Tetanus toxin-mediated cleavage of cellubrevin inhibits proton secretion in the male reproductive tract

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Tetanus toxin-mediated cleavage of cellubrevin inhibits proton secretion in the male reproductive tract. Am J Physiol Renal Physiol 278: F717–F725, 2000.—Our laboratory has previously shown that the vacuolar H+-ATPase, located in a subpopulation of specialized cells establishes a luminal acidic environment in the epididymis and proximal part of the vas deferens (Breton S, Smith PJ S, Lui B, and Brown D. Nat Med 2: 470–472, 1996). Low luminal pH is critical for sperm maturation and maintenance of sperm in a quiescent state during storage in these organs. In the present study we examined the regulation of proton secretion in the epididymis and vas deferens. In vivo microtubule disruption by colchicine induced an almost complete loss of H+-ATPase apical polarity. Endocytotic vesicles, visualized by Texas red-dextran internalization, contain H+-ATPase, indicating active endocytosis of the pump. Cellubrevin, an analog of the vesicle soluble N-ethyl maleimide-sensitive factor attachment protein (SNAP) receptor (v-SNARE) synaptobrevin, is highly enriched in H+-ATPase-rich cells of the epididymis and vas deferens, and tetanus toxin treatment markedly inhibited bafilomycin-sensitive proton secretion by 64.3 ± 9.0% in the proximal vas deferens. Western blotting showed effective cleavage of cellubrevin by tetanus toxin in intact vas deferens, demonstrating that the toxin gained access to cellubrevin. These results suggest that H+-ATPase is actively endocytosed and exocytosed in proton-secreting cells of the epididymis and vas deferens and that net proton secretion requires the participation of the v-SNARE cellubrevin.

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The molecular mechanisms responsible for the regulation of endocytic and exocytic processes in transporting epithelia are still poorly understood. The notion that these processes require components similar to those involved in the shuffling of synaptic vesicles in the central nervous system has emerged from recent studies showing the implication of the so-called “SNAREs” [soluble N-ethyl maleimide-sensitive fusion factor attachment protein (SNAP) receptor] proteins in the recycling of specialized transporting vesicles (2, 21–23, 33, 34, 37). In the original model, the specificity of docking and fusion of cytoplasmic vesicles with the plasma membrane is mediated by a family of receptor-like proteins, the SNAREs present on vesicles (v-SNAREs) and in the target membrane (t-SNAREs), and requires the participation of soluble factors such as N-ethyl maleimide-sensitive fusion factor (NSF) and α-SNAP (soluble NSF attachment protein) (42, 56).

Recent studies showing the implication of the so-called SNAREs in the shuttling of synaptic vesicles in those involved in the recycling of specialized transporting vesicles (2, 21–23, 33, 34, 37). In the original model, the specificity of docking and fusion of cytoplasmic vesicles with the plasma membrane is mediated by a family of receptor-like proteins, the SNAREs present on vesicles (v-SNAREs) and in the target membrane (t-SNAREs), and requires the participation of soluble factors such as N-ethyl maleimide-sensitive fusion factor (NSF) and α-SNAP (soluble NSF attachment protein) (42, 56).

Vesicle-associated membrane protein 2 (VAMP2 or synaptobrevin-2), cellubrevin, the ubiquitously expressed N-ethyl maleimide-sensitive fusion factor (NSF) and α-SNAP (soluble NSF attachment protein) (42, 56). Vesicle-associated membrane protein 2 (VAMP2 or synaptobrevin-2), cellubrevin, the ubiquitously expressed N-ethyl maleimide-sensitive fusion factor (NSF) and α-SNAP (soluble NSF attachment protein) (42, 56).

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In the present study, we examined whether proton secretion in the male reproductive tract is also regulated via endocytic and exocytic processes and whether the v-SNARE protein cellubrevin is involved in this process. Our work shows that the H^+-ATPase is actively endocytosed and that cellubrevin plays a significant role in the mechanisms underlying net acid secretion in the proximal vas deferens.

