Effect of acidification on the location of \( \text{H}^+ \)-ATPase in cultured inner medullary collecting duct cells

EDWARD A. ALEXANDER,1 DENNIS BROWN,2 THEODORA SHIH,1 MARY MCKEE,2 AND JOHN H. SCHWARTZ1

1Renal Section, Boston University Medical Center and Departments of Medicine, Physiology, and Pathology, Boston University School of Medicine, Boston, 02118-2908; and 2Renal Unit and Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02129

IN PREVIOUS STUDIES, our laboratory has utilized a cell line derived from the rat inner medullary collecting duct (IMCD) as a model system for mammalian renal epithelial cell acid secretion. We have provided evidence, from a physiological perspective, that acute cellular acidification stimulates apical exocytosis and elicits a rapid increase in proton secretion that is mediated by an \( \text{H}^+ \)-ATPase. The purpose of these experiments was to examine the effect of acute cellular acidification on the distribution of the vacuolar \( \text{H}^+ \)-ATPase in IMCD cells in vitro. We utilized the 31-kDa subunit of the \( \text{H}^+ \)-ATPase as a marker of the complete enzyme. The distribution of this subunit of the \( \text{H}^+ \)-ATPase was evaluated by immunohistochemical techniques (confocal and electron microscopy), and we found that there is a redistribution of these pumps from vesicles to the apical membrane. Immunoblot evaluation of isolated apical membrane revealed a 237 ± 34% (P < 0.05, n = 9) increase in the 31-kDa subunit present in the membrane fraction 20 min after the induction of cellular acidification. Thus our results demonstrate the presence of this pump subunit in the apical membrane. In addition, we found that acute cellular acidification markedly increases the amount of this subunit in the apical membrane. Thus cell acidification regulates the shuffling of cytosolic vesicles containing the 31-kDa subunit into the apical membrane.

Acid secretion; hydrogen-adenosinetriphosphatase; cell pH; exocytosis; protein trafficking

METHODS

Solutions and reagents. The following solutions were used: NaCl HEPES buffer (NHB; in mM: 110 NaCl, 50 HEPES acid, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), and 5 glucose, pH 7.2) and choline chloride HEPES buffer (CHB; identical to NHB except that 110 mM choline chloride and 5 mM potassium acetate were substituted for NaCl and KCl, pH 7.2). Buffers were titrated to the desired pH using NaOH (for NHB), KOH (for CHB), or HCl.

Cell culture. IMCD cells were obtained from rat papillae as described previously (17, 18). Aliquots of these isolations have been preserved at −70°C and activated as needed. Cells from passages 6–12 were grown to confluence in 75-cm\(^2\) plastic flasks or on 12 × 12-mm glass coverslips in DMEM plus 10% FCS and penicillin and streptomycin in an atmosphere of 95% air-5% CO\(_2\).

Antibodies. A synthetic polypeptide (NH\(_2\)-GANANRKFLD) corresponding to the COOH-terminal 10 amino acid residues of the bovine 31-kDa sequence with the addition of an

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NH$_2$-terminal Cys was prepared. The peptide was covalently coupled to maleimide-activated keyhole limpet hemocyanin before immunization of New Zealand White rabbits. High-titer sera were obtained after four boosts at 2-wk intervals (Charles River Biopharm, Westboro, MA). Sera were tested by peptide ELISA. The antibody against E-cadherin (uvomorulin), which was a rat monclonal antibody (DECMA-1), and the antibody for the β-subunit of the coat protein coatomer (β-COP), clone M3A5, were both obtained from Sigma Chemical (St. Louis, MO). The anti-GP-135 antibody, a mouse monoclonal antibody, was a gift of Dr. G. K. Ojakian.

Preparation of tissue homogenate. Confluent IMCD cells were washed three times in cold PBS and placed in NHB or CHB for 20 min, scraped from the growth surface, and pelleted by centrifugation at 1,000 g for 10 min. The pellet was suspended in four volumes of ice-cold homogenizing buffer containing 10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40, to which 4 µM phenylmethylsulfonyl fluoride, 0.5 µg/ml aprotinin, 2 µg/ml N-tosyl-L-phenylalanine chloromethyl ketone, 5 µg/ml DNase, and 5 µg/ml RNase were added just before use. The suspended pellet was homogenized by 10-1 s strokes in a Teflon homogenizer. To remove intact cells and nuclei, this homogenate was centrifuged for 10 min at 1,000 g at 4°C for 10 min.

