SNARE Proteins Regulate H⁺-ATPase Redistribution to the Apical Membrane in Rat Renal Inner Medullary Collecting Duct Cells*

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The interaction of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins provides the necessary steps for vesicle docking fusion. In inner medullary collecting duct (IMCD) cells, acid secretion is regulated in part by exocytotic insertion and endocytotic retrieval of an H⁺-ATPase to and from the apical membrane. We previously suggested a role for SNARE proteins in exocytotic insertion of proton pumps in IMCD cells. The purpose of the present study was to determine whether SNARE proteins are associated with the 31-kDa subunit of H⁺-ATPase in IMCD cells during exocytosis and to determine the effects of clostridial toxins on SNARE-mediated trafficking of H⁺-ATPase.

Cell acidification induced a marked increment of H⁺-ATPase in the apical membrane. However, pretreating cells with clostridial toxins blocked the cellular translocation of the 31-kDa subunit. Immuno precipitation of IMCD cell homogenate, using antibodies against either the 31-kDa subunit of H⁺-ATPase or vesicle-associated membrane protein-2, co-immunoprecipitated N-ethylmaleimide-sensitive factor, α-soluble NSF attachment protein (α-SNAP), synaptosome-associated protein-23, syntaxin, and vesicle-associated membrane protein-2. Pretreatment with clostridial toxin resulted in reduced co-immunoprecipitation of H⁺-ATPase and syntaxin. These experiments document, for the first time, a putative docking fusion complex in IMCD cells and a physical association of the H⁺-ATPase with the complex. The sensitivity to the action of clostridial toxin indicates the docking-fusion complex is a part of the exocytotic mechanism of the proton pump.

The vacuolar H⁺-ATPase is a ubiquitous multisubunit enzyme that participates in a wide variety of cellular functions (1). The renal collecting duct is populated by cells (α-intercalated cells and inner medullary collecting duct (IMCD) cells) that are specialized for H⁺ transport (2). The vacuolar H⁺-ATPase in these cells resides in high density in vesicles and is polarized to the apical membrane (3). Constitutive and regulated exocytotic insertion and endocytotic retrieval of H⁺-ATPase containing vesicles to and from the apical plasma membrane regulate, in part, not only the density of H⁺-ATPase in the apical membrane but also the rate of proton transport by these cells (4–7). A reduction in the intracellular pH followed by an elevation of cytosolic Ca²⁺ are the required stimuli that induce regulated exocytotic insertion of proton pumps into the apical membrane (8).

It is likely that the exocytotic event that regulates translocation of the H⁺-ATPase to the apical membrane in renal acid secretory cells utilizes a mechanism for exocytosis that is similar to that described in neuronal cells. The process in neuronal cells involves the participation of a subset of highly conserved, universally present membrane proteins (v- and t-SNAREs) and soluble factors (NSF and SNAP) (9). Consistent with this proposal is the observation that SNARE proteins are present in renal collecting duct cells (10–13) and our recent studies document that in cultured rat IMCD cells, H⁺ transport, mediated by an H⁺-ATPase, is inhibited by clostridial toxins (14). However, to date, no studies have described the exocytotic mechanisms regulating H⁺-ATPase insertion into the apical plasma membrane or provided direct evidence for the participation of the SNARE proteins in this process. Furthermore, the formation of a putative docking complex similar to the 20 S complex that has been described in neuronal and neuroendocrine cells (9) has not been demonstrated in renal epithelial cells.

To characterize the mechanisms involved in IMCD trafficking of H⁺-ATPase and to characterize the role of SNAREs in this process, we determined the effect of clostridial toxins on cell acidification-induced translocation of both H⁺-ATPase and SNAREs to the apical membrane, identified the v-SNARE proteins that participate in the translocation of H⁺-ATPase from vesicle to apical membrane following an acid load, and isolated a complex of proteins similar to the docking complex (20 S complex) that is required for docking and fusion of the vesicles. The present study, utilizing previously characterized cultured IMCD cells (15), provides direct evidence for the participation of SNARE proteins in H⁺-ATPase vesicle trafficking and elucidates an oligomeric protein complex that is comprised of the v- and t-SNAREs, NSF, SNAP, and H⁺-ATPase and that resembles the previously described 20 S docking complex in neuronal cells.

