Genomic and nongenomic stimulatory effect of aldosterone on H⁺-ATPase in proximal S3 segments

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Leite-Dellova DC, Malnic G, Mello-Aires M. Genomic and nongenomic stimulatory effect of aldosterone on H⁺-ATPase in proximal S3 segments. Am J Physiol Renal Physiol 300: F682–F691, 2011. First published December 29, 2010; doi:10.1152/ajprenal.00172.2010.—The genomic and nongenomic effects of aldosterone on the intracellular pH recovery rate (pHirr) via H⁺-ATPase and on cytosolic free calcium concentration ([Ca²⁺]i) were investigated in isolated proximal S3 segments of rats during superfusion with an Na⁺-free solution, by using the fluorescent probes BCECF-AM and FLUO-4-AM, respectively. The pHirr, after cellular acidification with a NH₄Cl pulse, was 0.064 ± 0.003 pH units/min (n = 17/74) and was abolished with concanamycin. Aldosterone (10⁻¹², 10⁻¹⁰, 10⁻⁸, or 10⁻⁶ M with 1-h or 15- or 2-min preincubation) increased the pHirr. The baseline [Ca²⁺]i was 103 ± 2 nM (n = 58). After 1 min of aldosterone preincubation, there was a transient and dose-dependent increase in [Ca²⁺]i, and after 6-min preincubation there was a new increase in [Ca²⁺], that persisted after 1 h. Spironolactone [mineralocorticoid receptor (MR) antagonist], actinomycin D, or cycloheximide did not affect the effects of aldosterone (15- or 2-min preincubation) on pHirr and on [Ca²⁺], but inhibited the effects of aldosterone (1-h preincubation) on these parameters. RU 486 [glucocorticoid receptor (GR) antagonist] and dimethyl-BAPTA (Ca²⁺ chelator) prevented the effect of aldosterone on both parameters. The data indicate a genomic (1 h, via MR) and a nongenomic action (15 or 2 min, probably via GR) on the H⁺-ATPase and on [Ca²⁺]. The results are compatible with stimulation of the H⁺-ATPase by increases in [Ca²⁺], (at 10⁻¹²-10⁻⁶ M aldosterone) and inhibition of the H⁺-ATPase by decreases in [Ca²⁺], (at 10⁻¹² or 10⁻⁶ M aldosterone plus RU 486).

mineralocorticoid stimulatory action; renal vacuolar H⁺-ATPase; proximal tubule; pH; [Ca²⁺];

Although the effects of aldosterone on its target cells have for a long time been considered to be mediated exclusively though the genomic pathway, only a few years ago the nongenomic effect of this mineralocorticoid started to be described. The proximal tubule is responsible for the reabsorption of 80% of the filtered bicarbonate, a process that depends on the secretion of H⁺ ions by two major mechanisms: the Na⁺/H⁺ exchanger and H⁺-ATPase (4, 35, 39). Recently, studies from our laboratory demonstrated genomic and nongenomic effects of aldosterone on the Na⁺/H⁺ exchanger in vivo S2 (25) and in isolated S3 (19) segments of proximal tubule. However, the action of this hormone on H⁺-ATPase in the proximal tubule is not known. In the cortical and outer medullary collecting duct, prolonged exposure to aldosterone stimulates H⁺ secretion directly through the H⁺-ATPase and indirectly through increased absorption of Na⁺ (9, 13, 33); in addition, a rapid activation of H⁺-ATPase has also been described in intercalated renal tubule cells (38). The nongenomic effect of aldosterone, which has been shown in several epithelial and nonepithelial tissues, has the following characteristics: it is not sensitive to inhibitors of gene transcription and synthesis of protein and, in general, has a rapid effect (seconds to a few minutes) (11, 20, 36).

However, the receptor involved in the rapid responses to aldosterone is unknown and has been the theme of discussions (13); recently, data from our laboratory suggest that the glucocorticoid receptor (GR) can participate in nongenomic effects of aldosterone on the proximal tubule (19, 25). A role for aldosterone in regulating calcium homeostasis is uncertain, but a rise in intracellular Ca²⁺ concentration ([Ca²⁺]) is a component of several effects of aldosterone, as a second messenger on the hormonal nongenomic effects and as a prerequisite for the genomic action (3, 13, 19, 25, 29, 36, 38).

Considering that the physiological dose of aldosterone in blood is 10⁻¹⁰–10⁻⁸ M and that it can increase or decrease in conditions of extracellular volume modification, the purpose of this work was to investigate the genomic and nongenomic effects of aldosterone (10⁻¹², 10⁻¹⁰, 10⁻⁸, or 10⁻⁶ M with 1-h or 15- or 2-min preincubation) on H⁺ extrusion of proximal tubule through the H⁺-ATPase, the role of [Ca²⁺], in these processes, and the receptors responsible for initiating these hormonal responses. The experiments were done in isolated proximal S3 segments of rats, a less studied tubular portion, during tubular superfusion with Na⁺-free solution. The activity of the H⁺-ATPase was evaluated by the intracellular pH recovery rate (pHirr) after the acidification of pH, by an NH₄Cl pulse.