Apical membrane isolation. The apical membrane was selectively isolated by a modification of a method that induces vesiculation of this membrane (19, 20). IMCD cells grown to confluence in 150-mm culture dishes were washed three times with PBS (pH 7.4) and then exposed to either NHB (control) or CHB (acid) for 20 min. The monolayers were then incubated for 90 min at 37°C in vesiculation medium (in mM: 1 CaCl$_2$, 1 MgCl$_2$, 50 paraformaldehyde, and 2 dithiothreitol). At the end of the incubation period, vesiculation medium was filtered through a 37-µm nylon mesh to remove whole cells, and then the filtrate was centrifuged at 25,000 rpm at 4°C in an RC5B Sorvall centrifuge for 1 h to pellet the vesicles. The pellet was dissolved in SDS sample buffer, and aliquots were saved for protein and immunoblot analysis. Nine separate experiments were performed and analyzed.

Immunoblot. Whole cell homogenates and isolated plasma apical membrane samples prepared as described above were heated at 100°C for 5 min before the coat protein coatomer subunit of the H$_2$-ATPase. The diluent was PBS containing 1% normal goat serum and 1% BSA, which was also used for subsequent rinses. Preimmune serum (1:100) and diluent alone served as negative controls. After rinsing, the grids were incubated for 1 h at room temperature on drops of goat-anti-rabbit IgG coupled to 10-nm gold particles (Ted Pella, Redding, CA; 1:25 in rinse buffer). Grids were then rinsed in PBS rinse buffer, followed by rinses in PBS alone and then in distilled water. The grids were then fixed for 10 min at room temperature on drops of 1% glutaraldehyde in distilled water (Electron Microscopy Sciences, Fort Washington, PA). Sections were rinsed in distilled water and stained with 2% uranyl acetate for 7 min, followed by 2 min in lead citrate. Sections were examined and photographed at 80 kV on a Philips CM10 electron microscope.

Preembedding immunoperoxidase staining. Cells grown to confluence in 55-mm plastic petri dishes were incubated in NHB or CHB (pH 7.2) for 20 min and then fixed for 1 h in 4% paraformaldehyde plus 1% glutaraldehyde at room temperature. The dishes were rinsed several times in PBS, and then the cells were permeabilized for 1 h at room temperature on a shaker in 1% BSA, 0.2% animal gelatin, and 0.05% saponin (this solution was also used in all rinsing steps unless otherwise noted). The 31-kDa antibody described above was applied to the dishes 1 h in PBS-1% BSA in a humidified chamber, overnight at 4°C on a shaker. One dish each of control and acidotic cells had no primary antibody as a negative control. After being rinsed, the cells were incubated in biotinylated goat-anti-rabbit IgG (1:100 in PBS-BSA) for 2 h at room temperature. The cells were then rinsed and incubated with Vector ABC reagent solution in PBS-BSA for 2 h at room temperature (Vector Laboratories, Burlingame, CA). After several rinses in PBS, the cells were additionally fixed in 1% glutaraldehyde in PBS plus 5% sucrose for 30 min at room temperature. These were then rinsed in PBS plus 5% sucrose, followed by 0.05 M Tris (pH 7.6) plus 7.5% sucrose. The 3,3'-diaminobenzidine (DAB) reaction was carried out as follows. The cells were incubated for 5 min at room temperature in a solution of 0.1% DAB (Electron Microscopy Sciences) in Tris-sucrose, and then hydrogen peroxide was added to a final concentration of 0.01%, and the incubation was continued for 10 min in the dark. The cells were then rinsed in Tris-sucrose and then in Tris alone. The cells were then rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) and postfixed for 1 h in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer at room temperature (EMS). After being rinsed in sodium cacodylate and distilled water, the cells were stained en bloc for 1 h in 2% aqueous uranyl acetate at room temperature. The cells were then rinsed in distilled water, dehydrated through a graded series of ethanol, and infiltrated in a 1:100...
ethanol-EPON solution overnight on a shaker. The following day, after two changes of 100% EPON, a thin layer of fresh EPON (Ted Pella) was added to the dishes and they were polymerized at 60°C overnight. Small pieces of the EPON, with cells embedded in it, were cut out of the dish and reembedded in the tips of flat-embedded molds. Thin sections were collected onto Formvar-coated slot grids using a Retchert ultramicrotome, and the sections were examined on a Phillips CM 10 electron microscope and photographed.

RESULTS

To acidify cells, they were bathed in CHB for 20 min. As previously demonstrated (21), this reduced intracellular pH (pHi) from 7.35 ± 0.04 to 6.49 ± 0.05.

