Regulation of V-ATPase recycling via a RhoA- and ROCKII-dependent pathway in epididymal clear cells

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Submitted 26 May 2010; accepted in final form 11 March 2011

Shum WW, Da Silva N, Belleannée C, McKee M, Brown D, Breton S. Regulation of V-ATPase recycling via a RhoA- and ROCKII-dependent pathway in epididymal clear cells. Am J Physiol Cell Physiol 301:C31–C43, 2011. First published March 16, 2011; doi:10.1152/ajpcell.00198.2010.—Luminal acidification in the epididymis is critical for sperm maturation and storage. Clear cells express the vacuolar H+–ATPase (V-ATPase) in their apical membrane and are major contributors to proton secretion. We showed that this process is regulated via recycling of V-ATPase-containing vesicles. We now report that RhoA and its effector ROCKII are enriched in rat epididymal clear cells. In addition, cortical F-actin was detected beneath the apical membrane and along the lateral membrane of “resting” clear cells using a pan-actin antibody or phalloidin-TRITC. In vivo luminal perfusion of the cauda epididymal tubule with the ROCK inhibitors Y27632 (10–30 μM) and HA1077 (30 μM) or with the cell-permeable Rho inhibitor Clostridium botulinum C3 trans- ferase (3.75 μg/ml) induced the apical membrane accumulation of V-ATPase and extension of V-ATPase-labeled microvilli in clear cells. However, these newly formed microvilli were devoid of ROCKII. In addition, Y27632 (30 μM) or HA1077 (30 μM) decreased the ratio of F-actin to G-actin detected by Western blot analysis in epididymal epithelial cells, and Y27632 also decreased the ratio of F-actin to G-actin in clear cells isolated by fluorescence-activated cell sorting from B1-enhanced green fluorescence protein (EGFP) transgenic mice. These results provide evidence that depolymerization of the cortical actin cytoskeleton via inhibition of RhoA or its effector ROCKII favors the recruitment of V-ATPase from the cytosolic compartment into the apical membrane in clear cells. In addition, our data suggest that the RhoA-ROCKII pathway is not locally involved in the elongation of apical microvilli. We propose that inhibition of RhoA-ROCKII might be part of the intracellular signaling cascade that is triggered upon agonist-induced apical membrane V-ATPase accumulation.

actin; proton pump; luminal acidification; proton ATPase; epididymis

SEVERAL SPECIALIZED biological tissues, such as kidney tubules (8–10, 66), the male reproductive tract (1, 6, 32, 43, 57), the inner ear (27, 59), salivary glands (51, 65), and osteoclasts (6–10, 31) establish an acidic extracellular local environment. These tissues all have in common a high expression of the proton pumping V-ATPase, which is localized in the plasma membrane of specialized acidifying cells. In the male reproductive tract, luminal acidification is a critical process for sperm maturation and for their storage in a quiescent state. We have shown that the V-ATPase localizes in the apical membrane of clear cells in the epididymis (2, 3, 6, 42, 43, 57) and that these cells are important players in luminal acidification, especially in the distal region of the epididymis, where they are present in high numbers. Impairment of the acidification process in the epididymis, either by genetic mutations or environmental factors, such as the heavy metal cadmium, reduces male fertility (4, 5, 11, 22, 64).

We have shown previously that luminal bicarbonate via bicarbonate-regulated adenyl cyclase (sAC)-dependent intracellular cAMP production (42), or luminal angiotensin II via the nitric oxide (NO)/soluble guanylate cyclase (sGC)-dependent pathway (41), induce the apical membrane recruitment of V-ATPase from an intracellular pool of subapical vesicles. This process is correlated with an increase in proton secretion and the formation and elongation of numerous V-ATPase-labeled microvilli, which contain a high density of V-ATPase complexes (2, 6, 42, 57). Our studies also suggested that dynamic remodeling of the actin cytoskeleton plays an important role in the subcellular localization of V-ATPase (2). Subunits B1, B2, and C of the V-ATPase can all interact directly with actin (12, 24, 65). In addition, B1 can interact indirectly with the actin cytoskeleton via its association with NHERF1, a PDZ protein that contains a merlin-ezrin-radixin moesin actin-binding domain (7). We have shown that clear cells express very high levels of the calcium-dependent, actin-capping, and severing protein gelsolin (2). Inhibition of actin polymerization using a permeant peptide that prevents uncapping of gelsolin from the barbed end of actin filaments (F-actin) induced a marked accumulation of the V-ATPase in clear cell microvilli. These results suggested that gelsolin-dependent actin depolymerization in clear cells favors either the inhibition of V-ATPase endocytosis or stimulation of exocytosis, leading to an increase in V-ATPase accumulation in the plasma membrane (2).

Actin is required for vesicle recycling at multiple steps, including endocytosis and exocytosis, and helps maintain a reservoir of specialized transporter-containing vesicles ready for stimulation (17, 41, 53). Key regulators of actin cytoskeletal dynamics are the 22 members of the family of mammalian Rho GTPases, of which RhoA, Rac1, and Cdc42 are the best studied (19, 21, 47, 50). These signaling proteins act as molecular switches to regulate the cytoskeleton of eukaryotic cells, and they indirectly control almost all cellular activities, including cell polarity, membrane protrusion, endocytosis, vesicle trafficking, and cytokinesis (19, 47, 50). Most Rho GTPases locally modulate the membrane-associated actin cytoskeleton in specialized compartments while maintaining global cellular actin dynamics (35, 44, 47). RhoA exerts its function by activating the serine/threonine-specific protein kinases (ROCKs) (28, 31, 38). Mammalian ROCKs consist of
two isoforms: ROCKI (also known as ROKβ or p160ROCK) and ROCKII (also known as ROKα). Abnormal activation of the Rho-ROCK pathway has been observed in several disorders, and therapeutic beneficial effects of ROCK inhibitors have been reported in animal models of stroke, inflammation, and erectile dysfunction (13, 40, 48). However, the role of the Rho-ROCK pathway in the physiology of the male excurrent duct, particularly luminal acidification in the epididymis, is still unknown. Our recent proteomic analysis showed the presence of RhoA in epididymal clear cells isolated by fluorescence-activated cell sorting (FACS) from B1-enhanced green fluorescence protein (EGFP) mice (14). Interestingly, several Rho GTPase-regulating proteins as well as proteins involved in active remodeling of the actin cytoskeleton were detected in these cells.

