Direct action of aldosterone on bicarbonate reabsorption in in vivo cortical proximal tubule

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Pergher PS, Leite-Dellova D, de Mello-Aires M. Direct action of aldosterone on bicarbonate reabsorption in vivo cortical proximal tubule. Am J Physiol Renal Physiol 296:F1185–F1193, 2009. First published February 18, 2009; doi:10.1152/ajpren.09217.2008.—The direct action of aldosterone (10^{-12} M) on net bicarbonate reabsorption (J_{HCO_3}^{\text{in}}) was evaluated by stationary microperfusion of an in vivo middle proximal tubule (S2) of rat kidney, using H ion-sensitive microelectrodes. Aldosterone in luminally perfused tubules caused a significant increase in J_{HCO_3}^{\text{in}} from a mean control value of 2.84 ± 0.08 [49/19 (n of measurements/n of tubules)] to 4.20 ± 0.15 mmol·cm^{-2}·s^{-1} (58/10). Aldosterone perfused into peritubular capillaries also increased J_{HCO_3}^{\text{in}}, compared with basal levels during intact capillary perfusion with blood. In addition, in isolated perfused tubules aldosterone causes a transient increase of cytosolic free calcium ([Ca^{2+}]_i), monitored fluorometrically. In the presence of ethanol (in similar concentration used to prepare the hormonal solutions), spironolactone (10^{-6} M, a mineralocorticoid receptor antagonist), actinomycin D (10^{-6} M, an inhibitor of gene transcription), or cycloheximide (40 mM, an inhibitor of protein synthesis), the J_{HCO_3}^{\text{in}} and the [Ca^{2+}]_i were not different from the control value; these drugs also did not prevent the stimulatory effect of aldosterone on J_{HCO_3}^{\text{in}} and on [Ca^{2+}]_i. However, in the presence of RU 486 alone [10^{-6} M, a classic glucocorticoid receptor (GR) antagonist], a significant decrease on J_{HCO_3}^{\text{in}} and on [Ca^{2+}]_i was observed; this antagonist also inhibited the stimulatory effect of aldosterone on J_{HCO_3}^{\text{in}} and on [Ca^{2+}]_i. These studies indicate that luminal or peritubular aldosterone (10^{-12} M) has a direct nongenomic stimulatory effect on J_{HCO_3}^{\text{in}} and on [Ca^{2+}]_i, in proximal tubule and that probably GR participates in this process. The data also indicate that endogenous aldosterone stimulates J_{HCO_3}^{\text{in}} in middle proximal tubule.

S2 bicarbonate reabsorption

IN ADDITION TO THE CLASSICAL genomic actions of aldosterone in the stimulation of Na^+ reabsorption and K^+ and H^+ secretion by the distal nephron, mediated through binding to the mineralocorticoid receptor (MR) (1), it is now firmly established that aldosterone acts through nongenomic pathways to regulate many different ion transport proteins and signaling pathways in a variety of renal epithelial cells, like proximal tubule cells derived from human renal cortex, MDCK-C11 cells (a cell line that exhibits properties of collecting duct intercalated cells), and principal cells isolated from rabbit cortical collecting duct and both M-1 and RCCD2 cortical collecting duct cell lines (12). Studies using isolated perfused tubules also demonstrate that aldosterone, via nongenomic mechanisms, regulates the transepithelial transport function of different nephron segments, such proximal S3 segment (19), renal medullary thick ascending limb (MTAL), type α intercalated cells of outer medullary collecting ducts, and principal cells in the connecting tubule and inner medullary collecting ducts (12). Nongenomic sites of regulation include not only classical aldosterone targets, such as epithelial sodium channel (ENaC) (37) and Na^+-K^+-ATPase (33), but also renal proximal tubule cells (5, 18) and Na^+/H^+ exchanger [both NHE1 (19) and NHE3 (11) isoforms], not considered previously to be direct targets for aldosterone-induced regulation. However, it is important to apply the information from segment-derived cell lines or isolated perfused tubules to intact native tubules, to identify nongenomic responses that are relevant to aldosterone-induced regulation of renal proximal tubule function.

It has been known that apical NHE3 mediates the majority of NaCl, NaHCO_3, and fluid reabsorption by the renal proximal tubule (24) and virtually all of NaHCO_3 reabsorption by the MTAL (11). In microperfused rat MTAL, the addition of 1 mM aldosterone rapidly decreased HCO_3^- absorption by 30%. This inhibition was unaffected by maneuvers that inhibit basolateral Na^+/H^+ exchange and was preserved in MTALs from NHE1 knockout mice, ruling out the involvement of NHE1. In contrast, exposure to aldosterone for 15 min caused a 30% decrease in apical Na^+/H^+ exchange activity due to a decrease in the exchanger’s maximal velocity. This inhibition was not affected by 0.1 mM lumen Zn^{2+} or 1 mM lumen DIDS, arguing against the involvement of an apical H^+ conductance or apical K^+-HCO_3^- cotransport. These results demonstrate that aldosterone inhibits HCO_3^- absorption in the MTAL through inhibition of apical NHE3, and identify NHE3 as a target for nongenomic regulation by aldosterone (11), but whether aldosterone influences NHE3 activity through nongenomic actions in the proximal tubule in vivo remains to be determined.

