similar results were obtained for light endosomes (data not shown). The internal pH of the lysosomes decreased 0.3 unit with the addition of ATP, again, only in the presence of magnesium (Fig. 3 *A* and *B*). Fig. 3*C* shows the effects of GTP on the acidification of heavy endosomes and lysosomes. GTP had no effect on the pH of the heavy endosomes. Similar results were obtained with light endosomes (data not shown). In contrast, addition of 1 mM Na_2GTP to lysosomes in the presence of 5 mM $MgCl_2$ resulted in a rapid decrease of 0.2 pH unit (Fig. 3*C*). Thus, GTP appeared almost as effective as ATP in stimulating acidification of lysosomes. The ability of GTP to stimulate acidification of the lysosome but not the endosome suggests that the systems acidifying these two compartments are distinct.

ATP-Dependent Acidification of Vesicles Isolated from Cross-Resistant Mutants. Control and mutant CHO cells were incubated with FITC-dextran (15 mg/ml) in EME medium for 15

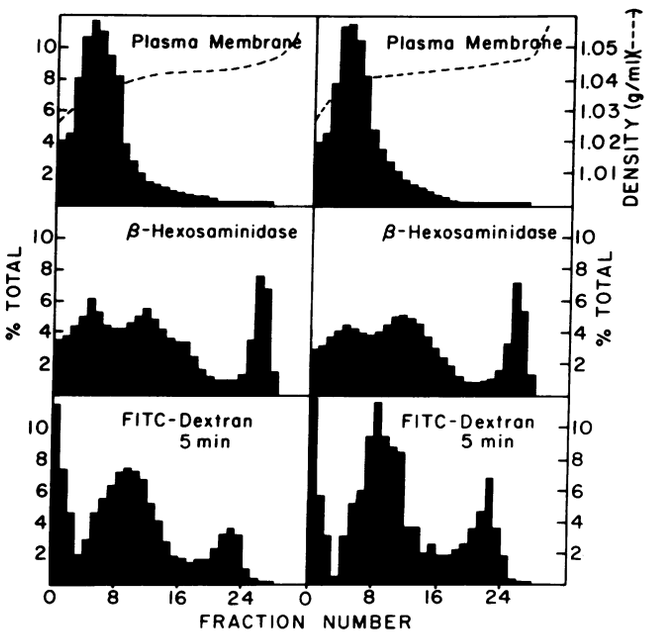

FIG. 2. Second Percoll density gradient fractionation. (*Left*) CHO-K1 cells. (*Right*) RPE.28. Fractions 9–14 from the first Percoll density gradient (Fig. 1) were pooled and fractionated on a second Percoll density gradient. Fractions were collected by upward flow displacement and plasma membrane, β-hexosaminidase, and fluorescence activities of the fractions were determined.

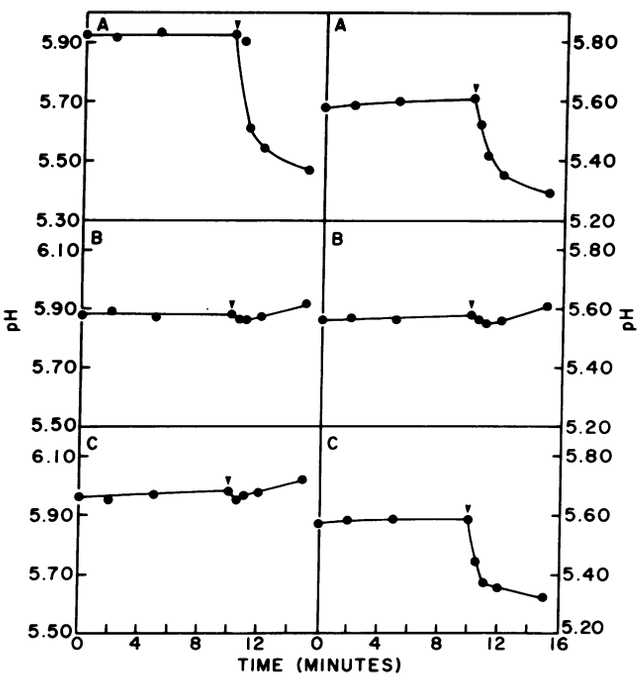

FIG. 3. Mg^{2+} and nucleotide dependence of acidification of isolated endosomes and lysosomes from CHO-K1 cells. CHO-K1 cells were incubated with FITC-dextran (15 mg/ml) for 15 min at 37°C and then fractionated by the two-step Percoll density gradient procedure. Heavy endosome (*Left*; Fig. 2, fractions 20–24) and secondary lysosome (*Right*; Fig. 1, fractions 21–24) fractions were buffered with 50 mM Hepes/KOH, pH 7.0/5 mM $MgCl_2$ (*A* and *C*), or with 50 mM Hepes/KOH, pH 7.0 (B). The pH was monitored for 10 min, and then either 1 mM K_2ATP (*A* and *B*) or 1 mM Na_2GTP (*C*) was added (▼) and the pH was monitored for an additional 5 min.

