Angiotensin II stimulates vacuolar H+ -ATPase activity in renal acid-secretory intercalated cells from the outer medullary collecting duct

Rothenberger, F; Velic, A; Stehberger, P A; Kovacikova, J; Wagner, C A
Angiotensin II stimulates vacuolar H+ -ATPase activity in renal acid-secretory intercalated cells from the outer medullary collecting duct

Abstract

Final urinary acidification is mediated by the action of vacuolar H(+)-ATPases expressed in acid-secretory type A intercalated cells (A-IC) in the collecting duct. Angiotensin II (AngII) has profound effects on renal acid-base transport in the proximal tubule, distal tubule, and collecting duct. This study investigated the effects on vacuolar H(+)-ATPase activity in A-IC in freshly isolated mouse outer medullary collecting ducts. AngII (10 nM) stimulated concanamycin-sensitive vacuolar H(+)-ATPase activity in A-IC in freshly isolated mouse outer medullary collecting ducts via AT(1) receptors, which were also detected immunohistochemically in A-IC. AngII increased intracellular Ca(2+) levels transiently. Chelation of intracellular Ca(2+) with BAPTA and depletion of endoplasmic reticulum Ca(2+) stores prevented the stimulatory effect on H(+)-ATPase activity. The effect of AngII on H(+)-ATPase activity was abolished by inhibitors of small G proteins and phospholipase C, by blockers of Ca(2+)-dependent and -independent isoforms of protein kinase C and extracellular signal-regulated kinase 1/2. Disruption of the microtubular network and cleavage of cellubrevin attenuated the stimulation. Finally, AngII failed to stimulate residual vacuolar H(+)-ATPase activity in A-IC from mice that were deficient for the B1 subunit of the vacuolar H(+)-ATPase. Thus, AngII presents a potent stimulus for vacuolar H(+)-ATPase activity in outer medullary collecting duct IC and requires trafficking of stimulatory proteins or vacuolar H(+)-ATPases. The B1 subunit is indispensable for the stimulation by AngII, and its importance for stimulation of vacuolar H(+)-ATPase activity may contribute to the inappropriate urinary acidification that is seen in patients who have distal renal tubular acidosis and mutations in this subunit.
Angiotensin II Stimulates Vacuolar H⁺-ATPase Activity in Renal Acid-Secretory Intercalated Cells from the Outer Medullary Collecting Duct

Florina Rothenberger, Ana Velic, Paul A. Stehberger, Jana Kovacikova, and Carsten A. Wagner

Institute of Physiology and Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

ABSTRACT

Final urinary acidification is mediated by the action of vacuolar H⁺-ATPases expressed in acid-secretory type A intercalated cells (A-IC) in the collecting duct. Angiotensin II (AngII) has profound effects on renal acid-base transport in the proximal tubule, distal tubule, and collecting duct. This study investigated the effects on vacuolar H⁺-ATPase activity in A-IC in freshly isolated mouse outer medullary collecting ducts. AngII (10 nM) stimulated concanamycin-sensitive vacuolar H⁺-ATPase activity in A-IC in freshly isolated mouse outer medullary collecting ducts via AT₁ receptors, which were also detected immunohistochemically in A-IC. AngII increased intracellular Ca²⁺ levels transiently. Chelation of intracellular Ca²⁺ with BAPTA and depletion of endoplasmic reticulum Ca²⁺ stores prevented the stimulatory effect on H⁺-ATPase activity. The effect of AngII on H⁺-ATPase activity was abolished by inhibitors of small G proteins and phospholipase C, by blockers of Ca²⁺-dependent and -independent isoforms of protein kinase C and extracellular signal-regulated kinase 1/2. Disruption of the microtubular network and cleavage of cellubrevin attenuated the stimulation. Finally, AngII failed to stimulate residual vacuolar H⁺-ATPase activity in A-IC from mice that were deficient for the B1 subunit of the vacuolar H⁺-ATPase. Thus, AngII presents a potent stimulus for vacuolar H⁺-ATPase activity in outer medullary collecting duct IC and requires trafficking of stimulatory proteins or vacuolar H⁺-ATPases. The B1 subunit is indispensable for the stimulation by AngII, and its importance for stimulation of vacuolar H⁺-ATPase activity may contribute to the inappropriate urinary acidification that is seen in patients who have distal renal tubular acidosis and mutations in this subunit.

