Mechanisms through which ammonia regulates cortical collecting duct net proton secretion

AMY E. FRANK,1 CHARLES S. WINGO,1 PETER M. ANDREWS,2 SHANA AGELOFF,3 MARK A. KNEPPER,3 AND I. DAVID WEINER1

1Division of Nephrology, Hypertension, and Transplantation, University of Florida, and Gainesville Veterans Affairs Medical Center, Gainesville, Florida 32610-0224; 2Department of Cell Biology, Georgetown University, Washington, District of Columbia 20007; and 3Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

Received 27 August 2001; accepted in final form 5 January 2002

Frank, Amy E., Charles S. Wingo, Peter M. Andrews, Shana Ageloff, Mark A. Knepper, and I. David Weiner. Mechanisms through which ammonia regulates cortical collecting duct net proton secretion. Am J Physiol Renal Physiol 282: F1120–F1128, 2002.—Ammonia stimulates cortical collecting duct (CCD) net bicarbonate reabsorption by activating an apical H+-K+-ATPase through mechanisms that are independent of ammonia’s known effects on intracellular pH and active sodium transport. The present studies examined whether this stimulation occurs through soluble N-ethylmaleimide-sensitive fusion attachment receptor (SNARE) protein-mediated vesicle fusion. Rabbit CCD segments were studied using in vitro microperfusion, and transepithelial bicarbonate transport was measured using microcalorimetry. Ammonia’s stimulation of bicarbonate reabsorption was blocked by either chelating intracellular calcium with 1,2-bis(2-aminophenoxy)ethane-N,N,N,N′,N′,N′-tetraacetic acid acetoxymethyl ester or by inhibiting microtubule polymerization with colchicine compared with parallel studies performed in the absence of these inhibitors. An inactive structural analog of colchicine, lumicolchicine, did not alter ammonia’s stimulation of bicarbonate reabsorption. Tetanus toxin, a zinc endopeptidase specific for vesicle-associated SNARE (v-SNARE) proteins, prevented ammonia from stimulating net bicarbonate reabsorption. Consistent with the functional evidence for v-SNARE involvement, antibodies directed against a conserved region of isoforms 1–3 of the tetanus toxin-sensitive, vesicle-associated membrane protein (VAMP) members of v-SNARE proteins labeled the apical and subapical region of collecting duct intercalated cells. Similarly, antibodies to NSF protein, a protein involved in activation of SNARE proteins for subsequent vesicle fusion, localized to the apical and subapical region of collecting duct intercalated cells. These results indicate that ammonia stimulates CCD bicarbonate reabsorption through an intracellular calcium-dependent, microtubule-dependent, and v-SNARE-dependent mechanism that appears to involve insertion of cytoplasmic vesicles into the apical plasma membrane of CCD intercalated cells.

intracellular calcium; 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′,N′-tetraacetic acid acetoxymethyl ester; microtubule; soluble N-ethylmaleimide-sensitive fusion attachment receptor protein; hydrogen-potassium-adenosine triphosphatase

AMMONIA PLAYS AN IMPORTANT role in renal acid-base homeostasis (17, 19, 24). The proximal tubule utilizes glutamine to produce equimolar amounts of ammonia and bicarbonate (9). The bicarbonate produced buffers acids produced by endogenous acid production (33). Acidosis stimulates ammoniagenesis and consequent bicarbonate generation, enabling changes in ammonia’s metabolism to mediate a central role in acid-base homeostasis (10, 19).

In addition, ammonia regulates collecting duct proton, potassium, and sodium transport (12, 13, 18). Recent studies show that ammonia stimulates collecting duct proton secretion, measured as net bicarbonate reabsorption, in the cortical collecting duct (CCD) (13, 25), in the outer medullary collecting duct (OMCD) (11), and in the inner medullary collecting duct (IMCD) (44). This stimulation is due to activation of apical H+-K+-ATPase (13). Ammonia also regulates urinary potassium excretion (22, 40), at least in part by stimulating CCD unidirectional potassium reabsorption and inhibiting potassium secretion (18). Ammonia’s stimulation of CCD potassium reabsorption can be explained by its stimulation of H+-K+-ATPase (13), a proton-secreting, potassium-reabsorbing protein. Thus ammonia regulates collecting duct proton secretion and potassium reabsorption, at least in part, by stimulating H+-K+-ATPase-mediated ion transport.