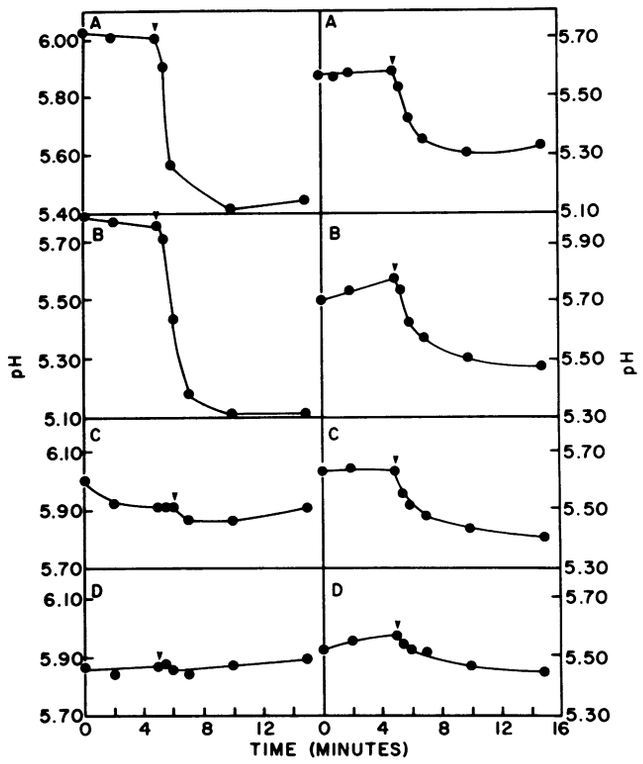

FIG. 4. Mg^{2+}/ATP-dependent acidification of isolated endosomes and lysosomes from control and cross-resistant mutant cells. Cells were incubated with FITC-dextran (15 mg/ml) for 15 min at 37°C and then fractionated by the two-step Percoll density gradient procedure. Heavy endosome (*Left*; Fig. 2, fractions 20–24) and secondary lysosome (*Right*; Fig. 1, fractions 21–24) fractions were buffered in 50 mM Hepes/KOH, pH 7.0/5 mM $MgCl_2$. The pH was monitored for 5 min, 1 mM K_2ATP was added, and the pH was monitored for an additional 10 min. (*A*) Parental CHO-K1 cells. (*B*) RE.31 cells (resistant only to DT). (*C*) Cross-resistant cell line RPE.28. (*D*) Cross-resistant cell line RPE.44.

min at 37°C and the endosome and lysosome fractions were isolated. Fractions were buffered with 50 mM Hepes/KOH, pH 7.0, in the presence of 5 mM $MgCl_2$. After the pH was monitored for 5 min, 1 mM K_2ATP was added and the pH was monitored for an additional 10 min (Fig. 4). The addition of ATP to endosomes and lysosomes isolated from normal CHO-K1 cells (Fig. 4*A*) resulted in a rapid acidification of both compartments. The same was true for RE.31 cells, a cell line resistant only to DT (Fig. 4*B*). However, the addition of ATP to endosomes isolated from cross-resistant RPE.28 and RPE.44 cells (Fig. 4 *C* and *D*), did not lower the pH. Lysosomes isolated from these two cell types (Fig. 4 *C* and *D*), like the lysosomes isolated from normal CHO-K1 and RE.31 cells (Fig. 4 *A* and *B*), became acidified in response to ATP. However, the extent of acidification of lysosomes in the two cross-resistant mutants was different. Although the acidification of lysosomes from RPE.28 cells was almost as great as that of those from CHO-K1, the extent of acidification of lysosomes from RPE.44 cells was less. Since both RPE.28 and RPE.44 cells are cross-resistant and have, in common, a defect in the acidification of the endosome, these results suggest that a defect in acidification of the endosome is the biochemical lesion that confers cross-resistance.

The Kinetics of Acidification of Endosomes in Intact Cells. The cells were loaded for 5 min and washed, and the pH of the intracellular vesicles was determined over the next 20 min. Each cell type was studied at least three times. One experiment is shown in Fig. 5. Normal CHO-K1 cells acidified rapidly, reaching a stable pH of 5.15 ± 0.05 by 10 min. Acidification of the cross-resistant strains RPE.28 and RPE.44 was much slower, reaching values of 5.65 ± 0.2 and 5.75 ± 0.15 by 10 min. RE.31 appeared to acidify nearly normally, reaching a pH of 5.45 ± 0.02 by 10 min (data not shown). By 20 min, CHO-K1, RPE.28, and RE.31 (data not shown) had reached stable pH values of 5.1 ± 0.1, 5.3 ± 0.2, and 5.29 ± 0.03, respectively. Only RPE.44 failed to acidify to a pH of less than 5.5 in 20 min. These studies show that both cross-resistant mutants, RPE.28 and RPE.44, have a defect in the normal process of acidification of the endocytic compartment(s).