The aim of the present study was, therefore, to determine the role of RhoA and its downstream effector ROCKII in regulating actin cytoskeleton organization and V-ATPase trafficking in polarized clear cells of rat epididymis. Our results provide evidence that RhoA and ROCKII are enriched in clear cells, and that depolymerization of the actin cytoskeleton via inhibition of RhoA or ROCKs favors apical membrane V-ATPase accumulation.

METHODS

Tissue fixation and preparation. Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were acquired, retained, and used in compliance with the Institutional Animal Care and Use Committees of the Massachusetts General Hospital. Adult male rats were anesthetized with Nembutal (60 mg/kg body wt ip). The epididymis and vas deferens were fixed by perfusion through the left ventricle with phosphate-buffered saline (PBS) followed by fixative containing 4% paraformaldehyde, 10 mM sodium periodate, 75 mM lysine, and 5% sucrose in 0.1 M sodium phosphate buffer (PLP), as we have described previously (3, 42, 57). The epididymis and vas deferens were harvested and further fixed by immersion in PLP overnight. Tissues were then washed three times in PBS and stored at 4°C until use.

In vivo microperfusion of cauda epididymidis. Rats were anesthetized with Nembutal (60 mg/kg body wt ip). The cauda epididymis was exposed by an abdominal incision under a dissecting microscope, and a small piece of connective tissue sheath (about 1 mm2) was removed in the middle part of the cauda epididymidis. The duct was cannulated by insertion of a small catheter with a tip diameter of ∼200–250 μm was also inserted into the lumen of the vas deferens and was ligated in place to collect the perfusate. The lumen of the cauda epididymidis was flushed with a physiological solution at a rate of 0.5 ml/h using a syringe infusion pump (model 100, Kel Scientific), as we have described previously (3, 57). The perfusing solution consisted of (in mM) 50 NaCl, 50 K gluconate, 1.2 MgCl2, 0.6 CaCl2, 4 Na acetate, 1 Na-citrate, 6.4 NaH2PO4, 3.6 Na2HPO4, 102 raffinose, with a pH of 6.6, and osmolarity of 330 mosmol/kg H2O. Horseradish peroxidase (HRP, 1 mg/ml; Type VI-A, Sigma) was then added in the perfusate and perfused for 10 min to detect endocytosis in the presence or absence of agonists and/or antagonists, as described in RESULTS. The luminal solution was then changed for HRP-free ice-cold perfusate for 3 min (in the continued presence of agonists or antagonists, if applicable) to wash the lumen free of HRP, followed by PLP fixative for 10 min. Vas deferens and cauda epididymidis were then harvested and further fixed by immersion in PLP overnight at 4°C. Tissues were then processed as described in Tissue fixation and preparation.

Immunofluorescence and antibodies. Single-, double-, and triple-immunofluorescence labeling was performed as we have described previously (2, 3, 42, 57). All antibodies were diluted in Dako antibody diluent (Dako). Affinity-purified rabbit polyclonal antibodies against the B1 (56 kDa) (diluted 1:1,000) and affinity-purified chicken polyclonal antibody against the B1-subunit (56 kDa) or E-subunit (31 kDa) (diluted 1:20) of the V-ATPase were used. These V-ATPase antibodies have been characterized previously (7, 46). A goat-anti-HRP antibody (Jackson Immuno-research Laboratories) (1:2,500) was used to detect the endocytosed HRP in the perfused epididymis. Commercial monoclonal anti-RhoA antibody (Cytoskeleton, Denver, CO) (1:100 dilution) against a peptide sequence corresponding to amino acids 120–150, and a goat-anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (1:50 dilution) against the COOH-terminus of human RhoA, were used to detect RhoA protein. The peptide used to generate the goat-anti-RhoA antibody was provided by the manufacturer (Santa Cruz Biotechnology) and used five times in excess of IgG (µg/µl) in competition assays to confirm the specificity of this polycyclonal antibody. Rabbit-anti-ROCKII (1:1,000 dilution) antibody and the immunopeptide [used 10 times in excess of IgG (µg/µl)] provided by the manufacturer (Bethyl Laboratories) were used to determine ROCKII protein expression. A mouse monoclonal antibody against pan-actin (Chemicon) and TRITC-conjugated phalloidin (Sigma) were used to label F-actin by immunofluorescence.

All secondary antibodies used in this study were affinity purified and were purchased from Jackson Immunoresearch Laboratories: FITC- and CY3-conjugated donkey- or goat-anti-rabbit IgG, FITC-, CY3-, and CY5-conjugated donkey-anti-chicken, and FITC- and CY3-conjugated goat-anti-mouse IgG.

The slides were mounted in Vectashield medium (Vector Labs, Burlingame, CA) with or without 4′,6-diamidino-2-phenylindole (DAPI). Immunolabeled sections were examined using a Nikon E800 microscope (Nikon Instruments, Melville, NY) equipped with an Orca-100 CCD camera (Hamamatsu, Bridgewater, NJ). Digital images were acquired with IPLab Spectrum software (Scanalytics, Fairfax, VA) and were imported into Adobe Photoshop. Sections were also examined using a Bio-Rad Radiance 2000 confocal microscope (version 4.1, Zeiss Laboratories). For three-dimensional reconstruction, Z-series images were imported into Velocity software (version 4.5), and final animations were exported as TIFF or QuickTime movies.