The mechanism(s) through which ammonia regulates CCD proton secretion and potassium reabsorption has not been completely identified. Ammonia’s stimulation of bicarbonate reabsorption is independent of its effect on intracellular pH or active sodium transport, and, in contrast to rat IMCD (45), does not require ammonium (NH4+) uptake by basolateral Na+-K+-ATPase (13). Thus ammonia’s effect on CCD ion transport cannot be explained solely through its role as a solute transported by CCD intercalated cells.

One mechanism by which renal epithelial cells regulate ion transport is via vesicular trafficking of pro-
teins between the apical plasma membrane and cytosolic vesicles (2–4). This appears, for example, to be an important mechanism for the regulation of H\(^+-K^+\)-ATPase in both the stomach (6) and, at least in response to acute elevations in PCO\(_2\), in rabbit CCD (52) and for the regulation of H\(^+-\)ATPase in the collecting duct (15, 16).

The purpose of the present studies was to determine whether ammonia stimulates CCD net bicarbonate reabsorption through insertion of intracellular vesicles into the apical plasma membranes of CCD intercalated cells. We determined whether ammonia’s stimulation of CCD net bicarbonate reabsorption exhibited physiological characteristics of vesicular trafficking, specifically, intracellular calcium-dependent, microtubule-dependent activation that involved soluble N-ethylmaleimide-sensitive fusion attachment receptor (SNARE) proteins. Our results show that ammonia stimulates CCD net bicarbonate reabsorption through an intracellular calcium-dependent, microtubule-dependent and vesicle-associated SNARE (v-SNARE)-dependent mechanism. Furthermore, immunohistochemical studies demonstrate the presence of at least two components of SNARE protein-mediated vesicle docking, vesicle-associated membrane protein (VAMP) and N-ethylmaleimide-sensitive fusion protein (NSF), in collecting duct intercalated cells. Thus ammonia appears to regulate CCD net bicarbonate transport, at least in part, by a mechanism that appears to involve vesicle-mediated insertion of proteins, most likely H\(^+-\)K\(^+-\)ATPase, into the apical plasma membrane of CCD intercalated cells.

**METHODS**

Microperfusion. We used standard in vitro microperfusion techniques and female New Zealand White rabbits (1.5–2 kg) as previously described in detail (12, 13, 47, 48, 50). The solutions used were artificial solutions, and, unless otherwise mentioned, contained (in mM) 119.2 NaCl, 3 KCl, 25 NaHCO\(_3\), 2 KH\(_2\)PO\(_4\), 1 Na-acetate, 1.2 CaCl\(_2\), 1 MgSO\(_4\), 5 alanine, and 8.3 glucose. Osmolarity was adjusted to 290 ± 7 mosmol/kgH\(_2\)O with NaCl. Ammonia chloride (10 mM) substituted for NaCl in both the luminal and the peritubular solutions used were artificial solutions, and, unless otherwise described in detail (12, 13, 47, 48, 50). The solutions were gassed with 95% O\(_2\)-5% CO\(_2\) for at least 1 h before use. CCD were equilibrated at 37°C for at least 30 min before the initial set of measurements and for at least 30 min after ammonia addition before repeat measurements were made. All inhibitors were added 30 min before the initial set of measurements and were present throughout the remainder of the experiment.

