Aldosterone stimulates vacuolar H\(^+\)-ATPase activity in renal acid-secretory intercalated cells mainly via a protein kinase C-dependent pathway

Christian Winter,¹,² Nicole B. Kampik,¹ Luca Vedovelli,³,⁵ Florina Rothenberger,¹ Teodor G. Păunescu,³,⁴ Paul A. Stehberger,¹ Dennis Brown,³,⁴ Hubert John,⁶ and Carsten A. Wagner¹

¹Institute of Physiology, Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland; ²Clinics of Urology, University Hospital Düsseldorf, Düsseldorf, Germany; ³MGH Center for Systems Biology, Program in Membrane Biology and Division of Nephrology, Massachusetts General Hospital, Boston, Massachusetts; and ⁴Harvard Medical School, Boston, Massachusetts; ⁵Department of Pharmacology and Anesthesiology, University of Padova, Padua, Italy; and ⁶Clinic of Urology, Kantonsspital, Winterthur, Switzerland

Submitted 14 March 2011; accepted in final form 3 August 2011

Renal acid secretion and urinary acidification are tightly regulated by a number of factors including several hormones such as endothelin, angiotensin II, and aldosterone (15, 27, 70–71). The influence of the renin-angiotensin II-aldosterone system (RAAS) on the ability of the kidney to excrete protons is well recognized. Both angiotensin II and aldosterone have been demonstrated to directly and indirectly influence transport pathways involved in acid secretion such as Na\(^+\)/H\(^+\) exchangers and vacuolar H\(^+\)-ATPases in different nephron segments (1, 16, 19–23, 30, 51–52, 58, 75). Aldosterone deficiency or defects in its receptor activation are causative for the hyperkalemic form of distal renal tubular acidosis (type IV dRTA) (15).

In the connecting segment and cortical collecting duct, aldosterone stimulates the activity of the electrogenic vacuolar H\(^+\)-ATPase, an effect that has been proposed to be of a primarily indirect nature. Increased reabsorption of Na\(^+\) through the epithelial Na\(^+\) channel ENaC in neighboring principal cells renders the tubule lumen more negative and could, thus, facilitate proton pumping (15, 27). We have recently demonstrated that this interaction occurs mainly in the connecting tubule (40). Thus, the exact mechanism by which Na\(^+\) absorption, H\(^+\) secretion, and aldosterone effects are linked in the cortical collecting duct is not fully understood. In contrast, in the OMCD, aldosterone stimulates proton secretion and this effect occurs even in the absence of significant Na\(^+\) absorption, suggesting a direct effect on vacuolar H\(^+\)-ATPase activity (65). Consistent with these observations, long-term stimulatory effects of aldosterone on vacuolar H\(^+\)-ATPase activity have been described in rabbit and rat OMCDs (14, 19, 30, 52).

Cellular responses to aldosterone in the classic long-term range (i.e., responses requiring several hours or days) are mediated by alterations in gene expression driven by the binding of the steroid hormone to a cytosolic or nuclear receptor acting as a transcription factor (50, 64, 68). However, not all biological effects of aldosterone can be explained by transcriptional or genomic effects. Over the past years an increasing number of studies have identified nongenomic effects of aldosterone in both epithelial and nonepithelial tissues (for review, see refs. 17, 46–50, 64). Taken together, these studies suggest the existence of novel steroid hormone receptors or possibly classical receptors embedded in the membrane that initiate nongenomic signaling cascades (17–18, 35, 49). Activation of these putative receptors initiates several intracellular signaling cascades that appear to be tissue or cell specific.
and the involvement of phospholipase C, inositol 1,4,5-trisphosphate (IP$_3$), protein kinase C pathways as well as adenyllyl cyclases, cAMP and PKA-dependent pathways has been described (17, 48–49). In addition, activation of ERK1/2 MAPK kinases has been observed (20, 41, 57). However, most of these studies were performed in cell culture models and only little information exists if similar pathways are activated in freshly isolated cells and tissues.

Recently, we described the direct stimulation of vacuolar H$^+$-ATPase activity in freshly isolated mouse acid-secretory intercalated cells by aldosterone. In OMCD intercalated cells, aldosterone increased vacuolar H$^+$-ATPase activity in the presence of the mineralocorticoid receptor blocker spironolactone and did not require transcription or translation. The stimulatory effect of aldosterone was associated with a transient rise in intracellular Ca$^{2+}$ and required intact protein kinase C (PKC) (75). Here we further investigated the mechanism by which aldosterone stimulates vacuolar H$^+$-ATPase activity. The results present pharmacological evidence supporting the hypothesis that aldosterone acts through both PLC/ERK1/2 activity. Furthermore, the rapid effects of aldosterone may require a G protein-coupled membrane receptor sensitive to suramin and GP-2A but not pertussis toxin.

**MATERIALS AND METHODS**

**Animals.** C57BL/6J mice (Jackson Laboratory) (male, 12–15 wk old, 30–35 g) were housed in climate- and humidity-controlled light-cycled rooms, fed standard rodent chow, and allowed free access to water. All studies carried out in Zurich were approved by the local Swiss Veterinary Authority (Veterinäramt, Zurich, Switzerland) and were performed according to Swiss Animal Welfare laws.