The kidney plays a central role in controlling systemic acid-base homeostasis by reabsorbing bicarbonate, synthesis and excretion of ammonia, and regulated secretion of acid or base equivalents.¹,² The importance of these processes is underlined by both genetic and acquired defects in proteins that are involved in proton and bicarbonate transport, such as the vacuolar H⁺-ATPase subunits a4 (ATP6V0A4) and B1 (ATP6V1B1) and the Cl⁻ / HCO₃⁻ exchanger AE1 (SLC4A1).³ Acidification of the urine occurs in several distal nephron segments, but final urinary acidification occurs along the cortical and outer medullary collecting duct (OMCD), largely through the action of vacuolar H⁺-ATPase in acid-secretory type A intercalated cells (A-IC).²,⁴

Final urinary acidification and H⁺-ATPase activity in the collecting duct are controlled by several factors, including endothelin, angiotensin II (AngII), and aldosterone.¹,²,⁴,⁵ The mechanism(s) through which AngII stimulates net acid secretion in the medullary collecting duct has not been fully explored. AngII, however, has been shown to stimulate acid excretion and bicarbonate reabsorption in the proximal tubule by acting on Na⁺/H⁺ exchangers, Na⁺/bicarbonate

Received July 18, 2006. Accepted April 10, 2007.

Published online ahead of print. Publication date available at www.jasn.org.

F.R. and A.V. contributed equally to this work and therefore share first authorship.

Correspondence: Dr. Carsten A. Wagner, Institute of Physiology and Centre for Integrative Human Physiology, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. Phone: +41-1-63-50659; Fax: +41-1-63-56814; Email: wagnerca@access.unizh.ch

Copyright © 2007 by the American Society of Nephrology
co-transporters, and H$^+$-ATPases. In vivo experiments, AngII stimulates also final urinary acidification. Thus, AngII may stimulate H$^+$-ATPases in the collecting duct.

Vacuolar H$^+$-ATPases are composed of at least 13 subunits in mammals of which multiple isoforms exist. The B subunit belongs to the cytosolic V$_i$ domain and is essential for vacuolar H$^+$-ATPase function in yeast. In mammals, two isoforms of the B subunit, B1 (ATP6V1B1) and B2 (ATP6V1B2), have been identified. H$^+$-ATPases that contain the B1 subunit were detected in the inner ear, in lung and epididymis, and in the kidney. In kidney, expression of the B1 subunit is restricted to IC in the late distal tubule, connecting segment, and cortical and medullary collecting ducts. In these cells, the B1 subunit is found in the plasma membrane and seems to be involved in acid extrusion. Mutations in the B1 subunit in human cause distal renal tubular acidosis with sensorineural deafness. This type of metabolic acidosis is thought to be caused by the partial or complete lack of proton secretion in the collecting duct. Accordingly, a mouse model that is deficient for the B1 subunit is not able to acidify urine appropriately and remove an acid load. H$^+$-ATPase activity in IC from B1-deficient mice is attenuated. The B2 subunit, in contrast, is found in various tissues and can be detected both in the plasma membrane and in intracellular organelles and may serve a housekeeping function. In mouse and rat kidney, B2 is also found in IC with some labeling of the apical plasma membrane, suggesting that B2 may take part in acid excretion. The exact function of the B1 and B2 isoforms, however, has not been fully understood to date.

Here we investigated the stimulatory effect of AngII H$^+$-ATPase activity in A-IC of mouse OMCD. Moreover, we tested whether the B1 subunit is important for the hormonal stimulation that was observed with AngII. We present evidence for a specific function of the B1 subunit in the stimulation of H$^+$-ATPase activity by AngII.

**RESULTS**

**Stimulation of H$^+$-ATPase Activity in Mouse OMCD A-IC by AngII**

In single mouse OMCD A-IC, the mean initial intracellular pH (pH$_i$) was acidified to 6.35 ± 0.012 using the NH$_4$Cl prepulse in the absence of extracellular Na$^+$. Intracellular pH$_i$ recovered slowly (alkalinized) during the subsequent superfusion with Na$^+$-free solution with a rate of 0.032 ± 0.002 units pH/min. Upon re-addition of Na$^+$, pH$_i$ increased rapidly to the initial value mediated by Na$^+$/H$^+$ exchange as described previously (Table 1).