Bicarbonate transport. We measured transepithelial bicarbonate transport using standard techniques previously described in detail (13). Briefly, total CO\(_2\) (tCO\(_2\)) concentration, which is predominantly HCO\(_3\) at physiological pH, was measured using a picocnophotometer (WPI, Sarasota, FL). We calculated net transepithelial bicarbonate transport (J\(_{\text{CO}_2}\)) with the formula J\(_{\text{CO}_2}\) = \(V_o \times (C_1 - C_2)/l\), where \(V_o\) is the collected fluid rate (in nl/min), \(C_1\) and \(C_2\) are the tCO\(_2\) content in the perfusate and collected fluid, respectively, and \(l\) is the tubule length (in mm). The luminal fluid flow was adjusted to ~4 nl/min by regulating the hydrostatic perfusion pressure. J\(_{\text{CO}_2}\) was measured two to four times during each experimental period and averaged to yield a single measurement. Exhaustively dialyzed \(^{14}\)H\(\)ulin was added to the perfusate in all experiments, and experiments with leak rates >5% were discarded.

Antibody production. To generate an antibody to a conserved region of VAMP1–3, a synthetic peptide was produced to the sequence H\(_2\)N-CNVKVLREDQKLSELDARRADACOOH and conjugated to keyhole limpet hemocyanin (KLH) for immunization of rabbits as previously described (29). The antisera were affinity purified using a column on which 2 mg of the synthetic peptide were immobilized via sulphydryl linkage to activated agarose beads (SulfoLink Immunobilization Kit 2, Pierce, Rockford, IL).

Polyclonal antisera were raised in rabbits against an HPLC-purified synthetic peptide corresponding to the COOH-terminal 24 amino acids of rat NSF (GenBank accession no. NP_068516; sequence H-CPDEYVRKPLMLRREEGASPLDFD-COOH). The peptide was synthesized with the addition of a cysteine residue at the amino terminus to facilitate conjugation to KLH via a cysteine sulphydryl linkage. Two rabbits were immunized with the peptide-KLH conjugate. Both rabbits (L248 and L249) developed ELISA titers of >1:32,000. The resulting antibodies were affinity purified on a column on which the same synthetic peptide was immobilized. Although the two antisera gave similar labeling on both immunoabsorbs, the studies reported here were carried out with antiserum L249.

**Immunoblotting.** After euthanasia, kidneys were quickly removed and cortical and outer and inner medullary sections were obtained. All sections were placed in chilled isolation solution (pH 7.6) containing 250 mM sucrose, 10 mM triethanolamine (Calbiochem, La Jolla, CA), 1 µg/ml leupeptin (Bachem, Torrance, CA), and 0.1 mg/ml phenylmethylsulfonyl fluoride (US Biochemical, Toledo, OH) and homogenized using a tissue homogenizer (Omni 1000) at 4°C. After homogenization, protein concentration was measured using a Pierce bicinchoninic acid protein assay reagent kit. After homogenization, protein concentration was measured using a Pierce bicinchoninic acid protein assay reagent kit and adjusted to 2 µg/µl with isolation solution. Samples were then solubilized by adding 5× Laemmli sample buffer and heating to 60°C for 15 min. To assess the equality of loading, a single electrophoresis was initially run on a 12% gel that was then stained with Coomassie blue. Major bands were then analyzed using densitometry (Molecular Dynamics, San Jose, CA). SDS-PAGE was done using 12% polyacrylamide minigels (Bio-Rad, Hercules, CA). Gels were then transferred electrophoretically to nitrocellulose membranes and subsequently blocked with 5 g/dl nonfat dry milk. Proteins were probed overnight at 4°C with primary antibody. The primary antibody was prepared in an antibody diluent containing 150 mM NaCl, 50 mM Na phosphate, 10 mg/dl Na azide, 50 mg/dl Tween 20, and 1 g/dl bovine serum albumin (pH 7.5). After being washed, membranes were exposed to secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase; Pierce 31463) at a concentration of 0.16 µg/ml and incubated for 1 h at room temperature. The membrane was stained with Coomassie blue. Major bands were identified using densitometry (Molecular Dynamics, San Jose, CA).

