Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2011;28:513-520

Accepted: September 21, 2011

Angiotensin II Stimulates H^+-ATPase Activity in Intercalated Cells from Isolated Mouse Connecting Tubules and Cortical Collecting Ducts

Carsten A. Wagner[1,3]*, Nilufar Mohebbi[3]*, Ulrike Uhlig[1,2], Gerhard H. Giebisch[1], Sylvie Breton[4], Dennis Brown[4] and John P. Geibel[1,2]

[1]Department of Cellular and Molecular Physiology, School of Medicine, Yale University, [2]Department of Surgery, School of Medicine, Yale University, [3]Institute of Physiology and Zurich Center for Integrative Human Physiology, University of Zurich, [4]Massachusetts General Hospital Center for Systems Biology, Program in Membrane Biology and Division of Nephrology, Massachusetts General Hospital, Boston, MA; and Harvard Medical School, Boston, MA, *C.A. Wagner and N. Mohebbi contributed equally to this study and therefore share first authorship

Key Words
Pendrin • Bicarbonate secretion • Chloride absorption

Abstract
Intercalated cells in the collecting duct system express V-type H^+-ATPases which participate in acid extrusion, bicarbonate secretion, and chloride absorption depending on the specific subtype. The activity of H^+-ATPases is regulated by acid-base status and several hormones, including angiotensin II and aldosterone. Angiotensin II stimulates chloride absorption mediated by pendrin in type B intercalated cells and this process is energized by the activity of H^+-ATPases. Moreover, angiotensin II stimulates bicarbonate secretion by the connecting tubule (CNT) and early cortical collecting duct (CCD). In the present study we examined the effect of angiotensin II (10 nM) on H^+-ATPase activity and localization in isolated mouse connecting tubules and cortical collecting ducts. Angiotensin II stimulated Na^+-independent intracellular pH recovery about 2-3 fold, and this was abolished by the specific H^+-ATPase inhibitor concanamycin. The effect of angiotensin II was mediated through type 1 angiotensin II receptors (AT_1-receptors) because it could be blocked by saralasin. Stimulation of H^+-ATPase activity required an intact microtubular network - it was completely inhibited by colchicine. Immunocytochemistry of isolated CNT/CCDs incubated *in vitro* with angiotensin II suggests enhanced membrane associated staining of H^+-ATPases in pendrin expressing intercalated cells. In summary, angiotensin II stimulates H^+-ATPases in CNT/CCD intercalated cells, and may contribute to the regulation of chloride absorption and bicarbonate secretion in this nephron segment.

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Introduction

Vacuolar type H^+-ATPases are expressed along the entire nephron in various cell types and are involved in proton secretion into the urine thereby promoting bicarbonate reabsorption in the proximal tubule and thick ascending limb of Henle [1-2]. In the segments of the collecting duct system (e.g. connecting tubule, cortical

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1015-8987/11/0283-0513$38.00/0

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Carsten A. Wagner
Institute of Physiology, University of Zurich
Winterthurerstrasse 190, CH-8057 Zurich (Switzerland)
Tel. +41-44-63 55023, Fax +41-44-63 56814
E-Mail Wagnerca@access.uzh.ch

and medullary collecting ducts), H^+-ATPases are abundant in intercalated cells and can be found at the luminal, basolateral, or both membranes depending on the subtype of intercalated cells [1, 3]. In type A intercalated cells, H^+-ATPases are found at the luminal membrane secreting protons into urine and thereby energizing ammonium secretion and bicarbonate regeneration [1]. These cells are characterized by the additional presence of the basolateral chloride/bicarbonate exchanger AE1 [3, 4]. In contrast, in non-type A intercalated cells (i.e. type B and non-A/non-B intercalated cells), H^+-ATPases can be localized at the luminal, basolateral or both membranes and act in concert with the apical chloride/bicarbonate exchanger pendrin [1, 3, 5]. Several subtypes of non-type A intercalated cells may exist but all forms express pendrin and vary with respect to their subcellular distribution of H^+-ATPases [6, 7]. Non-type A intercalated cells mediate bicarbonate secretion (in the absence of luminal H^+-ATPases) and reabsorb chloride (independently of the subcellular distribution of H^+-ATPases).