Quantification of V-ATPase apical membrane accumulation in clear cells. The level of accumulation of V-ATPase in microvilli was quantified using IPLab software as we have described previously (2, 3, 42, 57). Sections (10 µm) of microperforated cauda epididymis were double-immunolabeled for the V-ATPase and HRP under identical conditions, and for a given incubation, confocal images were acquired using identical parameters. The segmentation function of IPLab software was used to measure the area of V-ATPase-positive microvilli, which was normalized against the width of the cell measured along the border between the base of microvilli and apical cytoplasm (2, 3, 42, 57). This border was determined from the localization of HRP, which was mainly present in the apical pole of the cell (red channel). The total area occupied by the microvilli, divided by the apical width, is referred to as the average “length of the apical microvilli domain.” Only vertically sectioned cells, in which both the apical and basal poles were visible, were selected for quantification. For each set of data, at least three epididymides from different animals were perfused for each condition, and a minimum of 10 cells/tissue were examined for a total of at least 30 cells/condition.
**RHOA AND ROCKII REGULATE PLASMA MEMBRANE V-ATPASE RECYCLING**

Immunogold electron microscopy and quantification of gold particle labeling. Immunogold electron microscopy was performed to localize the V-ATPase. As we have previously described (2, 15, 57), small pieces of PLP-fixed epididymis were embedded at −45°C using HM20 resin (Electron Microscopy Sciences) in a Leica EM ATS, and then ultrathin sections were cut using a Leica Ultracut EM UC7 ultramicrotome (Leica Microsystems, Bannockburn, IL) and collected on Formvar-coated, gilded nickel grids. Sections were immunolabeled for the V-ATPase A subunit, followed by a goat anti-rabbit antibody coupled to 15-nm gold particles (Ted Pella, Redding, CA). Grids were examined in a JEOL 1011 transmission electron microscope at 80 kV, and images were acquired using an AMT digital imaging system. The number of V-ATPase-associated gold particles located on the apical membrane (including microvilli) was counted for each clear cell examined. To determine the density of V-ATPase molecules along the apical membrane, the number of gold particles was divided by the total length of apical membrane, including microvilli, measured in each cell (using IPLab software). This value is referred to as “gold particles/μm apical membrane.” To determine the relative density of the V-ATPase at the cell surface, the number of gold particles was normalized for the width of the cell measured at the base of microvilli (gold particles/μm cell width).

Isolation of clear cells by FACS from B1-EGFP transgenic mice. We have previously reported the generation of B1-EGFP transgenic mice that express EGFP under the control of the B1-V-ATPase promoter. In these mice, EGFP fluorescence is detectable only in cells that strongly express the B1 subunit, including epididymal clear cells and renal intercalated cells (39). These cells were isolated by FACS, as we have described previously (14, 57). Briefly, the epididymides or kidney were dissected from anesthetized mice, then minced, and dissociated in a shaking water bath (100 rpm) at 37°C for 45 min in RPMI 1640 medium (GIBCO Invitrogen, Carlsbad, CA) containing 1 mg/ml collagenase type I, 1 mg/ml collagenase type II (GIBCO Invitrogen), and 1 mg/ml hyaluronidase (Sigma) in 10 mM HEPES buffer, pH 7.5. Cell mixtures were then passed through a cell strainer with a 70-μm nylon mesh to remove undigested material. The collected cells were then washed once with RPMI 1640 medium and once with calcium-free PBS and passed through a 35-μm mesh. Populations of EGFP-positive cells from epididymis and kidney were isolated immediately by FACS based on their green fluorescence using a modified FACSVantage SE/DiVa SORP V cell sorter (BD Biosciences, San Jose, CA) at the MGH Flow Cytometry Core facility. EGFP-positive cell samples were collected in PBS and used without delay for RNA isolation or treatment with Rho kinase inhibitors followed by cell fractionation, as described below.

Total RNA extraction, reverse transcription, and polymerase chain reaction. FACS-isolated B1-EGFP-positive epididymal and renal cells were centrifuged briefly and cell pellets were resuspended in RNA extraction buffer (PicoPure RNA isolation, Molecular Devices, Sunnyvale, CA) and stored at −80°C, as we described previously (14, 57). Total RNA was also isolated from whole epididymides and kidneys. Tissues were frozen in liquid nitrogen, then powdered in a pestle and mortar, and homogenized in RLT lysis buffer (Qiagen). RNA samples were aliquoted and stored at −80°C without delay for RNA isolation or treatment with Rho kinase inhibitors. Total protein extracts from rat and mouse epididymis and kidney were amplified in an ice-cold buffer containing 160 mM NaCl, 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.05% IGEPAL, 1% Triton X-100, and complete protease inhibitors (Roche Applied Science, Indianapolis, IN). Homogenates were resuspended in reducing [liiithium dodecyl sulfate (LDS) plus DTT] sample buffer (NuPAGE, Invitrogen) and heated for 5 min at 99°C before electrophoresis. Electrophoresis was performed using 4–12% Bis-Tris precast gels with MES/SDS running buffer (NuPAGE, Invitrogen), and proteins were transferred to PVDF membrane (Bio-Rad). Overnight incubation was performed at 4°C with either a mouse-anti-RhoA antibody (1:500) or rabbit-anti-RockII antibody (1:1,000) followed by HRP-conjugated goat-anti-mouse or anti-rabbit secondary antibodies at a dilution of 1:5,000 for 1 h at room temperature. Proteins were detected using Western Lightning Western Blot Chemiluminescence Reagent Plus (PerkinElmer Life Sciences) or SuperSignal West Dura Extended Duration Substrate (Thermo Scientific).