Immunocytochemistry. Rat kidneys were fixed by retrograde vascular perfusion of Bouin’s fixative. Rats were used instead of rabbits for these studies because the primary antibodies were generated in rabbits against rat SNARE protein sequences. After a brief rinse in PBS, the kidneys were dehydrated and embedded in paraffin. Semithick sections (i.e., 2–5 µm) were cut and mounted on glass slides.

AJP-Renal Physiol • VOL 282 • JUNE 2002 • www.ajprenal.org
After deparaffinization, the sections were rinsed in PBS, blocked with goat serum, briefly rinsed in PBS, and incubated for 2 h at 37°C with affinity-purified primary antibodies directed against either NSF or conserved regions present in VAMP 1–3. Following a brief rinse in PBS, a gold-labeled secondary antibody was applied for 2 h at 37°C. The slides were then rinsed in water, reacted with silver to enhance the localization of the immunogold (BBI silver-enhancing kit, Cardiff, UK), counterstained with hematoxylin, and viewed and photographed using an Olympus BH-2 light microscope equipped with a 35-mm camera.

Chemicals. Tetanus toxin was obtained from Boehringer Mannheim and dissolved directly into experimental solutions when used. All other chemicals were from Sigma (St. Louis, MO).

Statistics. Results are presented as means ± SE. The data were analyzed using a paired Student’s t-test and ANOVA as appropriate, and P < 0.05 was evidence of statistical significance.

RESULTS

Role of intracellular calcium. Intracellular calcium is necessary for the activation of vesicular insertion into plasma membranes (5, 27). In the first set of experiments, we determined the effect of chelating intracellular calcium with the membrane-permeant calcium-chelator 1,2-bis(2-aminophenoxy)ethane- N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM) on ammonia’s stimulation of net bicarbonate reabsorption. BAPTA-AM (15 μM) was present in the peritubular solution throughout the experiment. In the presence of BAPTA-AM, basal net bicarbonate reabsorption averaged 2.9 ± 1.2 pmol·mm⁻¹·min⁻¹ in the absence of ammonia and only 5.0 ± 3.3 pmol·mm⁻¹·min⁻¹ (n = 6) in the presence of ammonia. Figure 1 summarizes these results. Ammonia, in the presence of the intracellular calcium chelator BAPTA-AM did not significantly stimulate bicarbonate reabsorption [P = not significant (NS), n = 6]. This contrasts with previous findings that ammonia substantially stimulates CCD bicarbonate reabsorption (13, 25). Thus ammonia appears to stimulate CCD net bicarbonate reabsorption through mechanisms that are blocked by BAPTA-AM and therefore appear related to involvement of intracellular calcium.

The lack of stimulation by ammonia could be related to BAPTA-AM or could be due to spontaneous variations in the magnitude of ammonia’s stimulation of bicarbonate transport. To differentiate between these possibilities, the next set of experiments sought to reconfirm the magnitude of ammonia’s stimulation of bicarbonate reabsorption (13). No inhibitors were used in this set of studies. Net bicarbonate reabsorption averaged 4.4 ± 1.8 pmol·mm⁻¹·min⁻¹ in the absence of ammonia and 13.5 ± 3.1 pmol·mm⁻¹·min⁻¹ in the presence of ammonia (n = 3). Figure 2 summarizes these results. In the absence of inhibitors, ammonia significantly stimulated bicarbonate reabsorption (P < 0.02 by ANOVA), and the degree of stimulation was similar to that we reported previously (13). Basal transport rates were not altered by the presence or absence of BAPTA-AM (P = NS by ANOVA). These results confirm that ammonia stimulates net bicarbonate reabsorption, and, in conjunction with the first set of studies in this report, demonstrate that this stimulation occurs through an intracellular calcium-dependent, BAPTA-AM-inhibitable mechanism.