MATERIALS AND METHODS

Tubule Preparation

The approach for obtaining and superfusing rat S3 proximal tubules was similar to that described by Burg et al. (6), modified by Schafer et al. (30), and by our laboratory (19). This study was approved by the Biomedical Sciences Institute/USP-Ethical Committee for Animal Research (CEEA).

Male Wistar rats (90 g) were anesthetized with Tiletamine/Zolazepam (Zoletil-Virbac). Their kidneys were removed, and slices 2 mm in thickness were prepared and transferred to ice-cold normal Ringer solution. The segments were isolated by microdissection and transferred to glass coverslips prepared with poly-d-lysine (for tubule adhesion). The coverslips were mounted on an inverted microscope (Olympus IX70) in a thermostatically regulated perfusion chamber whose solutions were changed by means of valves. The integrity of the S3 segments was confirmed by histological analysis after the experiments.
Measurement of pHr

The S3 segments were first superfused with 140 mM Na⁺ control solution (solution 1), exhibiting the basal pHr. The pHr was examined after the acidification of pHr, with the NaHCl pulse technique (5) after 2-min exposure to 20 mM NH₄Cl (solution 3). The pHr was measured in the presence of 140 mM or 0 external Na⁺ (Na⁺; solution 4) alone or plus Schering 28080, concanamycin, ethanol, or aldosterone (10⁻¹², 10⁻¹⁰, 10⁻⁸, or 10⁻⁶ M, respectively, with 1-h or 15- or 2-min preincubation) alone or plus spironolactone, actinomycin D, cycloheximide, RU 486, or dimethyl-BAPTA. These drugs were added 15 min before the application of aldosterone. In all the experiments in the absence of Na⁺, we calculated the pHr starting from the time we observed the recovery of pHr, and for a period of 2 min. With the H⁺-ATPase, the recovery of pHr does not begin immediately after the acid pulse. Observing Fig. 1B, we note that, soon after the acid load no recovery of pHr is observed, but after ~1 min, a small and slow recovery of pHr begins. From this point on, we calculated pHr (dpHr/dt, pH units/min) using linear regression. Calculations and drawings were performed by an Excel program.

Measurement of [Ca²⁺].

The S3 segments were loaded for 15 min with 10 μM of the calcium-sensitive probe fluo 4-AM (23) at 37°C and rinsed in Tyrode’s 0 Na⁺ solution (solution 5). Fluo 4 intensity emitted above 505 nm was imaged by using laser excitation at 488 nm on a Zeiss LSM 510 confocal microscope. The images were continuously acquired (at time intervals of 2 s) before and after substitution of experimental solutions. Transformation of the fluorescence signal to [Ca²⁺] was performed by calibration with ionomicyn (5 μM; maximum Ca²⁺ concentration) followed by EGTA (2.5 mM; minimum Ca²⁺ concentration) according to the Grynkiewicz equation (12). This equation was originally used for fura 2 fluorescence, but in previous studies we have results indicating that the basal, low, and high levels of [Ca²⁺], measured from single-wavelength fluo 4 were similar to those measured from dual-wavelength fura 2 (23).

Solutions and Reagents

The solutions utilized are described in Table 1. They had an osmolarity of ~300 mosmol/kgH₂O and pH 7.4. BCECF-AM and fluo 4-AM were obtained from Molecular Probes (Eugene, OR). The other chemicals were purchased from Sigma (St. Louis, MO).

Statistics

The results are presented as means ± SE; N/ n is the number of tubules/number of tubular areas, and N is the number of tubules (each tubule is the average of 10 cell areas). Date were analyzed statistically by analysis of variance followed by the Bonferroni’s contrast test. Differences were considered significant if P < 0.05.

RESULTS

Acid Extrusion

Figure 1A shows a representative experiment in which S3 segments were first bathed with 140 mM Na⁺ solution, exhibiting the basal pHr. After 2-min exposure to NH₄Cl, during which cell pHr increased transiently, the removal of NH₄Cl caused a rapid acidification of pHr as a result of NH₃ efflux. In the presence of external 140 mM Na⁺, the initial fall in pHr was followed by a recovery toward the basal value. Figure 1B shows that, in the absence of external Na⁺, the pHr was markedly decreased (and the final pHr was lower than the basal value). This effect is reversed with the return of Na⁺ to the bathing solution (and the final pHr was not different from the basal value). Figure 1C indicates that in the absence of external Na⁺ plus concanamycin (a H⁺-ATPase inhibitor) the pHr was abolished. Tables 2, 3, and 4 show the basal pHr found in the experimental groups. The results indicate that the S3 segment in pH 7.4 HCO₃⁻-free solution has a mean pHr of 7.09 ± 0.008 (N/ n = 364/1578). Figure 2 indicates that in the control situation (140 mM Na⁺) the pHr was 0.176 ± 0.007 pH units/min (N/ n = 23/115). In the absence of Na⁺, the pHr

