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Regulation of endocytic pH by the Na^+,K^+-ATPase in living cells

(endosomal acidification/ouabain/vacuolation/flow cytometry)

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ABSTRACT **Acidification of endocytosed ligands destined for lysosomes is biphasic, with a rapid drop to pH 6, followed by a slow decrease to pH 5. Continuous measurements of transferrin acidification have confirmed that the pH minimum in early (presorting) endosomes is approximately pH 6. On the basis of measurements of endosomal acidification *in vitro*, it has been proposed that the pH in the early endosome is limited by the internalization of the Na^+,K^+-ATPase, which generates an interior-positive membrane potential in this compartment [Fuchs, R., Schmid, S. & Mellman, I. (1989) *Proc. Natl. Acad. Sci. USA* 86, 539–543]. We present two lines of evidence that strongly implicate the Na^+,K^+-ATPase as a major regulatory element of endocytic pH *in vivo*. First, ouabain, a specific inhibitor of the Na^+,K^+-ATPase, interferes with the regulation of acidification in early endocytic compartments. Transferrin is normally rapidly acidified to pH 6.0–6.2, followed by alkalinization during recycling. In the presence of ouabain, the minimum pH of transferrin-containing endosomes decreases from 6.0–6.2 to <5.3. Second, ouabain eliminates the resistance to both the growth inhibitory and vacuologenic effects of chloroquine in the lysosomal acidification defective cell line CHL60-64. The phenotype of this cell line is consistent with a defect in the removal or inactivation of the early acidification regulatory elements from the late endocytic compartments. The ouabain data suggest that the defect in this cell line is due to improper localization of the Na^+,K^+-ATPase. A model for pH regulation and vacuolation by weak bases is discussed.**

Endocytosis is the primary process by which cells take up macromolecular material from their environment. This process includes both fluid-phase pinocytosis of extracellular fluid and specific concentrative uptake of ligands via cell-surface receptors. Endocytosis is involved in many cellular processes, including the scavenging of acid hydrolases (mannose receptor and α_2-macroglobulin), clearance of damaged proteins (asialoglycoprotein receptor), nutrient uptake (low density lipoprotein and transferrin), antigen processing, and regulation of peptide hormone response (epidermal growth factor and insulin) (for review, see refs. 1–3). Acidification of endocytic compartments has been implicated in the proper segregation of receptor–ligand complexes to the recycling or degradative pathways and in the regulation of lysosomal hydrolase activity (for review, see refs. 4 and 5).

We have previously used flow cytometry to measure the kinetics of acidification of a number of endocytic probes. Results in 3T3 cells with insulin (6), epidermal growth factor (7), and dextran (8), and in activated T lymphocytes with anti-major histocompatibility complex antibodies (9) showed that the acidification of ligands that are delivered to lysosomes is biphasic, with a rapid initial acidification to pH 6 within 5 min of internalization, followed by a subsequent slow acidification to pH 5 over the next 30–40 min. Unlike most other ligands, transferrin, a major iron transport protein, escapes lysosomal degradation by recycling with its receptor back to the cell surface after the release of iron in the endosome (10, 11). In 3T3 cells, transferrin follows the same initial phase of acidification to pH 6 but is alkalinized during recycling rather than further acidified (12). This implies that the endocytic pathway is maintained at a mildly acidic pH until after recycling has taken place (12).

Recently, evidence has been presented, which suggests that the pH of endocytic compartments may be regulated by the Na^+,K^+-ATPase (13, 14). This model offers a simple explanation for biphasic acidification. The endocytic H^+-ATPase is electrogenic (15–17) and is limited by both membrane potential and pH. Na^+,K^+-ATPase internalized with receptors and ligands during endocytosis is proposed to generate an interior-positive membrane potential in the endosome. The resulting potential would limit acidification by the H^+-ATPase. Removal of the Na^+,K^+-ATPase (by recycling or degradation) would relieve the membrane potential and permit acidification to lysosomal levels. The accompanying paper by Fuchs *et al.* (18) describes *in vitro* evidence for electrogenic inhibition of endosomal acidification, but not lysosomal acidification, by the Na^+,K^+-ATPase. In this paper, we describe two separate lines of evidence for the role of the Na^+,K^+-ATPase as a major regulator of endocytic pH *in vivo*.