In renal epithelial cells, an elevation in cytosolic Ca^{2+} serves as a second messenger for the nongenomic Na^+/H^+ exchanger activation initiated by aldosterone (8) and in Xenopus laevis oocyte nuclei this elevation is a prerequisite for the genomic action of aldosterone, with strong evidence that the pregenomic hormone response can influence the genomic processes (32). However, whether this regulation occurs in native proximal tubule is unknown.

In addition, a recent study from our laboratory (19) in an isolated superfused proximal S3 segment of rats indicated that aldosterone (10^{-8}, 10^{-10}, or 10^{-12} M with 2-min preincubation) causes a nongenomic dose-dependent increase in the cellular pH recovery rate after acidification by the NH_4Cl pulse, but aldosterone (10^{-6} M with 2-min preincubation) decreases it.

Considering these findings, the present study was designed to determine whether aldosterone (10^{-12} M) added to intratubular or peritubular capillary perfusions has a direct action on HCO_3^- transport in vivo and on [Ca^{2+}]_i in isolated superfused proximal tubule.
METHODS

Male Wistar rats, obtained from Biomedical Sciences Institute-University of São Paulo, received a rat pellet diet and water ad libitum until the time of the experiment. The animals, weighing 200–340 g, were anesthetized with 100 mg/kg ip tiletamine/zolazepam (Zoletil-Virbac) and prepared for in vivo micropuncture as described previously (20). Briefly, a tracheostomy was performed and the left jugular vein and left carotid artery were cannulated for infusions and blood withdrawal, respectively. The kidney was isolated by a lumbar approach, immobilized in situ by Ringer-agar in a lucite cup under a microscope, and adequately illuminated. During the experiment, the rats received venous saline containing 3% mannitol at 0.05 ml/min.

The kinetics of HCO₃⁻ resorption was studied by means of a stationary micropuncture technique of using an in vivo cortical proximal tubule, with continuous measurement of intratubular pH (20). The S2 segment was perfused by means of a double-barreled micropipette, one barrel filled with Sudan black-colored castor oil, and the other with the luminal perfusion solution colored with 0.05% FD & C green. The intratubular pH was measured as the voltage difference between the two barrels of the microelectrode made from Hilgenberg (Malsfeld, Germany) double-barreled asymmetric glass capillaries. The larger barrel contained an H⁺-sensitive ion exchange resin (Fluka), and the smaller one contained 1 M KCl colored by FD & C green (reference barrel). Transepithelial electrical potential difference was the difference between the reference barrel and ground. This parameter was an additional criterion for the recognition of S2 segments (10). Intratubular pH changes were recorded continuously in the same tubule with a microcomputer equipped with an analog-to-digital conversion board (Lynx, São Paulo, Brazil) for data acquisition and processing.

The kinetics of HCO₃⁻ resorption was measured by injecting a droplet of the perfusion solution between oil columns and following the intratubular pH changes toward the steady-state level [(pH)s]. The pH microelectrodes were calibrated before and after every experiment, immobilized in situ by Ringer-agar in a lucite cup under a microscope, and adequately illuminated. During the experiment, the luminal perfusion solution contained (in mM) 100 NaCl, 25 NaHCO₃, 5 KCl, 1 CaCl₂, 1.2 MgSO₄, and 5 Na⁻-acetate at pH 7.4. This solution was preequilibrated with 5% CO₂ in air. In each tubule, several measurements of JHCO₃⁻ were done with control luminal perfusion first during intact capillary perfusion with blood (basal values), then in the presence of capillary perfusion solution (experimental values), and then again during capillary perfusion with blood (recovery of basal levels).

Measurements with aldosterone-containing solution were performed within ~1 to 7 min of the start of luminal or peritubular perfusions.

Measurement of [Ca²⁺]i

Tube preparation. The kidneys were removed from anesthetized male Wistar rats (80 g) and slices 2 mm in thickness were prepared and transferred to ice-cold normal Ringer solution. In continuation, proximal tubules were isolated by microdissection and transferred to glass coverslips prepared with poly-D-lysine (for tubule adhesion). The coverslips were mounted on a Zeiss LSM 510 confocal microscope in a thermostatically regulated perfusion chamber whose solutions were changed by means of valves. Changes in [Ca²⁺]i were monitored fluorometrically by using the calcium-sensitive probe FLUO-4-AM (28). The proximal tubular segments were loaded for 15 min with 10 μM FLUO-4-AM at 37°C and rinsed in a solution containing (in mM) 137 NaCl, 12 NaHCO₃, 2.68 KCl, 1.36 CaCl₂, 0.49 MgCl₂, 0.36 Na₂HPO₄, 5.6 glucose, and 5.0 HEPES at pH 7.4. 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. For each tubular Ca²⁺ measurement, the maximum fluorescent signal for 10 cells of the same tubular segment was averaged and then used to calculate the [Ca²⁺]i of this segment. Transformation of the fluorescence signal to [Ca²⁺]i was performed by calibration with ionomycin (5 μM; maximum Ca²⁺ concentration) followed by EGTA (2.5 mM; minimum Ca²⁺ concentration) according to the Grynkiewicz equation (14). This equation was originally used for FURA-2 fluorescence, but in previous studies we have results indicating that the basal and both low and high levels of [Ca²⁺]i measured from single-wavelength FLUO-4 were similar to those measured from dual-wavelength FURA-2 (29, 30).