**MATERIALS AND METHODS**

Immunofluorescence. Sexually mature Sprague-Dawley rats (10 wk old) were perfused via the left ventricle with a physiological Hanks' buffer bubbled with a mixture of 5% CO2-95% O2 at 37°C to reach pH 7.4, followed by 150 ml of fixative solution containing 4% paraformaldehyde, 10 mM sodium periodate, 75 mM lysine, and 5% sucrose (PLP), as previously described (9). A longitudinal incision was made, the opened vas deferens was washed free of sperm, and the apical surface of the epithelium was exposed. The vas deferens was mounted on a petri dish and pinned down, its lumen open, on a block of dental wax. Incubation with the fluid phase marker Texas red-dextran was performed at room temperature for 30 min, at a concentration of 5 mg/ml in PBS, pH 7.4. The vas deferens was then washed as above. Goat anti-mouse IgG conjugated with FITC (20 µg/ml; Kirkegaard & Perry, Gaithersburg, MD) was applied for 1 h at room temperature and washed. Sections were mounted in Vectashield diluted 2:1 in 0.1 M Tris·HCl, pH 8.0.

Sections were photographed on color Kodak Ektachrome 400 Elite film pushed to 2800 ASA by using Nikon FXA or Nikon 800 microscopes. Slides were scanned by using a slide scanner (SprintScan 35, Polaroid) and Adobe Photoshop software and were stored on 1-GB Jaz Cartridge (Omega). An Optronics 3-bit charge-coupled device color camera or a Hamamatsu Orca camera was also used to capture images directly, which were stored on an Apple Macintosh Power PC 8650 by using IP Lab Spectrum software (Scanalytics, Vienna, VA). The digitized images were printed on a Tektronix Phaser 440 dye sublimation color printer.

Detection of endocytosis with Texas red-dextran. Sexually mature Sprague-Dawley rats were anesthetized, proximal vas deferens were dissected, and most of the surrounding connective and muscular tissue was removed, as previously described (9). A longitudinal incision was made, the opened vas deferens was washed free of sperm, and the apical surface of the epithelium was exposed. The vas deferens was mounted on a petri dish and pinned down, its lumen open, on a block of dental wax. Incubation with the fluid phase marker Texas red-dextran was performed at room temperature for 30 min, at a concentration of 5 mg/ml in PBS, pH 7.4. The vas deferens was then washed as above. Goat anti-mouse IgG conjugated with FITC, as described above.

Detection of proton secretion. Proximal vas deferens were dissected, most of the connective and muscular tissue was removed, and they were cut longitudinally to expose the apical surface of the epithelium, as described above. Proton secretion by the vas deferens was detected by using an extracellular proton-selective, self-referencing electrode, as described previously (6, 9, 54, 55). The vas deferens was bathed in the same low PBS (2 mM phosphate) that we used in our previous reports (6, 9), and no bath perfusion was performed, to allow the establishment of a proton gradient near the apical surface of the H^+-ATPase-rich cells. The vas deferens was mounted on the stage of an inverted microscope, and the electrode was positioned close (~5 µm) to the apical surface of the epithelium.
Proton-selective microelectrodes were constructed from 1.5-mm borosilicate tubes (TW150-4; World Precision Instruments, New Haven, CT) pulled on a Sutter model PC90 pipette puller to reach a final tip diameter of 2–4 μm (6, 9, 54, 55). Electrodes were silanized, front-filled with a proton-selective liquid ion exchanger (30-μm column, Fluka Hydrogen ionophore cocktail B), and back-filled with 100 mM KCl. Electric potentials were measured with a high-input-impedance preamplifier with unity gain (model AD515; Analog Devices), followed by a 1,000-fold gain amplifier and low- and high-pass filters. The circuit was completed with a 3 M KCl-agar bridge. Signals were recorded by using an analog-to-digital board (DT 2800 series; Data Translations) and stored and analyzed in a Pentium computer by using the IonView software developed at the BioCurrents Research Laboratory (MBL, Woods Hole, MA). To detect prostatic secretion, square-wave oscillations of the electrode were performed, perpendicular to the apical membrane, with an amplitude of 50 μm and a frequency of 0.3 Hz. Proton flux was estimated from the difference in proton equilibrium potentials (ΔV) detected by the selective electrode at the extreme points of oscillation (6, 9). The Nernstian slope of the electrode was determined before and after each experiment by using calibration solutions at pH 6.0, 7.0, and 8.0. Amplifiers, motion controllers, and micromanipulators used in the present study are products of the BioCurrents Research Center (MBL; www.mbl.edu/BioCurrents).

