**H^+ secretion is inhibited by clostridial toxins in an inner medullary collecting duct cell line**

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**Recent evidence supports the importance of the phenomenon of exocytosis-endocytosis in renal epithelial cell proton secretion (9, 12, 13). In an inner medullary collecting duct (IMCD) cell line, which we have utilized as a model of renal epithelial cell acid secretion, we demonstrated both constitutive and cell pH-regulated exo-endocytosis. Specifically, we found that acidification increased exocytosis and alkalization increased endocytosis (9, 13). It is likely, therefore, that the rate of proton secretion is regulated by the membrane insertion and retrieval of proton pumps.**

There is abundant evidence from studies in the nerve terminal and the chromaffin cell that vesicle docking, membrane fusion, and discharge of vesicular contents (exocytosis), involves a series of interactions among so-called trafficking proteins (1, 4, 10, 16). Recently, evidence for the participation of some of these proteins, especially synaptobrevin/vesicle-associated membrane proteins (VAMP), has been suggested in the membrane fusion of vesicles that contain the antidiuretic hormone-sensitive water channel in the rat IMCD (7, 8). The clostridial toxins, botulinum and tetanus, are proteases that inactivate some of these proteins by cleavage. The specificity of this reaction between the botulinum serotypes or tetanus and the target synaptic protein synaptobrevin/VAMP, synaptosomal-associated protein (SNAP)-25, or syntaxin has provided a powerful tool in the understanding of the role of these proteins in the neuro-exocytic process (2, 3, 11).

Of particular importance concerning the present work, no information is available relating these synaptic proteins to renal epithelial cell proton secretion. The results of these experiments provide strong evidence that the specific proteins cleaved by these toxins are present in IMCD cells and that these proteins play a significant role in acid secretion by the IMCD.

**Methods**

Solutions and reagents. The following solutions were used. NaCl-N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) buffer (NHB) contained (in mM) 110 NaCl, 50 HEPES acid, 5 KCl, 1 MgCl2, 1 CaCl2, and 5 glucose (pH 7.2). Choline chloride HEPES buffer (CHB) was identical to NHB, except that 110 mM choline chloride was substituted for NaCl (pH 7.2). KCl HEPES buffer (KHB) contained (in mM) 130 KCl, 25 HEPES, 5 NaCl, 1 MgCl2, 1 CaCl2, and 5 glucose (pH 7.2). Buffers were titrated to the desired pH using NaOH (for NHB), KOH (for CHB and KHB), or HCl. 2',7'-Bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was prepared as stock solutions in dimethyl sulfoxide (DMSO). The total DMSO content to which the cells were exposed was less than 0.7%. Nigericin was prepared in ethanol. All other inhibitors employed in this study were dissolved in NHB or CHB. Bafilomycin was obtained from Calbiochem (San Diego, CA). Clostridial and botulinum toxins were purchased from Sigma Chemicals (St. Louis, MO).

Cell culture. IMCD cells were obtained from rat papillae as described previously (12, 14). 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-cm² plastic flasks or on 12 × 12-mm glass coverslips in Dulbecco’s modified Eagle’s medium in an atmosphere of 95% air-5% CO2.

Cell pH. Quiescent cells grown on glass coverslips were incubated for 1 h at 37°C in NHB containing 10 µM of the
acetoxyethyl ester of BICEF (BCECF-AM). The coverslip was then placed in a plastic cuvette containing 1 ml of NHB and secured by means of a device designed to hold the coverslip at a 35° angle to the excitation beam (12). The monolayer was washed three times with NHB and then suspended in 1 ml NHB. Fluorescence intensity was measured in a Perkin-Elmer model LS 650-10 fluorospectrophotometer equipped with a thermostatically controlled (37°C) cuvette holder, at excitation wavelengths 505 and 455 nm with a slit width of 2 nm and emission wavelength 560 nm with a slit width of 4 nm. At the end of each experiment, the fluorescence intensity ratio (FIR) was calibrated to cell pH (pHi) using KHB buffer containing nigericin 10 mg/ml (12, 14). The FIR varied linearly with pH over the range 6.3–7.6. Autofluorescence of probe-free monolayers was less than 10% of the fluorescent signal of BCECF-loaded monolayers at excitation of both 505 and 455 nm, and a correction for this was not made. Na+-independent and Na+-dependent pHi recovery after a 20 mM NH4Cl-induced acid load when incubated in CHB was determined as previously described (14, 15). After an initial control measurement of active H+-adenosinetriphosphatase (H+-ATPase)-mediated proton transport (J\textsubscript{H-act}), the monolayer was exposed to various transport (14). The FIR varied linearly with pH over the range 6.3–7.6. Autofluorescence of probe-free monolayers was less than 10% of the fluorescent signal of BCECF-loaded monolayers at excitation of both 505 and 455 nm, and a correction for this was not made. Na+-independent and Na+-dependent pHi recovery after a 20 mM NH4Cl-induced acid load when incubated in CHB was determined as previously described (14, 15). After an initial control measurement of active H+-adenosinetriphosphatase (H+-ATPase)-mediated proton transport (J\textsubscript{H-act}), the monolayer was exposed to various transport inhibitors or just the diluent used to dissolve them, and J\textsubscript{H-act} was redetermined.