Role of microtubules. Intracellular calcium can regulate renal ion transport through a variety of mechanisms. At least one mechanism is by stimulating membrane transporter insertion from cytoplasmic vesicles into plasma membranes (5, 27), a process that involves microtubule-dependent movement of the vesicles. To...
test whether ammonia stimulates bicarbonate reabsorption through this mechanism, we examined whether inhibiting microtubule polymerization would prevent the ammonia-stimulated increase in net bicarbonate reabsorption. In these studies, we used the highly specific microtubule polymerization inhibitor colchicine (50 μM). In the presence of colchicine, basal bicarbonate reabsorption averaged 2.5 ± 1.6 pmol·mm⁻¹·min⁻¹ and was not significantly increased by ammonia, averaging only 6.1 ± 1.6 pmol·mm⁻¹·min⁻¹ (P = NS, n = 4). Figure 3 summarizes the magnitude of ammonia’s stimulation of bicarbonate reabsorption in the absence and presence of colchicine. Colchicine significantly inhibited ammonia’s stimulation of net bicarbonate reabsorption (P < 0.05 by ANOVA) but did not alter basal bicarbonate reabsorption (P = NS by ANOVA). These results demonstrate that ammonia stimulates net bicarbonate transport through a colchicine-sensitive mechanism, most likely involving microtubule polymerization.

To confirm that colchicine’s effects were related to inhibition of microtubule polymerization, we tested the effect of a structurally related but inactive analog, lumicolchicine. These results are summarized in Fig. 3. When lumicolchicine (50 μM) was present, basal net bicarbonate reabsorption averaged 9.8 ± 7.8 pmol·mm⁻¹·min⁻¹ (n = 4), and adding ammonia significantly increased net bicarbonate reabsorption to 20.5 ± 8.6 pmol·mm⁻¹·min⁻¹ (P < 0.003 vs. without ammonia, n = 4). In contrast to colchicine, lumicolchicine did not significantly alter ammonia’s stimulation of net bicarbonate reabsorption (P = NS by ANOVA). Thus ammonia stimulates CCD net bicarbonate reabsorption through a colchicine-sensitive, lumicolchicine-insensitive mechanism, most likely involving microtubule polymerization.

**Effect of inhibiting SNARE proteins.** The observation that intracellular calcium and microtubule polymerization are required for ammonia’s stimulation of net bicarbonate reabsorption suggests that ammonia stimulates insertion of cytoplasmic vesicles into the apical plasma membrane. If so, this likely requires the interaction of fusion proteins, such as SNARE proteins. To test this hypothesis, we examined the effect of a SNARE protein inhibitor, tetanus toxin, on ammonia’s ability to stimulate CCD net bicarbonate reabsorption. Tetanus toxin (25 nM) was present in the luminal fluid throughout the experiment. Bicarbonate reabsorption is expressed as pmol·mm⁻¹·min⁻¹.

**SNARE proteins present in CCD intercalated cells.** Tetanus toxin inhibits vesicle fusion by cleaving members of the VAMP family of v-SNARE proteins (21, 37). To begin identifying which SNARE proteins are involved in ammonia-induced insertion of CCD intercalated cell vesicles, we determined whether VAMP proteins were present in collecting duct intercalated cells. An antibody to a conserved region of VAMP1–3 was generated. Figure 5 demonstrates that the antibody recognizes a single band in protein from rat cortex and outer and inner medulla. Not shown is that no band was seen with preimmune sera.

![Fig. 3. Effects of colchicine, an inhibitor of microtubule polymerization, and lumicolchicine, an inactive structural analog, on ammonia’s stimulation of CCD net bicarbonate reabsorption. Results plotted reflect the increase in net bicarbonate reabsorption (in pmol·mm⁻¹·min⁻¹) after ammonia addition. Control data are from Fig. 1. *P = NS vs. 0 change. **P < 0.003 vs. 0 change.](http://ajprenal.physiology.org/)