MATERIALS AND METHODS

Cell Culture and Toxic Incubation—IMCD cells were obtained from rat papillae as described previously (15). Cells from passages 6–12 were grown to confluence in 150-cm² plastic culture dishes in DMEM + 10% fetal calf serum and penicillin and streptomycin in an atmosphere of 95% air and 5% CO₂. Just prior to study, the confluent monolayers were incubated for 30 min in fresh DMEM containing either 25 nM clostridial toxin (BotD/TeTx (light chain) or no toxin. Following this incubation the toxin-containing media were removed, and the monolayers were briefly washed with fresh DMEM.

Manipulation of Cell pH—Toxin-free and toxin-treated monolayers had their cytosolic pH (pHc) adjusted to 7.2 or 6.5. To obtain these pH, values IMCD monolayers were initially washed with PBS to remove the
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DMEM from the external solution. To obtain a pH of 7.2, the normal resting pH of IMCD cells, monolayers were incubated for 20 min prior to harvest in NHB (110 mM NaCl, 50 mM HEPES acid, 5 mM KCl, 1 mM MgCl₂, 5 mM KH₂PO₄, 1 mM CaCl₂, 5 mM glucose, pH 7.2). To reduce pH, to 6.5 other monolayers were incubated for 20 min in CHB. CHB is identical to NHB except that 110 mM NaCl was replaced with choline chloride and for the addition of 10 mM potassium acetate, pH 7.2). In NHB intracellular pH has been shown to be maintained at approximately 7.2, and in CHB pH, rapidly declines to 6.5 because of reversal of the Na⁺/H⁺ exchanger and diffusion of acetic acid into the cell (16).

Cell Homogenization—At the end of the incubation period, cells were scraped once with a plastic spatula into a homogenization buffer consisting of CHB or NHB containing protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 1 mM aprotinin, and 1 mM leupeptin. Cells were passed 20 times through a 23-gauge needle and then homogenized 10 times using a Teflon-coated Dounce homogenizer. The homogenate was centrifuged at 1000 × g in a Sorvall refrigerated RC5B centrifuge for 20 min at 4°C to pellet intact cells and large fragments. The supernatant was further centrifuged at 13,000 × g for 30 min, the pellet was resuspended in homogenizing buffer, and aliquots were saved for protein determination, immunoprecipitation, and immunoblot analysis.

Two 100-μg aliquots of each homogenate from cells not treated with clostridial toxin, one incubated with diluent alone and the other with 25 nM TeTx (light chain), were resuspended in CHB at 37°C for 30 min. After the incubation period EDTA and EGTA (4 mM) were added to each aliquot to inactivate the toxin, and the suspension was further processed for immunoprecipitation and immunoblot analysis. This method has been shown previously to yield relatively purified apical membrane (17, 18, 21).

Preparation of Apical Membranes and Immunoblots—In separate studies, apical membrane was isolated from IMCD cells by a vesiculation method recently developed by our laboratory for polarized epithelial cells (17, 18, 21). 20 min after the pH was adjusted to 7.2 or 6.5, monolayers were incubated for an additional 90 min at 37°C in a vesiculation medium that contained, in addition to CHB or NHB, 1 mM CaCl₂, 1 mM MgCl₂, 50 mM parafomaldehyde, 2 mM dithiothreitol, and protease inhibitors. Parafomaldehyde and dithiothreitol induce the formation of vesicles from the apical membrane that are released into the incubation medium. At the end of the incubation period vesiculation medium was removed and 15-μm nylon mesh was used to remove vesicles and then the filtrate was centrifuged at 25,000 rpm at 4°C in a Sorvall RC5B 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. This method has been shown previously to yield relatively purified apical membrane (17, 18, 21).