The activity of intercalated cells is tightly regulated by a variety of factors including acid-base status, dietary electrolyte intake, and various hormones including angiotensin II, aldosterone, and endothelin [3]. Angiotensin II is a potent regulator of urinary acidification *in vivo* [8-11] and has been shown by us and others to stimulate H^+-ATPase activity in proximal tubule cells [12], type A intercalated cells in the outer medullary collecting ducts [13, 14], and renal cell lines [15-17]. Recent studies by Pech and Wall demonstrated that angiotensin II stimulates chloride absorption in isolated mouse cortical collecting ducts. Chloride absorption was dependent on the presence of pendrin and blocked by an H^+-ATPase inhibitor suggesting that H^+-ATPases in non-type A intercalated cells may also be stimulated by angiotensin II [18]. Along the same lines, angiotensin II enhances bicarbonate secretion in rabbit early cortical collecting ducts [19]. Taken together, these observations suggest that angiotensin II may have a direct stimulatory effect on H^+-ATPases in type B intercalated cells and may thereby drive bicarbonate secretion and chloride absorption.

We examined in the present study whether angiotensin II directly stimulates H^+-ATPase activity in isolated mouse cortical collecting duct intercalated cells and which receptor subtype may be involved. Our results reveal a potent stimulation of H^+-ATPases by angiotensin II via AT_1 receptors and suggest that stimulated H^+-ATPases may be important for type B intercalated cell function.

Materials and Methods

Animals

Male C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME, USA), 12-14 weeks old, were kept under standard conditions with free access to food and water.

The generation, breeding, and genotyping of mice expressing eGFP under the control of the B1 H^+-ATPase (ATP6V1B1) promoter has been described previously [20]. B1-eGFP mice were kindly provided by Dr. Lance Miller and Rauol Nelson, University of Utah, Salt Lake City, USA, and bred in Zurich.

The use of mice was according to local Animal Welfare Laws and approved by the Yale University Committee for the Use of Animals and the Zurich Veterinary Office (Kantonales Veterinäramt).

Preparation of isolated cortical collecting duct fragments and intracellular pH measurements

C57Bl/6J mice were sacrificed with pentobarbital (150 mg/kg), both kidneys removed and transferred into ice-cold HEPES solution. After the removal of the capsula, vessels and pelvis, the kidneys were cut into slices 2-3 mm in thickness. The cortex was prepared under a stereo microscope (5x magnification) and the cortical slices were then incubated in a digestion solution (4 ml Minimum Essential Medium, 5 mM glycine, 6 mg/ml trypsin inhibitor, and 250 µg/ml collagenase (Sigma)) at 37 °C in a water bath for 15 min. After 15 min the digestion was stopped by transferring the tubules onto ice, gently removing the supernatant and replacing it with 4 ml ice-cold 1 % BSA HEPES solution. The BSA HEPES solution was replaced by ice-cold HEPES solution after 10 min and tubules kept on ice for the experiments. Cortical collecting ducts were selected under the preparation microscope and transferred to a perfusion chamber containing coverslips precoated with the cell-adhesive Cell-Tak (Becton-Dickinson). The temperature of the chamber was maintained at 37 ± 0.5 °C by an electronic feedback circuit. The control bath solution was initially a HEPES solution (sol. 1), flowing continuously at ≈ 3 ml/ min. The chamber volume was ≈ 180 µl. Single tubule fragments were loaded with the pH-sensitive dye 2',7'-bis(2carboxylethyl)-5(6)-carboxyflourescein (BCECF) (10 µM) for 20 min at room temperature as described [12, 21-23]. pH_i was measured microflourometrically by alternately exciting the dye with a 10 µm diameter spot of light at 440 and 490 nm while monitoring the emission at 532 nm [12, 21-23]. Each experiment was calibrated for pH_i using the nigericin/ high K^+ method [24] that converted the obtained ratios to pH_i.