All applied chemicals were of analytic grade and obtained from Sigma (St. Louis, MO).

The pH and Pco₂ in samples of blood were measured with a Radiometer ABL 5 blood-gas system.

Data are means ± SE; N = n° of measurements/n° of tubules. Statistical comparisons were made by the t-test between parameters of intact, experimental, and postexperimental capillary perfusions. Differences between experimental groups were evaluated by ANOVA (1-way) with contrasts by the Bonferroni technique.

This study was approved by the Biomedical Sciences Institute, University of São Paulo-Ethical Committee for Animal Research (CEEA).

RESULTS

In all experimental groups, no significant changes between transepithelial electrical potential differences (~2 mV) were
observed, indicating that during peritubular capillary perfusions with an artificial solution at physiological ion concentrations, tubular transport capacity was maintained at normal levels.

In all experimental groups no significant changes between intratubular stationary (pH) or (HCO₃⁻), were observed, suggesting that the main driving force for H⁺ secretion (i.e., the Na⁺ gradient across the apical membrane) is not significantly altered. Thus, intratubular (pH) values found during capillary microperforations were not significantly different from basal data obtained during capillary perfusion with blood, indicating that during peritubular capillary perfusions with an artificial solution at physiological pH and PCO₂, tubular acidifying capacity was maintained at normal levels.

Mean half-time (t₁/₂) of the reduction of the luminally injected HCO₃⁻ levels to their stationary level is given in Table 1 (during luminal microperfusion with aldosterone and/or others agents) and Table 2 (during peritubular capillary microperfusion with aldosterone).

Figure 1 gives mean data of HCO₃⁻ reabsorption in proximal S2 segments to which control solution alone or plus aldosterone (10⁻¹² M) or ethanol was applied in the tubular lumen. These results indicate that in the presence of aldosterone in luminal perfused tubules a significant rapid direct increase in J_HCO₃⁻ was observed. Figure 1 also shows that in the presence of ethanol alone the J_HCO₃⁻ was different from the control value, indicating that the stimulatory effect we observed on J_HCO₃⁻ is only due to aldosterone.

Figure 2, A and B, shows a sequence of perfusions in two different S2 segments to which capillary control solution alone or plus aldosterone was applied in the peritubular capillary perfusion. During capillary perfusion with the control solution, it is apparent that over a period of several minutes no significant change of the HCO₃⁻ reabsorption occurs compared with basal levels during intact capillary perfusion with blood (Fig. 2A), confirming the capacity of the S2 segment to maintain an adequate rate of acidification during capillary perfusion with an artificial solution at physiological pH and PCO₂. It is clear that aldosterone (10⁻¹² M) added to the capillary solution has a rapid direct stimulatory effect on HCO₃⁻ reabsorption compared with basal levels during intact capillary perfusion with blood (Fig. 2B); however, recovery of basal levels did not occur after the hormonal capillary perfusion, indicating the maintenance of residual aldosterone effects. Figure 2, C and D, summarizes the mean data of HCO₃⁻ reabsorption during peritubular capillary microperfusion with control or aldosterone solutions, respectively.

Figure 3 indicates that in the presence of spironolactone, actinomycin D, or cycloheximide alone in luminally perfused tubules, the J_HCO₃⁻ was not different from the control value. Figure 3 also shows that these drugs did not prevent the stimulatory effect of aldosterone on J_HCO₃⁻.

In addition, we performed measurements to investigate the effect of the glucocorticoid corticosterone (4-pregnene-11β, 21-diol-3, 20-dione) on bicarbonate reabsorption. We used in the luminal perfusion corticosterone (0.3 × 10⁻⁸ M) alone or plus spironolactone (10⁻⁶ M) or RU 486 (10⁻⁶ M). Our results show that corticosterone stimulated the bicarbonate reabsorption [t/₂ = 2.44 ± 0.11 s and J_HCO₃⁻ = 3.77 ± 0.15 mmol·cm⁻²·s⁻¹, N = 28/12, P < 0.01 vs. control group (t/₂ = 3.23 ± 0.10 s and J_HCO₃⁻ = 2.84 ± 0.08 mmol·cm⁻²·s⁻¹, N = 49/19)], spironolactone had no effect on the action of corticosterone (t/₂ = 2.35 ± 0.08 s and J_HCO₃⁻ = 4.20 ± 0.17 mmol·cm⁻²·s⁻¹, N = 27/8), but RU 486 inhibited the stimulatory effect of the corticosterone (t/₂ = 4.92 ± 0.34 s and J_HCO₃⁻ = 1.61 ± 0.15 mmol·cm⁻²·s⁻¹, N = 13/9, P < 0.01 vs. control or corticosterone groups).