Preparation of endocytotic vesicles and detection of H^+ influx. Endocytic vesicles were isolated from kidney cortex homogenates by differential and Percoll density centrifugation, as described previously (46, 47). The endosome preparation was preloaded in KCl buffer containing (in mM) 300 mannitol, 100 KCl, 5 MgSO_4_, and 5 HEPES-Tris, pH 7.0.

ATP-dependent acidification of the endosome lumen was measured by using the pH-sensitive dye acridine orange, which accumulates in acidic compartments and whose fluorescence is quenched at acidic pH (46, 47). Acridine orange fluorescence was monitored at 37°C over 1-s intervals in an SLM-J-Åminco 8000 fluorimeter (Urbana, IL), interfaced to an IBM/PC computer. For each assay, an aliquot of endosome preparation corresponding to 100 μg protein was added to 2 ml of KCl buffer containing 6 μM acridine orange and 5 μM valinomycin. ATP was added at a final concentration of 1.5 mM, nigericin was used at a final concentration of 2.5 μM, and 1 μM baflomycin was applied in some experiments. For the experiments in which the effect of tetanus toxin was examined, the endosome preparation was preincubated in KCl buffer containing 100 mM toxin for 20 min before addition of ATP, and acridine orange fluorescence was measured in the presence of the toxin.

Western blotting. To confirm specificity of the anti-cellubrevin antibodies, and to determine the effect of tetanus toxin on cellubrevin, immunoblotting of proximal vas deferens and cauda epididymis was performed. Sexually mature Sprague-Dawley rats were anesthetized, and both proximal vas deferens were harvested. Most of the connective and muscular tissue was removed, and the lumen was cut open and washed free of sperm as for the detection of proton secretion described above. One vas deferens was treated in vitro with 50 nM of tetanus toxin in PBS (Calbiochem, La Jolla, CA), at room temperature for 30 min, and the second vas deferens from the same rat was incubated in control solution (PBS) at room temperature for the same period of time. Each vas deferens was then homogenized in 250 μl of Laemmli sample buffer (Boston Bioproducts Ashland Technology Center, Ashland, MA). The distal part of the cauda epididymis was also dissected, most of the connective and muscular tissue surrounding epididymal ducts was removed, and the tissue was immediately homogenized in 1 ml of Laemmli sample buffer. The homogenates were heated to 95°C for 5 min and centrifuged at 14,000 rpm for 2 min in an Eppendorf centrifuge (model 5415C). For separation by SDS-PAGE, 20 μl of the supernatant for each preparation were loaded onto a 12% acrylamide gel. Proteins were transferred to Immobilon membranes by using a semidry electrophoretic transfer cell (Bio-Rad) for immunoblotting. Membranes were blocked overnight at 4°C in Blotto buffer (0.05% Tween-20 and 5% nonfat milk in PBS). Anti-cellubrevin antibodies were applied to Immobilon strips at a concentration of 1:1,000 for 1 h at room temperature. Membranes were then washed 4 × 10 min in Blotto buffer, and goat anti-rabbit IgG coupled to horseradish peroxidase was applied at a dilution of 1:10,000 for 1 h at room temperature. After four washes in Blotto buffer and one wash in PBS, recognized bands were visualized by enhanced chemiluminescence.

RESULTS

Microtubule disruption. The steady-state distribution of membrane proteins that rapidly recycle between intracellular vesicles and the plasma membrane is microtubule dependent (13, 17, 26, 48). Therefore, the effect of microtubule disruption on the localization of H^+-ATPase in the cauda epididymis was examined. Microtubule disruption was induced by an intraperitoneal injection of colchicine (0.5 mg/100 g body wt) for 12 h. The efficacy of colchicine treatment was monitored by labeling the microtubule network with an anti-α-tubulin antibody. In all colchicine-treated epididymis, the typical linear microtubule organization that is seen in control tissue was abolished, leading to an almost complete disappearance of microtubule staining (data not shown). As shown in Fig. 1, colchicine treatment resulted in a marked redistribution of H^+-ATPase from the apical membrane and subapical vesicles to numerous intracellular vesicles scattered throughout the cytoplasm, leading to a loss of apical H^+-ATPase polarity. This result suggests that the H^+-ATPase is an actively recycling membrane protein in these cells.