MATERIALS AND METHODS

All chemicals were obtained from Sigma unless otherwise indicated.

Transferrin Acidification. The kinetics of transferrin acidification were determined by flow cytometry as described (12). A549 human epidermoid carcinoma cells (19) were plated at $\approx 3 \times 10^5$ cells per 100-mm dish in Dulbecco's modified Eagle's medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, and 10% (vol/vol) calf serum (GIBCO) (cDMEM) at least 15 hr before use. Cells were treated with either 6 mM or 10 μM ouabain 4–6 hr prior to acidification measurements. Cells were incubated in either phosphate-buffered saline (PBS; 140 mM NaCl/3 mM KCl/8 mM NaH_2PO_4/1.5 mM KH_2PO_4) or low K^+ buffer (160 mM NaCl/0.6 mM KCl/9 mM NaH_2PO_4/0.3 mM KH_2PO_4) as indicated.

Growth Curves. The cytotoxicity of ouabain and chloroquine were determined as described (20). After a 5-day incubation with 60 μM chloroquine, 1 mM ouabain, or both, the number of cell doublings was calculated relative to the number of cells at the time of the additions (1.5×10^4 for Swiss 3T3; 1.1×10^4 for CHL60-64). The values listed are the averages of two counts each on duplicate plates.

Microscopy. Cells were plated at a density of 2×10^4 (Swiss 3T3) or 3×10^4 (CHL60-64) cells per well on two-chamber tissue culture slides (Lab-Tek, Naperville, IL) in 2 ml of

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Abbreviations: FITC, fluorescein isothiocyanate; LRSC, lissamine rhodamine sulfonyl chloride.

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cDMEM 1 day before use. The cells were refed with cDMEM containing 60 μM chloroquine, with or without 1 mM ouabain, and incubated at 37°C overnight. Phase contrast micrographs were taken with a ×40 water immersion lens.

RESULTS

Ouabain Eliminates pH Regulation in the Endosome. If the Na^+,K^+-ATPase were responsible for limiting early endocytic acidification to a pH above 6, then inhibition of the Na^+,K^+-ATPase should result in a decrease in the pH of the presorting compartment. Therefore, transferrin acidification in the presence of ouabain, a specific inhibitor of the Na^+,K^+-ATPase, should reflect the minimum pH achievable by the endocytic H^+-ATPase and should equal that measured in the lysosome.

To test directly the role of the Na^+,K^+-ATPase in the regulation of endosomal pH *in vivo*, acidification kinetics for transferrin were measured in the presence and absence of ouabain, following the method described previously (12). This method permits continuous measurement of the pH of a cohort of internalized transferrin. Since mouse 3T3 cells (in which we have previously characterized the transferrin cycle) have a Na^+,K^+-ATPase with a low affinity for ouabain (21), measurements were made in A549, a human epidermoid carcinoma cell line (19). A549 cells were first incubated with a mixture of fluorescein isothiocyanate (FITC)- and lissamine rhodamine sulfonyl chloride (LRSC)-labeled transferrins at 4°C, washed, and analyzed by flow cytometry at 0°C. The cells were warmed to 37°C to allow endocytosis and acidification to begin, and analysis was continued. The ratio of FITC to LRSC fluorescence was then calculated. This ratio is a monotonic function of pH and therefore reflects intracellular acidification.

Fig. 1 shows results for cells in the presence or absence of ouabain in low K^+ buffer (6 mM ouabain) or PBS (10 μM ouabain). Under both of these incubation conditions, ouabain decreased the minimum FITC/LRSC ratio, which occurred 5 min after warm-up, from 0.64 to 0.54.

By determining the internalization kinetics of transferrin under each condition (using anti-transferrin antibodies), the individual FITC and LRSC fluorescence values can be corrected for the fraction of transferrin remaining on the cell surface. The ratio of the corrected fluorescences can then be converted to pH with calibration curves obtained from surface-labeled cells analyzed in buffers of known pH. Although a slight lag in the onset of internalization was observed in the presence of ouabain, the internalization curves were otherwise quite similar (data not shown). The calculated internal pH shown in Fig. 2 indicates that for control cells, the average pH of a synchronous cohort of internalized transferrin decreases rapidly after a 2- to 3-min lag, reaches a minimum pH of 6.0–6.2 at 4–5 min, and then increases to the initial value (corresponding to the external pH). When cells are incubated with ouabain, the pH drops to below 5.3 within 4 min. The slight difference in internalization rates results in a difference between the calculated internal pH values in the presence and absence of ouabain (Fig. 2) that is even larger than the difference between the fluorescence ratios (Fig. 1). Note that although ouabain decreased the minimum pH from >6 to <5.3, transferrin was still subsequently alkalinized, suggesting that the recycling of transferrin was not affected by ouabain or by this abnormally low pH.