Immunostaining. Confocal microscopy of the same control cells revealed intracellular punctate labeling of the 31-kDa subunit of the H⁺-ATPase that was most intense in the perinuclear region, presumably the Golgi apparatus (Fig. 1A; cell center), while there was minimal apical membrane staining (Fig. 1B; cell apex). After acute cellular acidification, the Golgi continued to stain positively (Fig. 1C), but, in addition, immunostaining of the apical membrane was strikingly positive (Fig. 1D). Control immunohistochemical studies with preimmune serum instead of the primary antibody did not result in the staining of any structures. In addition, when β-COP, a Golgi marker (8), was examined, only immunostaining of the Golgi apparatus in control and acid cells was seen, without apical membrane or vesicular staining. Double labeling with β-COP and 31-kDa antibodies revealed partial overlap (not shown).

Electron microscopic immunocytochemistry. Further confirmation of the localization of the 31-kDa subunit is seen in the representative electron micrographs in Fig. 2. In control cells, there is little 31-kDa staining of the apical membrane (Fig. 2A), but there is staining of the cytosolic membrane and the Golgi (Fig. 2A and B). After acute cellular acidification, there is a marked increase in the H⁺-ATPase staining of the apical membrane and the microvilli (Fig. 2C). In addition, with immunogold staining (Fig. 2D), the 31-kDa subunit was also demonstrated in the microvilli and apical membrane of acid-loaded IMCD cells.

Apical membrane analysis. To confirm and quantify these immunocytological observations, we isolated apical membrane and analyzed it for H⁺-ATPase subunits by immunoblot. Apical membrane was isolated using an adaptation of a vesiculation technique (19, 20). With this technique, we have been able to obtain a preparation that is enriched in apical membrane and has no demonstrable basolateral membrane contamination. The degree of purification was demonstrated by Western blot analysis of apical vesicles and whole cell homogenate (Fig. 3). The amount of GP-135, an apical protein marker (14), was enriched 9.2 ± 0.4-fold (P < 0.05, n = 9) in the vesicle fraction compared with the whole cell homogenate whereas, E-cadherin, a basolateral marker, was barely detectable in the vesicle preparation but was present in whole cell homogenate. With this method, we have identified the 31-kDa subunit of the H⁺-ATPase in the apical membrane. In addition, after cells were acidified to stimulate proton secretion, there was a striking increase in the amount of the 31-kDa subunit of the H⁺-ATPase detected in the apical membrane fraction in all nine experiments. Densitomet-

![Fig. 1. Immunolocalization of 31-kDa subunit of H⁺-ATPase by confocal microscopy in same cells: control at center of cell (A) and at cell apex (B) and after acute acidification at center of cell (C) and at cell apex (D). See text for descriptions. Arrows identify the same cells. Scale bars, 1 µm.](http://apcell.physiology.org/)

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ric evaluation of these data revealed an increase of 237 ± 34% in 31-kDa subunit (P < 0.05, n = 9), whereas there was no change in GP-135 when control and acid incubated cells were compared.

**DISCUSSION**

These experiments, utilizing immunohistochemical and immunoblot techniques, provide new information that IMCD cells, in culture, express the 31-kDa subunit of the proton pump and that an acute acid challenge to these cells initiates a redistribution of these pumps to the apical membrane. We have previously demonstrated that these cells exhibited pH$_i$-mediated exocytosis that is enhanced by cell acidification (17) and that disruption of microtubules or microfilaments reduced this exocytic response (17). In addition, we have re-

![Fig. 2](image)

**Fig. 2.** Composite plate showing electron micrographs of inner medullary collecting duct (IMCD) cells in which there is immunolocalization of 31-kDa subunit of H$^+$-ATPase in control cells (A and B; arrowheads identify Golgi apparatus) and in acutely acidified cells (C and D). A, B, and C were stained using pre-embedding immunoperoxidase technique, whereas D was obtained with immunogold labeling. Scale bars, 1 µm.

![Fig. 3](image)