Demonstration of endocytosis. The recycling process involves endocytotic and exocytotic events. To determine whether apically derived endosomes internalized H^+-ATPase in H^+-ATPase-rich cells, fluid phase markers of endocytosis were applied in vitro to the vas deferens for 30 min. After this time, the H^+-ATPase-rich cells were identified by double staining with antibodies against the 31-kDa subunit of the H^+-ATPase. As shown in Fig. 2, H^+-ATPase-positive cells showed a higher rate of Texas red-dextran endocytosis than did surrounding principal cells. These endosomes were brightly stained with anti-H^+-ATPase antibodies (cf. Fig. 2, A and B). These results show that the H^+-ATPase is retrieved from the apical membrane by endocytosis in these cells.

Detection of cellubrevin in the cauda epididymis and vas deferens. Western blotting of control epididymis and proximal vas deferens, as well as in a kidney cortex homogenate, revealed a major band at ~12 kDa, showing high expression of cellubrevin in these tissues (Fig. 3).
Localization of cellubrevin in the cauda epididymis and vas deferens. Vesicle fusion events involve a battery of proteins, including the so-called v-SNAREs. These vesicle-associated proteins interact with soluble proteins and with t-SNAREs on the target membrane to allow specific and selective targeting of membrane proteins. As shown in Fig. 4, double labeling of cauda epididymis by using H^+-ATPase and cellubrevin antibodies showed that all H^+-ATPase-rich cells express a high level of cellubrevin. Adjacent principal cells are negative for cellubrevin. H^+-ATPase is located on the apical membrane and on tightly packed subapical vesicles. Whereas cellubrevin is generally diffuse throughout the cytoplasm, in many cells it is more concentrated in the apical pole and shows some degree of colocalization with H^+-ATPase. This result indicates a potential role for cellubrevin in proton pump recycling.

Enrichment of cellubrevin in H^+-ATPase-rich cells was also shown in the proximal vas deferens, in which we also observed a partial overlap of the pattern of immunofluorescent staining for cellubrevin with H^+-ATPase staining (Fig. 5).

Effect of tetanus toxin on proton secretion in the vas deferens. To determine a potential role for cellubrevin in vesicle fusion and, therefore, in apical acidification, we examined the effect of tetanus toxin on proton secretion by the vas deferens. Tetanus toxin is a specific metalloenzyme that proteolytically cleaves cellubrevin. Each vas deferens was scanned initially for the presence of H^+-ATPase-rich cells, using the proton-selective electrode. By measuring ΔV at different locations along the surface of the tissue, variable rates of proton secretion were detected within the same vas deferens, as previously reported (9). Acidification at these "hot spots" is strongly inhibited by bafilomycin, the specific inhibitor of the vacuolar H^+-ATPase, indicating that they correspond to proton secretion by H^+-ATPase-rich cells located beneath the tip of the electrode (9). All experiments were conducted after one region of high acidification (hot spot) was located; the electrode then remained at this location. We have previously shown that, under control conditions, proton secretion remains stable for periods of up to 1 h (6, 9). Figure 6 shows a representative trace of the effect of tetanus toxin on proton secretion. In this series of six experiments, addition of 50 nM tetanus toxin markedly reduced total proton secretion by 32.6 ± 5.4% (P < 0.005), and when 1 µM bafilomycin was applied at the end of the experimental period, an additional inhibition of 19.4 ± 6.2% was observed (P < 0.05). Therefore,
64.3 ± 9.0% of the bafilomycin-sensitive proton secretion was inhibited by tetanus toxin (Fig. 7).

Effect of tetanus toxin on H\textsuperscript{+}-ATPase activity. To determine whether H\textsuperscript{+}-ATPase was directly inhibited by tetanus toxin, we measured proton-pumping activity on isolated kidney cortical endosomes, which contain abundant H\textsuperscript{+}-ATPase, as described previously (47). Using acridine orange as a marker of proton influx into endosomes, we detected a marked acidification on addition of ATP (Fig. 8, control trace). On addition of nigericin, intravesicular pH is equilibrated back to external pH. ATP-dependent acidification is inhibited by bafilomycin, indicating the participation of H\textsuperscript{+}-ATPase in this process (bafilomycin trace). To examine the effect of tetanus toxin on the H\textsuperscript{+}-pumping activity of the H\textsuperscript{+}-ATPase, we preincubated endosomes that were derived from the same control preparation with KCl buffer containing 100 nM of toxin for a period of 20 min, and acridine orange fluorescence was measured in the continued presence of tetanus toxin. Tetanus toxin did not affect ATP-dependent acidification in this preparation, which renders unlikely the formal possibility that tetanus toxin might have a direct effect on H\textsuperscript{+}-ATPase activity in the vas deferens.