Table 1. Half-time of reduction of luminally injected HCO₃⁻ (t/₂ s) in proximal S2 segment during luminal microperfusion with control solution alone or plus aldosterone and/or other agents

<table>
<thead>
<tr>
<th>Luminal Microperfusion</th>
<th>(t/₂, s)</th>
<th>(N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control solution</td>
<td>3.23±0.10</td>
<td>49/19</td>
</tr>
<tr>
<td>Ethanol*</td>
<td>3.44±0.09</td>
<td>30/11</td>
</tr>
<tr>
<td>Aldosterone (10⁻¹² M)</td>
<td>2.24±0.14*</td>
<td>58/10</td>
</tr>
<tr>
<td>Spironolactone (10⁻⁶ M)</td>
<td>3.62±0.05</td>
<td>28/10</td>
</tr>
<tr>
<td>Aldo + Spironolactone</td>
<td>2.06±0.10†</td>
<td>65/14</td>
</tr>
<tr>
<td>Actinomycin D (10⁻⁶ M)</td>
<td>3.62±0.05</td>
<td>34/11</td>
</tr>
<tr>
<td>Aldo + Actinomycin D</td>
<td>2.00±0.20†</td>
<td>24/05</td>
</tr>
<tr>
<td>Cycloheximide (40 mM)</td>
<td>3.69±0.11</td>
<td>11/05</td>
</tr>
<tr>
<td>Aldo + Cycloheximide</td>
<td>2.24±0.09†</td>
<td>33/06</td>
</tr>
<tr>
<td>RU 486 (10⁻⁶ M)</td>
<td>4.68±0.08*</td>
<td>28/7</td>
</tr>
<tr>
<td>Aldo + RU 486</td>
<td>4.53±0.08‡</td>
<td>30/13</td>
</tr>
</tbody>
</table>

Values are means ± SE; N = (n/2) of measurements/n of tubules. *Ethanol is in similar concentration used to prepare the solution of aldosterone (Aldo; 10⁻¹² M). No statistical differences were observed between the control and the ethanol groups. In the presence of aldosterone, the spironolactone, actinomycin D, or cycloheximide groups were similar to aldosterone alone group, i.e., without effect on the action of the hormone (indicating a non-genomic hormonal action). No statistical difference was observed between the RU 486 alone and RU 486 plus aldosterone groups. *P < 0.01 vs. control group; †P < 0.01 vs. respective drug group in the absence of aldosterone; ‡P < 0.01 vs. aldosterone group.
DISCUSSION

The cortical proximal tubule of mammalian kidney has an important role in the renal control of \( \text{H}^+ \) secretion and \( \text{HCO}_3^- \) reabsorption. The major fraction of apical \( \text{H}^+ \) secretion is mediated by the Na\(^+\)/\( \text{H}^+ \) exchanger (isoform NHE3), although \( \text{H}^+\)-ATPase also contributes (15). The apical NHE3 isoform mediates the majority of NaCl, \( \text{NaHCO}_3\), and fluid reabsorption by the renal proximal tubule. So, regulation of NHE3 is critical for the normal maintenance of extracellular fluid volume, blood pressure, and acid-base balance. Studies using renal proximal tubules of adrenalectomized rats (18) and a human proximal tubule cell line (5) indicate that aldosterone stimulates, respectively, surface expression and activity of apical NHE3. However, the nature of the mechanism underlying aldosterone action in vivo proximal tubule bicarbonate reabsorption is not yet clearly defined.

The existence of a nongenomic action of aldosterone is in general attributed to conditions where this action is observed over a short period of time (minutes) against the much longer time (hours, days) needed for a genomic action. In our experiments, the observed effect occurs within \( 1 \) to \( 7 \) min, the period during which the hormonal perfusions are performed. This is a strong reason to argue for a nongenomic action of aldosterone, supported by several other evidences, such as the absence of an effect of spironolactone (a MR antagonist), actinomycin D (an inhibitor of gene transcription), or cycloheximide (an inhibitor of protein synthesis) on the experiments with aldosterone.

Considering the possible existence of a direct hormonal action, in the present work we studied the effect of aldosterone (10\(^{-12}\) M) on the kinetics of \( \text{HCO}_3^- \) reabsorption in the cortical proximal segment. We examined the effect of luminal or peritubular capillary perfusion of this hormone in vivo, by a stopped-flow microperfusion technique, which is not affected by glomerular filtration rate. Previous results from our laboratory indicated that this procedure avoids systemic and renal tubular alterations caused by luminal or peritubular angiotensin II (10\(^{-12}\) M) (26) or arginine vasopressin (10\(^{-11}\), 10\(^{-9}\) M) (25) concentration changes during microperusions, confirmed by the absence of changes in urine flow and pH, Na\(^+\) and K\(^+\) excretion, and systemic acid-base parameters. On the basis of these findings, we may conclude that in this present preparation also the systemic effects of aldosterone (10\(^{-12}\) M) during luminal or peritubular capillary microperfusion were eliminated. Furthermore, the luminal perfusion solution contained raffinose (a nonreabsorbable molecule) to reach isotonicity, to prevent fluid reabsorption induced by the hormone.

