Angiotensin II Stimulates Vacuolar H\(^+\)-ATPase Activity in Renal Acid-Secretory Intercalated Cells from the Outer Medullary Collecting Duct

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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 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.
co-transporters, and H+-ATPases. In in vivo experiments, AngII stimulates also final urinary acidification. Thus, AngII may stimulate H+-ATPases in the collecting duct. Vacular H+-ATPases are composed of at least 13 subunits in mammals of which multiple isoforms exist. The B subunit belongs to the cytosolic V₁ domain and is essential for vacular 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 are not able to acidify urine appropriately. The B2 subunit, in contrast, is 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 (pHi) was acidified to 6.35 ± 0.012 using the NH₄Cl prepulse in the absence of extracellular Na⁺. Intracellular pHi 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⁺, pHi increased rapidly to the initial value mediated by Na+/H⁺ exchange as described previously (Table 1).

Table 1. Summary of pHi measurements in single intercalated cells in mouse OMCD fragments

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Na⁺-Independent pHᵢ</th>
<th>Recovery Rate (ΔpH/min)</th>
<th>No. of Cells, OMCD, and Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.032 ± 0.002</td>
<td>61 (5/4)</td>
<td></td>
</tr>
<tr>
<td>AngII, 1 nM</td>
<td>0.035 ± 0.002</td>
<td>96 (7/3)</td>
<td></td>
</tr>
<tr>
<td>AngII, 10 nM</td>
<td>0.080 ± 0.005</td>
<td>127 (11/6)</td>
<td></td>
</tr>
<tr>
<td>AngII, 100 nM</td>
<td>0.047 ± 0.005</td>
<td>15 (4/2)</td>
<td></td>
</tr>
<tr>
<td>Concannamycin</td>
<td>0.013 ± 0.001</td>
<td>101 (6/3)</td>
<td></td>
</tr>
<tr>
<td>Concannamycin + AngII</td>
<td>0.011 ± 0.001</td>
<td>88 (5/3)</td>
<td></td>
</tr>
<tr>
<td>SCH28080 + AngII</td>
<td>0.065 ± 0.006</td>
<td>16 (6/2)</td>
<td></td>
</tr>
<tr>
<td>Saralasin</td>
<td>0.020 ± 0.002</td>
<td>81 (6/3)</td>
<td></td>
</tr>
<tr>
<td>Saralasin + AngII</td>
<td>0.019 ± 0.001</td>
<td>99 (6/3)</td>
<td></td>
</tr>
<tr>
<td>Losartan + AngII</td>
<td>0.027 ± 0.002</td>
<td>19 (5/2)</td>
<td></td>
</tr>
<tr>
<td>PD123.319</td>
<td>0.038 ± 0.003</td>
<td>60 (5/4)</td>
<td></td>
</tr>
<tr>
<td>PD123.319 + AngII</td>
<td>0.057 ± 0.006</td>
<td>84 (6/3)</td>
<td></td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>0.025 ± 0.002</td>
<td>85 (6/3)</td>
<td></td>
</tr>
<tr>
<td>Pertussis toxin + AngII</td>
<td>0.023 ± 0.001</td>
<td>83 (5/3)</td>
<td></td>
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<tr>