**Human kidney samples.** Human kidney samples were collected from patients undergoing nephrectomy for tumor removal (Department of Urology, University of Zurich, Switzerland). Informed consent was obtained from all patients and all studies were approved by the local ethics committee. Kidney samples were taken from the tumor was obtained from all patients and all studies were approved by the exclusion of bicarbonate. The initial solution was a HEPES-buffered Ringer solution (in mM: 125 NaCl, 3 KCl, 1 CaCl$_2$, 1.2 MgSO$_4$, 2 KH$_2$PO$_4$, 32.2 HEPES, pH 7.4). Cells were acidified by using the NH$_4$Cl (20 mM) prepulse technique and washed into a Na$^+$-free solution [Na$^+$ was replaced by equimolar concentrations of N-methyl-d-glucamine (NMDG)]. Intercalated cells were identified by their rapid uptake of BCECF as described previously (72). The rate of H$^+$-ATPase activity was determined as the concanamycin-sensitive pHi alkalization rate in the absence of Na$^+$. Rates were calculated over the same range of pHi (6.55–6.75) for all cells studied. All chemicals were of highest purity and were purchased from Sigma (Buchs, Switzerland) and Calbiochem (Merck, Darmstadt, Germany). Chemicals were added to the solutions from stock solutions at the given concentrations for each experimental protocol (Table 1).

**Intrinsic buffering power and calculation of proton fluxes.** The intrinsic buffering power ($\beta_i$) is defined by the amount of acid or base that has to be added to a solution to change its pH by one unit. The $\beta_i$ of OMCD cells was measured as described previously (7, 73), but changes in the pHi were induced by superfusion with a weak acid and the exclusion of Cl$^-$ from the bath. The cellular H$^+$-dependent regulatory mechanisms were excluded as a buffering system by using the inhibitors
c

<table>
<thead>
<tr>
<th>Substance</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pertussis toxin</td>
<td>G, and Gs, G protein inhibitor</td>
</tr>
<tr>
<td>Suramin</td>
<td>G protein inhibitor. Also directly blocks purinergic and adrenergic receptors.</td>
</tr>
<tr>
<td>G2A</td>
<td>G$_{q/11}$ inhibitor</td>
</tr>
<tr>
<td>U-73122</td>
<td>Inhibitor of phospholipase C</td>
</tr>
<tr>
<td>U-73343</td>
<td>Inactive analog to U-73122</td>
</tr>
<tr>
<td>BAFTA-AM</td>
<td>Chelator of intracellular calcium</td>
</tr>
<tr>
<td>Cherythrine</td>
<td>Pan-PKC inhibitor</td>
</tr>
<tr>
<td>DOG (1,2-dioctanoyl-sn-glycerol)</td>
<td>Diacylglycerol analog, PKC activator</td>
</tr>
<tr>
<td>PD098059</td>
<td>Prevents activation of mitogen-activated protein kinase-1</td>
</tr>
<tr>
<td>H89</td>
<td>Inhibitor of protein kinase A</td>
</tr>
<tr>
<td>[N-(2-p</td>
<td>bronomcaminylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride</td>
</tr>
</tbody>
</table>

**Table 1. Pharmacological agents used to modulate the activity of intracellular signaling molecules**
ratio of the change in intracellular acetate concentration to the change in pH; \( \beta_i = \Delta A / \Delta \text{pH}_i \), where \( \Delta \text{pH}_i \) is the decrease in pH, resulting from weak acid addition and \( \Delta A \) is the change in intracellular acetate concentration calculated from its \( \text{pK}_a \) (4.74 at 37°C) and \( \text{pH}_i \).

Proton fluxes \( J_H \) (in pmol·min\(^{-1} \cdot \text{mm}^{-2} \)) were calculated following the equation:

\[
J_H = \Delta \text{pH}_i / (\Delta t \times \beta_i \times V),
\]

where \( \Delta \text{pH}_i / \Delta t \) is the rate of \( H^+ \)-ATPase activity and \( V \) is cell volume. Net proton efflux is indicated by positive \( J_H \) values.

**Tissue preparation and treatment of rats with aldosterone and DOG.** Adult male Sprague-Dawley rats (Charles River, Wilmington, MA) were housed under standard conditions and fed a standard rodent diet. Animal studies were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care, in accordance with the National Institutes of Health, Department of Agriculture, and Association for Assessment and Accreditation of Laboratory Animal Care International requirements.

Rats were anesthetized with pentobarbital sodium (Nembutal, Abbott Laboratories, Abbott Park, IL, 50 mg/kg body wt ip) and treated using a modification of the protocol we described previously (55). A 200-μl bolus injection of 200 nM or 200 μM aldosterone (Sigma-Aldrich, St. Louis, MO) or 20 μM DOG (Cayman Chemical, Ann Arbor, MI), a cell membrane-permeable diacylglycerol analog and activator of protein kinase C, dissolved in physiological buffer was injected intravenously into the tail vein. Immediately following the bolus injection, a steady infusion of the respective drug at the same concentration was made for 15 min into the tail vein. After the infusion, the animal was perfused via the left cardiac ventricle with phosphate-buffered saline (PBS, 0.9% NaCl in 10 mM phosphate buffer, pH 7.4) followed by paraformaldehyde-lysine-periodate fixative (PLP) as previously described (54). Three control rats were injected and infused with buffer alone, and three rats each were injected and infused with aldosterone at both concentrations or with DOG as described above. Following the PLP perfusion, the kidneys were dissected, sliced, and further fixed by immersion in PLP for 4 h at room temperature and then overnight at 4°C. Kidneys were rinsed extensively in PBS the following day and stored at 4°C in PBS containing 0.02% sodium azide.