The solutions used are given in Table 1. H^+-ATPase activity was measured as described previously [12, 14, 21-23, 25-28]. Briefly, bicarbonate free solutions were used and Na^+ removed to abolish Na^+/H^+ exchanger activity. For these experiments Na^+ was replaced by equimolar amounts of NMDG (N-Methyl-D-Glucamine). To induce a strong intracellular acidification and elicit H^+-ATPase activation, NH_4Cl pulses were performed in the absence of Na^+ as described previously [22, 29]. All chemicals were obtained from Sigma. Stimulators and inhibitors were added to the BCECF incubation

	Standard HEPES	Na^+-free HEPES	Na^+-free HEPES + NH_4Cl	Na^+ and K^+-free HEPES	High K^+ calibration
NaCl	125	-	-	-	-
NMDG	-	125	105	130	32.8
NH_4Cl	-	-	20	-	-
KCl	3	3	3	3	105
$MgSO_4$	1.2	1.2	1.2	1.2	1.2
$CaCl_2$	1	1	1	1	1
KH_2PO_4	2	2	2	2	-
Glucose	5	5	5	5	-
HEPES	32.2	32.2	32.2	32.2	32.2
pH	7.4	7.4	7.4	7.4	6.0, 7.0, 8.0

Table 1. Composition of solutions used for functional experiments. NMDG is N-Methyl-*D*-Glucamine, all solutions were titrated to pH 7.4 at 37 °C using either NaOH or KOH. NMDG was titrated with HCl.

	Initial pH_i	Na^+-independent pH_i recovery (ΔpH/min)	Na^+-dependent pH_i recovery (ΔpH/min)	Final pH_i	Number Cells (tubules)
Control	7.29 ± 0.01	0.037 ± 0.003	0.184 ± 0.012	7.24 ± 0.02	36 (7)
Concanamycin	7.31 ± 0.01	0.006 ± 0.001	0.262 ± 0.018	7.23 ± 0.02	53 (5)
Angiotensin II	7.27 ± 0.03	0.088 ± 0.007	0.351 ± 0.031	7.26 ± 0.04	20 (6)
Angiotensin II Concanamycin	7.27 ± 0.01	0.009 ± 0.001	0.262 ± 0.039	7.23 ± 0.04	35 (5)
Saralasin Angiotensin II	7.29 ± 0.02	0.031 ± 0.007	0.189 ± 0.023	7.19 ± 0.03	24 (4)
Colchicine Angiotensin II	7.33 ± 0.02	0.032 ± 0.002	0.119 ± 0.055	7.18 ± 0.02	22 (4)

Table 2. Summary of functional data from pH measurements. Data are summarized as mean ± SEM. Shown are initial pH_i in the presence of sodium, pH_i recovery rates after removal of NH_4Cl in the absence of sodium and after readdition of sodium to the bath, final pH_i before calibration, and the number of intercalated cells and isolated CNT-CCDs investigated.

solution and all other solutions at the concentrations stated.

Immunostaining

Tubules were prepared as described above, eGFP positive CNT/CCD fragments collected under a dissection microscope equipped with a fluorescent lamp, and staining was performed as described previously [22]. Briefly, isolated tubules were transferred onto cover-slips precoated with Cell-Tak and allowed to adhere for 15 min. Next, isolated tubules were incubated with 10 nM Angiotensin II for 15 min at 37 °C and then fixed with 2% paraformaldehyde in PBS for 5 min at room temperature, tubules were washed 3 times with PBS, permeabilized with 0.1% Triton X, washed twice with PBS, treated with 1% SDS [30], washed 3 times with PBS, and incubated with PBS containing 1% bovine serum albumin for 15 min prior to addition of the primary antibodies. The primary antibodies were diluted in PBS and applied overnight at 4 °C: guinea-pig anti-mouse pendrin (1:2000 raw serum) [31], and rabbit anti-human ATP6V0B1 (B1) (1:500 whole serum) [22]. Tubules were then washed twice for 5 min with high-NaCl PBS (PBS+2.7% NaCl), once with PBS, and incubated with the secondary antibodies (goat anti-guinea-pig Alexa 647 1:500, donkey anti-rabbit Alexa 594 1:1000, Jackson ImmunoResearch Laboratories Europe Ltd., Suffolk, UK), and DAPI at a dilution of 1:500 for 1 h at room temperature. Tubules were again washed twice with high-NaCl PBS and once with PBS. Cover-slips were mounted with Glycergel mounting medium (DakoCytomation, Glostrup, Denmark). Slides were examined using a Leica SP2 confocal microscope (Zurich Center for Microscopy and Imaging), and the images assembled with Photoshop (Adobe, San Jose, Calif., USA) software.