<td>U73122</td>
<td>0.017 ± 0.002a</td>
<td>77 (5/3)</td>
<td></td>
</tr>
<tr>
<td>U73122 + AngII</td>
<td>0.012 ± 0.001h,c</td>
<td>80 (5/3)</td>
<td></td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>0.026 ± 0.009</td>
<td>64 (5/3)</td>
<td></td>
</tr>
<tr>
<td>BAPTA-AM + AngII</td>
<td>0.013 ± 0.001h,c</td>
<td>87 (5/3)</td>
<td></td>
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<tr>
<td>Thapsigargin</td>
<td>0.027 ± 0.002</td>
<td>60 (5/3)</td>
<td></td>
</tr>
<tr>
<td>Thapsigargin + AngII</td>
<td>0.036 ± 0.002</td>
<td>74 (5/4)</td>
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<tr>
<td>Chelerythrine</td>
<td>0.026 ± 0.002</td>
<td>68 (5/3)</td>
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<tr>
<td>Chelerythrine + AngII</td>
<td>0.030 ± 0.002</td>
<td>96 (7/3)</td>
<td></td>
</tr>
<tr>
<td>G6976 10 μM + AngII</td>
<td>0.018 ± 0.002h,c</td>
<td>60 (5/4)</td>
<td></td>
</tr>
<tr>
<td>G6976</td>
<td>0.019 ± 0.002h,c</td>
<td>54 (5/3)</td>
<td></td>
</tr>
<tr>
<td>G6976 1 μM + AngII</td>
<td>0.029 ± 0.003c</td>
<td>77 (5/3)</td>
<td></td>
</tr>
<tr>
<td>Ro 31-2880 10 μM + AngII</td>
<td>0.022 ± 0.002c</td>
<td>103 (7/3)</td>
<td></td>
</tr>
<tr>
<td>Ro 31-2880 1 μM + AngII</td>
<td>0.018 ± 0.003h,c</td>
<td>55 (6/4)</td>
<td></td>
</tr>
<tr>
<td>Ro 31-2880 1 μM + AngII</td>
<td>0.040 ± 0.003c</td>
<td>74 (5/2)</td>
<td></td>
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<tr>
<td>PD98059</td>
<td>0.027 ± 0.002</td>
<td>80 (6/3)</td>
<td></td>
</tr>
<tr>
<td>PD98059 + AngII</td>
<td>0.017 ± 0.002h,c</td>
<td>74 (6/3)</td>
<td></td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.013 ± 0.002h,c</td>
<td>79 (5/3)</td>
<td></td>
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<tr>
<td>Colchicine + AngII</td>
<td>0.013 ± 0.001h,c</td>
<td>89 (5/3)</td>
<td></td>
</tr>
<tr>
<td>Tetanus toxin</td>
<td>0.037 ± 0.002</td>
<td>112 (9/4)</td>
<td></td>
</tr>
<tr>
<td>Tetanus toxin + AngII</td>
<td>0.020 ± 0.002c</td>
<td>74 (6/3)</td>
<td></td>
</tr>
<tr>
<td>Wortmannin</td>
<td>0.026 ± 0.004</td>
<td>54 (7/4)</td>
<td></td>
</tr>
<tr>
<td>Wortmannin + AngII</td>
<td>0.027 ± 0.002c</td>
<td>89 (6/3)</td>
<td></td>
</tr>
<tr>
<td>B1 0.1 M control</td>
<td>0.032 ± 0.002</td>
<td>89 (5/3)</td>
<td></td>
</tr>
<tr>
<td>B1 0.1 M + AngII</td>
<td>0.035 ± 0.003c</td>
<td>78 (5/3)</td>
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</tr>
<tr>
<td>B1 0.1 M + Concannamycin</td>
<td>0.027 ± 0.001h,c</td>
<td>100 (5/3)</td>
<td></td>
</tr>
<tr>
<td>B1 0.1 M + Concannamycin + AngII</td>
<td>0.024 ± 0.001h,c</td>
<td>85 (5/3)</td>
<td></td>
</tr>
</tbody>
</table>

*Data are means ± SEM. OMCD, outer medullary collecting duct; pHᵢ, intracellular pH; BAPTA-AM, (1,2-bis(2-amino-5-fluoro-phenoxo)ethane-N,N′,N″-tetracetic acid tetrakis (acetoxymethyl)ester. hSignificant difference from control.

*Significant difference from 10 nM angiotensin II alone.

Preincubation of mouse OMCD with 10 nM AngII for 10 min increased the Na⁺-independent alkalization rate 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 enhanced rate of pH recovery in the absence of sodium was mediated by vacular 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 alkalization 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 vacular 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 Gs and Gq, 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 alkalization 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 tetralaks (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-ε 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<sub>6976</sub> (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-δ and PKC-ε isoforms, attenuated the stimulatory effect partially (Figure 5). Using Go<sub>6976</sub> 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<sub>6976</sub> 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 isofrom, 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 (0.03 ± 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_αo or G_αi). 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 concentrations (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-β 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.

Angiotensin Stimulates H^+-ATPase in Intercalated Cells
mechanisms, an effect that may occur at least in part via stimulation of AT₁ receptors.45,46 Some reports indicated that AngII may inhibit urinary acidification, bicarbonate reabsorption, or H⁺-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.

AT₁ receptors couple to phospholipase C via small G proteins; increase intracellular Ca²⁺ 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 H⁺-ATPase activity involving PTX-sensitive G proteins (Gαo, Gai), phospholipase C, intracellular Ca²⁺, 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 Ca²⁺-dependent and Gö 6976-sensitive PKC-α and/or PKC-β1 and a novel Ca²⁺-independent and Ro-31-2880–sensitive PKC-δ and/or PKC-ε isoform. Immunohistochemistry demonstrated the expression of PKC-α, PKC-β1, PKC-ε, and PKC-δ in IC of the collecting duct.25,27 Evidence from freshly isolated OMCD IC as well as from cell culture models19,31,34 suggests that PKC has a stimulatory effect on H⁺-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 H⁺-ATPases in renal cell lines derived from the proximal tubule.34 Also, AngII stimulates Na⁺/H⁺ exchange activity in a proximal tubular cell line via PI3-K.35

DISCUSSION

Here we describe two related findings: The stimulation of H⁺-ATPase activity in acid-secretory IC by AngII and that stimulation requires the presence of the 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 H⁺-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 CO\(_2\), 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

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*Figure 9.* The B1 subunit of the vacuolar H\(^+\)-ATPase is necessary for its stimulation with AngII. (A) Preincubation of OMCD from B1-deficient mice with AngII (10 nM) did not show any significant stimulatory effect on the rate of intracellular alkalinization. Concanaamycin (200 nM), a specific inhibitor of vacuolar H\(^+\)-ATPases, reduced the rate of intracellular alkalinization in the absence and presence of AngII to the same extent, demonstrating that AngII did not stimulate proton pumps that lacked the B1 subunit. (B) The presence of functional AngII receptors in OMCD IC from Atp6v1b1-deficient mice was confirmed by measurement of intracellular Ca\(^{2+}\) changes in response to AngII (10 nM) and ATP (100 μM) as a positive control. Intracellular Ca\(^{2+}\) increased in OMCD IC to a similar extent as shown in Figure 4 (n = 12 cells, four OMCD). (C) Staining for the AT\(_1\) receptor (red) and AQP-2 water channel (green) in the OMCD of B1-deficient mice. *Significantly different from control; #significantly different from 10 nM AngII.
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 Ca2+ Measurements
Cover slips were transferred to a thermostatically controlled perfusion chamber (approximately 3 ml/min flow rate) maintained at 37°C on an inverted microscope (Zeiss Axiovert 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-carboxylethyl)-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. pH1 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 pH1 using the nigericin/high K+ method, and the obtained ratios were converted to pHc.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+], calculated. 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+-free solution: NaCl was replaced by equimolar Na+-free solution; NH4Cl was added). Cells were incubated at 37°C on a thermostatically controlled perfusion chamber (approximately 3 ml/min flow rate) maintained at 37°C. 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 (NaCl was replaced by equimolar NaCl). The rate of H+-ATPase activity was determined as the concanamycin-sensitive pH1 alkalization rate in the absence of NaCl. Rates were calculated over the same range of pH1 (6.55 to 6.75) for all cells studied. OMCD were used only for one single experiment (one NH4Cl prepulse as repetitive intracellular acidification altered recovery rates in our hands). For the inhibitor studies, OMCD were preincubated with the inhibitors alone for 10 min, then co-incubated with BCECF for another 10 min. The inhibitors were also added to all solutions in the absence or presence of AngII, respectively. All chemicals were from Sigma (Buchs, Switzerland) and Calbiochem (Dietikon, Switzerland).

Immunohistochemistry
Immunostaining on paraformaldehyde/lysine/periodate-perfused kidneys was carried out as described previously20 using rabbit anti-AQP1 receptor (Santa Cruz Biotechnology, Santa Cruz, CA),21 1:200, and goat anti-human AQP-2 (Santa Cruz Biotechnology), 1:200, antibodies.

Statistical Analyses
Data are presented as means ± SEM. All data were tested for significance using the one-way ANOVA test, and only results with P < 0.05 were considered statistically significant.

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DISCLOSURES
None.

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