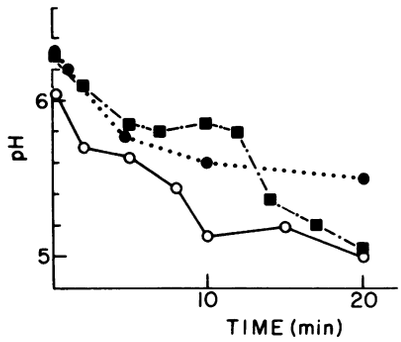

FIG. 5. Kinetics of acidification of the endocytic compartment(s) in intact cells. Cells on glass plates were loaded with FITC-dextran, washed, and placed in a thermostated cuvette containing HBSS/Hepes, pH 7.4, and the pH was determined at the indicated times by using emission at 516 nm and excitation at 495 or 450 nm. Media (HBSS/Hepes, pH 7.4) were replaced 15 sec before each determination to remove extracellular FITC-dextran. ○, CHO-K1; ■, RPE.28; ●, RPE.44 cells.

DISCUSSION

The data presented here indicate that the internal pH of isolated endosomes and lysosomes is acidic and that the addition of ATP in the presence of magnesium results in further acidification of both compartments. Thus, at least in part, both endosomes and lysosomes are acidified through a magnesium/ATP-dependent process. Ohkuma *et al.* (15) have shown that both ATP and GTP stimulate acidification of rat liver lysosomes, and we confirmed this observation in CHO cells. However, we have shown that acidification of endosomes, in contrast to lysosomes, was not stimulated by GTP. This result suggests that these two acidification mechanisms are distinct.

It has been proposed that DT (6, 7) and several enveloped viruses (9–13) gain access to the cytosol through an acidic intracellular compartment(s). We postulated that certain mutant cells that are cross-resistant to these agents would prove to be defective in acidification of the intracellular compartment(s) through which these agents gain access to the cytosol (19, 20, 28). The present studies on the DPV^r mutants of CHO-K1 cells support this hypothesis. Endosomes isolated from both parental CHO-K1 cells and mutant RE.31 cells, which are resistant only to DT, displayed an ATP-dependent acidification. However, the endosomes isolated from both DPV^r mutants were defective for this process. Furthermore, studies of the kinetics of acidification of DPV^r mutant cells revealed a substantial delay in the acidification of vesicles containing newly internalized FITC-dextran. Subcellular fractionation studies suggested that, at early times, when the difference between the control and DPV^r cells was greatest, the majority of FITC-dextran was contained within endosomes. The defect in the acidification of endosomes in cross-resistant mutants may represent a defect in the proton pump itself—i.e., the proton-generating ATPase—or a defect in another component of the membrane essential for maintaining a pH gradient.

By 25 min after internalization, the vesicles containing FITC-dextran in intact RPE.28 cells were as acidic as those in CHO-K1 and RE.31 cells. Subcellular fractionation studies showed that, by this time, a large portion of the internalized marker was localized in the lysosomes. These observations suggest that the lysosomes in RPE.28 cells acidify normally or at least that they reach the same final pH. This conclusion is supported by studies of lysosomes isolated from these cells that show normal ATP-dependent acidification. Thus, cell strain RPE.28 appears to have a defect that affects acidification of the endosome but not acidification of lysosomes. Studies with intact RPE.44 cells indicated that vesicles containing internalized dextran had not reached the pH of lysosomes in CHO-K1 cells even after 25 min. Studies of lysosomes isolated from these cells also showed a diminished response to the addition of ATP. Thus, the RPE.44 cells clearly have a defect in acidification of endosomes, but their defect may also affect acidification of lysosomes. The relationship between the systems responsible for acidification of these two compartments is not yet clear.

Although the DPV^r mutants have a defect in the ATP-dependent acidification of endosomes, the internal pH of their endosomes is nearly 1 pH unit below that of the surrounding buffer when isolated. One factor contributing to their acidification might be a Donnan equilibrium created by impermeant anions (sialic acid residues of membrane glycolipids and glycoproteins and polar head groups of phospholipids) on one side of the vesicle membrane coupled with a selective permeability for cations. Another system that might contribute to their acidification is the Na^+/H^+ exchange system known to be present in the plasma membrane (29). However, this degree of acidification is not sufficient for DT toxicity (30) and successful infection by several animal viruses (9, 11, 12). The studies reported here suggest that the additional acidification required for virus penetration and toxin action is produced by the ATP-dependent acidification system for which the DPV^r mutants are defective.

We gratefully acknowledge Drs. K. Creek and V. Talkad for helpful discussions, Joanne Nielsen for cell culture, and Sabra Lovejoy and Margaret Kane for help in manuscript preparation. This work was supported by U.S. Public Health Service Grants GM 21096 and GM 31988 to W.S.S., HL 26300 to P.S., and A1-09100 to T.J.M. and J.M.M. and by a gift from the Ranken Jordan Trust.

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