Statistics

Data are presented as mean and standard error of the mean (SEM). All data were tested for statistical significance using unpaired student's t-test and results were considered significant if $P < 0.05$.

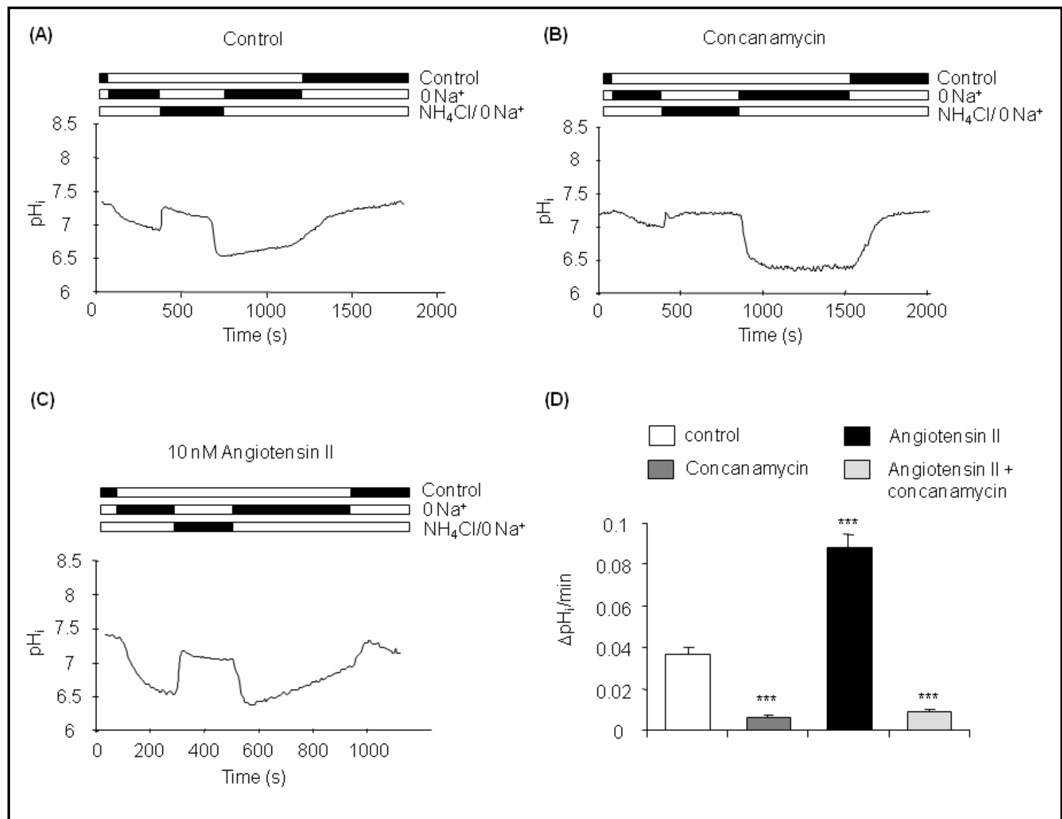

Fig. 1. Angiotensin II stimulates H^+-ATPase activity in CNT-CCD intercalated cells. (A-C) Original tracings of intracellular pH measurements in intercalated cells in isolated CNT-CCD fragments under control conditions (A), during incubation with the H^+-ATPase inhibitor concanamycin (100 nM) (B), and during exposure to 10 nM angiotensin II (C). The rate of intracellular pH recovery (alkalinization) after removal of NH_4Cl from the bath and in the absence of sodium was analyzed. (D) Summary of Na^+-independent pH_i recovery rates. Mean ± SEM, ***indicates $p < 0.001$ compared to control.