The level of actin polymerization was examined in epithelial cells of the distal cauda epididymis as we have described previously (2). The distal cauda epididymis was harvested from anesthetized rats and washed free of sperm by either cutting open the tubule or by luminal perfusion. The epithelium was peeled off the surrounding connective and muscle tissues and incubated with 2 mM phosphate saline (pH 7.4) containing Rho-kinase inhibitors for 30 min. The epithelium of the epididymis from the other side of the same animal was incubated without inhibitors (Control). Control and treated epithelia were freeze-thawed three times in 100 μl of 2 mM phosphate saline (pH 7.4) containing protease inhibitors and then centrifuged at 4°C at 16,000 g for 17 min. The supernatant was transferred into a new tube, and 100 μl of 2 mM phosphate saline (pH 7.4) were added to the pellet and centrifuged again. Both supernatants were pooled together and were designated as the cytosolic soluble monomeric actin (G-actin) fraction. The pellet was then homogenized in an equal volume (200 μl) of the same buffer containing 1% Triton X-100 and 1% Igepal. This pellet fraction was designated as the filamentous actin (F-actin) fraction. All fractions were further homogenized with a 19-gauge needle syringe after resuspension in LDS/DDT buffer (NuPAGE, Invitrogen) and heated for 5 min at 99°C. Equal volumes of each fraction (10 μl) were subjected to gel electrophoresis and semi-quantitative immunoblotting analysis, using a mouse-anti-pa-
RESULTS

Clear cells have a distinct apical band of cortical F-actin labeling that is weaker than the apical microvillar labeling of adjacent principal cells. The actin cytoskeleton in clear cells was examined on PLP-fixed rat cauda epididymis cryostat sections immunolabeled using a pan-actin monoclonal antibody. As shown in Fig. 1, A and A’, actin immunoreactivity (red) in V-ATPase-positive (green) clear cells was weak (arrows) compared with adjacent principal cells, in which strong labeling of apical stereocilia was detected, suggesting that the actin cytoskeleton is much less organized in clear cells. However, higher magnification images (Fig. 1, B and B’) clearly revealed a pool of actin beneath the apical membrane of clear cells (arrows). Epididymal sections were also labeled with the F-actin marker phalloidin-TRITC. While a very strong labeling was detected in principal cell stereocilia (arrowheads) and surrounding smooth muscle cells (asterisks), a weak but clearly visible tight apical band (arrows) was detected along the apical membrane of clear cells (Fig. 1, C and C’).

Enriched expression of RhoA and ROCKII in clear cells. Conventional epifluorescence analysis of PLP-fixed rat cauda epididymis cryostat sections using a commercial monoclonal antibody against RhoA (generated against amino acids 120–150) revealed a substantial enrichment of this protein in clear cells (Fig. 2A and red in A’), identified by their positive immunoreactivity for the V-ATPase (Fig. 2A’ and green in A’). Weaker immunoreactivity was seen in adjacent epithelial cells. This monoclonal antibody for RhoA is specific for RhoA GTPase and does not cross-react with other Rho proteins including RhoB, RhoC, Rac1–3, Cdc42, or H-Ras, according

Fig. 1. Actin labeling in the apical pole of clear cells and adjacent principal cells. A and A’: double labeling of rat cauda epididymis using a pan-actin antibody (red) and an anti-B1-V-ATPase antibody (green). A weak actin labeling is detected in the apical pole of V-ATPase-labeled clear cells (arrows) compared with adjacent principal cells, indicating that the actin cytoskeleton is much less organized in the apical pole of clear cells. B and B’: higher magnification revealed a tight apical band of actin labeling in the apex of clear cells (arrows). Arrowheads show actin-rich stereocilia in principal cells. C and C’: epididymis sections (cauda) labeled with the F-actin marker, phalloidin-TRITC (red) and the V-ATPase (B1 subunit: green). Very strong labeling is seen in principal cell stereocilia (arrowheads) as well as in smooth muscle cells located at the base of the epithelium (asterisks). A tight F-actin labeling is detected beneath the apical membrane of clear cells (arrows). Nuclei and sperm are visualized with DAPI (blue) in the merged panels (A’, B’, C’). Bars: 50 μm (A and A’) and 5 μm (B–C).
to the manufacturer. Semiquantitative analysis of the immuno-fluorescence labeling for RhoA using IPLab software demonstrated that the RhoA intensity in the apical pole of clear cells is about two times brighter than in principal cells (Fig. 2B). Western blot analysis using this antibody detected a single band at 20 kDa, corresponding to the expected size of RhoA, in protein extracts from rat epididymis (RE) and kidney (RK), as well as in a positive control consisting of human platelet extract (Ctl) provided by the manufacturer (Fig. 2C). A similar labeling pattern was also observed by immunofluorescence using a polyclonal antibody against the COOH-terminus of RhoA (Fig. 2D). RhoA epithelial cell immunolabeling was almost abolished using antibody that had been preabsorbed with an excess amount of the immunizing peptide, confirming specificity of this polyclonal antibody (Fig. 2D').

Expression of the Rho kinase ROCKII, a key downstream effector of RhoA, was then examined in rat and mouse epididymis. Figure 3A shows that ROCKII transcripts were detected in mRNA extracts from rat (RE) and mouse (ME) epididymis, as well as rat (RK) and mouse (MK) kidney. ROCKII transcripts were also detected in EGFP-positive epididymal clear cells (ME EGFP) and kidney intercalated cells (MK EGFP) isolated by FACS from our B1-EGFP transgenic mice (14, 39).