Immunocolocalization of SNARE Protein Complexes—Immunoprecipitation was performed on the cell homogenate fraction prepared as described above. This homogenate was incubated overnight at 4°C with antibody to either the 31-kDa subunit of H⁺-ATPase (a gift from Dennis Brown), VAMP-2 (Stressgen and T. F. J. Martin), 2 or syntaxin/HPC-1 (Sigma) respectively purified apical membrane (17, 18, 21).

Immunoblot—Whole cell homogenates or immunoprecipitated or apical membrane samples prepared as described above were added to sample buffer, heated at 100°C for 5 min before loading on a 12 or 15% SDS-polyacrylamide gel electrophoresis. Immunoblotting of proteins transferred to nitrocellulose was conducted with antibodies to syntaxin (Sigma), SNAP-23 (P. Roche), synaptotagmin (T. F. J. Martin), NSF, SNAP, SNAP-25 (P. T. Sollner and J. E. Rothman), the 31-kDa subunit of H⁺-ATPase, and GP-135 (G. Ojakian).

RESULTS

H⁺-ATPase Translocation to Apical Membrane and Toxin Action—After acute cell acidification, a maneuver that enhances the rate of H⁺ transport by IMCD cells (2), the mass of the immunodetectable 31-kDa subunit of H⁺-ATPase increased in apical membrane by a factor of 2.5, compared with that present in apical membrane of control cells (Fig. 1, A and B). In contrast, the mass of GP-135, a resident protein of the apical membrane, is unaffected by cell-acidification cells (Fig. 1, A and B). After intact cells are exposed to either 25 nM TeTx (Fig. 1, A and B) or 25 nM BotD (Fig. 1C) for 30 min, the mass of the 31-kDa subunit and GP-135 detectable in the apical membrane is not changed. However the increment in H⁺-ATPase observed with cell acidification is abolished. In addition, when intact IMCD cells are exposed to BotD, the immunodetectable level of VAMP-2 in whole cell homogenate and apical membrane is reduced (Fig. 1C). Thus, cell acidification induces clostridial toxin-sensitive amplification of apical membrane H⁺-ATPase.

Identification of the Protein Components and Isolation of a 20 S-like Complex—We first determined if IMCD cells express the proteins typically observed in 20 S docking complexes. By immunoblot analysis of whole cell homogenate as depicted in Fig. 2A, lanes 3 and 4, both brain and IMCD cells express NSF, synaptotagmin, syntaxin, SNAP, and VAMP-2. In addition, IMCD cells, but not brain cells, express SNAP-23, a 30-kDa protein that is an isoform of brain SNAP-25. Next we determined if these proteins formed an immunoprecipitable complex in cells subjected to an acute acid load. Antibody to vesicular proteins such as the 31-kDa subunit of H⁺-ATPase or VAMP-2 co-immunoprecipitated NSF, SNAP, syntaxin, SNAP-23, synaptotagmin, VAMP-2, and the 31-kDa subunit of H⁺-ATPase, respectively, from the IMCD cell homogenate (Fig. 2A). In addition, antibody to the apical plasma membrane protein, syntaxin, also co-immunoprecipitated synaptotagmin, the 31-kDa subunit of H⁺-ATPase, and VAMP-2 (Fig. 2B).

The observation that the immunodetectable amount of synaptotagmin co-immunoprecipitated by antibody against either VAMP or the 31-kDa subunit appears to be significantly greater than that identified in whole cell homogenate (Fig. 2A) was unexpected. One possible explanation is that after complexes are formed, the antigenicity of this protein is enhanced.

Toxin Sensitivity of the 20 S-like Complex—To evaluate the stability and specificity of the complexes isolated, we determined the in vitro effect of tetanus toxin on the immunoprecipitability of complexes present in a homogenate derived from acid-loaded cells. Aliquots containing equal amounts of protein (100 μg) of this homogenate were incubated in the presence of either diluent or 25 nM tetanus toxin for 25 min. At the end of the incubation period, the toxin was inactivated by chelation of Zn²⁺ with EDTA and EGTA. Antibodies to the VAMP-2, 31-kDa H⁺-ATPase subunit, or syntaxin were employed to co-immunoprecipitate the oligomeric complex from these toxin treated and control IMCD cell homogenates. There is a reduction in the mass of the proteins that are co-immunoprecipitated by a VAMP-2 antibody from homogenates exposed to TeTx as compared with those co-immunoprecipitated from aliquots of the same homogenate not treated with TeTx (Fig. 3A). Although the toxin-induced change is likely to be because of the effect of tetanus toxin on the integrity of the complex, it is also possible that the complex remains intact, but TeTx alters the immunogenicity of VAMP by its proteolytic action on this target protein. However, when antibodies to either the 31-kDa subunit of
Fig. 1. Effect of clostridial toxins on H⁺-ATPase translocation to the apical membrane of IMCD cells. A, IMCD cells were incubated with or without 25 nM TeTx in DMEM for 30 min at 37 °C and then incubated for 20 min in buffer (NHB) with Na⁺ (Control), or in buffer (CHB) without Na⁺ (Tetanus Toxin). Apical membrane was isolated by incubating the cells in either NHB or CHB containing 50 mM paraformaldehyde and 2 mM dithiothreitol for 90 min at 37 °C. These buffers induce vesiculation of the apical membrane and release of membrane fragments into the incubation medium. The fragments were centrifuged, and the pellet was redisolved in NHB or CHB. Aliquots were used for protein assay and immunoblotted (40 μg of protein/lane) for the 31-kDa subunit of the H⁺-ATPase and GP-135. B, densitometric analysis depicting the relative abundance of the 31-kDa subunit of H⁺-ATPase and GP-135 (for immunoblot, see A) in the apical membrane of control- and toxin-treated IMCD cells (*, p < 0.05 versus all other lanes, n = 5) is shown. C, IMCD cells incubated with 25 nM BotD and apical membrane isolated as described above are shown. The apical membrane (40 μg of protein/lane) was immunoblotted for the 31-kDa subunit of H⁺-ATPase, GP-135, and VAMP-2. The cells remaining in the culture dish after apical membrane isolation (Cell Homogenate) were harvested and dissolved in sample buffer and immunoblotted to detect VAMP-2.

the H⁺-ATPase or syntaxin are used, the amount of the primary protein immunoprecipitated (the 31-kDa subunit of the H⁺-ATPase or syntaxin) is unchanged by exposure to toxin, whereas the proteins that are associated with these molecules by complex formation are reduced (Fig. 3, B and C). This observation confirms that specific complexes are present and suggests that the 20 S-like docking complex requires intact VAMP-2 for stability.

**DISCUSSION**

The rate of proton secretion by the collecting duct is regulated by changes in cytosolic pH (4). Because proton pump activity is relatively insensitive to pH changes (19), pH must affect this transport through alternative mechanisms. It is widely believed that pH controls this secretory process by regulating exocytic amplification and endocytic retrieval of proton pumps from the apical membrane of acid secretory collecting duct cells. We have utilized a tissue culture model system, the IMCD cell, to characterize the process of regulated exocytosis of H⁺-ATPase (20). In recent studies we have demonstrated in these cells that pH-regulated exocytosis results in the translocation of H⁺-ATPase to the apical membrane (21) and that clostridial toxins inhibit pH recovery after an acute acid load (14).

In the present study, evidence is presented that directly supports the hypothesis that SNAREs are involved in the targeting fusion of H⁺-ATPase vesicles with the apical plasma membrane. Cell acidification-induced regulated exocytosis with translocation of H⁺-ATPase to the apical membrane is inhibited by either TeTx or BotD (Fig. 1, A–C). These toxins proteolytically cleave VAMP-2 at different sites (22), and both reduce the amount of VAMP-2 detected in apical membrane and in IMCD cell homogenate. Although these two toxins may have additional ill-defined effects on IMCD cells, the observation that both toxins have equivalent effects in H⁺-ATPase
translocation and VAMP-2 expression is strong evidence indicating that VAMP-2 has a role in this process. In contrast to the effect of toxin on pH$_i$-induced amplification of apical membrane H$^+$-ATPase, the mass of H$^+$-ATPase present in the unacidified cell is not reduced by exposure to toxin. This observation is consistent with the proposal that the constitutive delivery of H$^+$-ATPase may be VAMP-2-independent.