Results

Angiotensin II stimulates H^+-ATPase activity

CNT-CCD intercalated cells had an initial intracellular pH (pH_i) under control conditions of pH 7.29 ± 0.01 (n = 36 cells in 7 CNT-CCDs) (Table 2). Removal of Na^+ from the bath caused a slow acidification of pH_i to pH 6.90 ± 0.02 which was further acidified after removal of an NH_4Cl (20 mM) load from the bath to pH 6.54 ± 0.04 (Fig. 1A). A slow alkalinization of 0.037 ± 0.003 units pH/ min. was observed in the nominal absence of Na^+ and K^+ from the bath. We and others have previously shown that this type of intracellular pH recovery under these conditions is mostly mediated by the activity of plasma membrane H^+-ATPases [12-14, 21-23, 26-28].

Accordingly, preincubation with the H^+-ATPase inhibitor concanamycin (100 nM) [32-34] significantly reduced the rate of Na^+-independent pH_i recovery to 0.006 ± 0.001 units pH/min. demonstrating that most of the alkalinization rate was due to H^+-ATPase activity (Table 2, Fig. 1A and 1D). The small remaining rate may be mediated by H^+/K^+-ATPase activity [35, 36].

When the collecting tubules were preincubated with 10 nM angiotensin II for 15 min, the initial pH (7.27 ± 0.03), the extent of acidification after Na^+ removal and after the NH_4Cl pulse were not altered (data not shown). However, the rate of pH_i recovery under nominally Na^+ and K^+ free conditions was significantly accelerated to 0.088 ± 0.007 pH units/ min, 2-3 times control rates (Table 2, Fig. 1C,D). Again, inhibition of H^+-ATPase activity with concanamycin (100 nM) reduced the rate of pH_i recovery to 0.009 ± 0.001 units pH/min demonstrating that increased H^+-ATPase activity was responsible for the accelerated alkalinization in the presence of angiotensin II (Table 2, Fig. 1D).

Angiotensin II stimulates H^+-ATPase via the AT_1 receptor

Bicabonate reabsorptipon and urinary acidification along the nephron and collecting duct is stimulated by angiotensin II involving the AT_1 receptor subtype [13-14, 17, 37, 38]. Similarly, AT_1 receptors are involved in the stimulation of bicarbonate secretion by the initial cortical collecting duct by angiotensin II [19]. Thus, we tested whether angiotensin II stimulated H^+-ATPase activity via AT_1 receptors in our preparation. Isolated CNT-CCDs were preincubated *in vitro* with the AT_1 receptor antagonist saralasin (1 µM) for 5 min before angiotensin II (10 nM) was added. Preincubation with saralasin completely abolished the stimulatory effect of angiotensin