Conventional immunofluorescence microscopy detected a strong ROCKII labeling in clear cells of rat cauda epididymis, identified by their positive V-ATPase immunofluorescence labeling (Fig. 3, B and B'). A weak and more uniform labeling was also detected in adjacent principal cells. Similar immunoreactivity patterns for ROCKII were observed in adult mouse epididymis (data not shown), indicating that the enriched ROCKII expression in clear cells occurs across species. Complete inhibition of the immunofluorescence labeling was observed after preabsorption of the antibody with a 10-fold excess of the immunizing peptide (Fig. 3, C and C'), confirming specificity of the ROCKII antibody. A strong band at ~160 kDa corresponding to the expected size of ROCKII was detected by Western blot analysis using a rat epididymis protein extract (Fig. 3D, arrow). Additional smaller molecular mass bands were also detected, indicating potential degradation products in this tissue. All detected bands were abolished using antibody that had been preabsorbed with the immunizing peptide. Regional examination of rat epididymis sections revealed that RhoA and ROCKII proteins were enriched in clear cells of the caput (not shown), corpus (not shown), and cauda (Fig. 4, A, A', C, and C') epididymis but not in V-ATPase-expressing narrow cells in the initial segments (Fig. 4, B, B', D, and D'). This implies that these proteins might play a more important role in proton-secreting cells of the distal regions of the epididymis compared with the proximal region. This expression pattern also suggests that clear cells exhibit different
protein expression patterns compared with narrow cells, as was previously suggested (23).

Role of RhoA and ROCKII on V-ATPase localization in clear cells. We then determined whether RhoA and ROCKII play a role in the recycling and apical membrane accumulation of V-ATPase in clear cells. We have previously shown that activation of V-ATPase-dependent proton secretion in these cells correlates with apical membrane V-ATPase accumulation, which induces a marked elongation of V-ATPase-labeled microvilli (2, 8, 42, 57). We, therefore, assessed the role of RhoA and ROCKII on V-ATPase apical recruitment in clear cells by measuring the extension of V-ATPase-labeled microvilli after in vivo epididymis luminal perfusion with various RhoA and ROCKII inhibitors. We first used a cell-permeable Rho-GTPase inhibitor Clostridium botulinum C3 transferase (TxC3), which inactivates RhoA, RhoB, and RhoC through ADP ribosylation at Asp41 (20, 54) but does not inhibit Rac-1 or Cdc42 GTPases according to the manufacturer (Cytoskeleton). To allow enough time for TxC3 penetration into the cells, the cauda epididymidis was perfused with 3.75 μg/ml of TxC3 for 2 h. The epididymis from the opposite side of the same animal was used as a parallel control and was perfused for the same period of time with a phosphate-buffered saline adjusted to the physiological luminal pH 6.6. Under these "resting" control conditions, the V-ATPase in clear cells was distributed between short microvilli and subapical vesicles (Fig. 5, A and A'). In the presence of TxC3, the V-ATPase was mainly located in well-developed microvilli (Fig. 5, B and B'). Quantitative analysis revealed a significant increase in the average length of V-ATPase-labeled microvilli from a value of 1.6 ± 0.2 μm under control conditions to 2.5 ± 0.2 μm after perfusion with TxC3 (Fig. 5C), corresponding to an increase of 51 ± 15% versus control (P < 0.05, unpaired t-test).

Fig. 3. ROCKII is enriched in clear cells. A: RT-PCR analysis showed that ROCKII transcripts are present in RNA extracts isolated from whole rat and mouse epididymis and kidney (RE, ME, RK, and MK, respectively). ROCKII mRNA was also detected in enhanced green fluorescent protein (EGFP)-positive clear cells in epididymis (ME EGFP) or EGFP-positive intercalated cells in kidney (MK EGFP) isolated by fluorescence activated cell sorting (FACS) from B1-EGFP transgenic mice. NTC1 and NTC2: no template control for rat and mouse primers, respectively. B and B': double-immunofluorescence labeling of rat epididymis using a rabbit polyclonal antibody against the COOH-terminus of ROCKII (B and B'; red) and a chicken anti-E-subunit V-ATPase antibody (green). ROCKII is highly abundant in intracellular structures of V-ATPase-positive clear cells. A weak and more uniform labeling is also detected in adjacent principal cells. Nuclei and spermatozoa are visualized in blue with DAPI in B'. Bars: 5 μm. C and C': specificity of the anti-ROCKII antibody was verified by preincubating the antibody with a 10-fold excess of the immunizing peptide. The ROCKII labeling was completely abolished (compare C' with C) using the antibody preabsorbed with the immunizing peptide (+ Peptide). Bar: 50 μm. D: Western blot detection of ROCKII in total homogenates from rat epididymis. A 160-kDa band (arrow) corresponding to the molecular mass of ROCKII was detected. Additional bands were also detected, indicating potential degradation products in this tissue. All bands were abolished using antibody that had been preabsorbed with the immunizing peptide (+ Peptide).
(data not shown), 100 μM Y27632 induced a slightly greater response (Fig. 6C). Quantification showed that V-ATPase-labeled microvilli in clear cells increased from a value of 1.2 ± 0.1 μm under control conditions to 1.7 ± 0.2, 1.8 ± 0.2, and 2.0 ± 0.2 μm in the presence of 10, 30, and 100 μM Y27632, respectively (Fig. 6E), corresponding to increases of 46 ± 17%, 50 ± 10%, and 71 ± 26% versus controls (*P < 0.05 and **P < 0.01 vs. control, one-way ANOVA).