Table 1. Composition of solutions

<table>
<thead>
<tr>
<th>Solution 1 (Control)</th>
<th>Solution 2 (K⁺-HEPES)</th>
<th>Solution 3 NH₄Cl</th>
<th>Solution 4 (0 Na⁺)</th>
<th>Solution 5 (0 Na⁺ Tyrode’s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>140</td>
<td>20.0</td>
<td>120</td>
<td>140</td>
</tr>
<tr>
<td>K2HPO4</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO4</td>
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<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5.5</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>HEPEs</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>130</td>
<td>130</td>
<td>130</td>
<td>130</td>
</tr>
<tr>
<td>KCl</td>
<td>2.68</td>
<td>2.68</td>
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<tr>
<td>NaHCO³</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Na2HPO4</td>
<td>0.36</td>
<td>0.36</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>Nigericin</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Values are expressed in mM. Na⁺, external Na⁺; NMDG, N-methyl-D-glucamine. HCl or NaOH were used in all Na⁺-containing solutions to titrate to the appropriate pH, and KOH was used in Na⁺-free solution.
Effect of Aldosterone on H⁺-ATPase

One hour of hormonal preincubation. The main pHᵢ responses found in these groups are in Table 2. Figure 3 indicates that aldosterone (10⁻¹², 10⁻¹⁰, 10⁻⁸, or 10⁻⁶ M, 1-h preincubation) caused a significant increase in pHᵢrr (of ~100, 108, 73, and 46% of the control value, respectively). Figure 3 also indicates that with ethanol alone (in the concentration of hormonal solvent we used), 1-h preincubation) the pHᵢrr was not different from the control value. Figure 4A shows that in presence of 1-h preincubation with spironolactone [10⁻⁶ M, a mineralocorticoid receptor (MR) antagonist], actinomycin D (10⁻⁶ M, an inhibitor of gene transcription), or cycloheximide (40 mM, an inhibitor of protein synthesis) alone the pHᵢrr was not different from the control value. However, these agents prevented the effect of aldosterone (10⁻¹² or 10⁻⁶, 1-h preincubation) on the H⁺-ATPase. These results show that the stimulatory effect of a 1-h preincubation with aldosterone on the H⁺-ATPase is genomic.

Fifteen minutes of hormonal preincubation. The main pHᵢ responses found in these groups are in Table 3. Figure 5 shows that in the presence of 15-min preincubation with spironolactone, actinomycin D, or cycloheximide alone the pHᵢrr was not different from the control value. These drugs also did not prevent the effect of aldosterone (10⁻¹² or 10⁻⁶, 15-min preincubation) on the H⁺-ATPase. These data indicate that the stimulatory effect of aldosterone 15-min preincubation on the H⁺-ATPase is nongenomic. RU 486 also prevented the effect of aldosterone (10⁻¹² or 10⁻⁶, 15-min preincubation) on the H⁺-ATPase. These results indicate that the nongenomic stimulatory effect of aldosterone 15-min preincubation on the H⁺-ATPase probably is via GR.

Two minutes of hormonal preincubation. The main pHᵢ responses found in these groups are in Table 4. Figure 7A indicates that aldosterone (10⁻¹² or 10⁻⁶ M, 2-min preincubation) increased the pHᵢrr (of ~98 and 85% of the control value, respectively). Figure 7A also indicates that spironolactone, actinomycin D, or cycloheximide did not prevent the effect of aldosterone (10⁻¹² or 10⁻⁶ M, 2-min preincubation) on the H⁺-ATPase. RU 486 also prevented the effect of aldosterone (10⁻¹² or 10⁻⁶ M, 2-min preincubation), confirming that this GR antagonist affects the rapid nongenomic stimulatory effect of aldosterone on H⁺-ATPase.

Effect of Aldosterone on \([Ca^{2+}]_i\)

Figure 8 shows cell calcium fluorescent signal tracing during three representative experiments. The basal value did not change after 1 or 15 min or 1 h of preincubation with Tyrode
Table 2. Summary of pH$_i$ measurements in proximal S3 segments of a rat after aldosterone 1-h preincubation and acute acid load