![Fig. 4. Effect of tetanus toxin, an inhibitor of N-ethylmaleimidesensitive fusion attachment receptor (SNARE) proteins, on ammonia’s stimulation of CCD net bicarbonate reabsorption. Tetanus toxin (25 nM) was present in the luminal fluid throughout the experiment. Bicarbonate reabsorption is expressed as pmol·mm⁻¹·min⁻¹.](http://ajprenal.physiology.org/)
Figure 6 shows representative light micrographs of VAMP1–3 immunolocalization. VAMP proteins appeared to be present in the apical and subapical region of all collecting duct cells. Control experiments examining results when the primary antibody was omitted revealed no immunoreactivity (not shown). Thus tetanus toxin-sensitive v-SNAREs, VAMP1–3, are present in both intercalated cells and principal cells. These proteins are likely candidates for proteins that mediate, at least in part, ammonia's stimulation of 

Docking of cytoplasmic vesicles with plasma membranes is a complicated process that involves multiple protein components that typically includes NSF. Antibodies directed against the COOH-terminal 24 amino acids of rat NSF were generated and affinity purified. As shown in Fig. 7, the antibody strongly labeled an appropriately sized band (93 kDa) in the lane loaded with brain homogenate and recombinant NSF protein. The antibody also labeled a weak band of the same molecular mass in the renal cortex. Not shown is that no band was seen with preimmune sera.

Figure 8 shows a representative section of rat kidney examined for NSF. Specific labeling of the intercalated cell apical and subapical regions (arrowheads) was present. Intercalated cells were recognized by their characteristic morphological characteristics, apical protrusion into the lumen and a darker appearance. Low levels of localization of NSF also appeared to be present in principal cell apical membranes. Control experiments that examined staining when the primary antibody was omitted were negative (not shown). Thus multiple components of the SNARE protein system for vesicle docking and fusion are present in collecting duct intercalated cells. These results are consistent with ammonia stimulating net bicarbonate reabsorption through a process that involves multiple components of SNARE protein-mediated vesicle, including a tetanus toxin-sensitive v-SNARE, VAMP1–3, and NSF.

DISCUSSION

The present studies extend our series of studies examining the cellular mechanisms through which ammonia stimulates renal H⁺-K⁺-ATPase. In previous studies, our laboratory has shown that ammonia exerts a profound effect to stimulate CCD net bicarbonate reabsorption and that this occurs almost entirely through activation of H⁺-K⁺-ATPase (13). This activation is independent of ammonia's effects on interca-
lated cell intracellular pH (12, 13) and on principal cell-mediated sodium transport (13, 18). Moreover, ammonia's stimulation of H^+\text{}/\text{H}^{1001}/\text{K}^{+}/\text{H}^{1001}/\text{ATPase} is a component of the integrated regulation of the CCD proton and bicarbonate transporters involved in the separate process of luminal bicarbonate reabsorption and bicarbonate secretion (12). The present studies show that ammonia stimulates CCD net bicarbonate reabsorption through an intracellular calcium-dependent, microtubule-dependent process that is blocked by inhibition of SNARE proteins. These findings, in association with the demonstration of multiple SNARE protein components in the apical and subapical region of collecting duct intercalated cells, suggest that ammonia stimulates net bicarbonate transport through a mechanism that appears to involve insertion of cytoplasmic vesicles containing H^+\text{}/\text{K}^{+}/\text{ATPase} into the apical membrane of CCD intercalated cells.

Changes in intracellular calcium play a role in cellular responses to a wide variety of stimuli. For example, trafficking of aquaporin-2 to the apical membrane of collecting duct principal cells in response to vasopressin is dependent on intracellular calcium release from ryanodine-sensitive stores (7). Intracellular calcium also regulates H^+\text{}/\text{K}^{+}/\text{ATPase} in both the stomach and the colon (20, 26, 28, 39), 10% CO_2-dependent stimulation of CCD H^+\text{}/\text{K}^{+}/\text{ATPase} (52), and collecting duct H^+\text{}/\text{ATPase} stimulation by metabolic acidosis (38, 43). Finally, intracellular calcium plays a role in SNARE-protein-mediated vesicle fusion with plasma membranes (27). The present studies extend these findings by demonstrating that intracellular calcium also participates in ammonia's stimulation of CCD net bicarbonate reabsorption.