Our results show that in the presence of capillary perfusion with control solution alone, no significant change of \( \text{HCO}_3^- \) reabsorption occurs compared with basal levels in the presence of intact capillary perfusion with blood (Fig. 2C), indicating that the short-term substitution of peritubular capillary blood by artificial solutions appears to be a valid method for the study of hormonal peritubular effects on tubular acidification. Furthermore, repeated luminal perfusion procedures did not significantly alter the transepithelial electrical potential difference, intratubular steady-state pH values, and net \( \text{HCO}_3^- \) reabsorption, indicating that sequential comparisons in the same nephron segment can be used to accurately assess the effect of experimental capillary perfusions. This represents a considerable experimental advantage, since it permits paired measurements of basal and experimental conditions in the same nephron segment.

In addition, no significant differences were observed between intratubular steady-state pH values of capillary perfused tubules and the respective control tubules. Changes in this parameter are related to several factors, including the function of \( \text{H}^+ \) and \( \text{HCO}_3^- \) transporters. These findings suggest that the main driving force for \( \text{H}^+ \) secretion across the apical membrane is not altered during the experiments with peritubular capillary perfusion, indicating that, during peritubular capillary perfusions at physiological pH and \( \text{PCO}_2 \), tubular pH/\( \text{HCO}_3^- \) gradients were maintained at normal levels, and modifications of \( \text{HCO}_3^- \) reabsorption rates were only due to changes in the rate \( (t_1/2) \) at which these gradients were reached.

Our results indicate that luminal or peritubular aldosterone (10\(^{-12}\) M) has a direct effect stimulating \( \text{HCO}_3^- \) reabsorption in the cortical proximal segment, expressed by a significant fall in acidification half-time and consequent increase in \( J_{\text{HCO}_3^-} \).

### Table 2. Half-time of reduction of luminally injected \( \text{HCO}_3^- \) \((t/2\ s)\) in proximal S2 segment during capillary perfusion with control solution alone or plus aldosterone

<table>
<thead>
<tr>
<th>Condition</th>
<th>Blood (Basal Values)</th>
<th>Capillary Perfusion ((t/2,\ s))</th>
<th>Blood (Recovery)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Capillary control solution</td>
<td>3.41±0.12 (7/6)</td>
<td>3.38±0.10 (12/6)</td>
<td>3.39±0.09 (11/6)</td>
</tr>
<tr>
<td>Aldosterone (10(^{-12}) M)</td>
<td>3.43±0.13 (8/6)</td>
<td>2.29±0.04* (13/4)</td>
<td>2.39±0.15* (7/4)</td>
</tr>
</tbody>
</table>

Values are means ± SE; \((n°\ of\ measurements/n°\ of\ tubules)\). *\(P < 0.01\) vs. respective capillary perfusion with blood (basal values).

### Fig. 1. Direct effect of aldosterone (10\(^{-12}\) M) tubular luminal perfusion on net bicarbonate reabsorption \((J_{\text{HCO}_3^-})\) in in vivo rat proximal S2 segments. Values are means ± SE; \(N = n°\ of\ measurements/n°\ of\ tubules\). \(\#P < 0.01\) vs. control solution. \&\(P < 0.01\) vs. aldosterone. No statistical differences were observed between the luminal perfusion with control solution and with ethanol. \#In similar concentration used to prepare the 10\(^{-12}\) M solution of aldosterone.

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and the experiment occurred within postexperimental blood capillary perfusion (recovery of basal levels; Fig. 2). *P<0.01 vs. initial peritubular capillary perfusion with blood (basal values).}

Fig. 2. A and B: sequence of measurements of $J_{\text{HCO}_3}$ in 2 different proximal S2 segments. In each tubule, several measurements of $J_{\text{HCO}_3}$ were done first during initial peritubular capillary perfusion with blood (basal values) and then in the presence of experimental capillary perfusion [control solution alone (A) or plus aldosterone (10^{-12} M; B)], and then again during capillary perfusion with blood (recovery of basal levels). In each tubule, 17 measurements of $J_{\text{HCO}_3}$ were done and the experiment occurred within −17 min. C and D: mean data of $J_{\text{HCO}_3}$ during peritubular capillary micropерfusion with control or aldosterone (10^{-12} M) solutions, respectively. Values are means ± SE. N = n° of measurements/n° of tubules; in each tubule 5–7 measurements of $J_{\text{HCO}_3}$ were done, and each experiment occurred within −7 min. No difference was observed between initial peritubular capillary perfusion with blood (basal values) and the respective postexperimental blood capillary perfusion (recovery of basal levels; C); however, recovery of basal levels did not occur after aldosterone (10^{-12} M) capillary perfusion (D). *P<0.01 vs. initial peritubular capillary perfusion with blood (basal values).