**Immunofluorescence and confocal microscopy.** PLP-fixed kidney slices were cryoprotected in PBS containing 0.9 M sucrose, embedded in Tissue-Tek OCT compound 4583 (Sakura Finetek USA, Torrance, CA) and frozen at −20°C, and 5-μm sections were cut on a Leica CM3050 S cryostat (Leica Microsystems, Bannockburn, IL). Sections were rehydrated in PBS and treated with 1% (wt/vol) SDS for 4 min for retrieval of antigenic sites (9). We subsequently followed a previously described immunostaining protocol which includes a 20-min blocking step in 1% (wt/vol) bovine serum albumin in PBS, a 90-min incubation with a primary antibody against the V-ATPase 70-kDa “A” subunit (34) diluted in Dako antibody diluent (Dako, Carpinteria, CA), and a 60-min incubation with Alexa Fluor 594 goat anti-rabbit IgG (H+L) (Invitrogen, Carlsbad, CA) at 2.5 μg/ml as secondary antibody, all at room temperature (55).

Digital images were acquired using a Nikon 80i epifluorescence microscope (Nikon Instruments, Melville, NY) equipped with an Orca 100 CCD camera (Hamamatsu, Bridgewater, NJ). Images were acquired using IPLab version 3.2.4 (Scanalytics, Fairfax, VA) and final figures were produced using Adobe Photoshop version CS2 image editing software (Adobe Systems, San Jose, CA) (55).

**Statistics.** Data are presented as means ± SE. One-way ANOVA with Bonferroni post test was performed when comparing means across three or more groups. All other data were tested for significance using the unpaired Student’s t-test, and only results with \( P < 0.05 \) were considered as statistically significant.

---

**RESULTS**

**Rapid stimulation of \( H^+ \)-ATPase activity in mouse OMCD intercalated cells by aldosterone.** In type A acid-secreting intercalated cells of mouse OMCD, the mean initial \( \text{pH}_i \) was 7.31 ± 0.01 (Table 2), \( \text{pH}_i \) acidified after removal of sodium from the bath and alkalinized after the addition of an NH\(_4\)Cl pulse (20 mM) (Fig. 1A). After removal of NH\(_4\)Cl from the perfusion chamber, the OMCD intercalated cells were acidified to a mean \( \text{pH}_i 6.42 ± 0.01 \). In the continued absence of Na\(^+ \) the intracellular \( \text{pH}_i \) recovered slowly. The observed intracellular alkalization rate was 0.031 ± 0.002 \( \text{pH}_i \) units/min. This alkalization is mainly due to the activity of \( H^+ \)-ATPases and is mostly inhibited by the specific inhibitor concanamycin as previously shown (72, 75). Readaptation of Na\(^+ \) to the bath led to a rapid increase of \( \text{pH}_i \) to the initial value mediated by Na\(^+ \)/H\(^+ \) exchange (Fig. 1A).

Preincubation of mouse OMCDs with 10 nM aldosterone for 20 min at 37°C increased the Na\(^+ \)-independent alkalization rate 2–3 fold to 0.069 ± 0.002 \( \text{pH}_i \) units/min as observed previously (75). To test for the immediate onset of stimulation, 10 nM aldosterone was added to the bath 3 min before the solution was switched to Na\(^+ \)-free conditions (75). The stimulation of \( H^+ \)-ATPase activity was similar to the 20-min preincubation (0.081 ± 0.006 pH units/min) (Table 2 and Fig. 1), suggesting that the nongenomic stimulation of \( H^+ \)-ATPase activity occurs within a very short time frame. Addition of 1 nM aldosterone in the same experimental series (3-min preincubation) led to a small but significant increase of the Na\(^+ \)-independent alkalization rate (0.041 ± 0.004 pH units/min, Table 2 and Fig. 1). The steroid hormone hydrocortisone had no effect at similar concentrations (10 nM) with preincubation periods of 20 min (0.030 ± 0.002 pH units/min, Table 2 and Fig. 1). Thus, aldosterone specifically stimulates \( H^+ \)-ATPase activity in OMCD intercalated cells in a concentration-dependent manner and this effect occurs very rapidly, consistent with a rapid and nongenomic effect (75).

We also measured intrinsic buffering power (\( \beta_i \)) in OMCD intercalated cells. Incubation with 10 nM aldosterone for 20 min decreased \( \beta_i \) significantly from 42.8 ± 1.5 \((n = 61)\) to 34.0 ± 1.4 \((n = 58)\) (\( P < 0.001 \)). Net proton fluxes were calculated and found to be significantly stimulated by aldosterone (Fig. 1C).

**Aldosterone stimulates \( H^+ \)-ATPases in human OMCD cells.** Similar to the effect observed in freshly isolated mouse OMCD

---

**Table 2. Summary of Na\(^+ \)-independent \( \text{pH}_i \) alkalization rates of single intercalated cells in freshly isolated mouse outer medullary collecting ducts**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Initial ( \text{pH}_i )</th>
<th>( \text{Na}^+ )-Independent ( \text{pH}_i ) Recovery, ( \Delta \text{pH}/\text{min} )</th>
<th>Number of Cells, OMCDs, Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.31 ± 0.01</td>
<td>0.031 ± 0.002</td>
<td>122 (11/6)</td>
</tr>
<tr>
<td>Aldosterone 10 nM</td>
<td>7.30 ± 0.02</td>
<td>0.069 ± 0.002*</td>
<td>123 (15/13)</td>
</tr>
<tr>
<td>(20 min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldosterone 1 nM</td>
<td>7.39 ± 0.03</td>
<td>0.041 ± 0.004*</td>
<td>32 (3/3)</td>
</tr>
<tr>
<td>(3 min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldosterone 10 nM</td>
<td>7.29 ± 0.01</td>
<td>0.081 ± 0.006†</td>
<td>72 (4/3)</td>
</tr>
<tr>
<td>(3 min)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>7.26 ± 0.02</td>
<td>0.030 ± 0.002</td>
<td>82 (5/3)</td>
</tr>
</tbody>
</table>