H^+\text{}/\text{K}^{+}/\text{ATPase} is likely to be at least one of the proteins inserted into CCD intercalated cell apical plasma membranes in response to ammonia. Previous studies from our laboratory indicate that ammonia stimulates both CCD H^+\text{}/\text{K}^{+}/\text{ATPase}-mediated net bicarbonate reabsorption (13) and intercalated cell apical H^+\text{}/\text{K}^{+}/\text{ATPase}-mediated proton secretion (12). Elevated PCO_2 appears to stimulate CCD H^+\text{}/\text{K}^{+}/\text{ATPase} through vesicular insertion into CCD intercalated cell apical plasma membranes (52). Recycling of H^+\text{}/\text{K}^{+}/\text{ATPase} between cytosolic vesicles and plasma membranes is a major mechanism of its regulation in both the stomach (23, 34, 41) and the kidney (46) and appears to involve a tyrosine-based signal in the cytoplasmic tail of the H^+\text{}/\text{K}^{+}/\text{ATPase} β-subunit (8, 46) and involvement of SNARE proteins (Ref. 32 and present study). Thus it is likely that both ammonia and elevated PCO_2 stimulate CCD bicarbonate reabsorption by inducing insertion of H^+\text{}/\text{K}^{+}/\text{ATPase} into the apical plasma membrane of CCD intercalated cells. An alternative explanation that we cannot exclude at present is

Fig. 7. Immunoblot of N-ethylmaleimide-sensitive fusion protein (NSF) antibody. SDS-PAGE was run on a 10% polyacrylamide gel, and proteins were transferred electrophoretically to nitrocellulose membranes. Blot was probed with rabbit polyclonal antibody L249. Fifteen micrograms of homogenate protein were loaded for brain and kidney; 0.5 μg of recombinant NSF was loaded in last lane. Antibody was used at a concentration of 0.6 μg/ml IgG.

Fig. 8. Localization of NSF in the collecting duct. Gold-labeled, affinity-purified antibody to NSF complexes was amplified with silver to be visualized at the light microscopic level. Arrowheads, apical localization of NSF in intercalated cells.
that ammonia might induce vesicular trafficking of an H+\textsuperscript{-}K\textsuperscript{+}-ATPase regulatory protein, either instead of or in addition to H+\textsuperscript{-}K\textsuperscript{+}-ATPase.

SNARE proteins are highly conserved proteins involved in the insertion of proteins into the plasma membrane of a wide variety of cells (35, 36). Our results identify that both NSF and VAMP, ubiquitous components of SNARE protein-mediated vesicle trafficking, are present in collecting duct cells. Because tetanus toxin is a highly specific protease that inactivates VAMP proteins (31, 37), tetanus toxin is likely to inhibit ammonia-stimulated, H+\textsuperscript{-}K\textsuperscript{+}-ATPase-mediated ion transport through cleavage of VAMP proteins.

NSF is a ubiquitous component of SNARE protein-mediated vesicle trafficking. Identifying that NSF is present in collecting ducts intercalated cells in the present study is consistent with the previous demonstration that NSF is present in cultured IMCD cells (1) and in the amphibian bladder, model epithelia for the mammalian collecting duct (14). In the IMCD, NSF appears to participate in acidosis-induced shuttling of H+\textsuperscript{-}ATPase, but not H+\textsuperscript{-}K\textsuperscript{+}-ATPase, to apical plasma membranes (1). In view of the extensive evidence that vesicle-mediated shuttling regulates H+\textsuperscript{-}ATPase-mediated proton secretion in the collecting duct (15, 16), determining whether NSF participates in vesicular shuttling of H+\textsuperscript{-}K\textsuperscript{+}-ATPase, H+\textsuperscript{-}ATPase, or both will be an important field of future studies.