(respectively, Tables 1 and 2 and Figs. 1 and 2). The present demonstration of aldosterone-induced enhancement of HCO$_3^-$ reabsorption by proximal tubules is not the first study of this kind since it has been known that aldosterone stimulates activity and surface expression of NHE3 in proximal tubule cells (5, 18) and causes a dose-dependent increase in the cellular pH recovery rate after cellular acidification in isolated superfused proximal S3 segment (19). However, the present study is the first demonstration, to our knowledge, that aldosterone (10^{-12} M) has a direct action on net $J_{\text{HCO}_3}$ in the in vivo cortical proximal tubule.

Therefore, it is interesting to consider 1) the physiological level of aldosterone in blood is 10^{-10}-10^{-9} M but it may increase or decrease in conditions of extracellular volume modification, 2) cardiovascular tissues produce aldosterone, with the result that this hormone is more concentrated in vascular tissue than in the circulation (2), 3) high-salt intake increases aldosterone production in cardiovascular tissues (34), and 4) at present time, we do not have information about the aldosterone levels in the tubular luminal compartment or in the peritubular capillary, but it is necessary to remember that it is probable that aldosterone may concentrate in the tubular lumen due to fluid reabsorption. So, another advantage of the luminal or peritubular capillary perfusion is to ascertain that the measurements of acidification kinetics are performed at well-determined in vivo hormonal levels.

Several studies identify the Na$^+$/H$^+$ exchanger as a target for genomic and nongenomic regulation by aldosterone and show that this mineralocorticoid can control the absorptive function of epithelial tissues through regulation of this exchanger (6, 8, 9, 11, 21, 23, 27). In the proximal tubule the principal fraction of H$^+$ secretion is by the Na$^+$/H$^+$ exchanger but H$^+$-ATPase also contributes, since even in the absence of Na$^+$ or in presence of HMA (a Na$^+$/H$^+$ exchanger inhibitor) a significant rate of H$^+$ extrusion was observed (15). The purpose of the present investigation was to clarify the nongenomic effect of aldosterone on HCO$_3^-$ reabsorption in the in vivo cortical proximal tubule. However, whether aldosterone participates in the two apical H$^+$ secretion mechanisms responsible for the cortical proximal tubular HCO$_3^-$ reabsorption is a question to be determined. In this regard, it is interesting to comment that a recent study from our laboratory (19), in isolated superfused proximal S3 segment of rats, indicates that the cellular pH recovery rate after acidification by the NH$_4$Cl pulse is mostly dependent on Na$^+$/H$^+$ exchanger, but the vacular H$^+$-ATPase also participates in the pH$_i$ recovery. However, the Na$^+$-independent H$^+$ extrusion mechanism initiates −2.5 min after cellular acidification and does not interfere in the evaluation of the rate of pH$_i$ recovery dependent on the Na$^+$/H$^+$ exchanger (since it is calculated from the first 2 min after the NH$_4$Cl pulse). In our present study, during each intraluminal micropерfusion the time of the reduction of the
Caused a significant decrease in for aldosterone is lower. Our results showed that RU 486 alone solution, since it is known that aldosterone-induced effects we used RU 486 (a GR antagonist) in the luminal perfusion prevent this increase (Fig. 3). Also not to occur by MR, because spironolactone failed to the apical NHE3 we observed in presence of aldosterone seems the present study, the rapid increase in nongenomic activity of the most convincing from the MR knockout mouse (3), indicating the presence of these receptors in proximal tubules. However, the possible physiological role of GR action should also be considered since the RU 486 alone in the luminal perfusion solution reduces \( J_{\text{HCO}_3} \) below the basal levels (Fig. 4). This could be due to an unspecific inhibitory action of this GR antagonist; but it might also indicate that, in basal conditions, a basal level of luminal aldosterone (or some glucocorticoid) binding to MR may exist, causing some tonic activation of the NHE3. This question remains to be determined. In this regard, it is interesting to observe that our present results indicate that corticosterone increases the bicarbonate proximal (S2) reabsorption; in addition, similar to aldosterone, the stimulatory corticosterone effect is not affected by spironolactone but is inhibited by RU 486 below the basal levels.

The stimulatory nongenomic effect of aldosterone we observed on \( J_{\text{HCO}_3} \) during luminal or peritubular capillary hormonal perfusion raises the question of the distribution of receptors for this mineralocorticoid on the cell surface. Several lines of evidence support the view that nongenomic actions of aldosterone are mediated through nonclassical receptors that may be membrane-associated proteins (12). So, it appears reasonable to believe that the nongenomic effect of aldosterone during luminal or capillary perfusion occurs due to the presence of nonclassical receptors, respectively, at the proximal cell apical or basolateral membrane surface; however, it is also possible that in leaky epithelia such as the proximal cortical tubule this mineralocorticoid could reach the apical or basolateral membrane via the paracellular shunt path during, respectively, peritubular or luminal perfusion.