<table>
<thead>
<tr>
<th></th>
<th>Basal pH</th>
<th>pH$_i$ Acid Load</th>
<th>Final pH$_i$</th>
<th>N/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (140 mM Na$_+]i$)</td>
<td>7.17 ± 0.015</td>
<td>6.35 ± 0.062</td>
<td>7.12 ± 0.024</td>
<td>23/115</td>
</tr>
<tr>
<td>ONa$_-$</td>
<td>7.16 ± 0.019</td>
<td>6.00 ± 0.036</td>
<td>6.36 ± 0.045*</td>
<td>17/74</td>
</tr>
<tr>
<td>Concanamycin</td>
<td>7.10 ± 0.021</td>
<td>6.11 ± 0.028</td>
<td>6.15 ± 0.032*</td>
<td>6/24</td>
</tr>
<tr>
<td>Ethanol</td>
<td>7.33 ± 0.042</td>
<td>6.03 ± 0.028</td>
<td>6.37 ± 0.088*</td>
<td>9/30</td>
</tr>
<tr>
<td>Aldosterone 10$^{-12}$ M</td>
<td>7.19 ± 0.016</td>
<td>6.20 ± 0.049</td>
<td>6.49 ± 0.036*</td>
<td>9/47</td>
</tr>
<tr>
<td>Aldosterone 10$^{-10}$ M</td>
<td>7.03 ± 0.039</td>
<td>6.00 ± 0.072</td>
<td>6.47 ± 0.049*</td>
<td>6/37</td>
</tr>
<tr>
<td>Aldosterone 10$^{-8}$ M</td>
<td>7.06 ± 0.034</td>
<td>6.06 ± 0.069</td>
<td>6.52 ± 0.056*</td>
<td>7/47</td>
</tr>
<tr>
<td>Aldosterone 10$^{-6}$ M</td>
<td>7.16 ± 0.042</td>
<td>6.12 ± 0.044</td>
<td>6.46 ± 0.067*</td>
<td>8/40</td>
</tr>
<tr>
<td>Spironolactone 10 mM</td>
<td>7.13 ± 0.019</td>
<td>6.00 ± 0.057</td>
<td>6.31 ± 0.036*</td>
<td>5/18</td>
</tr>
<tr>
<td>+ Aldo 10$^{-12}$ M</td>
<td>7.10 ± 0.018</td>
<td>6.15 ± 0.080</td>
<td>6.56 ± 0.087*</td>
<td>5/22</td>
</tr>
<tr>
<td>+ Aldo 10$^{-6}$ M</td>
<td>7.09 ± 0.024</td>
<td>6.14 ± 0.059</td>
<td>6.41 ± 0.060*</td>
<td>5/29</td>
</tr>
<tr>
<td>Actinomycin D 10$^{-6}$ M</td>
<td>7.02 ± 0.030</td>
<td>6.00 ± 0.042</td>
<td>6.35 ± 0.045*</td>
<td>5/14</td>
</tr>
<tr>
<td>+ Aldo 10$^{-12}$ M</td>
<td>7.12 ± 0.015</td>
<td>6.10 ± 0.041</td>
<td>6.48 ± 0.049*</td>
<td>11/56</td>
</tr>
<tr>
<td>+ Aldo 10$^{-6}$ M</td>
<td>7.09 ± 0.007</td>
<td>6.05 ± 0.093</td>
<td>6.23 ± 0.049*</td>
<td>5/12</td>
</tr>
<tr>
<td>Cycloheximide 40 mM</td>
<td>7.11 ± 0.019</td>
<td>6.12 ± 0.044</td>
<td>6.39 ± 0.041*</td>
<td>5/19</td>
</tr>
<tr>
<td>+ Aldo 10$^{-12}$ M</td>
<td>7.18 ± 0.034</td>
<td>6.11 ± 0.044</td>
<td>6.30 ± 0.030*</td>
<td>6/26</td>
</tr>
<tr>
<td>+ Aldo 10$^{-6}$ M</td>
<td>7.13 ± 0.023</td>
<td>6.21 ± 0.095</td>
<td>6.43 ± 0.099*</td>
<td>5/22</td>
</tr>
</tbody>
</table>

Values are means ± SE; N/s = no. of tubules/no. of areas; pH$_i$, intracellular pH; Aldo, aldosterone. Spironolactone is a mineralocorticoid receptor antagonist. Actinomycin D is an inhibitor of gene transcription. Cycloheximide is an inhibitor of protein synthesis. Ethanol is shown in the concentration of hormonal solvent we used. *P < 0.001 vs. respective to basal pH$_i$.

0 Na$_+]i$ solution. However, after ~1 min of addition of aldosterone (10$^{-12}$ or 10$^{-6}$ M) to the bath there was a transient (~1.5 min) and dose-dependent increase in cell calcium fluorescent signal, followed by a recovery toward the basal value. After ~5 min of preincubation with aldosterone (10$^{-12}$ or 10$^{-6}$ M), the cell calcium fluorescent signal begins to increase, after 15 min it was significantly higher, mainly for 10$^{-6}$ M aldosterone, and after 1 h of hormonal preincubation these values did not significantly change. The data show that the S3 segment exhibited a mean baseline [Ca$_{2+]i$] of 103 ± 2 nM (n = 58). In the presence of spironolactone, actinomycin D, or cycloheximide (2- or 15-min or 1-h preincubation) alone, the [Ca$_{2+]i$] was not different from the control value (Figs. 7B, 6B, and 4B, respectively). Also, the stimulatory effect of aldosterone 2- or 15-min preincubation on [Ca$_{2+]i$] was not modified by these drugs (Figs. 7B and 6B, respectively). However, after 1 h of hormonal preincubation the [Ca$_{2+]i$] was significantly reduced by these inhibitors (Fig. 4B). In addition, RU 486 alone caused a significant decrease in the [Ca$_{2+]i$] (of ~26% of the control value) and prevented the stimulatory effect of aldosterone (10$^{-12}$ or 10$^{-6}$ M, 2- or 15-min preincubation) on the [Ca$_{2+]i$] (Figs. 7B and 6B, respectively).