Values are means ± SE. \( \text{pH}_i \), intracellular \( \text{pH}_i \); OMCDs, outer medullary collecting ducts. *\( P < 0.01 \), †\( P < 0.001 \) as compared with the respective control.
intercalated cells, aldosterone also stimulated H\(^+\)-ATPase activity in freshly isolated human OMCDs prepared from human kidney samples after nephrectomy. The basal Na\(^+\)-independent pH\(_{i}\) recovery rate was 0.028 ± 0.002 pH\(_{i}\) units/min (Table 3 and Fig. 2). After preincubation with 10 nM aldosterone for 20 min the intracellular pH\(_{i}\) recovery rate increased twofold to 0.067 ± 0.004 pH\(_{i}\) units/min (Table 3 and Fig. 2). Because of the limited access to human tissue, further studies were performed with mouse tissues. However, the results demonstrate that the stimulatory effect of aldosterone occurs also in human renal tissue and is not restricted to mouse OMCD intercalated cells.

The rapid stimulatory effect of aldosterone requires \(G\) proteins and phospholipase C activity. Aldosterone has been shown to require phospholipase C activity for its rapid effects in other tissues (6, 11). To investigate whether \(G\) proteins might be involved in the aldosterone-induced stimulation of H\(^+\)-ATPase activity in mouse OMCDs, suramin (200 μM), a general and relatively unspecific inhibitor of \(G\) proteins (32), was used. Preincubation with suramin (200 μM) alone had no effect on the basal rates of alkalinization, whereas it prevented completely the stimulatory effect of aldosterone (Table 4 and Fig. 3). Pertussis toxin (200 ng/ml), which inactivates more

Table 3. Summary of Na\(^+\)-independent pH\(_{i}\) alkalinization rates of single intercalated cells in freshly isolated human outer medullary collecting ducts

<table>
<thead>
<tr>
<th>Na(^+)-Independent pH, Number of Cells, Initial pH, Recovery, (\Delta)pH/min, OMCDs, Patients</th>
<th>Human OMCD, control</th>
<th>7.20 ± 0.01</th>
<th>0.028 ± 0.002</th>
<th>65 (5/4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human OMCD, aldosterone (10 nM)</td>
<td>7.19 ± 0.02</td>
<td>0.067 ± 0.004*</td>
<td>39 (3/1)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. *\(P < 0.001\) as compared with control.

Fig. 1. Aldosterone (Aldo) stimulates vacuolar H\(^+\)-ATPase activity in freshly isolated mouse outer medullary collecting duct (OMCD) intercalated cells. A: original intracellular pH (pH\(_{i}\)) tracing of single intercalated cells in freshly isolated mouse OMCDs. OMCDs were incubated with 10 nM aldosterone for 20 min or left untreated (control). The dotted line marks the increase in pH\(_{i}\) measured and used to calculate \(\Delta\)pH/min values. The bars above the tracing indicate solutions used. Initially, OMCD fragments were bathed in a solution containing sodium; after removal of sodium, an initial pH\(_{i}\) acidification could be observed. Addition of NH\(_4\)Cl to the bath caused a transient alkalinization, and upon its removal from the bath, a strong acidification, pH\(_{i}\) recovered slowly in the absence of sodium and more rapidly after readdition of sodium to the bath. B: summary bar graph of intracellular Na\(^+\)-independent pH\(_{i}\) alkalinization rates of mouse single intercalated cells. OMCDs were either incubated for 20 min with 10 nM aldosterone or aldosterone was added to the superfusate during the measurement 3 min before switching to Na\(^+\)-free solutions after the NH\(_4\)Cl-prepulse. Hydrocortisone (10 nM) was incubated for 20 min. C: summary of net proton fluxes (\(J_{p}\)) calculated from intracellular buffering power and \(\Delta\)pH/min for control and aldosterone incubated OMCD intercalated cells. Values are presented as means ± SE. **\(P < 0.01\), ***\(P < 0.001\).
Aldosterone (Table 4 and Fig. 3). Therefore, the rapid nongenomic (10 nM Aldosterone) was unable to prevent the stimulatory effect of aldosterone in mouse OMCD intercalated cells (75). Moreover, we have previously shown that cAMP stimulates H^+-ATPase activity and luminal accumulation in type A intercalated cells (55). Thus, in a last set of experiments, the role of cAMP and PKA in the aldosterone-specific Gq and Go G proteins, had no effect alone and did not prevent the stimulatory effect of aldosterone (Table 4 and Fig. 3). In contrast, GP-2A (20 μM), a small peptide inhibitor of Gq proteins (33), abolished the stimulatory effect of aldosterone, whereas it did not affect the basal activity (Table 4 and Fig. 3). Inhibition of phospholipase C activity with U-73122 (10 μM) completely impeded the stimulation by aldosterone (Table 4 and Fig. 3B). In contrast, the inactive analog U-73343 (10 μM) was unable to prevent the stimulatory effect of aldosterone (Table 4 and Fig. 3C). Thus, the rapid nongenomic action of aldosterone may require a Gq protein-coupled membrane receptor and phospholipase C activity.

Intracellular calcium, protein kinase C, and ERK1/2 participate in the stimulatory action of aldosterone. We had previously shown that aldosterone acutely and transiently raised intracellular Ca^{2+} in mouse OMCD intercalated cells (75). Therefore, we tested whether intracellular Ca^{2+} is involved in the stimulatory effect of aldosterone. Chelation of intracellular Ca^{2+} by preincubation of isolated OMCDs with BAPTA-AM abolished the stimulation of H^+-ATPase activity without altering the basal activity (Table 4 and Fig. 4).

Inhibition of PKC activity with chelerythrine (1 μM) prevented completely the stimulatory effect of aldosterone in mouse OMCDs. On the other hand, incubation with the PKC activator DOG (1 μM) mimicked the effect of aldosterone and increased H^+-ATPase activity twofold (Table 4 and Fig. 4).

We had previously shown that acute application of aldosterone increased luminal localization of H^+-ATPases in vivo (75). Here, collecting duct A-type intercalated cells of rats exhibited a similar response to aldosterone as after exposure to the cell-permeable cAMP analog CPT-cAMP (55). H^+-ATPase localization following aldosterone became more polarized, and apical H^+-ATPase immunostaining intensity increased markedly in most kidney regions, especially in the outer medulla (Fig. 5, A and C). Infusion of DOG had a similar effect as aldosterone alone (Fig. 5B). As previously reported for cAMP, the brighter apical staining was associated in many cells with the development of extensive microvilli that were easily detectable by conventional wide field fluorescence microscopy (Fig. 5C, see inset). The effect of aldosterone is augmented upon increasing its concentration from 200 nM to 200 μM (Fig. 5, see inset).

Aldosterone can interact in a nongenomic fashion with the mitogen-activated protein kinase (MAPK) signaling pathway involving ERK1/2 (20, 41, 51, 57). The stimulating effect of aldosterone on H^+-ATPase activity in intercalated cells of OMCDs was completely inhibited when the activation of ERK1/2 was prevented using PD098059 (20 μM), a specific inhibitor. Preincubation with PD098059 alone had no effect on H^+-ATPase activity (Table 4 and Fig. 6). To investigate the interaction between PKC and ERK1/2, we performed experiments using the PKC activator DOG (1 μM) together with the ERK1/2 inhibitor PD098059 (20 μM). The stimulation of H^+-ATPase activity was attenuated (Table 4 and Fig. 6), suggesting that ERK1/2 may act downstream of PKC.

Protein kinase A participates in aldosterone signaling. Conflicting reports exist concerning the role of cAMP and protein kinase A (PKA) activity in the rapid effects of aldosterone, suggesting that this pathway may be cell and target specific (12, 28, 38, 62). Moreover, we have previously shown that cAMP stimulates H^+-ATPase activity and luminal accumulation in type A intercalated cells (55). Thus, in a last set of experiments, the role of cAMP and PKA in the aldosterone-dependent pHi alkalinization rates in single intercalated cells in freshly isolated human OMCD intercalated cells. OMCDs were incubated with 10 nM aldosterone (10 nM, 20 min preincubation) is shown. Values are presented as means ± SE. ***P < 0.001.

### Table 4. Summary of Na^+-independent pH, alkalinization rates of single intercalated cells in freshly isolated mouse outer medullary collecting ducts in the absence and presence of different inhibitors of intracellular signaling molecules