**Fig. 3.** Western blot analysis showing effect of acute acidification on 31-kDa subunit of H$^+$-ATPase in apical plasma membrane of IMCD cells utilizing vesiculation technique. A: an apical vesicle preparation (lanes 1, 3, and 5) is compared with a whole cell homogenate (lanes 2, 4, and 6). Partial purification of an apical membrane fraction from control cells is demonstrated with apical marker GP-135 (lanes 1 and 2), basolateral marker E-cadherin (lanes 3 and 4), and 31-kDa subunit of H$^+$-ATPase (lanes 5 and 6). Paraformaldehyde-isolated vesicles are enriched with GP-135 compared with whole cell homogenate (lane 1 vs. lane 2), nearly devoid of E-cadherin (lane 3 vs. 4), and have equivalent distribution of H$^+$-ATPase between vesicle and whole cell homogenate (lane 5 vs. lane 6). B: effect of acute acidification is depicted in duplicate experiments. Lanes 1 and 2, control cells; lanes 3 and 4, acid cells. An increase in staining intensity of 31-kDa band is clearly visible after acid loading. Immunoblot for GP-135 is provided as an internal control. All lanes were loaded with 40 µg protein.
ciently demonstrated that the exocytotic response to acid is probably mediated by the vesicle membrane SNAP-25 (v-SNARE) and target membrane SNAP-23 (t-SNARE) system (1), a mechanism for exocytosis best described in neural cells (2, 6). The present data provide additional support for the thesis that proton secretion by this IMCD cell line is mediated by an H^+-ATPase and that the rate of proton transport is regulated by exocytic insertion and endocytic retrieval of this H^+-ATPase from the apical membrane (4, 10, 16, 17).

In the control cells, examined by immunohistochemistry with confocal microscopy, the H^+-ATPase was not detectable in the apical membrane, and positive staining was found only in the perinuclear region of the cells, including the Golgi apparatus. However, with the more sensitive techniques of electron microscopy and immunolabeling of the isolated apical membrane, some H^+-ATPase was detectable in the apical membrane even in control cells. This is consistent with the likelihood that proton pump-containing vesicles are continuously recycled from the cytosol to the apical membrane and participate in constitutive acid secretion (7).

The response to acute cellular acidification was striking. We have previously shown that exposure to CHB reduces pH, to 6.5 and that recovery occurs at 0.05–0.07 pH units/min (1, 17, 21). In the present studies under these same conditions, a markedly increased staining was noted in the apical membrane by immunohistochemical methods, and this increase was greater than 200% when quantified on Western blot of isolated apical membrane. Thus acute cell acidification appears to be a signal for shuttling of the 31-kDa subunit of the H^+-ATPase from internal structures (cytosolic vesicles) to the apical membrane. Whether acidification also increased trafficking of proton pumps from the Golgi apparatus cannot be determined from our experiments.

These data are consistent with findings previously demonstrated in other acid secretory cells such as the mitochondrion-rich cell of the turtle bladder and the cortical collecting duct A or α-intercalated cell of the rat or rabbit. In these cell types, acidification leads to an increase in the surface area of the apical membrane because of the insertion of acidic vesicles containing proton pumps (12, 22). It is of interest that we have previously demonstrated acidic vesicles in our IMCD cell line also (17).

Studies examining the effect of acute and chronic acidosis on the distribution of H^+-ATPase in cortical collecting duct tissue sections are also consistent with our findings (3, 15). Sabolic et al. (15) found that, after 6 h of metabolic acidosis in rats, there was a redistribution of H^+-ATPase as demonstrated by an enhancement in apical membrane staining in type A intercalated cells (15). Bastani and colleagues (3) examined the effect of chronic acid feeding on the distribution of the 31-kDa subunit in tissue sections of rat IMCD. The shortest period they studied was 3–5 days of acid feeding. They found that acid loading produced a marked redistribution of the H^+-ATPase staining. Under control conditions the proton pump was predominantly found in the cytosolic vesicles, whereas in acid-fed animals the apical membrane staining was much more prominent. These data are also consistent with the present findings. Although it is possible that in their chronic studies there was time for redistribution of new pumps to the apical locus, this issue remains highly controversial (11). In our studies, given the rapidity of response, only the redistribution of existing pumps could have occurred.

One alternative hypothesis that would also be consistent with the present data is the concept that some degree of regulation of the proton pump occurs through assembly of pump units upon cellular acidification. The 31-kDa subunit is thought to be a part of the stalk domain of the H^+-ATPase that connects the catalytic unit (V_o) to the transmembrane (V_i) domain of the protein. The appearance of the 31-kDa subunit in the apical membrane after acidification could represent, at least in part, pump assembly at this location.

These data demonstrate that the proton pump can be identified in cultured IMCD cells and that these cells respond to acidification in a manner that is consistent with that observed in intercalated cells of animals with acute or chronic acid feeding in vivo. The data provide further support for the proposal that changes in cell pH regulate acid secretion by insertion of proton pump units into the apical membrane of acid-secreting collecting duct cells. In addition, they provide further support for the use of this cell line as a model for renal cell acidification.

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Address for reprint requests: E. A. Alexander, Evans 401, One Boston Medical Center Place, Boston, MA 02118-2908.

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