Effect of Dimethyl-BAPTA on H$^+$-ATPase and [Ca$_{2+]i$].

To confirm whether blocking the increase in [Ca$_{2+]i$] would affect the aldosterone stimulatory effect on H$^+$-ATPase, we performed experiments in which dimethyl-BAPTA (5 × 10$^{-5}$ M) was added to our preparation, since this agent is known to markedly decrease cell Ca$_{2+]i$} in many tissues. Table 5 shows that dimethyl-BAPTA-AM (2-min or 1-h preincubation) alone does not affect the pH$_{irr}$, although it causes a significant decrease in [Ca$_{2+]i$]. Table 5 also indicates that dimethyl-BAPTA-AM decreases the stimulatory effect of aldosterone (10$^{-12}$ or 10$^{-6}$ M, 2-min or 1-h preincubation) on the pH$_{irr}$ and on [Ca$_{2+]i$]. These results suggest a role of the increase in...
[Ca\(^{2+}\)] in regulating the dose-dependent stimulatory effect of aldosterone on H\(^{+}\)-ATPase.

### DISCUSSION

The present results demonstrate that the cells of the rat proximal S3 segment, in pH 7.4 HCO\(_3^{-}\)-free solution, maintain a baseline pHi of 7.09 ± 0.008 (N/n = 364/1578), a value compatible with the data found in the S3 segment of the rabbit (21). Our results, indicating that in the control situation (140 mM Na\(^{+}\)) the mean pHirr is 0.176 ± 0.007 pH units/min (N/n = 23/115), are in accordance with the values found by us in Madin-Darby canine kidney (MDCK) cells, a cell line with similarities to the mammalian distal nephron (22).

The present data show that the removal of Na\(^{+}\) resulted in inhibition of the pHirr that is subsequently reversed with the return of Na\(^{+}\) to the bathing solution, indicating that the pHirr in the S3 segment is mostly dependent on the Na\(^{+}\)/H\(^{+}\) exchanger. However, even in the absence of Na\(^{+}\) a significant pHirr of 0.064 ± 0.003 pH units/min (N/n = 17/34) was still observed. This pHirr observed in the absence of Na\(^{+}\) was abolished by concanamycin and was not changed by Schering 28080, indicating that the H\(^{+}\)-ATPase is solely responsible for the Na\(^{+}\)-independent H\(^{+}\) extrusion mechanism in S3 segment of normal rats.

Our data show that aldosterone (1-h, 15-min, or 2-min preincubation) at a low (10^{-12}, 10^{-8} M) or high (10^{-6} M) concentration causes a significant increase in pHirr. This stimulatory effect we observed is only due to aldosterone since in the presence of ethanol alone (1-h preincubation), in a similar concentration we used to prepare the hormonal solution, the basal pHi and the pHirr were not different from the control values. Our present results are in accordance with data indicating that aldosterone (10^{-8} M for 24 h) increases H\(^{+}\)-ATPase activity in MDCK-C11 cells (7) and that this hormone (10 mM, 15-min preincubation) causes a rapid stimulatory effect on H\(^{+}\)-ATPase activity in isolated outer medullary collecting ducts (38) and increases the activity of vacuolar H\(^{+}\)-ATPase in turtle urinary bladder (1). However, the present study is the first demonstration, to our knowledge, that aldosterone (10^{-12}, 10^{-10}, 10^{-8}, or 10^{-6} M, 1-h preincubation) or ethanol (in the concentration of hormonal solvent we used, 1-h preincubation) on the initial pHi recovery rate after cellular acidification, in the presence of 0 Na\(^{+}\) external solution (Na\(^{+}\)), in S3 segment of a rat proximal tubule. Values are means ± SE; N/n = no. of tubules/no. of areas. *P < 0.001 vs. respective to basal pHi.