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Initial pH</th>
<th>Na^+-Independent pH</th>
<th>No. of Cells, OMCDs, Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suramin</td>
<td>7.39 ± 0.02</td>
<td>0.029 ± 0.002</td>
<td>74 (5/3)</td>
</tr>
<tr>
<td>Suramin + aldosterone</td>
<td>7.40 ± 0.02</td>
<td>0.030 ± 0.002$§$</td>
<td>51 (4/3)</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>7.20 ± 0.01</td>
<td>0.031 ± 0.001</td>
<td>84 (5/3)</td>
</tr>
<tr>
<td>Pertussis toxin + aldosterone</td>
<td>7.28 ± 0.01</td>
<td>0.084 ± 0.004†</td>
<td>83 (5/5)</td>
</tr>
<tr>
<td>GP-2A</td>
<td>7.13 ± 0.01</td>
<td>0.024 ± 0.002</td>
<td>68 (4/2)</td>
</tr>
<tr>
<td>GP-2A + aldosterone</td>
<td>7.26 ± 0.01</td>
<td>0.017 ± 0.001$§$</td>
<td>92 (5/2)</td>
</tr>
<tr>
<td>U-73122</td>
<td>7.27 ± 0.01</td>
<td>0.031 ± 0.002</td>
<td>53 (3/5)</td>
</tr>
<tr>
<td>U-73122 + aldosterone</td>
<td>7.26 ± 0.01</td>
<td>0.026 ± 0.001$§$</td>
<td>116 (8/3)</td>
</tr>
<tr>
<td>U-73343 + aldosterone</td>
<td>7.22 ± 0.01</td>
<td>0.078 ± 0.004†</td>
<td>112 (12/8)</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>7.42 ± 0.02</td>
<td>0.036 ± 0.002</td>
<td>68 (4/3)</td>
</tr>
<tr>
<td>BAPTA-AM + aldosterone</td>
<td>7.35 ± 0.02</td>
<td>0.032 ± 0.002$§$</td>
<td>60 (5/4)</td>
</tr>
<tr>
<td>Chelerythrine</td>
<td>7.23 ± 0.02</td>
<td>0.028 ± 0.001</td>
<td>68 (4/3)</td>
</tr>
<tr>
<td>Chelerythrine + aldosterone</td>
<td>7.25 ± 0.01</td>
<td>0.026 ± 0.002$§$</td>
<td>77 (4/3)</td>
</tr>
<tr>
<td>DOG</td>
<td>7.38 ± 0.02</td>
<td>0.060 ± 0.003$†$</td>
<td>123 (10/5)</td>
</tr>
<tr>
<td>DOG + PD098059</td>
<td>7.42 ± 0.01</td>
<td>0.050 ± 0.003$§$</td>
<td>87 (5/3)</td>
</tr>
<tr>
<td>DOG + H89</td>
<td>7.33 ± 0.03</td>
<td>0.060 ± 0.005$†$</td>
<td>39 (3/5)</td>
</tr>
<tr>
<td>PD098059</td>
<td>7.23 ± 0.02</td>
<td>0.042 ± 0.003</td>
<td>86 (7/4)</td>
</tr>
<tr>
<td>PD098059 + aldosterone</td>
<td>7.27 ± 0.01</td>
<td>0.039 ± 0.003$§$</td>
<td>73 (6/3)</td>
</tr>
<tr>
<td>H89</td>
<td>7.16 ± 0.03</td>
<td>0.028 ± 0.003</td>
<td>26 (2/2)</td>
</tr>
<tr>
<td>H89 + aldosterone</td>
<td>7.26 ± 0.03</td>
<td>0.030 ± 0.003$§$</td>
<td>63 (5/3)</td>
</tr>
<tr>
<td>KT5720</td>
<td>7.16 ± 0.09</td>
<td>0.031 ± 0.002</td>
<td>82 (7/3)</td>
</tr>
<tr>
<td>KT5720 + aldosterone</td>
<td>7.22 ± 0.01</td>
<td>0.036 ± 0.005$§$</td>
<td>114 (7/4)</td>
</tr>
<tr>
<td>8-Br-cAMP</td>
<td>7.43 ± 0.01</td>
<td>0.047 ± 0.002$††$</td>
<td>110 (7/3)</td>
</tr>
<tr>
<td>8-Br-cAMP + chelerythrine</td>
<td>7.10 ± 0.08</td>
<td>0.024 ± 0.002$§$</td>
<td>92 (7/3)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Incubation with aldosterone (10 nM) was for 20 min before measurement of alkalinization rates. *P < 0.05 and †P < 0.001, significantly different from control; ‡P < 0.01 and $P < 0.001, significantly different from aldosterone alone.

**AJP-Cell Physiol • VOL 301 • NOVEMBER 2011 • www.ajpcell.org**
induced stimulation of H\(^+\)-ATPase activity in OMCD intercalated cells was examined. Inhibition of PKA activity with H89 (10 \(\mu\)M) or KT5720 (1 \(\mu\)M) completely prevented the aldosterone-induced stimulation of H\(^+\)-ATPase activity (Fig. 7 and Table 4). Incubation with the cell-permeable cAMP analog 8-bromoadenosine-cAMP (8-Br-cAMP, 50 \(\mu\)M) increased H\(^+\)-ATPase activity but to a lesser extent than aldosterone or DOG (Table 4 and Fig. 7). Inhibition of PKA with H89 in the presence of the PKC activator DOG (1 \(\mu\)M) did not prevent the stimulatory effect of DOG alone (Fig. 7B). In contrast, activation of PKA with 8-Br-cAMP in the presence of the PKC inhibitor chelerythrine (1 \(\mu\)M) did not stimulate H\(^+\)-ATPase activity. These results suggest that the cAMP/PKA-dependent pathway is involved in the aldosterone action and may act upstream of PKC.

DISCUSSION

Nongenomic effects of several steroid hormones including vitamin D\(_3\), sex hormones, glucocorticoids, and aldosterone have been identified in various tissues and cells (for review, see refs. 25–26, 48–50, 59, 74). Aldosterone is mainly involved in maintaining extracellular Na\(^+\) and K\(^+\) homeostasis by regulating intestinal and renal Na\(^+\) absorption and K\(^+\) excretion (68). Moreover, in the kidney, aldosterone also regulates acid secretion by acting on proximal tubular Na\(^+/\)H\(^+\) exchange and most importantly by stimulating H\(^+\)-secretion along the collecting
duct. Several studies have documented the chronic effects of aldosterone and aldosterone deficiency in rat and rabbit animal models as well as in cell culture and freshly isolated turtle bladder on H+/HCO₃⁻ exchanger, and the ability of the collecting duct to excrete protons (1, 13–14, 16, 19, 30, 36, 39, 42, 52, 61, 65). Some of these effects are likely mediated by changes in transcription and translation of proteins involved in acid-base transport or acting on these transporters (36).

The biological actions of these steroid hormones are mediated not only by changes in transcription but also by much faster nongenomic mechanisms. Distinct rapid effects of aldosterone on acid-base transport in several nephron segments and kidney-derived cell lines have been described. H⁺-ATPase activity is stimulated by aldosterone in freshly isolated rat late proximal tubule and mouse OMCD intercalated cells (44, 75), and Na⁺/H⁺ exchanger activity increased in the rat late proximal tubule (45), MDCK, and M1 cell lines (20, 22, 51). Similarly, aldosterone stimulates Na⁺/H⁺ exchange in freshly isolated colonic crypts (76). In contrast, in the thick ascending limb, aldosterone acutely inhibits bicarbonate absorption (23).