The direct nongenomic aldosterone-induced effects, many by second messengers, may occur via the classical MR. The possible involvement of MR in mediating some rapid responses has been suggested for steroid hormones (7) and Grossmann et al. (13) demonstrated that MR contributes to rapid aldosterone activation of the ERK 1/2 pathway in Chinese hamster ovary cells. However, several lines of evidence, the most convincing from the MR knockout mouse (3), indicate that aldosterone induces nongenomic responses independently of the MR. Nevertheless, the receptor for the nongenomic aldosterone-induced mechanism is still unknown. In the present study, the rapid increase in nongenomic activity of the apical NHE3 we observed in presence of aldosterone seems also not to occur by MR, because spironolactone failed to prevent this increase (Fig. 3).

To improve our knowledge of this nongenomic mechanism, we used RU 486 (a GR antagonist) in the luminal perfusion solution, since it is known that aldosterone-induced effects may also occur via GR receptors, although the affinity of GR for aldosterone is lower. Our results showed that RU 486 alone caused a significant decrease in \( J_{\text{HCO}_3} \) and in [Ca\(^{2+}\)] (of ~33 and 28% of the control value, respectively; Figs. 4 and 5, Tables 1 and 3). So, our results indicate that probably the GR receptors participate in the direct nongenomic effect of aldosterone on the NHE3 isoform. Our data are in accordance with studies in opossum kidney cells, a cell culture model of renal proximal tubule, showing that glucocorticoids induced an acute increase in maximal velocity of NHE3 that was not blocked by cycloheximide, consistent with nongenomic regulation and participation of GRs (4). Our present results also are in accordance with the studies of Todd-Turla et al. (35) indicating the presence of these receptors in proximal tubules. However, the possible physiological role of GR action should also be considered since the RU 486 alone in the luminal perfusion solution reduces \( J_{\text{HCO}_3} \) below the basal levels (Fig. 4). This could be due to an unspecific inhibitory action of this GR antagonist; but it might also indicate that, in basal conditions, a basal level of luminal aldosterone (or some glucocorticoid) binding to MR may exist, causing some tonic activation of the NHE3. This question remains to be determined. In this regard, it is interesting to observe that our present results indicate that corticosterone increases the bicarbonate proximal (S2) reabsorption; in addition, similar to aldosterone, the stimulatory corticosterone effect is not affected by spironolactone but is inhibited by RU 486 below the basal levels.

The stimulatory nongenomic effect of aldosterone we observed on \( J_{\text{HCO}_3} \) during luminal or peritubular capillary hormonal perfusion raises the question of the distribution of receptors for this mineralocorticoid on the cell surface. Several lines of evidence support the view that nongenomic actions of aldosterone are mediated through nonclassical receptors that may be membrane-associated proteins (12). So, it appears reasonable to believe that the nongenomic effect of aldosterone during luminal or capillary perfusion occurs due to the presence of nonclassical receptors, respectively, at the proximal cell apical or basolateral membrane surface; however, it is also possible that in leaky epithelia such as the proximal cortical tubule this mineralocorticoid could reach the apical or basolateral membrane via the paracellular shunt path during, respectively, peritubular or luminal perfusion.

![Fig. 3. Effect of spironolactone (10^{-6} M, a mineralocorticoid receptor antagonist), actinomycin D (10^{-6} M, an inhibitor of gene transcription), or cycloheximide (40 mM, an inhibitor of protein synthesis), alone or plus aldosterone (10^{-12} M) in tubular luminal perfusion on \( J_{\text{HCO}_3} \) in vivo rat proximal S2 segments. Values are means ± SE. No statistical differences were observed between the luminal perfusion with control solution and the different drug solutions in the absence of aldosterone. In its presence, the perfusions with the drugs were similar to aldosterone alone, i.e., without effect on the action of the hormone (indicating a nongenomic hormonal action). *P < 0.01 vs. control solution. &P < 0.01 vs. respective drug solution in the absence of aldosterone.](http://ajprenal.physiology.org/)