### Table 4. Summary of pHi measurements in proximal S3 segments of a rat after aldosterone 2-min preincubation and acute acid load

<table>
<thead>
<tr>
<th>Basal pHi</th>
<th>pH Acid Load</th>
<th>Final pHi</th>
<th>N/n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0Na(^{+})</td>
<td>7.16 ± 0.019</td>
<td>6.00 ± 0.056</td>
<td>6.36 ± 0.045</td>
</tr>
<tr>
<td>Aldosterone 10^{-12} M</td>
<td>7.11 ± 0.016</td>
<td>6.07 ± 0.048</td>
<td>6.36 ± 0.067*</td>
</tr>
<tr>
<td>Aldosterone 10^{-6} M</td>
<td>7.10 ± 0.017</td>
<td>6.07 ± 0.033</td>
<td>6.43 ± 0.063*</td>
</tr>
<tr>
<td>Spironolactone 10 µM</td>
<td>7.08 ± 0.019</td>
<td>6.22 ± 0.107</td>
<td>6.53 ± 0.066*</td>
</tr>
<tr>
<td>+ Aldo 10^{-12} M</td>
<td>7.08 ± 0.011</td>
<td>6.08 ± 0.094</td>
<td>6.50 ± 0.054*</td>
</tr>
<tr>
<td>+ Aldo 10^{-6} M</td>
<td>7.05 ± 0.015</td>
<td>6.05 ± 0.045</td>
<td>6.51 ± 0.075*</td>
</tr>
<tr>
<td>Actinomycin D 10^{-6} M</td>
<td>7.07 ± 0.022</td>
<td>6.13 ± 0.065</td>
<td>6.57 ± 0.041*</td>
</tr>
<tr>
<td>+ Aldo 10^{-12} M</td>
<td>7.06 ± 0.012</td>
<td>6.24 ± 0.024</td>
<td>6.43 ± 0.039*</td>
</tr>
<tr>
<td>+ Aldo 10^{-6} M</td>
<td>7.09 ± 0.047</td>
<td>6.15 ± 0.033</td>
<td>6.36 ± 0.055*</td>
</tr>
<tr>
<td>Cycloheximide 40 mM</td>
<td>7.08 ± 0.013</td>
<td>6.14 ± 0.161</td>
<td>6.40 ± 0.141*</td>
</tr>
<tr>
<td>+ Aldo 10^{-12} M</td>
<td>7.08 ± 0.017</td>
<td>6.14 ± 0.063</td>
<td>6.40 ± 0.070*</td>
</tr>
<tr>
<td>+ Aldo 10^{-6} M</td>
<td>7.09 ± 0.022</td>
<td>6.00 ± 0.025</td>
<td>6.30 ± 0.058*</td>
</tr>
<tr>
<td>RU 486 10^{-6} M</td>
<td>7.05 ± 0.008</td>
<td>6.05 ± 0.028</td>
<td>6.15 ± 0.055*</td>
</tr>
<tr>
<td>+ Aldo 10^{-12} M</td>
<td>7.04 ± 0.015</td>
<td>6.03 ± 0.021</td>
<td>6.14 ± 0.054*</td>
</tr>
<tr>
<td>+ Aldo 10^{-6} M</td>
<td>7.07 ± 0.015</td>
<td>6.04 ± 0.019</td>
<td>6.15 ± 0.075*</td>
</tr>
</tbody>
</table>

Values are means ± SE; N/n = no. of tubules/no. of areas. *P < 0.005 vs. respective to basal pHi.
Aldosterone has a stimulatory effect on proximal H^+-ATPase. Thus it is possible that the stimulatory effect of aldosterone on the H^+-ATPase may contribute to upregulation of HCO_3^- reabsorption along the proximal tubule. The present results indicate that spironolactone, actinomycin D, or cycloheximide alone did not show effects on the pHirr and on the [Ca^{2+}]_i and failed to prevent the effects of aldosterone (10^{-12} and 10^{-6} M, 15- or 2-min preincubation) on these parameters. However, these drugs prevent the effects of aldosterone (10^{-12} and 10^{-6} M, 1-h preincubation) on these parameters. Thus our study indicates that aldosterone has a stimulatory effect on the H^+-ATPase via a nongenomic (15- or 2-min preincubation) and a genomic (1-h preincubation) pathway. The present data are compatible with our recent demonstration of an aldosterone nongenomic (15- or 1-min preincubation) and genomic (1-h preincubation) effect on the Na^{+}/H^{+} exchanger in S2 (25) and S3 (19) segments of rat proximal tubule.

The genomic aldosterone-induced regulation of the H^+-ATPase seems to be a mineralocorticoid specific effect, since our data showed that spironolactone inhibits the aldosterone (1-h preincubation) effect on H^+-ATPase, as had been observed in MDCK-C11 cells (7). The present results are in accordance with our finding of MR in the S3 segment by RT-PCR (19). Our data are also consistent with the studies of Krug et al. (18) and Todd-Turla et al. (31) that had reported mRNA and protein expression of the MR in the proximal tubule, and with the results of Pinto et al. (27) that confirmed the presence of the MR transcript by PCR in proximal tubular cells of rats.