Ouabain Reverses the Acidification Defect in CHL60-64. We have previously described a Swiss 3T3 cell line, CHL60-64, with a defect in late endocytic acidification (14). Selected for resistance to the lysosomotropic amine chloroquine, CHL60-64 is also resistant to ammonium chloride and is resistant to vacuolation induced by either of these amines. This mutant

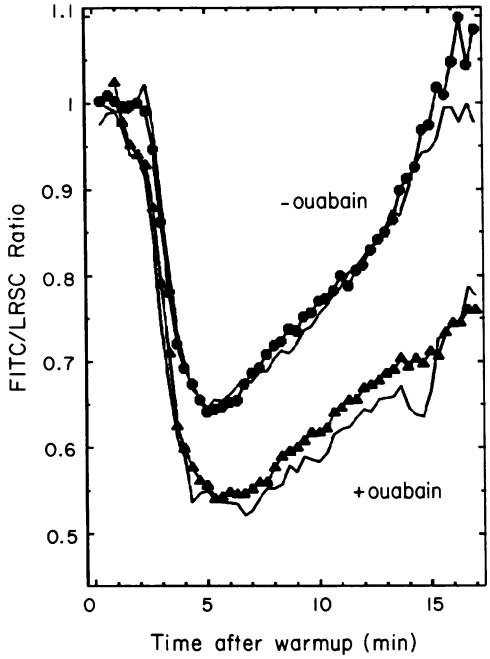

FIG. 1. Effect of ouabain on transferrin acidification. A549 cells were preincubated with either 6 mM ouabain in low K^+ buffer (▲) or 10 μM ouabain in PBS (—) for 4–6 hr. Acidification kinetics were then determined (in the presence of ouabain) as described (12). Control cells (minus ouabain) in low K^+ buffer (●) or PBS (—) were similarly labeled and analyzed. Flow cytometry was used to measure FITC-transferrin and LRSC-transferrin fluorescence for individual cells as a function of time after warm-up from 0°C to 37°C. Data were acquired continuously; mean values for both parameters were calculated for all cells collected in each 20-s interval. The ratio of FITC/LRSC fluorescence was then calculated for each time point.

does not fully acidify dextran (a marker for fluid-phase endocytosis) until 8 hr after uptake, compared to <1 hr in parental samples. The initial rapid phase of acidification, reflected by the kinetics of transferrin acidification, is unaffected in this mutant. Assuming that there is only one proton-translocating ATPase involved in endocytic acidification, this result implies that the defect in this cell line does not lie in the proton-ATPase itself but in a regulator of endocytic pH.

The phenotype of CHL60-64 is identical to that expected if the pH regulatory elements that maintain the mildly acidic pH in early endosomes were not removed from the late endocytic compartments. The presence of these regulatory elements would limit the acidification of late endocytic compartments both *in vivo* and *in vitro* without affecting the initial phase of acidification. We found previously that CHL60-64 is resistant to ouabain (22) and preliminary results indicate that ouabain-sensitive ATPase activity is amplified approximately 2- to 3-fold in CHL60-64 relative to the parental cell line (C.C.C., E. K. Wickert, and R.F.M., unpublished data). These results suggested that the Na^+,K^+-ATPase is affected in CHL60-64.

To determine whether the Na^+,K^+-ATPase is directly responsible for the phenotype of CHL60-64, we investigated whether ouabain would reverse the amine resistance of this mutant (Table 1). Although 1 mM ouabain inhibits growth in both cell lines, it does not cause cell death in either. At 60 μM chloroquine, CHL60-64 is capable of growth, while there is cell death in the parental samples. Incubation of cells with nonlethal doses of ouabain (1 mM) eliminated the resistance of CHL60-64 to 60 μM chloroquine.

It was further predicted that if improper localization of the Na^+,K^+-ATPase to postsorting endocytic compartments was the mechanism for the resistance to vacuolation in CHL60-64, then coincubation of cells with chloroquine and ouabain

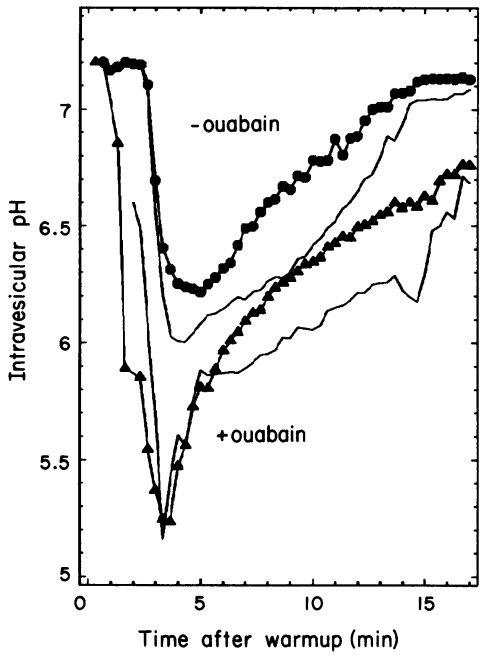

FIG. 2. Effect of ouabain on endosomal pH. Data from Fig. 1 were used to calculate pH values for the internalized transferrin cohort after subtraction of the fluorescence from surface-bound transferrin. The minimum pH measured in the control cells was 6.0–6.2 [for cells incubated in PBS (—) or low K^+ buffer (●), respectively], in good agreement with the value of 6.1 obtained in BALB/c 3T3 cells (12). For cells treated with either 10 μM (—) or 6 mM (▲) ouabain, the minimum pH observed was dramatically lower (≈5.2).

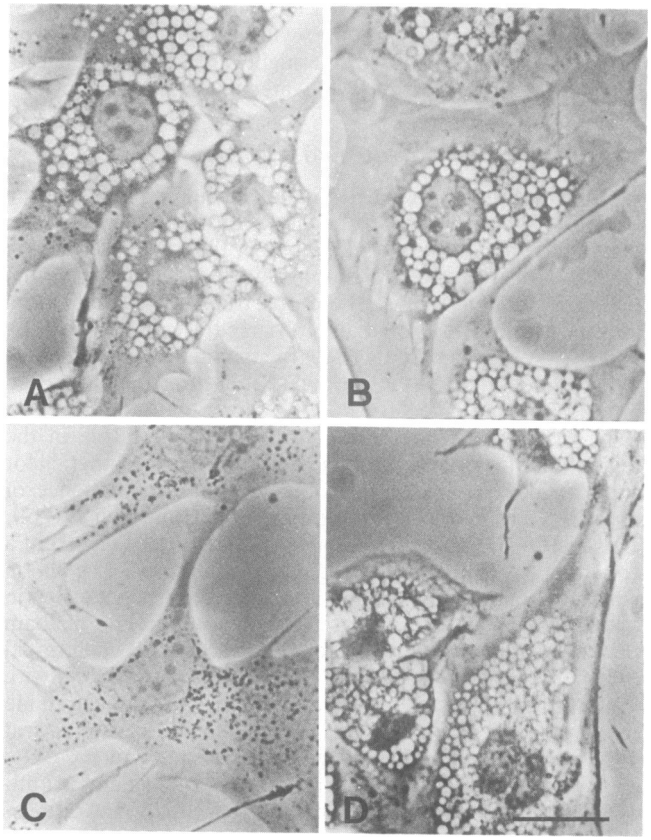

FIG. 3. Ouabain eliminates resistance to vacuolation in CHL60-64. Swiss 3T3 (*A* and *B*) or CHL60-64 (*C* and *D*) were incubated overnight in cDMEM with 60 μM chloroquine with (*B* and *D*) or without (*A* and *C*) 1 mM ouabain. Although ouabain did not itself induce vacuolation (not shown), it eliminated the resistance of CHL60-64 to vacuolation by chloroquine. (Bar = 10 μm.)

should result in vacuolation. This was tested by microscopic examination of CHL60-64 cells treated with 60 μM chloroquine and 1 mM ouabain. Representative photographs are shown in Fig. 3. Parental cells were extensively vacuolated in the presence of chloroquine alone (Fig. 3*A*) and in the presence of both chloroquine and ouabain (Fig. 3*B*). In contrast, CHL60-64 cells showed little vacuolation when incubated with chloroquine alone (Fig. 3*C*) but were extensively vacuolated when coincubated with both chloroquine and ouabain (Fig. 3*D*). This concentration of ouabain alone induced no vacuolation in either cell type (data not shown). These data indicate that the Na^+,K^+-ATPase is responsible for the phenotype observed in CHL60-64 and imply that the Na^+,K^+-ATPase is not normally found in the postsorting endocytic compartments.

DISCUSSION

The Na^+,K^+-ATPase in the Regulation of Endosomal pH. The transferrin acidification kinetics described above for A549 cells (without ouabain) are very similar to those reported in BALB/c 3T3 cells (12). In the presence of ouabain, however, the minimum fluorescence ratio is significantly lower than in control cells. This indicates that acidification in these cells is significantly greater in the presence of ouabain, even without correction for the fraction of transferrin remaining on the cell surface. To determine the extent of this change, internal pH values were calculated. In the presence of ouabain, rapid initial acidification to below pH 5.3 was observed, as would be predicted if the H^+-ATPase were no longer regulated in the early endosome. These results support and extend the *in vitro* results in the accompanying paper by Fuchs *et al.* (18) on isolated endosomes and lysosomes from CHO cells. Their data indicate that acidification in early endosomal compartments is inhibited relative to later compartments and that this inhibition can be relieved by vanadate or ouabain.

The Na^+,K^+-ATPase and Vacuolation. The phenotype of CHL60-64 is readily explained by the mislocalization of the Na^+,K^+-ATPase to late endocytic compartments. Na^+,K^+-ATPase internalized in endocytic vesicles would maintain a positive membrane potential inside the vesicles relative to the cytoplasm. This membrane potential would limit the development of a proton gradient by the electrogenic H^+-ATPase. The fact that the initial (transferrin) acidification is unimpaired in CHL60-64 implies that the endocytic H^+-ATPase is not defective in this cell line. Diversion of the Na^+,K^+-ATPase to the postsorting endosomal compartments in

Table 1. Ouabain reverses the chloroquine resistance of CHL60-64

	Cell doublings			
	Control	Ouabain (1 mM)	Chloroquine (60 μM)	+ ouabain + chloroquine
Parental	6.85 ± 0.13	−0.03 ± 0.22	−0.69 ± 0.15	−3.07 ± 0.41
CHL60-64	5.41 ± 0.04	0.91 ± 0.05	1.99 ± 0.19	−1.41 ± 0.38

Results are calculated as $\log_2$ (final number of cells per initial number of cells); mean ± SD (n = 4).