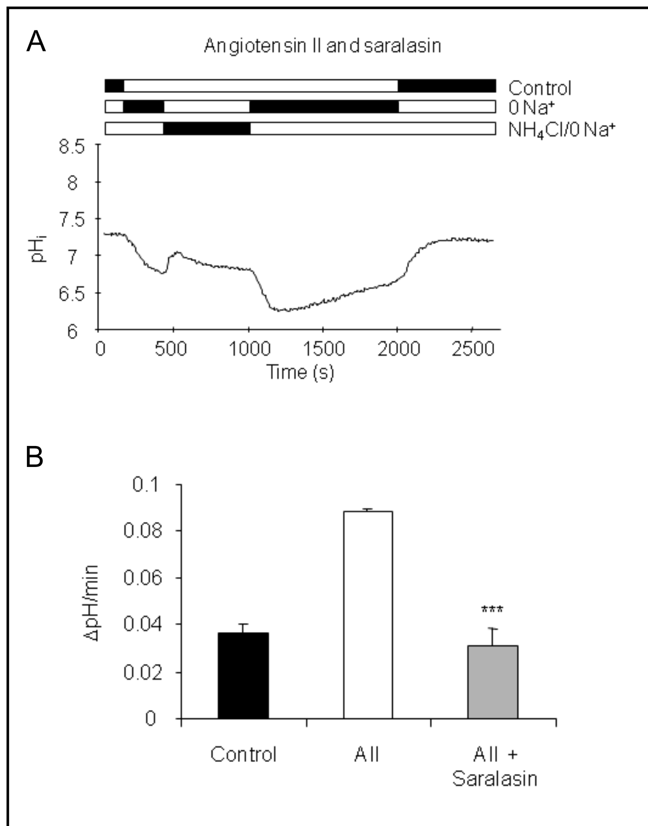

Fig. 2. The AT_1 receptor antagonist saralasin prevents the stimulation by angiotensin II. CNT-CCDs were preincubated with the AT_1 receptor antagonist saralasin (1 µM) and incubated with 10 nM angiotensin II. (A) Original pH_i tracing of an intercalated cell incubated with saralasin and angiotensin II. (B) Summary of pH_i recovery rates of intercalated cells under control conditions, angiotensin II, and saralasin with angiotensin II. pH_i recovery rates from control and angiotensin II are taken from Fig. 1 and shown for comparison. Mean ± SEM, ***indicates $p < 0.001$ compared to angiotensin II alone.

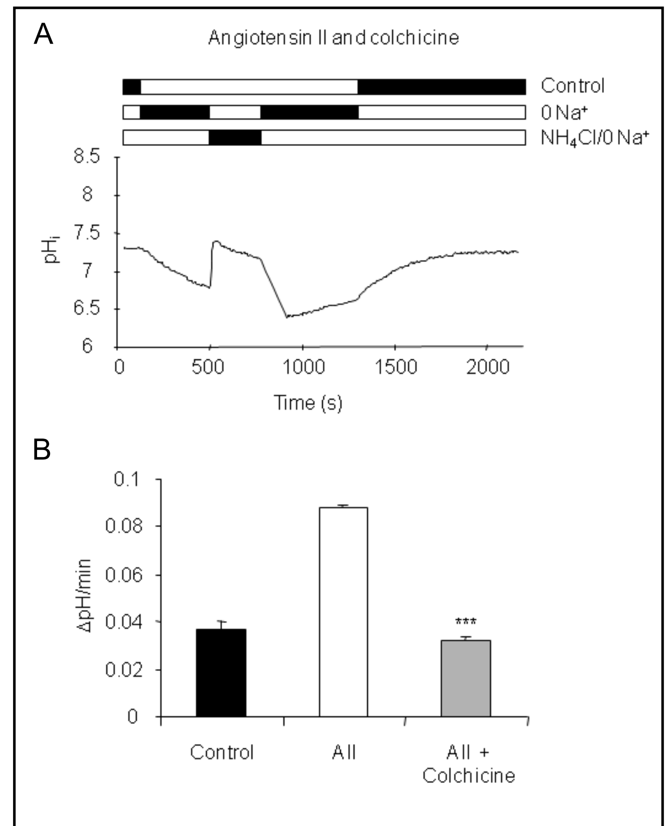

Fig. 3. Stimulation of H^+-ATPase activity requires an intact microtubular network. Isolated CNT-CCDs were preincubated with colchicine (20 µM) for 20 min prior to stimulation with 10 nM angiotensin II. (A) Original pH_i tracing of an intercalated cell incubated with colchicine and angiotensin II. (B) Summary of pH_i recovery rates of intercalated cells under control conditions, angiotensin II, and colchicine with angiotensin II. pH_i recovery rates from control and angiotensin II are taken from Fig. 1 and shown for comparison. Mean ± SEM, ***indicates $p < 0.001$ compared to angiotensin II alone.

II on H^+-ATPase activity. The Na^+-independent pH_i recovery rate in the presence of saralasin and angiotensin II was 0.031 ± 0.007 units pH/min (Table 2, Fig. 2), not significantly different from control.