The possible involvement of MR in mediating some rapid responses has been suggested for steroid hormones (8, 36), but some of the rapid responses of aldosterone are detected in MR...
knockout mice (15) or are not blocked by specific antagonists of the classic MR (38). The rapid increase in nongenomic activity of the H^{+}-ATPase that we observed seems not to occur via MR, because spironolactone failed to prevent this effect. To improve our knowledge of this mechanism, we used RU 486, since it is known that aldosterone-induced effects may also occur via GR and the cloning of the MR revealed that its sequence is highly homologous to the GR (2, 28), although the

![Graph A](http://ajprenal.physiology.org/)

**Fig. 6.** Effect of aldosterone (10^{-12} or 10^{-6} M, 15-min preincubation) alone or plus spironolactone (10 \mu M, a mineralocorticoid receptor antagonist), actinomycin D (10^{-6} M, an inhibitor of gene transcription), cycloheximide (40 mM, an inhibitor of protein synthesis), or RU 486 (10^{-6} M, a glucocorticoid receptor antagonist) on the initial pHi recovery rate after cellular acidification (A) and [Ca^{2+}]_i (B), in the presence of 0 Na^+, in S3 segment of rat proximal tubule. Values are means ± SE; N/n = no. of tubules/no. of areas; \(N\) = no. of tubules (each tubule is the average of 10 cells areas). *\(P < 0.01\) vs. respective control. &\(P < 0.001\) vs. aldosterone (10^{-12} M). *\(P < 0.001\) vs. aldosterone (10^{-6} M).
affinity of the GR for aldosterone is lower. Our results indicate that probably the GR participate in the direct nongenomic (2 or 15 min) stimulatory effect of aldosterone on the H⁺-ATPase. These results are also in accordance with our data indicating the presence of GR in S3 segments by RT-PCR (19). However, the possible physiological role of GR action should also be considered since RU 486 alone reduces the pHirr and the [Ca²⁺], below the basal levels.

The present data indicate that the baseline [Ca²⁺] was 103 ± 2 nM (n = 58), and the stimulatory effect of aldosterone on the H⁺-ATPase is associated with an increase in [Ca²⁺], similar to that observed with angiotensin II (35) and arginine vasopressin (7, 24). These results are also in accordance with several authors who found an aldosterone-induced increase in [Ca²⁺], (10, 14, 17, 19, 26, 37), indicating that intracellular calcium is a prerequisite for aldosterone action. The increase in [Ca²⁺], might initiate events that lead to activation of H⁺-ATPase (16), and the cell acidification stimulates a calcium-mediated exocytic insertion of proton pumps, a process important for pH_i regulation (34). Our results with RU 486 also confirm this behavior since 1) this GR antagonist alone caused a significant decrease in the pHirr and in [Ca²⁺], and 2) RU 486 prevented the stimulatory effect of aldosterone (10⁻¹² or 10⁻⁶ M, 15- or 2-min preincubation) on both parameters.

The high dose of aldosterone used in these experiments (10⁻⁶ M) was responsible for the higher increase in [Ca²⁺], but we did not observe the concomitant higher increase in H⁺-ATPase activity. 1) in the short period of hormonal preincubation (15 or 2 min), the low (10⁻¹² M) and the high (10⁻⁶ M) hormonal doses similarly stimulated the H⁺-ATPase, but 2) in the long period of hormonal preincubation (1 h), the high dose of aldosterone (10⁻⁶ M) caused a lower stimulation on the H⁺-ATPase compared with the low hormonal dose (10⁻¹² M). These observations suggest that an additional signaling mechanism is activated in the presence of a high dose of aldosterone that interferes with the well-known stimulatory effect of calcium on H⁺-ATPase. This question remains to be studied.

On the other hand, our present data showing a nongenomic effect of aldosterone on [Ca²⁺], in the S3 segment are similar to data showing that exposure of intercalated renal tubule cells to aldosterone (10⁻⁶ M) promotes a rapid transient increase in [Ca²⁺], to 34% of the control value (38). Additionally, our findings indicating a genomic effect of aldosterone (10⁻⁶ M, 1-h preincubation) in increasing [Ca²⁺], in the S3 segment are

Table 5. Effect of aldosterone alone or plus dimethyl-BAPTA-AM on pH_i recovery mediated by H⁺-ATPase (dpH_i/dt) and cytosolic calcium ([Ca²⁺]) in isolated proximal S3 segments of a rat