Similarly, luminal perfusion with 30 μM of the Rho kinase inhibitor HA1077 (16) for 30 min induced the extension of V-ATPase-labeled microvilli in clear cells compared with controls (Fig. 7, A and B). As shown in Fig. 7C, the average

![Fig. 4. Regional expression of RhoA and ROCKII in epididymis. Immunofluorescence labeling for RhoA (A and A'; red) and ROCKII (C and C'; red) showed expression of these proteins in V-ATPase-positive clear cells (green; arrows) in the cauda epididymidis. However, no RhoA (B and B'; red), or ROCKII (D and D'; red) immunoreactivity was detected in V-ATPase-positive narrow cells (green; arrows) in the initial segments of the epididymis. Nuclei and spermatozoa are visualized with DAPI (blue) in A', B', C' and D'. Bars: 5 μm.]

![Fig. 5. Effect of the Rho GTPase inhibitor Clostridium botulinum C3 transferase (TxC3) on V-ATPase apical accumulation and extension of microvilli in clear cells. A and B: confocal images showing clear cells of rat cauda epididymidis after perfusion for 2 h with PBS (pH 6.6) in the absence (A; Control) or presence of 3.75 μg/ml TxC3 (B). Horseradish peroxidase (HRP) was then added for 10 min, followed by 3 min washout of luminal HRP (still in the presence of TxC3 when applicable). V-ATPase and internalized HRP are shown in green and red, respectively. Arrows indicate the border between the base of microvilli and the cytoplasm of the cell. A' and B': average length of V-ATPase-labeled microvilli per cell was assessed by measuring the surface occupied by these microvilli (highlighted by the white line) normalized for the width of the cell measured along the apical pole of the cell, at the base of microvilli (blue line). Bars = 5 μm. C: histogram showing the length of V-ATPase-labeled microvilli expressed as mean values (± SE) obtained from at least 10 cells from 4 epididymides per group (for a total of at least 40 cells per group). TxC3 induced a significant elongation of V-ATPase-labeled microvilli. *P = 0.05 by unpaired Student’s t-test.]
Microvilli length per cell was increased from a control value of 1.4 ± 0.1 to 2.4 ± 0.1 μm, corresponding to an increase of 77 ± 8% versus control (P < 0.05, unpaired t-test).

Additionally, in the presence of TxC3, Y27632, and HA1077, we observed that the width of clear cells measured along the apical pole was significantly decreased (P < 0.001 for all, unpaired t-test). For TxC3, the apical width was decreased from 17.5 ± 0.6 μm for controls to 15.3 ± 0.6 μm, corresponding to a decrease of 24%. In the presence of 10, 30, and 100 μM of Y27632, the width of clear cells was decreased from 20.5 ± 0.6 μm in controls to 15.5 ± 0.6, 15.9 ± 0.9, and 14.5 ± 0.6 μm, corresponding to 24%, 22% and 29% decreases, respectively. The width was decreased from 23.7 ± 0.9 to 15.7 ± 1.1 μm in the presence of HA1077, corresponding to a 34% decrease.

The apical accumulation of V-ATPase following Rho kinase inhibition was confirmed by immunogold electron microscopy. Luminal Y27632 (30 μM for 30 min) induced a significant increase in V-ATPase gold particle labeling in microvilli (Fig. 8B) compared with control (Fig. 8A). Quantification of the number of V-ATPase-associated gold particles showed a significant increase in the density of V-ATPase in the apical membrane of clear cells by Y27632 compared with control (Fig. 8C; gold/μm apical membrane; P < 0.001). The total amount of V-ATPase located at the cell surface was also increased by Y27632 compared with controls (Fig. 8C; gold/μm cell width, P < 0.001).

Actin depolymerization in epididymal epithelial sheets and in isolated B1-EGFP clear cells by Rho kinase inhibitors. The level of polymerized versus depolymerized actin was first examined in epithelial sheets isolated from rat distal cauda epididymis, as we have previously described (2, 60). After incubation in PBS, pH 7.4, or in PBS containing Y-27632 or HA1077 for 30 min, cytosolic (G-actin) and pellet (F-actin) fractions were isolated, and the level of actin was assessed in each fraction by Western blot analysis. Y27632 (30 μM) induced a decrease in the amount of F-actin and an increase in G-actin compared with controls (Fig. 9A). Semiquantitative analysis revealed a significant decrease in the ratio of F-actin to G-actin after Y27632 treatment (Fig. 9C, left). HA1077 (30 μM) also decreased the amount of F-actin and increased the amount of G-actin (Fig. 9B). This was confirmed by quantification, which showed a significant decrease in the ratio of
F-actin to G-actin by HA1077 (Fig. 9C, right). These results indicate that inhibition of Rho kinases induced a significant depolymerization of the actin cytoskeleton in epithelial cells of the distal cauda epididymis.

The effect of Rho kinase inhibition on the ratio of F-actin to G-actin was further analyzed in clear cells, isolated by FACS from the epididymis of B1-EGFP transgenic mice. Here again, incubation with Y27632 (30 μM) decreased the ratio of F-actin to G-actin (Fig. 9D and E). A similar decrease in the ratio of F-actin versus G-actin was also observed after treatment of clear cells with another Rho kinase inhibitor HA1077 at 30 μM (data not shown).

Absence of ROCKII and weak F-actin labeling in the microvilli of clear cells. Double labeling for the V-ATPase and ROCKII followed by three-dimensional reconstruction of a stack of confocal images showed that ROCKII is absent from the newly formed microvilli that were induced by either Y27632 (30 μM) (Fig. 10A and A') or TxC3 (3.75 μM/ml) (Fig. 10B, and B') (See supplemental movies S1 and S2 online at the AJP-Cell Physiol website).