Preincubation of mouse OMCD with 10 nM AngII for 10 min increased the Na$^+$-independent alkalization rate to two- to three-fold to 0.080 ± 0.005 units pH/min. Addition of 1 nM AngII in the same experimental series did not significantly increase the alkalization rate (0.035 ± 0.002 units pH/min; Figure 1A). High supraphysiological concentrations of 100 nM AngII resulted in a small stimulation (0.047 ± 0.005 units pH/min). The B2 subunit, in contrast, is found in various tissues and can be detected both in the plasma membrane and in intracellular organelles and may serve a housekeeping function. In mouse and rat kidney, B2 is also found in IC with some labeling of the apical plasma membrane, suggesting that B2 may take part in acid excretion. The exact function of the B1 and B2 isoforms, however, has not been fully understood to date.

**Table 1. Summary of pH$_i$ measurements in single intercalated cells in mouse OMCD fragments**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Na$^+$-Independent pH$_i$ Recovery Rate (ΔpH/min)</th>
<th>No. of Cells, OMCD, and Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.032 ± 0.002b</td>
<td>61 (5/4)</td>
</tr>
<tr>
<td>AngII, 1 nM</td>
<td>0.035 ± 0.002</td>
<td>96 (7/3)</td>
</tr>
<tr>
<td>AngII, 10 nM</td>
<td>0.080 ± 0.005b</td>
<td>127 (11/6)</td>
</tr>
<tr>
<td>AngII, 100 nM</td>
<td>0.047 ± 0.005b,c</td>
<td>15 (4/2)</td>
</tr>
<tr>
<td>Concanamycin</td>
<td>0.013 ± 0.001b,c</td>
<td>101 (6/3)</td>
</tr>
<tr>
<td>Concanamycin + AngII</td>
<td>0.011 ± 0.001b,c</td>
<td>88 (5/3)</td>
</tr>
<tr>
<td>SCH28080 + AngII</td>
<td>0.065 ± 0.006b,c</td>
<td>16 (6/2)</td>
</tr>
<tr>
<td>Saralasin</td>
<td>0.020 ± 0.002</td>
<td>81 (6/3)</td>
</tr>
<tr>
<td>Saralasin + AngII</td>
<td>0.019 ± 0.001c</td>
<td>99 (6/3)</td>
</tr>
<tr>
<td>Losartan + AngII</td>
<td>0.027 ± 0.002c</td>
<td>19 (5/2)</td>
</tr>
<tr>
<td>PD123.319</td>
<td>0.038 ± 0.003</td>
<td>60 (5/4)</td>
</tr>
<tr>
<td>PD123.319 + AngII</td>
<td>0.057 ± 0.006b,c</td>
<td>84 (6/3)</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>0.025 ± 0.002b</td>
<td>85 (6/3)</td>
</tr>
<tr>
<td>Pertussis toxin + AngII</td>
<td>0.030 ± 0.001b,c</td>
<td>103 (7/3)</td>
</tr>
<tr>
<td>U73122</td>
<td>0.017 ± 0.002b,c</td>
<td>77 (5/3)</td>
</tr>
<tr>
<td>U73122 + AngII</td>
<td>0.012 ± 0.001b,c</td>
<td>80 (5/3)</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>0.026 ± 0.009</td>
<td>64 (5/3)</td>
</tr>
<tr>
<td>BAPTA-AM + AngII</td>
<td>0.013 ± 0.001b,c</td>
<td>87 (5/3)</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>0.027 ± 0.002</td>
<td>60 (5/3)</td>
</tr>
<tr>
<td>Thapsigargin + AngII</td>
<td>0.036 ± 0.002c</td>
<td>74 (5/4)</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>0.026 ± 0.002</td>
<td>68 (5/3)</td>
</tr>
<tr>
<td>Chelerythrine + AngII</td>
<td>0.030 ± 0.002c</td>
<td>96 (7/3)</td>
</tr>
<tr>
<td>G6976 10 μM + AngII</td>
<td>0.018 ± 0.002b,c</td>
<td>60 (5/4)</td>
</tr>
<tr>
<td>G6976</td>
<td>0.019 ± 0.002b,c</td>
<td>54 (5/3)</td>
</tr>
<tr>
<td>G6976 1 μM + AngII</td>
<td>0.029 ± 0.003c</td>
<td>77 (5/3)</td>
</tr>
<tr>
<td>Ro 31-2880 10 μM + AngII</td>
<td>0.022 ± 0.002b,c</td>
<td>103 (7/3)</td>
</tr>
<tr>
<td>Ro 31-2880</td>
<td>0.018 ± 0.003b,c</td>
<td>55 (6/4)</td>
</tr>
<tr>
<td>Ro 31-2880 1 μM + AngII</td>
<td>0.040 ± 0.003c</td>
<td>74 (5/2)</td>
</tr>
<tr>
<td>Ro 31-2880 + AngII</td>
<td>0.027 ± 0.002</td>
<td>80 (6/3)</td>
</tr>
<tr>
<td>PD098059</td>
<td>0.017 ± 0.002b,c</td>
<td>74 (6/3)</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.013 ± 0.002b,c</td>
<td>79 (5/3)</td>
</tr>
<tr>
<td>Colchicine + AngII</td>
<td>0.030 ± 0.001b,c</td>
<td>59 (5/3)</td>
</tr>
<tr>
<td>Tetanus toxin</td>
<td>0.037 ± 0.002</td>
<td>112 (9/4)</td>
</tr>
<tr>
<td>Tetanus toxin + AngII</td>
<td>0.020 ± 0.002c</td>
<td>74 (6/3)</td>
</tr>
<tr>
<td>Wortmann</td>
<td>0.026 ± 0.004</td>
<td>54 (7/4)</td>
</tr>
<tr>
<td>Wortmann + AngII</td>
<td>0.027 ± 0.002c</td>
<td>89 (6/3)</td>
</tr>
<tr>
<td>B1−/−control</td>
<td>0.032 ± 0.002</td>
<td>89 (5/3)</td>
</tr>
<tr>
<td>B1−/− + AngII</td>
<td>0.035 ± 0.003c</td>
<td>78 (5/3)</td>
</tr>
<tr>
<td>B1−/−Concanamycin</td>
<td>0.027 ± 0.001b,c</td>
<td>100 (5/3)</td>
</tr>
<tr>
<td>B1−/−Concanamycin + AngII</td>
<td>0.024 ± 0.001b,c</td>
<td>85 (5/3)</td>
</tr>
</tbody>
</table>

*Data are means ± SEM. OMCD, outer medullary collecting duct; pH$_i$, intracellular pH; BAPTA-AM, (1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(ethoxymethyl)ester. Significance difference from control. Significant difference from 10 nM angiotensin II alone.
pH/min). The enhanced rate of pH recovery in the absence of sodium was mediated by vacuolar H⁺-ATPase activity. OMCD were incubated for 10 min before experiments with the specific H⁺-ATPase inhibitor concanamycin (200 nM). In the presence of concanamycin, the rate of intracellular alkalinization was reduced 60 to 70% in control OMCD, and no stimulation was observed after incubation with AngII (Figure 1B). Inhibition of H⁺/K⁺-ATPases with SCH28080 (100 μM) reduced the rate of pH recovery only by approximately 10 to 15%, similar in extent to the residual pH recovery rate in the presence of concanamycin. Thus, AngII stimulates only vacuolar H⁺-ATPase activity in A-IC of isolated mouse OMCD.

**AT₁ Receptor Is Necessary for the Stimulatory Effect of AngII**

AngII mainly signals through two main receptor subtypes, AT₁ and AT₂. Both subtypes of receptors were previously identified in the OMCD. Saralasin (1 μM) and losartan (1 μM), two AT₁ receptor antagonists, prevented completely the stimulatory effect of AngII (Figure 2), whereas PD123,319, an inhibitor of AT₂ receptors, partially reduced the stimulatory effect of AngII (Figure 2), indicating that the stimulation of H⁺-ATPase activity by AngII is mainly mediated via AT₁ receptors.

Furthermore, immunohistochemistry with an affinity-purified antibody against the AT₁ receptor showed in the OMCD strong labeling of both principal cells and IC at the luminal pole. The basolateral membrane showed a weaker labeling. IC were identified as being negative for the aquaporin-2 water channel present only in principal/segment-specific cells (Figure 3). The antibody also stained arterial smooth muscle cells and apical and basolateral membranes of proximal tubules as described previously.