It has been suggested that VAMP plays a major role in the mechanism of vesicle trafficking (24). This is based upon previous knowledge of the substrate specificity of TeTx and BotD (23), the interaction of VAMP with other SNARE proteins during the events of vesicle fusion, and the ability of specific VAMP peptides to inhibit neurotransmitter release in neuronal and neuroendocrine cells (25). Recent studies demonstrated the regulatory role of VAMP isoforms in glucose transporter-4 trafficking in adipocytes, exocytosis of histamine in enterochromaffin-like cells, and in the sperm acrosome reaction (26–28). In renal cells, VAMP has been shown to co-localize with aquaporin 2-containing vesicles and participate in the fusion of antidiuretic hormone-regulated aquaporin 2 containing endosomes in vitro (12). Our results (Fig. 1, A–C) are consistent with prior observations regarding the role of VAMP in exocytosis and now document the participation of VAMP in another exocytic process, the translocation of H$^+$-ATPase in renal epithelial cells.

In neuronal cells, regulated exocytosis is thought to be mediated by the close interaction between the v- and t-SNAREs with the formation of a docking complex (20 S complex) comprised of the v- and t-SNAREs, NSF, and SNAP followed by ATP-mediated priming and Ca$^{2+}$-dependent fusion (29, 30). We have attempted to identify a similar 20 S-like complex in the IMCD cells. Immunoblots, utilizing rat brain as a control, indicate the presence of these proteins in IMCD cells (Fig. 2, A–B). We have employed antibodies directed against the 31-kDa subunit of H$^+$-ATPase and VAMP-2. Whole cell homogenate (30 μg) was used as control to identify the proteins of interest as in A.
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components of the 20 S complex suggests that the H⁺-ATPase per se may play some specific role in targeting and fusion of the vesicles they decorate.

In our studies, the oligomeric complex of SNAREs, NSF, SNAP, and the 31-kDa subunit isolated by immunoprecipitation from homogenate of IMCD cells was sensitive to the action of TeTx, an observation that has not been found universally (31). This is demonstrated by the reduced recovery of an immunoprecipitable oligomeric 20 S-like complex from a homogenate exposed in vitro to toxin (Fig. 3, A–C). These results not only demonstrate the ability of TeTx to destabilize the 20 S-like complex in the IMCD but also provide evidence that the molecules isolated in the supposed complex are not merely an aggregation but a specific complex. Finally, this effect of TeTx on 20 S-like complex documents a specific role of VAMP in docking events and clarifies the effect of toxins on the translocation of H⁺-ATPase to the apical membrane.

In summary, these data provide new evidence that both v- and t-SNAREs participate in the docking and fusion of H⁺-ATPase vesicles with the apical membrane. They demonstrate a close interaction between the participating SNAREs and the 31-kDa subunit of H⁺-ATPase. In addition, these data furnish evidence that a subunit of H⁺-ATPase is not only the cargo of the vesicle but a part of the 20 S-like complex and may function as an unique SNARE in this system for targeting this vesicle subtype.

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Fig. 3. Tetanus toxin disrupts the docking complex in IMCD cells. Cell homogenate from IMCD cells subjected to cellular acidification was resuspended in CHB (Control), and another was resuspended in CHB with 25 nM TeTx (Toxin). These homogenates were incubated for 30 min at 37 °C. After adding EDTA and EGTA to both tubes to inactivate toxin, 100-μg protein aliquots of these homogenates were immunoprecipitated with antibodies to A, VAMP (VAMP-IP); B, H⁺-ATPase (H⁺-ATPase-IP); or C, syntaxin (Syntaxin-IP). Immunocomplexes were analyzed for the recovery of NSF, SNAP, syntaxin, H⁺-ATPase, and VAMP-2.