Fig. 7. Effect of the Rho kinase inhibitor HA1077 on V-ATPase apical accumulation and extension of microvilli in clear cells. A and B: confocal images showing clear cells of rat cauda epididymides perfused in vivo under control conditions (A), or in the presence of 30 μM HA1077 for 30 min (B). Sections were double labeled for B1-V-ATPase (green) and clathrin (red). Arrows indicate the frontier between the base of microvilli and the cytoplasm of clear cells. Nuclei are visualized with TOPRO3 (blue). Bars: 5 μm. A’ and B’: average length of V-ATPase-labeled microvilli per cell was assessed by measuring the surface occupied by these microvilli (highlighted by the white line) normalized for the width of the cell measured along the apical pole of the cell, at the base of microvilli (blue line). C: histogram showing a significant elongation of V-ATPase-labeled microvilli induced by HA1077. Data are the mean value (± SE) measured from confocal images obtained with the same capturing parameters. The values represent at least 10 cells from 4 epididymides per group (for a total of at least 40 cells per group). *P < 0.01 by an unpaired Student’s t-test.

Fig. 8. Luminal Y27632 induces V-ATPase apical accumulation in clear cells. A and B: immunogold electron microscope images showing V-ATPase labeling in clear cells after luminal perfusion under control conditions (CTL), or in the presence of Y27632 (30 μM) for 30 min (Y27632). Longer and more numerous microvilli are detected in the presence of Y27632 compared with control. These microvilli contain a higher number of V-ATPase gold particles compared with the less numerous microvilli seen under control conditions. Scale bars = 500 nm. C, left axis: quantification showed that Y27632 significantly increased the number of V-ATPase-associated gold particles per unit length of apical membrane including microvilli (Gold/μm apical membrane). Right axis: Y27632 also significantly increased the total number of V-ATPase-associated gold particles located in the apical membrane of clear cells, normalized for the width of the cell (Gold/μm cell width). Data are means ± SE from 3 animals per group. A total of 35 cells were analyzed in the control group and 37 cells in the Y27632 group. *P < 0.001 vs. control using Student’s t-test for unpaired samples.
Fig. 9. Inhibition of Rho kinase activity by Y27632 or HA1077 depolymerized actin. A and B: Western blot showing the amount of actin present in the cytosolic fraction containing G-actin (G) and pellet fraction containing F-actin (F) of epithelial cell sheets isolated from cut open perfused distal cauda epididymis incubated under control conditions (Control) or Rho kinase inhibitors as indicated: 30 μM of Y27632 (30 μM Y) or HA1077 (30 μM HA) for 30 min. Equal volumes of each fraction were loaded onto each lane. Treatment with Y27632 and HA1077 induced a redistribution of actin from the F-actin fraction toward the G-actin fraction compared with controls. C: Semiquantitative analysis showed that the ratio of F-actin to G-actin was significantly decreased after incubation with Y27632 (30Y), or HA1077 (30HA) compared with controls (CTL). *P < 0.05 and **P < 0.01 using one-way ANOVA with Bonferroni post hoc test. D: Western blot showing the amount of actin present in the cytosolic fraction containing G-actin (G) and pellet fraction containing F-actin (F) of clear cells isolated by FACS from B1-EGFP transgenic mice. Incubation with Y27632 (30 μM) for 30 min induced the redistribution of actin from the F-actin fraction toward the G-actin fraction compared with controls. E: Semiquantitative analysis showed that the ratio of F-actin to G-actin was significantly decreased after incubation with Y27632 (30Y), compared with controls (CTL). *P < 0.05 using Student’s t-test for paired samples.

DISCUSSION

In this study we report that RhoA and ROCKII are enriched in acidifying clear cells of the epididymis, and we show that modulation of the actin cytoskeleton via the RhoA-ROCKII pathway plays a role in V-ATPase recycling. We have previously shown that V-ATPase accumulates in apical microvilli of clear cells in response to a variety of luminal stimuli, including elevation in luminal pH, increase in luminal bicarbonate concentration, cAMP and cGMP permissive analogs, or purinergic apical receptor activation (2, 3, 42, 57). V-ATPase apical membrane accumulation correlates with a significant increase in the number and extension of microvilli (which contain more V-ATPase per unit length of membrane) and an increase in net proton secretion (2, 8, 42, 57). In the absence of luminal stimuli, the V-ATPase constitutively recycles and is mainly located in subapical endosomes. V-ATPase apical membrane accumulation and microvilli extension following agonist stimulation occur rapidly, within 15 min, indicating dynamic remodeling of the apical membrane in clear cells.

The high expression of RhoA in clear cells that we describe here correlates with our recently published proteomic data showing the presence of this protein in clear cells isolated by FACS from B1-EGFP mice (14). In addition, several Rho GTPase regulating proteins were detected in these cells. The present study shows that inhibition of the RhoA-ROCKII pathway is sufficient to induce the accumulation of V-ATPase in elongated microvilli of clear cells, which contained a higher density of V-ATPase molecules, even in the absence of other extracellular stimuli. A significant reduction in the apical width of clear cells, measured along the base of microvilli, was also observed under these conditions. These results confirmed that clear cells have the ability to modify their shape extensively in a short period of time. It also appears that RhoA and ROCKII are active under resting conditions (PBS adjusted to the acidic pH of 6.6), which would result in the maintenance of a polymerized actin cytoskeleton. Indeed, we show here that, while actin labeling is weaker in clear cells versus adjacent principal cells, which have long F-actin-rich stereocilia, a web of actin cytoskeleton is nevertheless detected along the apical pole of resting clear cells, indicating the presence of polymerized cortical actin in these cells.