Antibodies. The following monoclonal antibodies were employed in these studies: anti-SNAP-25 (MAB331) obtained from Chemicon International (Temecula, CA); antibody C1 10.1, specific for synaptobrevin/VAMP and its homologs, was kindly provided by Prof. R. Jahn (Howard Hughes Medical Institute, New Haven, CT).

Preparation of tissue homogenate. Confluent IMCD cells were washed three times in cold PBS and scraped from the growth surface and pelleted by centrifugation at 1,000 g for 10 min. The pellet was suspended in 4 vol of ice-cold homogenizing buffer containing 10 mM tris(hydroxymethyl)aminomethane (Tris) hydrochloride, 150 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40, to which 4 mM phenylmethyisulfonyl fluoride, 0.5 µg/ml aprotinin, 2 µg/ml N-tosyl-L-phenylalnine chloromethyl ketone, 5 µg/ml deoxyriboseuclease, and 5 µg/ml ribonuclease were added just before use. The suspended pellet was homogenized by ten 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. Sample of rat brain (cortex) was washed with cold PBS and then suspended in 4 vol of ice-cold homogenizing buffer and homogenized in a Branson cell disrupter for 10 s. Intact tissue fragments and nuclei were removed by centrifugation at 1,000 g at 4°C for 10 min.

Immunoprecipitation. IMCD postnuclear homogenate was immunoprecipitated using one of the antibodies listed above, according to the following protocol. The homogenate was diluted to a protein concentration of 100 µg/ml with the homogenizing buffer that also contained 0.5% deoxycholate. To a 900-µl aliquot of this diluted homogenate was added 2 µl nonimmune serum and 30 µl of a 25% suspension of protein A-Sepharose 4B beads. This mixture was incubated at 4°C for 2 h, then centrifuged at 13,000 rpm in an Eppendorf centrifuge. The supernatant was incubated with 20 µl of primary antibody and 50 µl of a protein A-Sepharose 4B bead suspension for 12 h at 4°C. If the primary antibody was a mouse monoclonal antibody, then the beads were prereacted with rabbit anti-mouse immunoglobulin G (Sigma Chemicals) prior to use, since monoclonal antibodies do not bind to protein A. In preliminary studies, we determined that the quantity of primary antibody employed for immunoprecipita-
Recovery among each set of experiments did not differ statistically among control experiments or with toxin, but the rate of recovery was statistically reduced (~80%) with each toxin compared with control.

To determine whether botulinum toxin had any effect on the Na\(^+\)/H\(^+\) exchanger, pH\(_i\) recovery after an acid load was determined in the presence of Na\(^+\). In these studies, the experimental design was similar to that described above for assessing the effect of the neurotoxins on J\(_{\text{HCO}_3^-}\), with the exception that after the cells were acid loaded the extracellular solutions was changed from one that was Na\(^+\)-free to one containing 140 mM Na\(^+\) (14). Under these conditions, rate of pH\(_i\) recovery is primarily determined by Na\(^+\)/H\(^+\) exchange (14). Na\(^+\)/H\(^+\) exchange during the control period was 0.41 ± 0.02 pH U/min, and these monolayers recovered to a steady-state pH of 7.18 ± 0.04. The monolayers were then incubated with either botulinum A (n = 3) or botulinum B (n = 3), and the rate of Na\(^+\)/H\(^+\) exchange was determined after a second acute acid load. These toxins did not change significantly the pH\(_i\) recovery rate in the presence of Na\(^+\) (0.40 ± 0.01 pH U/min, P > 0.05) and the stable pH, to which they recovered (7.18 ± 0.05).

Immunoidentification of vesicular trafficking proteins. To determine the presence of some of the proteins expressed by our IMCD cell line and thought to be associated with exocytosis, we analyzed homogenates of these cells by immunoprecipitation and immunoblot analysis of the precipitate (synaptobrevin and synaptophysin) or by direct Western analysis of the homogenate (syntaxin and SNAP-25) (Fig. 2). Rat brain samples were also analyzed concurrently with the IMCD homogenates to provide positive controls for the analysis. Positive reactions were found to the antibodies for two vesicular proteins, synaptobrevin and synaptophysin, and two membrane proteins, syntaxin and SNAP-25 (Fig. 2).

DISCUSSION

In this study, we provide evidence relating the regulated Na\(^+\) independent H\(^+\) transport by the IMCD with proteins important in the process of regulated neurosecretion. This might have been anticipated, since the
secretion of protons by the IMCD is a calcium- and calmodulin-dependent exocytic process (13). Neurotransmitter release is similarly a calcium- and calmodulin-dependent exocytic process (1, 5).

Our data (Figs. 2 and 3) demonstrate the presence of four proteins that play a role in the incompletely understood but extensively investigated process of synaptic vesicle exocytosis. [A discussion of this process is beyond the scope of this report but can be found in recent excellent reviews (1, 5, 10, 16).] Synaptobrevin/VAMP and synaptophysin are vesicular proteins, whereas syntaxin and SNAP-25 are presynaptic membrane proteins. Previous studies have demonstrated synaptobrevin and its homolog, cellubrevin, in rat papillary cells associated with the fusion of the antidiuretic hormone-associated water channel (7, 8). In addition, other proteins such as N-ethylmaleimidesensitive factor and α-SNAP, which are part of the synaptic vesicle fusion process, have been identified in the rat IMCD (6).

These studies further demonstrate that tetanus toxin and four botulinum serotypes, A, B, C, and D, markedly inhibit the normal response of the IMCD cell to increase Na⁺-independent H⁺ secretion after an acid load. Activation of Na⁺-independent H⁺ secretion is dependent upon exocytic insertion of proton pump-containing vesicles into the apical membrane (13). In contrast, these toxins had no effect on Na⁺-dependent pHᵢ recovery, a process which is not dependent on exocytosis (13). The neurotoxicity of these agents has been well characterized. They are Zn-dependent proteins that cleave specific synaptic proteins involved in the neuroexocytic process. Tetanus and botulinum B and D cleave synaptobrevin/VAMP; botulinum A cleaves SNAP-25; and botulinum C cleaves syntaxin (16). The data presented in Fig. 3 provide additional support for the claim that the mechanism of action of these toxins in the IMCD cell acidification process is similar to that in neural tissue, since botulinum C cleaved syntaxin in a time course comparable to the physiological inhibition of J_H-act. The product, a protein of ~2–4 kDa smaller size, is similar to the results obtained in neural tissue (4, 17). We presume that the reduction in the J_H-act after an acid load in the presence of these toxins is due to the inhibition of exocytic insertion of additional pump units into the apical membrane. In a prior study, when exocytosis was inhibited by another means, i.e., disruption of cytoskeletal function with either cytochalasin or colchicine, the degree of inhibition of pHᵢ recovery from an acid load was similar (13). In the current and prior study (13), a residual rate of H⁺ transport was observed. This residual rate probably represents the activity of proton pumps that are constitutively expressed in the apical plasma membrane.

These data provide new insight into the mechanism for H⁺ secretion in the IMCD. Our results provide strong evidence for the presence of the specific proteins cleaved by clostridial toxins in the IMCD, as well as and the physiological result of this effect, impairment of J_H-act. Presumably, this effect is through the interruption of critical protein interactions in the sequential exocytic process. Given the results of these experiments, perturbations in the acid-base milieu may provide a very useful tool in defining the interaction of these specific proteins in the exocytic process.

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