**Role of Small G Proteins, Phospholipase C, and Intracellular Ca²⁺**

AT₁ receptors couple intracellularly mainly via pertussis toxin (PTX)-sensitive small G proteins to phospholipase C, releasing intracellular Ca²⁺ and activating the protein kinase C (PKC) pathway. PTX (200 ng/ml), an inhibitor of G₁ and G₅, small G proteins, had no effect alone but completely blocked the stimulatory effect of AngII (Figure 4A). Inhibition of phospholipase C activity with U73122 (10 μM) also impeded the stimulation by AngII. U73122 alone reduced the rate of alkalinization slightly but significantly (Figure 4A).

Intracellular calcium measurements demonstrated that AngII induced a rapid and transient increase in [Ca²⁺]ᵢ that was smaller than an ATP-induced (100 μM) rise in the same cells (Figure 4B). Chelation of intracellular Ca²⁺ by preincubation with 1,2-bis(2-amino-5-fluoro-phenoxy)ethane-N,N,N',N'-tetraacetic acid tetraakis (acetoxyethyl)ester (BAPTA-AM) (50 μM) abolished the stimulation of H⁺-ATPases by AngII. For examination of whether release of Ca²⁺ from endoplasmic reticulum (ER) stores was involved in this process, ER Ca²⁺ stores were depleted with thapsigargin (1 nM), which resulted in a significant inhibition of the AngII effect (Figure 4C). Thus, intracellular Ca²⁺ plays an important role in the stimulation of H⁺-ATPases by AngII.

**AngII Stimulates through Ca²⁺-Dependent and -Independent PKC**

The inhibition of PKC activity with chelerythrine (1 μM) prevented completely the effect of AngII. Several isoforms of PKC have recently been described in mouse OMCD IC by immunohistochemistry, including the classic Ca²⁺-dependent isoforms PKC-α and PKC-β1 and the novel Ca²⁺-independent PKC-δ and PKC-ɛ isoforms. On the basis of the observation that intracellular Ca²⁺ plays a critical role, we tested the involvement of
Ca<sup>2+</sup>-dependent and -independent PKC isoforms. Incubation with Go<sup>6976</sup> (1 μM), an inhibitor of Ca<sup>2+</sup>-dependent PKC subtypes PKC-α and PKC-β1, reduced the stimulation of H<sup>+</sup>-ATPases partially. Also Ro-31-2880 (1 μM), an inhibitor of Ca<sup>2+</sup>-independent PKC-ε and PKC-ζ isoforms, attenuated the stimulatory effect partially (Figure 5). Using Go<sup>6976</sup> and Ro-31-2880 each at a concentration of 10 μM, the stimulation that was caused by AngII was completely abolished. This may indicate that Go<sup>6976</sup> and Ro-31-2880 used at higher concentrations may be less isoform specific. Moreover, the effect of AngII was completely inhibited when the activation of extracellular signal–regulated kinase 1/2 (ERK1/2) was prevented using PD098059 (20 μM) (Figure 6). Preincubation with PD098059 alone had no effect on H<sup>+</sup>-ATPase activity.

Microtubular Network and SNARE Protein Are Involved in the Stimulatory Effect of AngII
Stimulation of H<sup>+</sup>-ATPase activity in various cells involves colchicine-sensitive trafficking and fusion of vesicles with the membrane depending on soluble not attachment receptor (SNARE) proteins and cellubrevin. Disruption of the microtubular network with colchicine (preincubation for 10 min, 10 μM) reduced the basal rate of alkalinization significantly possibly by inhibition of recycling of H<sup>+</sup>-ATPases. Colchicine completely abolished the effect of AngII (Figure 7). In addition, cleaving of cellubrevin, part of the membrane vesicle fusion complex, with tetanus toxin (50 nM) had no effect on basal activity but prevented the stimulation by AngII. Thus, the effect of AngII requires trafficking and SNARE-dependent fusion of vesicles that carry H<sup>+</sup>-ATPases or some of their subunits or possible accessory or regulatory proteins.

AngII Stimulates H<sup>+</sup>-ATPases via PI3 Kinases
Phosphatidylinositol-3 kinases (PI3-K) have been implicated in the assembly, trafficking, and stimulation of vacuolar H<sup>+</sup>-ATPases by glucose, and AT<sub>1</sub> receptor–mediated and PI3-K–dependent stimulation of Na<sup>+</sup>/H<sup>+</sup> exchanger NHE3 activity was described. The PI3-K inhibitor wortmannin (1 μM) prevented the stimulation of the intracellular alkalinization rate induced by AngII, without affecting basal activity (Figure 8).

B1 Subunit of the Vacuolar H<sup>+</sup>-ATPase Is Required for Stimulation
H<sup>+</sup>-ATPases contain either the B1 or the B2 isoform, forming part of the cytosolic V<sub>i</sub> domain. The B1 subunit is specifically expressed in IC. It has been speculated that the B subunit may be involved in cell-specific subcellular localization, trafficking, or enzyme regulation. Hence, we used mice that were deficient for the B1 subunit (Atp6v1b1<sup>−/−</sup>) and tested whether (1) OMCD IC express H<sup>+</sup>-ATPase activity and (2) activity can be stimulated by AngII.

The total rate of pH<sub>i</sub> alkalinization was similar in OMCD IC from B1-deficient mice (0.032 ± 0.002 units pH/min) as compared with wild-type mice (Figure 1). Inhibition of H<sup>+</sup>-ATPases
with concanamycin (200 nM) decreased the rate of alkalization significantly (0.027 ± 0.001 units pH/min), demonstrating that a vacuolar H\(^{+}\)-ATPase is functional in the plasma membrane of IC despite the lack of the B1 subunit (Figure 9A). However, the concanamycin-sensitive rate of alkalization was lower than that observed in wild-type mice (compare with Figure 1). AngII (10 nM) had no effect on the rate of alkalization in the absence or presence of concanamycin. To rule out that AT\(_{1}\) receptors were absent or unresponsive in the IC of B1-deficient mice, we measured intracellular calcium in response to AngII and ATP as detailed previously. Both AngII and ATP elicited a rise in intracellular calcium similar to what had been observed in wild-type OMCD (Figure 9B). Furthermore, AT\(_{1}\) receptor localization was not altered as assessed by immunohistochemistry (Figure 9C). Hence, vacuolar H\(^{+}\)-ATPases that lack the B1 subunit cannot be stimulated by AngII.

Figure 4. The stimulatory effect of AngII is mediated via small G proteins and phospholipase C (PLC) and requires intracellular Ca\(^{2+}\). (A) The stimulatory effect of angiotensin is mediated via pertussis toxin (PTX)-sensitive (200 ng/ml) small G protein (G\(_{\text{Gso}}\) or G\(_{\text{Gsi}}\)). U73122 (10 μM), an inhibitor of PLC activity, prevented the stimulation of vacuolar H\(^{+}\)-ATPase activity induced by AngII. (B) Superfusion of IC with 10 nM AngII induced a transient rise in intracellular calcium. ATP (100 μM) was used as a positive control (original tracing, left). Summary of data from 10 independent experiments showing the increase in intracellular calcium (right). (C) Chelation of intracellular Ca\(^{2+}\) with BAPTA-AM (50 μM) prevented the vacuolar H\(^{+}\)-ATPase stimulation. Depletion of endoplasmic reticulum Ca\(^{2+}\) stores with thapsigargin (1 μM) had a similar effect. The values of control and AngII-treated OMCD are shown again for comparison.*Significantly different from control; †significantly different from 10 nM AngII.

Figure 5. Different protein kinase C (PKC) isoforms are involved in the pathway activated by AngII. (A) Inhibition of PKC with chelerythrine (1 μM) completely prevented vacuolar H\(^{+}\)-ATPase stimulation. (B and C) Inhibition of classic Ca\(^{2+}\)-dependent and novel independent PKC isoforms PKC-α and PKC-β1 with Gö 6976 (1 μM) and PKC-δ and PKC-ε with Ro-31-2880 (1 μM), respectively, partially reduced stimulation. Higher concentrations of Gö 6976 and Ro-31-2880 (10 μM) completely blocked stimulation.*Significantly different from control; †significantly different from 10 nM AngII.
mechanisms, an effect that may occur at least in part via stimulation of \( \text{AT}_{2} \) receptors.\(^{45,46} \)

Some reports indicated that AngII may inhibit urinary acidification, bicarbonate reabsorption, or \( \text{H}^{+}-\text{ATPase} \) activity in the rabbit initial cortical collecting duct and the rat OMCD and inner medullary collecting duct, respectively.\(^{47-50} \) However, these studies are in contrast to a large number of observations demonstrating that AngII stimulates final urinary acidification in these nephron segments and that blockade of the angiotensin system reduces urinary acidification.\(^{5,10-12,39,44,51,52} \) We cannot exclude that the discrepancies may be either due to different species used or because we used superfusion of OMCD in contrast to perfused nephron segments, which may alter the access of AngII and other inhibitors to the luminal membrane.

\( \text{AT}_{1} \) receptors couple to phospholipase \( C \) via small \( G \) proteins; increase intracellular \( \text{Ca}^{2+} \) concentrations; and activate PKC, ERK1/2, and PI3-K as well as a variety of other signal pathways in a cell- and tissue-specific manner.\(^{21,53} \) Our results demonstrate that in OMCD IC, AngII signals through a distinct cascade to stimulate \( \text{H}^{+}-\text{ATPase} \) activity involving PTX-sensitive \( G \) proteins (\( G_{o6976} \), phospholipase \( C \), intracellular \( \text{Ca}^{2+} \), different PKC isoforms, ERK1/2 mitogen-activated protein kinases, and PI3-K. The data indicate that at least two isoforms of PKC may participate in the stimulatory effect of AngII: A classic \( \text{Ca}^{2+} \)-dependent and \( \text{Go}6976 \)-sensitive PKC-\( \alpha \) and/or PKC-\( \beta 1 \) and a novel \( \text{Ca}^{2+} \)-independent and Ro-31-2880 –sensitive PKC-\( \delta \) and/or PKC-\( \varepsilon \) isoform. Immunohistochemistry demonstrated the expression of PKC-\( \alpha \), PKC-\( \beta 1 \), PKC-\( \varepsilon \), and PKC-\( \delta \) in IC of the collecting duct.\(^{25,27} \) Evidence from freshly isolated OMCD IC as well as from cell culture models\(^{19,31,34} \) suggests that PKC has a stimulatory effect on \( \text{H}^{+}-\text{ATPase} \) activity. In addition, our experiments indicate that intact ERK1/2 mitogen-activated protein kinases and PI3-K are required for the stimulation to occur. PI3-K are implied in the glucose-stimulated assembly, trafficking, and stimulation of vacuolar \( \text{H}^{+}-\text{ATPases} \) in renal cell lines derived from the proximal tubule.\(^{34} \) Also, AngII stimulates \( \text{Na}^{+}/\text{H}^{+} \) exchange activity in a proximal tubular cell line via PI3-K.\(^{35} \)

**DISCUSSION**

Here we describe two related findings: The stimulation of \( \text{H}^{+}-\text{ATPase} \) activity in acid-secretory IC by AngII and that stimulation requires the presence of the \( \text{B1} \) isoform. The stimulatory effect of AngII on several renal acid-base transport systems has been extensively documented.\(^{6,7,9,38,39} \) These processes are mostly mediated via AT1 receptors. Here we show that AngII stimulates also vacuolar \( \text{H}^{+}-\text{ATPase} \) activity in the acid-secretory IC of the OMCD. Both pharmacologic evidence and immunohistochemistry demonstrate that AT1 receptors are present and involved. However, our preparation does not allow distinguishing between the stimulation of basolateral and/or luminal receptors. Urinary AngII concentrations in the OMCD are in the range of 10 to 30 nM,\(^{40,41} \) originating mainly from AngII synthesis along the nephron.\(^{40,42} \) During metabolic acidosis, an increase in AngII has been reported, and the ability of the kidney to adapt to changes in acid-base status is influenced by AngII.\(^{10,12,13,43,44} \) The less stimulatory effect of 100 nM AngII is consistent with high concentrations of AngII even inhibiting bicarbonate reabsorptive
Regulation of H⁺-ATPase activity can occur through several distinct mechanisms, and trafficking of vacuolar H⁺-ATPases plays a major role. Physiologic stimuli lead to increased proton secretion such as CO₂, acidification, or hormones such as AngII or aldosterone. Insertion of pumps or its subunits into the membrane requires an intact microtubular network and proteins that are involved in vesicle fusion, such as SNARE proteins. The inhibition of H⁺-ATPase stimulation by colchicine and tetanus toxin suggests strongly that AngII increases proton pump activity through trafficking of proton pumps or some of its subunits or accessory proteins into the membrane.

Evidence from yeast suggests that specific isoforms of some subunits could play a role in targeting, trafficking, and adaptive changes in the ratio between ATP hydrolysis and proton pumping. Particularly the two isoforms of the B subunit and various isoforms of the a subunit have been implicated. No evidence for isoform-specific functions for mammalian subunits has so far been described even though the cell- and organ-specific expression of some subunits has been noted. Organ-specific inherited diseases such as malignant infant osteopetrosis (mutations in the a3 subunit), distal renal tubular acidosis (a4 subunit), and distal renal tubular acidosis with sensorineural deafness (B1 subunit) underlined the concept of isoform-specific functions of vacuolar H⁺-ATPases. Here we find that H⁺-ATPases in the OMCD that lack the B1 subunit maintain a basic level of activity but cannot be stimulated by AngII. This activity is most likely due to substitution of lacking B1 subunits by the B2 isoform, which show enhanced luminal appearance in the B1-deficient mice. Apparently, the B1 isoform performs functions that cannot be compensated by the B2 isoform, suggesting that B1 is involved in the cell-specific stimulation that could involve trafficking or assembly of pumps. This interpretation is also in line with some recent data demonstrating that overexpression of some B1 mutations that were found in patients impairs assembly and trafficking of proton pumps to the apical membrane. The absence of a functional B1 subunit in patients and mice leads to defective urinary acidification. AngII has been shown to be involved in the adaptive increase in urinary acidification during acidosis, and that vacuolar H⁺-ATPases in IC that lacking the B1 subunit are insensitive to stimulation may help to explain this phenotype.

**CONCLUSION**

We identified AngII as a potent stimulus for H⁺-ATPases in OMCD A-IC. The stimulation is transmitted via a distinct signal cascade and requires the presence of the B1 subunit of the vacuolar H⁺-ATPase. The lack of stimulation by AngII may explain the inappropriate adaptive urinary acidification during acidosis of patients with mutations in the B1 subunit.

**CONCISE METHODS**

**Animals**

C57BL-6j (Jackson Laboratory, Bar Harbor, ME) and B1-deficient mice (Atp6v1b1 / / ; male 12 to 15 wk of age, 30 to 35 g) were housed under standard conditions. Breeding and genotyping of Atp6v1b1 / / mice has been described previously. All studies were approved by the Local Swiss
Veterinary Authority (Veterinäramt, Zurich, Switzerland) and were according to Swiss Animal Welfare Laws.

**Isolation of OMCD**

OMCD were prepared from mouse kidney as described previously.18,19

**Intracellular pH and Ca**2+ **Measurements**

Coverslips were transferred to a thermostatically controlled perfusion chamber (approximately 3 ml/min flow rate) maintained at 37°C on an inverted microscope (Zeiss Axiosvert 200, Feldbach, Switzerland) equipped with a video imaging system (Visitron, Munich, Germany). The isolated OMCD were incubated in a HEPES-buffered Ringer’s solution that contained either the pH-sensitive dye BCECF-AM (2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein ester; 10 μM; Molecular Probes, Eugene, OR) or the calcium-sensing dye FURA-2-AM (5 μM; Molecular Probes) for 20 min and were washed to remove all non de-esterified dye. pH was measured by alternately exciting the dye with a 10-mm-diameter spot of light at 495 and 440 nm for BCECF and 340 and 380 nm for FURA-2 while monitoring the emission at 532 or 510 nm, respectively, with a video imaging system. Each experiment was calibrated for pH using the nigericin/high K+ method, and the obtained ratios were converted to pH−.18,19,58 FURA-2 measurements were calibrated for Ca2+, with high (2 mM) and zero calcium solutions in the presence of a calcium ionophore and [Ca2+]i calculated.59 All experiments were performed in the nominal absence of bicarbonate. The initial solution was a HEPES-buffered Ringer’s solution (125 mM NaCl, 3 mM KCl, 1 mM CaCl2, 1.2 mM MgSO4, 2 mM KH2PO4, and 32.2 mM HEPES [pH 7.4]). Cells were acidified by using the NH4Cl (20 mM) prepulse technique and washed into a Na+-free solution (Na+ was replaced by equimolar concentrations of N-methyl-d-glucamine). The rate of H+ exchange and Na+/HCO3− cotransport in the rabbit proximal tubule. Proc Natl Acad Sci U S A 87: 7917–7920, 1990

**Acknowledgments**

This study was supported by a grant from the Swiss National Research Foundation to C.A.W. (31-068318) and the Hartmann-Müller Stiftung (Zurich, Switzerland).

**Disclosures**

None.

**References**

15. Paunesku TG, Da Silva N, Marshansky V, McKeel M, Breton S, Brown D: Expression of the S6 kDa B2 subunit isofrom of the vacuolar H+–ATPase in...