We have previously shown that another actin-modulating protein, gelsolin, is enriched in clear cells and that inhibition of actin assembly by a peptide that prevents the uncapping of gelsolin from the growing barbed end of actin filaments also induces the accumulation of V-ATPase in well-developed microvilli (2). Thus a balance between the actin depolymerizing property of gelsolin and the actin polymerizing action of RhoA and ROCKII probably contributes to maintaining the appropriate level of actin dynamics for the V-ATPase to recycle between the apical membrane and apical vesicles under resting conditions. RhoA regulates the phosphatidylinositol composition of membrane compartments and activates the phosphoinositide (PI) kinases PIPK type-I or type-II, leading to PI(4,5)P2 synthesis (53, 67). The actin capping action of gelsolin is inhibited by PI(4,5)P2 (53). Local variations in PI(4,5)P2 concentration have been reported at the sites of phagocytosis or actin-rich membrane ruffles (53). It is, therefore, possible that RhoA participates in the PI(4,5)P2-dependent regulation of gelsolin in clear cells.

Whereas actin depolymerization following inhibition of the RhoA-ROCKII pathway is sufficient to induce the apical membrane accumulation of V-ATPase followed by extension of long V-ATPase-rich microvilli, we found that ROCKII is absent from these newly formed microvilli. This result indicates that the RhoA-ROCKII pathway is not locally involved in
the elongation of microvilli. Several Rho GTPases, including Rac1, RhoA, and Cdc42, were shown to be activated at the leading edge of migrating cells, indicating their role in membrane protrusion (35). In addition to RhoA, our proteomic data showed expression of Cdc42 in clear cells (14). Future studies will be required to determine whether Rho GTPases other than RhoA, particularly Cdc42, are essential for the development and elongation of microvilli.

The present study confirms that the actin cytoskeleton is a key regulator of V-ATPase trafficking in clear cells. Apical membrane accumulation can occur either via activation of the exocytosis of V-ATPase-rich vesicles or inhibition of V-ATPase endocytosis from the plasma membrane. Several lines of evidence have indicated that the V-ATPase might participate directly in its own recycling (37). While V-ATPase-rich vesicles possess an elaborate cytoplasmic coat formed by the V-ATPase itself, they do not contain clathrin or caveolin-1 (9). In addition to interacting with the actin cytoskeleton (7, 12, 24, 65), the V-ATPase acts as a pH sensor that recruits small GTPases that are crucial for vesicle trafficking (25, 36). The $V_\text{0}$ domain of the V-ATPase was shown to participate in membrane fusion (45). Based on these considerations, the V-ATPase was recently proposed to be involved in mediating clathrin- and caveolin-independent endocytosis (9). Regulation of this novel V-ATPase-dependent pathway is not well characterized, but the role of actin in the regulation of clathrin-mediated endocytosis is well known. Actin polymerization provides the force required for producing clathrin-dependent membrane invaginations and scission of endocytic vesicles from the plasma membrane (26). In another recycling system involving the aquaporin 2 (AQP2) water channel in renal epithelial cells, inhibition of endocytosis by either depletion of membrane cholesterol (34) or expression of dominant-negative GTPase-deficient K44A dynamin (61) is sufficient to induce the plasma membrane accumulation of AQP2, a protein that is internalized via clathrin-mediated endocytosis. In addition, depolymerization of the actin cytoskeleton by vasopressin increases AQP2 membrane accumulation (58), whereas induction of actin polymerization by expression of constitutively active RhoA inhibits vasopressin-induced AQP2 membrane accumulation (29). In osteoclasts, V-ATPase binds to F-actin but not G-actin, and its internalization correlates with increased interaction with actin (12). Similarly, in the present study, cortical actin depolymerization followed by impairment of V-ATPase-dependent endocytosis upon RhoA-ROCKII inhibition would favor the redistribution of V-ATPase from a pool of recycling vesicles to the plasma membrane in clear cells.

As mentioned above, we have previously shown that V-ATPase accumulates in the apical membrane of clear cells after activation of the sAC-dependent or adenosine-dependent cAMP pathway (3, 42), as well as the ANG II-stimulated nitric oxide-dependent cGMP pathway (57). Both PKA and PKG inhibit RhoA by phosphorylation at Ser-188 (18, 49), and its internalization correlates with increased interaction with actin (12). Similarly, in the present study, cortical actin depolymerization followed by impairment of V-ATPase-dependent endocytosis upon RhoA-ROCKII inhibition would favor the redistribution of V-ATPase from a pool of recycling vesicles to the plasma membrane in clear cells.

As mentioned above, we have previously shown that V-ATPase accumulates in the apical membrane of clear cells after activation of the sAC-dependent or adenosine-dependent cAMP pathway (3, 42), as well as the ANG II-stimulated nitric oxide-dependent cGMP pathway (57). Both PKA and PKG inhibit RhoA by phosphorylation at Ser-188 (18, 49), a process that has been proposed for the regulation of several membrane transporters (55, 62, 63, 69). Thus cAMP- or cGMP-dependent inhibition of RhoA and ROCKII might be part of the intracellular signaling cascade that is triggered upon agonist-induced apical membrane V-ATPase accumulation and subsequent increase in V-ATPase-dependent proton secretion in clear cells.

Fig. 10. Absence of ROCKII in the microvilli of clear cells induced after ROCKII or RhoA inhibition. Three-dimensional reconstructions of a z-series of confocal images from rat cauda epididymis double-labeled for ROCKII (A, A', B, B'; red) and V-ATPase (A', B'; green). ROCKII is not detectable in the V-ATPase-labeled microvilli of clear cells (arrows) that were induced by perfusion with $30 \mu$M Y27632 (A, A') or TxC3 (B, B'). Bars: 5 $\mu$m. See also supplemental movies S1 and S2 online at the AJP-Cell Physiol website.
ACKNOWLEDGMENTS

We thank Eric Hill for excellent technical assistance.

GRANTS

This study was supported by National Institutes of Health Grants HD-40793 (to S. Breton) and DK-38452 (to D. Brown and S. Breton).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