Angiotensin II stimulates the trafficking of H^+-ATPases to the membrane

Stimulation of H^+-ATPase activity in intercalated cells by various hormones, acid, or CO_2 is at least in part mediated by trafficking and increased expression of H^+-ATPases at the plasma membrane [1]. Disruption of the microtubular network with colchicine has been shown to prevent the increased expression and activity of H^+-ATPases [12, 14, 23, 39, 40] in similar preparations. Here, preincubation of CNT-CCDs *in vitro* with colchicine (10 µM) for 20 min before application of 10 nM angiotensin II completely and significantly abolished the stimulatory effect of angiotensin II on H^+-ATPase activity (0.032 ± 0.002 units pH/min) (Table 2, Fig. 3).

We performed additional experiments with CNT-CCDs from mice expressing eGFP under the promoter of the B1 H^+-ATPase subunit to facilitate identification of the respective segments. CNT-CCDs were incubated in vitro with angiotensin II and stained with antibodies against the B1 H^+-ATPase subunit and pendrin (Fig. 4). In control CNT/CCDs, localization of the B1 H^+-ATPase in pendrin positive cells was mostly at the basolateral pole of cells with dispersed staining at the membrane and in the cytosol. In CNT/CCDs incubated *in vitro* with angiotensin II, staining for the B1 H^+-ATPase appeared

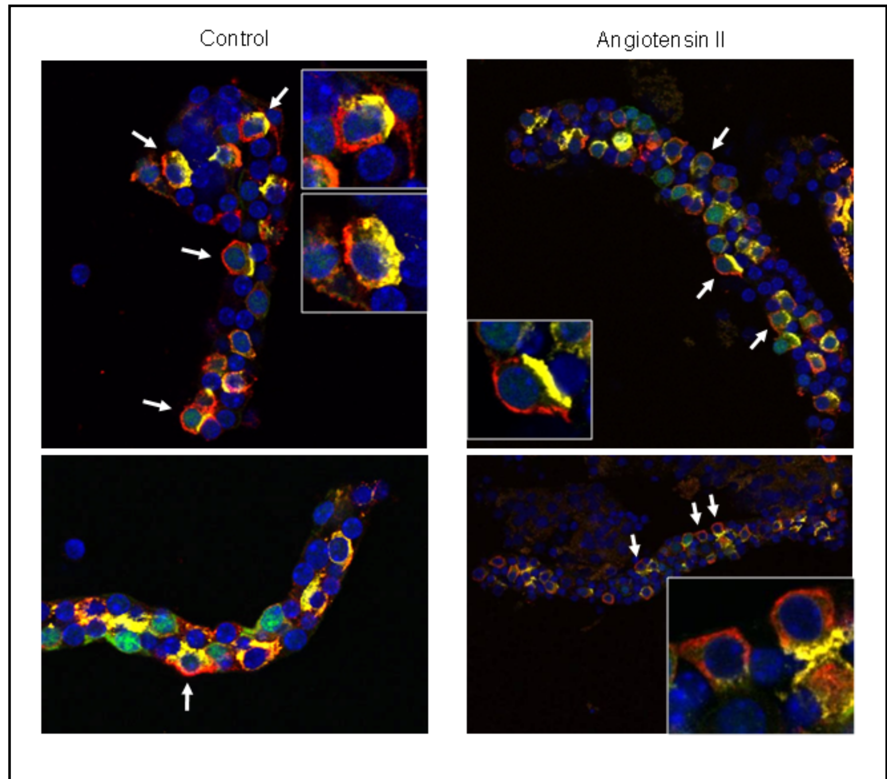

Fig. 4. Immunolocalization of H^+-ATPases in pendrin expressing CNT/CCD intercalated cells. CNT-CCDs expressing eGFP (green) in intercalated cells were incubated without (left panel) or with 10 nM angiotensin II (right panel) and stained with antibodies against the B1 H^+-ATPase subunit (red) and pendrin (yellow). Nuclei were visualized with DAPI (blue). Inserts show higher magnification of selected intercalated cells, arrows point to intercalated cells with typical patterns of H^+-ATPase staining. Original magnification 400 x.

to be more membrane associated whereas no difference in pendrin staining was noted consistent with previous experiments [18].