In the collecting duct, both H+\textsuperscript{-}K\textsuperscript{+}-ATPase and H+\textsuperscript{-}ATPase appear to be regulated, at least in part, by insertion of endocytic vesicles into intercalated cells apical plasma membranes. Some stimuli, such as ammonia and hypercapnia, stimulate H+\textsuperscript{-}K\textsuperscript{+}-ATPase-mediated ion transport (13, 53), whereas other stimuli, such as metabolic acidosis and acute intracellular acidosis, predominantly stimulate H+\textsuperscript{-}ATPase-mediated proton secretion (30, 42, 49). The finding that both H+\textsuperscript{-}K\textsuperscript{+}-ATPase and H+\textsuperscript{-}ATPase are regulated by plasma membrane insertion from cytoplasmic vesicles, yet are regulated by different stimuli, raises important issues regarding the cellular mechanisms underlying this differential regulation. One possibility is that H+\textsuperscript{-}K\textsuperscript{+}-ATPase and H+\textsuperscript{-}ATPase reside in different vesicle populations, the membrane insertion of which is separately regulated. This possibility is supported by the observation that in the CCD B-type intercalated cell, H+\textsuperscript{-}ATPase is inserted into the basolateral membrane whereas H+\textsuperscript{-}K\textsuperscript{+}-ATPase is inserted into the apical membrane (49). An alternative possibility is that H+\textsuperscript{-}K\textsuperscript{+}-ATPase and H+\textsuperscript{-}ATPase coexist in the same vesicles and that different stimuli have the ability to specifically stimulate insertion of either H+\textsuperscript{-}K\textsuperscript{+}-ATPase or H+\textsuperscript{-}ATPase, but not both, from the same vesicle into plasma membranes. At present, we cannot exclude the latter possibility.

In these studies, there was a tendency, although not statistically significant, for control bicarbonate reabsorption rates to be lowered by BAPTA-AM, colchicine, and tetanus toxin. One possible explanation is that these agents may interfere with vesicular trafficking of proteins involved in basal rates of CCD bicarbonate reabsorption.

CCD bicarbonate transport is the sum of the separate processes of bicarbonate reabsorption and bicarbonate secretion. Previous data from our laboratory suggest that both components of CCD bicarbonate transport are regulated by ammonia. In particular, ammonia inhibits B cell-mediated bicarbonate secretion (12). Ammonia also stimulates H+\textsuperscript{-}K\textsuperscript{+}-ATPase-dependent bicarbonate reabsorption (12, 13). The previous observation from our laboratory that inhibiting H+\textsuperscript{-}K\textsuperscript{+}-ATPase completely inhibits ammonia’s stimulation of net bicarbonate transport (13) suggests that stimulation of apical proton secretion is quantitatively more important than inhibition of bicarbonate secretion for the observed changes in net bicarbonate transport.

In summary, the present studies are the first to study the mechanisms through which ammonia stimulates H+\textsuperscript{-}K\textsuperscript{+}-ATPase-dependent ion transport in the CCD. Ammonia mediates this effect through an intracellular calcium-, microtubule-, and SNARE protein-dependent mechanism. These results combined with the localization of the SNARE proteins, NSF and VAMP, in collecting duct intercalated cells, suggests that ammonia stimulates CCD net bicarbonate reabsorption, at least in part, by a process that appears to involve fusion of H+\textsuperscript{-}K\textsuperscript{+}-ATPase-containing cytoplasmic vesicles into the apical plasma membranes of CCD intercalated cells.

The authors thank Dr. R. Tyler Miller for numerous helpful discussions and Dr. John H. Schwartz for assistance with the use of tetanus toxin.

These studies were supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-45788 (to I. D. Weiner) and DK-49750 (to C. S. Wingo), Department of Veterans Affairs Merit Review Grants (to I. D. Weiner and C. S. Wingo), the Florida Affiliate of the American Heart Association (I. D. Weiner), and the intramural budget of the National Heart, Lung, and Blood Institute.

REFERENCES


Ammonia and Proton Secretion