The intracellular signaling cascades mediating these non-genomic effects of aldosterone have been partly investigated and appear to differ between different cell types. However, most work has been done in cell culture models such as M1, MDCK, or vascular smooth muscle cells but much less information is available from freshly isolated cells and tissues. This may be important because it is well documented that cell lines harbor or lose characteristics not found or present in the native tissues (77). Thus, we investigated the signaling cascade mediating the stimulatory effect of aldosterone on H⁺-ATPase activity in freshly isolated mouse OMCD intercalated cells. We demonstrate that aldosterone stimulates within minutes H⁺-ATPase activity in mouse and human outer medullary collecting duct cells. Moreover, we show that in rat outer medullary collecting duct, aldosterone induces

Fig. 4. Aldosterone acts via intracellular Ca²⁺ and protein kinase C. Chelation of intracellular Ca²⁺ with BAPTA-AM (100 μM) prevented the aldosterone (10 nM) effect. Similarly, inhibition of protein kinase C activity with chelerythrine (Chel, 1 μM) completely blocked the stimulation by aldosterone. On the other hand, stimulation of PKC activity with DOG (1 μM) mimicked the effect of aldosterone. The values of control and aldosterone-treated OMCDs are shown again for comparison. Values are presented as means ± SE. ***P < 0.001 significantly different from control, ###P < 0.001 significantly different from aldosterone alone.

Fig. 5. Aldosterone or activation of protein kinase C enhances luminal H⁺-ATPase. Immunofluorescence labeling for the A subunit of the V-ATPase (red) in control (A), 20 μM DOG- (B), and 200 nM aldosterone-treated (C) rat OMCD intercalated cells. The nuclei are identified by DAPI staining (blue). While intercalated cells from control animals (A) exhibit extensive cytoplasmic staining and their apical membranes have a smooth appearance, extensively stained apical microvilli are readily detectable in the treated rats, and the cytosolic domain staining is less intense. An increased response is seen in the inset of C, which shows an intercalated cell from a rat treated with a higher concentration of aldosterone (200 μM). Bar = 20 μm.

Fig. 6. The stimulatory effect of aldosterone is mediated via ERK1/2 kinases. OMCDs were incubated with the ERK1/2 inhibitor PD098059 (20 μM) in the absence and presence of aldosterone (10 nM). PD098059 abolished the stimulatory effect of aldosterone. Coincubation of OMCDs with the PKC activator DOG (1 μM) and the ERK1/2 inhibitor PD098059 showed a small stimulation which was less pronounced than with DOG alone (see also Fig. 4). The values of control and aldosterone-treated OMCDs are shown again for comparison. Values are presented as means ± SE. ***P < 0.001 significantly different from control, ###P < 0.001 significantly different from aldosterone alone.
It has been postulated that specific receptors for steroid hormones may exist that initiate their nongenomic effects (6, 46). Some of the nongenomic effects of aldosterone and corticosterone involve membrane-bound mineralocorticoid receptors (25–26, 35). Moreover, GPR30, an orphan G protein-coupled receptor, has been recently identified as a receptor for estrogens and shown to mediate some of its nongenomic effects (31, 56, 67). GPR30 may also mediate the nongenomic effects of aldosterone in some tissues (24). Consistently, we found that the rapid stimulatory effect of aldosterone was inhibited by suramin and GP-2A, inhibitors of small G proteins. Suramin is an unspecific inhibitor of G proteins, whereas GP-2A specifically blocks Gaq proteins (33). Pertussis toxin was without effect, suggesting that Ga and Gs proteins are not involved and that the aldosterone effect involves Gaq proteins. In addition to G protein inhibition, blockade of phospholipase C activity by U-73122 also prevented the stimulatory effect of aldosterone. This effect of U-73122 was not achieved with the inactive analog U-73343. Recently, a direct stimulatory effect of U-73122 on human PLC has been described (37); however, it is unlikely that this occurred in our experiments since U-73122 had no effect on untreated OMCDs. Similarly, other groups have described evidence for a nongenomic receptor-mediated activation of intracellular signaling cascades by aldosterone that were abolished by inhibitors of G proteins and PLC (11, 22, 43, 76).

The effect of aldosterone on H+-ATPase activity was further mediated by Ca2+ and protein kinase C stimulation as evident from Fig. 8. Model of nongenomic signaling of aldosterone in mouse OMCD intercalated cells to stimulate H+-ATPase activity. Aldosterone stimulates H+-ATPase activity through a major pathway requiring Gaq proteins, phospholipase C, intracellular Ca2+, PKC, and ERK1/2. A second interlinked pathway involves cAMP and PKA. The PKA-dependent pathway is likely upstream of PKC and ERK1/2 because inhibition of this pathway blocks the cAMP/PKA-dependent stimulation of H+-ATPase activity. Chelation of Ca2+ may interfere at several stages because Ca2+ may be involved in stimulation of PKC isoforms as well as in the exocytic insertion of H+-ATPases into the membrane, which has been shown earlier to occur during stimulation of H+-ATPase activity (58, 75). Inhibitors and activators of signaling molecules used in this study are shown in italics.
from 1) the lack of stimulation after chelation of intracellular Ca\(^{2+}\) with BAPTA, 2) the previous observation that aldosterone induces an acute and transient rise in intracellular Ca\(^{2+}\) (75), 3) the inhibition of stimulation with the PKC inhibitor chelerythrine, and 4) the fact that the diacylglycerol analog DOG mimicked the stimulatory effect of aldosterone. Direct interactions of aldosterone with protein kinase C have been shown (3) but are unlikely to mediate the effect in OMCD intercalated cells since upstream inhibition of PLC completely abolishes the stimulatory effect. In some tissues and cells, the nongenomic effects of aldosterone involve activation of ERK1/2 MAPK kinases (20, 41, 57). In agreement with these observations, blockade of ERK1/2 phosphorylation by PD098059 strongly reduced the stimulation of H\(^{+}\)-ATPase activity. Furthermore, it appears that ERK1/2 acts downstream of PKC, because the concomitant incubation of OMCDs with DOG and PD098059 failed to stimulate H\(^{+}\)-ATPases. This blockade, however, was not complete, which may indicate either that ERK was not fully inhibited by PD098059 or that an alternative ERK-independent pathway may exist. Thus, H\(^{+}\)-ATPase activity in OMCD acid-secretory type A intercalated cells is stimulated by a PLC/Ca\(^{2+}\)/PKC-dependent pathway.