Discussion

Angiotensin II is a potent regulator of blood pressure and acid-base homeostasis. Angiotensin II acts on blood pressure by inducing vasoconstriction and stimulating renal NaCl reabsorption in the proximal tubule, distal convoluted tubule, and collecting duct system [37, 41, 42]. In addition, angiotensin II has multiple effects on renal acid-base handling. Angiotensin II stimulates bicarbonate reabsorption by the proximal tubule, the thick ascending limb of the loop of Henle, and the distal convoluted tubule [10, 11, 43-48]. In the late cortical collecting duct and outer medullary collecting duct, angiotensin II enhances proton secretion by type A intercalated cells [13, 14, 19]. Moreover, angiotensin II also increases luminal alkalinization in the connecting tubule and early cortical collecting duct and directly stimulates chloride absorption by type B intercalated cells [18, 19]. Moreover, stimulation of pendrin activity may also indirectly enhance electroneutral NaCl reabsorption via the recently described Na^+-driven Cl^-/HCO_3^- exchanger (NDCBE/SLC4A8) [49].

Here we demonstrate that angiotensin II in the physiological range [42, 50] stimulates the activity of H^+-ATPases in intercalated cells in isolated mouse CNT/CCDs. Angiotensin II most likely acts via AT_1 receptors since the AT_1 receptor antagonist saralasin completely abolished the effect. The distribution and localization of angiotensin II receptors along the nephron is not completely documented but AT_1 receptors may be present both on luminal and basolateral membranes of almost all cells along the collecting duct system as evident from various functional experiments and immunolocalization studies [9, 13, 14, 18, 19, 37, 38, 41, 42, 44, 50-52].

Angiotensin II acting in the collecting duct may come from various sources including circulating and filtered angiotensin II, angiotensin II produced in the proximal tubule, as well as angiotensin II activated locally in the collecting duct [53]. Rohrwasser et al. demonstrated the existence of all major components of a local renin-angiotensin system in the collecting duct [52] including angiotensin-converting enzyme (ACE).

Regulation of H^+-ATPase activity may involve various mechanisms including assembly and disassembly of proton pumps, trafficking of pumps into the membrane, and changes in the ATP: H^+-pumping coupling ratio [1, 54, 55]. In type A intercalated cells, angiotensin II stimulates H^+-ATPases by a process that requires an intact microtubular network sensitive to colchicine [14] and

leads to an accumulation of H^+-ATPases at the luminal membrane [13]. Similarly, many other hormones or stimuli increase H^+-ATPase trafficking and abundance at the membrane in the collecting duct, along the nephron, or in similar preparations [12, 23, 26, 39, 56-58]. Colchicine prevented the stimulation of H^+-ATPase activity by angiotensin II in the CNT/CCD intercalated cells suggesting a similar mechanism. Immunocytochemistry of isolated mouse CNT/CCD fragments stimulated i*n vitro* with angiotensin II suggested a more membrane associated H^+-ATPase staining in cells expressing pendrin consistent with retention or increased trafficking of pumps into the membrane and enhanced pump activity. The stimulated H^+-ATPase activity would then energize enhanced chloride absorption or luminal alkalinization via pendrin as observed in earlier studies [18, 19].

In summary, angiotensin II stimulates H^+-ATPase activity in isolated mouse CNT/CCD intercalated cells involving AT_1 receptors and leading to enhanced membrane abundance of H^+-ATPases. Increased H^+-ATPase activity is a prerequisite for enhancing pendrin activity by altering driving forces for bicarbonate secretion and chloride absorption.

Acknowledgements

This study was supported by NIH grants HD40793 to S. Breton, DK42956 to D. Brown, and DK-17433 to J.P. Geibel and the Swiss National Science Foundation (3100-068318 and 31003A-122217) to C.A. Wagner. C.A. Wagner was a Feodor Lynen fellow of the Humboldt foundation, Germany.

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