Immunoblotting for cellubrevin: effect of tetanus toxin. To confirm cleavage of cellubrevin by tetanus toxin in the vas deferens, we performed Western blotting on control and tetanus toxin-treated tissue. As shown in Fig. 9, preincubation of vas deferens for 30 min with 50 nM tetanus toxin resulted in the appearance of a 9-kDa band in addition to the control 12-kDa band (lanes 3 and 5). These bands are compatible with the theoretical molecular masses of intact cellubrevin (11.5 kDa) and its larger cleavage fragment (6.8 kDa). A previous report of a study in Chinese hamster ovary cells, using an affinity-purified anti-cellubrevin antibody derived from the same whole serum used in our present study (23), has shown a dose-dependent effect of tetanus toxin on cellubrevin, with a disappearance of cellubrevin immunoreactivity on Western blots at a concentration of 300 nM. Thus the use of 50 nM tetanus toxin in the present study might have produced a less extensive cleavage of cellubrevin. In addition, recent studies have shown that synaptobrevin and SNAP-25 are resistant to tetanus toxin and botulinum toxin, respectively, when they are complexed to other members of the SNARE family (40, 41). The partial cleavage of cellubrevin observed in the present study might, therefore, indicate that a significant fraction of cellubrevin is part of a v-SNARE-t-SNARE complex in the intact vas deferens. Nevertheless, the appearance of a smaller band after exposure of the tissue to tetanus toxin clearly indicates that the toxin gained access to, and cleaved, a significant amount of cellubrevin in the vas deferens.

DISCUSSION

We examined the mechanisms responsible for the regulation of proton secretion in the male reproductive system. We specifically addressed the question of whether H\textsuperscript{+}-ATPase in the male reproductive system is regulated, by analogy with other proton-secreting epi-
thelia, via vesicle-recycling pathways. The role of the v-SNARE cellubrevin in this recycling process was also determined.

Microtubules are important players in exocytotic events: they are involved in the delivery of newly synthesized membrane proteins to their respective plasma membrane and in the regulated recycling of some proteins via specialized vesicles (18, 36). Microtubule disruption inhibits exocytosis and results in an accumulation of endocytotic and transporting vesicles inside the cell. In the present study, the marked redistribution of H\(^{+}\)-ATPase from the apical membrane to numerous intracytoplasmic vesicles suggests that this protein undergoes significant endocytosis in the epididymis. In the absence of microtubules, these vesicles cannot recycle back to the apical membrane; instead, they accumulate inside the cell. An alternative explanation is that the accumulated vesicles are involved in the delivery of newly synthesized H\(^{+}\)-ATPase to the apical membrane. However, H\(^{+}\)-ATPase-rich cells, identified by positive staining for H\(^{+}\)-ATPase in the epididymis showed a vigorous uptake of the fluid phase marker Texas red-dextran, and H\(^{+}\)-ATPase is colocalized in the endosomes. These results provide further evidence that H\(^{+}\)-ATPase undergoes active endocytosis in these cells. Therefore, the intracellular H\(^{+}\)-ATPase-containing vesicles that are seen after microtubule disruption must include a significant proportion of endocytotic vesicles.

The molecular mechanisms responsible for the regulation of endocytotic and exocytotic mechanisms in epithelial cells are presently the subject of study by many laboratories. H\(^{+}\)-ATPase-containing vesicles in kidney intercalated cells possess an extensive cytoplasmic coat, indicating the involvement of vesicle-associated proteins in this process. We have previously shown that these vesicles are devoid of clathrin (14, 16) and that caveolin is not detectable in H\(^{+}\)-ATPase-coated vesicles in kidney intercalated cells (8). Epididymal epithelial cells are also negative for caveolin (data not shown), indicating that H\(^{+}\)-ATPase recycling is not mediated via caveolae. The coat protein \(\beta\text{-COP}\), a member of the COPI family, the presence of which on endosomes has been described in other cell types (57), is not expressed at detectable levels on endosomes from kidney intercalated cells and H\(^{+}\)-ATPase-rich cells of the epididymis (7). It therefore appears that H\(^{+}\)-ATPase recycling in intercalated cells as well as in H\(^{+}\)-ATPase-rich epididymal and vas deferens cells relies on different and possibly unique clathrin-independent mechanisms for the regulation of proton secretion.

Does the proton pump participate in its own trafficking? The 39-kDa subunit of the H\(^{+}\)-ATPase is homologous to physophilin, a cytosolic synaptophysin-binding
protein (52). Other subunits of the V0 sector of the H1-ATPase, including Ac39, Ac116, and the proton pore-forming subunit c, are associated with synaptobrevin and synaptophysin on synaptic vesicles (24). In addition, in collecting duct principal cells, the “kidney” isoform of the 56-kDa b1-subunit of the H1-ATPase is associated with AQP2 water channel-containing endosomes, which do not contain other subunits of the proton pump and do not acidify their lumen (49). It is possible, therefore, that some of the H1-ATPase subunits have a function independent of proton-pumping activity and that they might be involved, in a novel way, in the recycling machinery of H1-ATPase-rich cells, as well as some other cell types.

Our present study shows that cellubrevin, a v-SNARE protein, is highly expressed in H1-ATPase-rich cells of the epididymis and vas deferens, indicating a potential role in proton secretion. The marked inhibition of proton secretion after cleavage of cellubrevin by tetanus toxin strongly suggests that the acidification capacity of the vas deferens requires the participation of members of the SNARE family. Tetanus toxin does not directly inhibit H1-ATPase-pumping activity on endosomes isolated from rat kidney cortex. Therefore, the inhibition of proton secretion in the vas deferens after tetanus toxin treatment probably results from a decrease in the number of H1-ATPase molecules that are inserted into the apical membrane, due to an impairment of the exocytotic process. This result also indicates that H1-ATPase underwent continual endocytosis under our experimental conditions. Immunoblots for cellubrevin showed that, after tetanus toxin treatment, the control band at 12 kDa, although reduced in intensity, was still observed in addition to the smaller band, indicating the presence of some intact cellubrevin. This partial cleavage could explain the partial inhibition of proton secretion by tetanus toxin. Alternatively, some proton pump molecules might not have been endocytosed during the relatively short time course of the experiments and would therefore have remained unaffected by an inhibition of the vesicle docking/fusion process. Also, a tetanus neurotoxin-insensitive v-SNARE such as TI-VAMP (25) might be involved in the toxin-insensitive recycling of H1-ATPase.

According to the SNARE hypothesis, vesicle targeting to the appropriate plasma membrane domain involves sequential steps, including vesicle docking, activation, and fusion (45, 56). Specificity of docking was initially proposed to be ensured by the binding of a v-SNARE (present on the vesicle) with a t-SNARE (present on the target membrane). Activation of the
complex, which is essential for fusion of the two membrane domains, is provided by the association with α-SNAP and NSF proteins. Further studies have proposed that SNAREs cannot act alone in vesicle docking and that additional factors, including tethering proteins, participate in the targeting of exocytic vesicles to their appropriate membrane domain (42, 43). In neurons, tetanus or botulinum toxin treatment does not reduce the number of docked synaptic vesicles from the presynaptic plasma membrane (28). In mutated Droso phila lacking the neural v-SNARE synaptobrevin and the t-SNARE syntaxin, an increased number of docked vesicles on the presynaptic membrane were observed compared with wild type (11). In the same study, it was shown that, although syntaxin is absolutely required for the fusion of vesicles to the plasma membrane, synaptobrevin plays a facilitating role in this process. In the present study, we cannot distinguish between impairment of the fusion process or reduction in the number of docked H+-ATPase-containing vesicles after cleavage of cellubrevin, and further experiments will be required to address this issue.

Previous studies have proposed that permeabilization of nonneuronal cells is essential for proper internalization of tetanus toxin to occur (23). In the present study, the appearance of an additional smaller band on immunoblots for cellubrevin indicates that cleavage by tetanus toxin does occur in an intact tissue. A recent report has also shown inhibition of proton secretion by claudoidal toxins in an inner medullary collecting duct cell line. Am J Physiol Renal Physiol 273: F1054–F1057, 1997.

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REFERENCES


ROLE OF CELLUBREVIN IN PROTON SECRETION