We recently described a very similar stimulatory pathway for angiotensin II in mouse OMCD cells (58), suggesting that it may represent a common pathway of H\(^{+}\)-ATPase stimulation in these cells. Stimulation of H\(^{+}\)-ATPase activity has also been demonstrated in a cell line derived from the rat inner medullary collecting duct requiring intracellular Ca\(^{2+}\), PLC, and PKC activity (53, 60, 63). Similar observations have been made in the epididymis (5). The activation of protein kinase C is sufficient to induce the rapid translocation of H\(^{+}\)-ATPases to the luminal side of type A intercalated cells as demonstrated by the DOG in vivo experiments. Consistently, we have previously shown that disruption of the microtubular network with colchicine abolishes the stimulatory effect of aldosterone (75) and colchicine administration in vivo disrupts H\(^{+}\)-ATPase trafficking (10). Whether calcium is also directly required for the trafficking and insertion of H\(^{+}\)-ATPases into the membrane remains to be further examined.

We and others have recently shown that the cAMP-dependent pathway stimulates H\(^{+}\)-ATPase activity in renal intercalated cells and induces its translocation into the apical membrane (4, 55). Similarly, blockade with two different inhibitors of a protein kinase A-dependent pathway reduced the stimulatory effect of aldosterone. Incubation of OMCDs with the cAMP analog 8-Br-cAMP stimulated H\(^{+}\)-ATPase activity but to a lesser extent than the PKC-dependent pathway. The stimulation produced by 50 \(\mu\)M 8-Br-cAMP could not be increased with higher concentrations, indicating that maximal stimulation had been reached. However, pharmacologic stimulation of the PKC-dependent cascade appears to be sufficient for the increase in H\(^{+}\)-ATPase activity because DOG in the presence of H89 enhanced vacuolar H\(^{+}\)-ATPase activity. In contrast, activation of PKA with 8-Br-cAMP and concomitant inhibition of PKC with chelerythrine prevented the stimulation of H\(^{+}\)-ATPase activity, suggesting that PKA requires intact PKC to be effective and that PKA may act upstream of PKC (see hypothetical model Fig. 8). Taken together, it appears that PKA may rather have a modulatory effect and that PKC may transmit the main signal. A mild stimulatory effect of a PKA-dependent pathway on bicarbonate absorption has been described previously in perfused rabbit outer medullary collecting duct and may thus reflect a physiologically relevant route of regulation of vacuolar H\(^{+}\)-ATPase activity (29).

Besides its well-documented effects on renal Na\(^{+}\) and K\(^{+}\) transport, aldosterone is an important regulator of renal acid secretion acting mainly on H\(^{+}\)-ATPases along the collecting duct. The significance of the stimulatory effect of aldosterone is highlighted by the fact that various inborn or acquired syndromes associated either with aldosterone deficiency or with insufficient aldosterone signaling in the kidney are invariably associated with distal renal tubular acidosis and hyperkalemia (15). It is possible that the rapid nongenomic and slower genomic stimulatory effects of aldosterone on H\(^{+}\)-ATPase activity may interact in several ways as is becoming increasingly clear for the effects of aldosterone on the major Na\(^{+}\)-transporting mechanisms in the collecting duct (43, 64). Thus, mechanisms identified here may not only lead to the rapid stimulation of H\(^{+}\)-ATPase activity but may also contribute subsequently to the activation of genomic events.

In summary, aldosterone rapidly stimulates H\(^{+}\)-ATPase activity in acid-secretory OMCD intercalated cells from mouse and human kidney and induces the net translocation of pumps to the luminal membrane. This nongenomic effect is mediated via a PLC/Ca\(^{2+}\)/PKC-dependent pathway but is interconnected with the cAMP-PKA cascade. ERK1/2 kinases are essential for stimulation. A membrane G\(_{q}\) protein-coupled receptor may be involved in initiating these cascades. The rapid nongenomic stimulation of H\(^{+}\)-ATPases by aldosterone may be part of the biological action of aldosterone in the kidney and contribute to the increase in acid secretion and control of systemic acid-base homeostasis.

GRANTS

This study was supported by Swiss National Research Foundation Grants 31-608318 and 31-109677/1 (to C. A. Wagner). This work was also supported by National Institutes of Health (NIH) Grants DK-73266 (to T. G. Paunescu) and DK-42956 (to D. Brown). The Microscopy Core Facility of the Program in Membrane Biology received additional support from the Boston Area Diabetes and Endocrinology Research Center (NIH DK-57521) and from the Center for the Study of Inflammatory Bowel Disease (NIH DK-43351).

DISCLOSURES

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

REFERENCES

C1260

ALDOSTERONE SIGNALING