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>dpH_i/dt, pH units/min</th>
<th>[Ca²⁺], nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0Na⁺</td>
<td>0.067 ± 0.006 (N/n = 10/22)</td>
<td>104 ± 2 (n = 22)</td>
</tr>
<tr>
<td>Aldo 10⁻¹² M 2-min preincubation</td>
<td>0.12 ± 0.016 (N/n = 6/12)</td>
<td>160 ± 5 (n = 6)</td>
</tr>
<tr>
<td>Aldo 10⁻⁶ M 2-min preincubation</td>
<td>0.11 ± 0.006 (N/n = 6/11)</td>
<td>234 ± 5 (n = 7)</td>
</tr>
<tr>
<td>BAPTA 5 × 10⁻⁵ M 2-min preincubation</td>
<td>0.059 ± 0.006 (N/n = 6/17)</td>
<td>50 ± 1 (n = 11)</td>
</tr>
<tr>
<td>+ Aldo 10⁻¹² M 2-min preincubation</td>
<td>0.053 ± 0.005 (N/n = 5/19)</td>
<td>78 ± 3 (n = 5)</td>
</tr>
<tr>
<td>+ Aldo 10⁻⁶ M 2-min preincubation</td>
<td>0.071 ± 0.003 (N/n = 5/18)</td>
<td>113 ± 1.1 (n = 6)</td>
</tr>
<tr>
<td>Aldo 10⁻⁷ M 1-h preincubation</td>
<td>0.12 ± 0.005 (N/n = 5/18)</td>
<td>224 ± 3 (n = 5)</td>
</tr>
<tr>
<td>Aldo 10⁻⁸ M 1-h preincubation</td>
<td>0.12 ± 0.006 (N/n = 5/15)</td>
<td>353 ± 5 (n = 5)</td>
</tr>
<tr>
<td>BAPTA 5 × 10⁻⁵ M 1-h preincubation</td>
<td>0.066 ± 0.004 (N/n = 6/20)</td>
<td>51 ± 1 (n = 5)</td>
</tr>
<tr>
<td>+ Aldo 10⁻¹² M 1-h preincubation</td>
<td>0.068 ± 0.003 (N/n = 5/21)</td>
<td>113 ± 2.4 (n = 5)</td>
</tr>
<tr>
<td>+ Aldo 10⁻⁶ M 1-h preincubation</td>
<td>0.092 ± 0.003 (N/n = 5/24)</td>
<td>169 ± 2.6 (n = 5)</td>
</tr>
</tbody>
</table>

Values are means ± SE; N/n = no. of tubules/no. of tubular areas; n = no. of tubules (each tubule is the average of 10 cell areas). ¹P < 0.05 vs. 0Na⁺. ²P < 0.01 vs. Aldo 10⁻¹² M 2-min preincubation. ³P < 0.001 vs. Aldo 10⁻⁶ M 2-min preincubation. ⁴P < 0.001 vs. Aldo 10⁻¹² M 1-h preincubation. ⁵P < 0.01 vs. Aldo 10⁻⁸ M 1-h preincubation. ⁶P < 0.001 vs. BAPTA 1-h preincubation. ⁷P < 0.001 vs. 0Na⁺. ⁸P < 0.001 vs. Aldo 10⁻¹² M 2-min preincubation. ⁹P < 0.001 vs. Aldo 10⁻⁶ M 1-h preincubation. ¹⁰P < 0.001 vs. BAPTA 2-min preincubation.
similar to results indicating that exposure of A6D2 cells to aldosterone (10⁻⁶ M, 60- to 70-min preincubation) promotes an increase in [Ca²⁺], that was inhibited in magnitude by actinomycin and cycloheximide, suggesting that aldosterone induces an increase in [Ca²⁺], via a process dependent on mRNA and protein synthesis (26). However, the effect of a long-term increase in [Ca²⁺] is still not adequately explained.

Our present results suggest that dimethyl-BAPTA-AM alone does not affect the pHirr since it causes a decrease in [Ca²⁺], to ~50% of the control value that by itself does not impair cellular H⁺ secretion. However, dimethyl-BAPTA-AM impairs the effect of aldosterone on the pHirr since it impairs the increase in [Ca²⁺], in response to aldosterone, thus modulating the cellular action of this hormone. However, with aldosterone (10⁻⁶ M, 1-h preincubation) plus dimethyl-BAPTA-AM the pHirr shows a significant change (P < 0.001) against dimethyl-BAPTA-AM (1-h preincubation) alone. This finding is probably due to the significant increase in [Ca²⁺], observed with aldosterone (10⁻⁶ M, 1-h preincubation) plus dimethyl-BAPTA-AM (~60% above the control value), which is large enough to stimulate the H⁺-ATPase.

In summary, the present study on the proximal S3 segment of the rat suggests a role for cell calcium in regulating the process of pHirr after the acid load induced by NH₄Cl, mediated by the H⁺-ATPase and stimulated by aldosterone (10⁻¹², 10⁻¹⁰, 10⁻⁸ or 10⁻⁶ M). The data indicate a hormonal genomic (1 h) and nongenomic (15 or 2 min) action on the H⁺-ATPase and on [Ca²⁺]. They are compatible with stimulation of the H⁺-ATPase by increases in [Ca²⁺], at the lower (10⁻¹² M) and high (10⁻⁶ M) aldosterone levels or inhibition of the H⁺-ATPase by decreases in [Ca²⁺], (at 10⁻¹² or 10⁻⁶ M aldosterone, 15- or 2-min preincubation, plus RU 486). The present results indicating that MR and probably GR participate, respectively, in genomic and nongenomic effects of aldosterone on [Ca²⁺], and on the H⁺-ATPase are in accordance with our previous findings indicating the presence of these receptors in S3 segments (19). These aldosterone effects may represent physiologically relevant regulation of bicarbonate reabsorption along the proximal tubule.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES