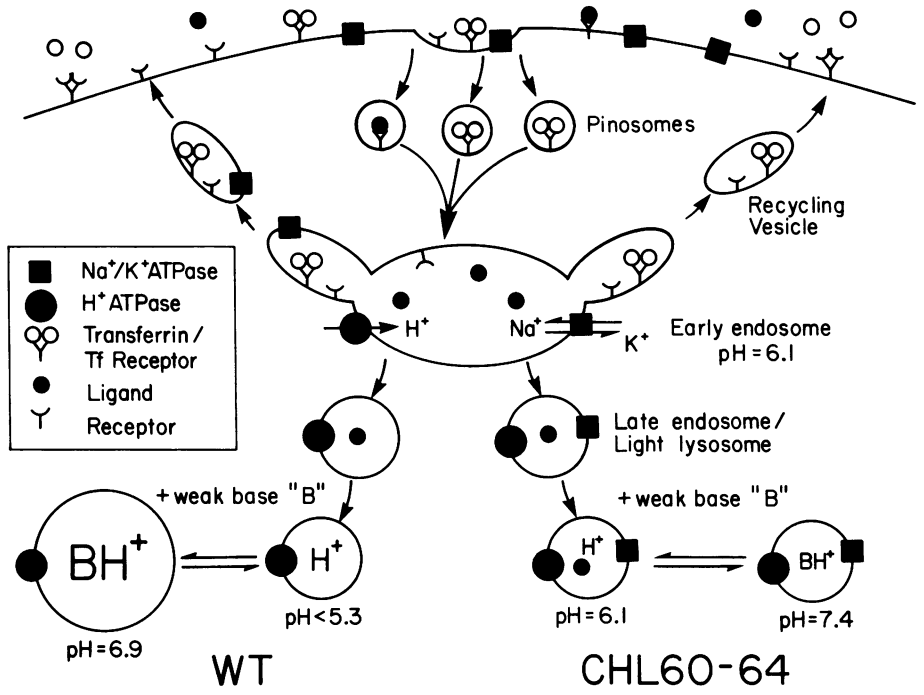

FIG. 4. A model for pH regulation and vacuolation in endosomes and lysosomes. Scheme for wild-type (WT) (*Left*) and CHL60-64 (*Right*) is shown (see text). Addition of a weak base to wild-type cells results in accumulation of the protonated base in the lysosomal compartments due to continuous pumping by the H^+-ATPase. This results in vacuolation of these compartments. Wild-type endosomes, as well as both endosomes and lysosomes of CHL60-64, are resistant to vacuolation by weak bases because of the presence of a limiting membrane potential. This potential prevents H^+ pumping by the H^+-ATPase in these compartments and the consequent vacuolation due to accumulation of weak base.

CHL60-64 would inhibit lysosomal acidification by maintaining the interior-positive membrane potential.

Mislocalization of the Na^+,K^+-ATPase can also account for the reduction in vacuolation in CHL60-64. The vacuologenic amines are weak bases that are freely membrane permeable in the unprotonated (uncharged) form but are much less permeable when protonated (23). The unprotonated base diffuses across cell membranes, but is protonated and trapped in acidic compartments. Thus, influx of base causes an increase in pH but does not change the membrane potential (since the trapped base is positively charged). In normal lysosomes, the decrease in the proton gradient allows for continued proton transport in an attempt to reestablish a proton gradient, and the membrane potential increases. This acidification causes further accumulation of weak base (24–26). When the concentration of base is sufficiently high, swelling (due to osmotic pressure) occurs. The uncoupling of the H^+-ATPase thus drives the formation of huge cytoplasmic vacuoles (Fig. 3). In contrast, in vesicles regulated by the Na^+,K^+-ATPase, the accumulation of base is inhibited (since the H^+-ATPase is limited by membrane potential and is not uncoupled), and vacuolation does not occur. We believe that this is the basis for the resistance of CHL60-64 to chloroquine and vacuolation.

A Model for pH Regulation and Vacuolation. A model consistent with the data reported in this and the accompanying paper is depicted in Fig. 4. Ligands bind to specific cell-surface receptors and are internalized together with the Na^+,K^+-ATPase via pinosomes. These compartments fuse with each other or preexisting endosomes. In the presorting (transferrin-containing) compartment, the Na^+,K^+-ATPase internalized with ligands maintains an interior-positive membrane potential that limits the pH to $\approx$6. This pH is sufficiently acidic to allow dissociation of ligands from receptors and the release of iron from transferrin. In wild-type cells (Fig. 4 *Left*), the Na^+,K^+-ATPase is removed from the endocytic pathway during recycling and is returned to the cell surface with recycled receptors and transferrin. The remaining endocytic compartment (the late endosome or light lysosome) becomes a dense lysosome and is further acidified below pH 5.3. In CHL60-64 (Fig. 4 *Right*), not all of the Na^+,K^+-ATPase is removed during recycling, and the pH remains high in the late endocytic compartments.

This model for the regulation of early endocytic pH is strongly supported by the observation that ouabain significantly reduces the pH in the early endosome. We have also presented evidence that misdirection of the Na^+,K^+-ATPase can result in incorrect regulation of acidification in late endocytic compartments. The data presented for CHL60-64 imply that the Na^+,K^+-ATPase is not normally found in the postsorting endocytic compartments *in vivo*, confirming the *in vitro* results on isolated cell fractions (18). Interestingly, preliminary results indicate that ouabain has little effect on the extent of transferrin acidification in a cell line (K562) that does not limit the pH in the transferrin-containing endosome (D.M.S., A. Jesurum, and R.F.M., unpublished data). This may be due to differential sorting and/or endocytosis of the Na^+,K^+-ATPase. Although other charged species in these compartments may affect acidification (through their effects on membrane potential), the results presented here strongly support a major role for the Na^+,K^+-ATPase in the regulation of endocytic pH *in vivo*.

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