![Fig. 4. Effect of RU 486 (10^{-6} M, a glucocorticoid receptor antagonist), alone or plus aldosterone (10^{-12} M), in tubular luminal perfusion on \( J_{\text{HCO}_3} \) in vivo rat proximal S2 segments. Values are means ± SE. *P < 0.01 vs. control solution. &P < 0.01 vs. aldosterone. No statistical difference was observed between the luminal perfusion with RU 486 alone and RU 486 plus aldosterone.](http://ajprenal.physiology.org/)
Our data show that after 1 min of addition of aldosterone (10^{-12} M), there was a transient (~1 min) increase of ~55\% of the [Ca^{2+}]_i control value; after 6 min the [Ca^{2+}]_i increased to ~129\% of the control value, and after 10 min this value did not significantly change (Fig. 5 and Table 3). These results are in accordance with several authors who found a direct aldosterone-induced increase in intracellular calcium (8, 13, 16, 17, 31, 36), indicating that intracellular calcium is a prerequisite for aldosterone action. Nongenomic and pregenomic actions usually involve second messengers, such as inorganic ions, cyclic AMP, or various protein kinases. Calcium is one of the second messengers and its increase is the first intracellular signal in response to aldosterone (32). This behavior is also compatible with our results showing that spironolactone, actinomycin, or cycloheximide did not affect the direct effects of aldosterone (10^{-12} M) on [Ca^{2+}]_i and on J_{HCO_3^{-}} (Table 3 and Fig. 3, respectively). Our results with RU 486 also confirm this behavior since 1) this GR antagonist alone caused a significant decrease of the [Ca^{2+}]_i and of J_{HCO_3^{-}} and 2) RU 486 prevented the stimulatory effect of aldosterone (10^{-12} M) on both parameters (Figs. 4 and 5, respectively). However, during this last situation the [Ca^{2+}]_i returned to control value but the J_{HCO_3^{-}} was significantly lower than control. Why this happened is a question to be determined. In addition, Fig. 2B indicates that in the presence of capillary perfusion with aldosterone over the time course of ~7 min, the J_{HCO_3^{-}} does not vary with intracellular calcium concentration (Fig. 5 and Table 3). These data show that the effect of an increase in cell calcium is not the direct cause for J_{HCO_3^{-}} stimulation, but may be a long range factor to activate the Na^+/H^+ exchanger.

In our present experiments, the possible physiological role of peritubular aldosterone perfusion should also be considered. Aldosterone may act through rapid nongenomic pathways (through nonclassical receptors), to modulate its own later genomic mechanisms and provide an integrated system of control of cellular responses. In addition, as a liphophilic hormone, aldosterone may diffuse through the plasma membrane and bind to cytosolic MR; then, the hormone receptor complex translocates to the nucleus where it promotes the later classical genomic actions. Our data show that there was no difference between the J_{HCO_3^{-}} of the basal group (in the presence of intact capillary perfusion with blood) and the control solution capillary perfusion group (Fig. 2C), which is apparently incompatible with the concept of endogenous aldosterone-stimulated bicarbonate reabsorption. However, Fig. 2B

Table 3. Cytosolic free calcium concentration in proximal segments of rat kidney after 1 or 10 min subjected to different agents

<table>
<thead>
<tr>
<th></th>
<th>[Ca^{2+}]_i, nM</th>
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<tbody>
<tr>
<td></td>
<td>1 min</td>
<td>10 min</td>
<td>1 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Control solution</td>
<td>100\pm 3</td>
<td>101\pm 8</td>
<td>164\pm 3</td>
<td>214\pm 10*</td>
</tr>
<tr>
<td>Aldosterone (10^{-12} M)</td>
<td>155\pm 8*</td>
<td>229\pm 4*</td>
<td>140\pm 3*</td>
<td>190\pm 8*</td>
</tr>
<tr>
<td>Spironolactone (10^{-6} M)</td>
<td>99\pm 5</td>
<td>99\pm 2</td>
<td>102\pm 3*</td>
<td>102\pm 3*</td>
</tr>
<tr>
<td>Aldo + Spironolactone</td>
<td>154\pm 5*</td>
<td>223\pm 7*</td>
<td>100\pm 4</td>
<td>102\pm 2</td>
</tr>
<tr>
<td>Actinomycin D (10^{-6} M)</td>
<td>99\pm 4</td>
<td>99\pm 2</td>
<td>101\pm 2</td>
<td>101\pm 2</td>
</tr>
<tr>
<td>Aldo + Actinomycin D</td>
<td>160\pm 5*</td>
<td>232\pm 7*</td>
<td>102\pm 3*</td>
<td>102\pm 3*</td>
</tr>
<tr>
<td>Cycloheximide (40 mM)</td>
<td>100\pm 4</td>
<td>102\pm 3*</td>
<td>100\pm 4</td>
<td>102\pm 3*</td>
</tr>
<tr>
<td>Aldo + Cycloheximide</td>
<td>100\pm 4</td>
<td>102\pm 3*</td>
<td>100\pm 4</td>
<td>102\pm 3*</td>
</tr>
<tr>
<td>RU 486 (10^{-6} M)</td>
<td>72\pm 2*</td>
<td>73\pm 3*</td>
<td>100\pm 4</td>
<td>102\pm 3*</td>
</tr>
<tr>
<td>Aldo + RU 486</td>
<td>102\pm 3*</td>
<td>102\pm 3*</td>
<td>102\pm 3*</td>
<td>102\pm 3*</td>
</tr>
</tbody>
</table>

Values are means \pm SE; N = 5 tubules in each group (the value of 1 tubule was the mean of 10 measurements). No statistical differences were observed between the control group and the spironolactone, actinomycin D, or cycloheximide groups in the absence of aldosterone. In its presence, these groups were similar to aldosterone alone group, i.e., without effect on the action of the hormone (indicating a nongenomic hormonal action). *P < 0.001 vs. control; †P < 0.01 vs. respective drug group in the absence of aldosterone; ‡P < 0.001 vs. aldosterone.
indicates that aldosterone washout (during the recovery period in the presence of capillary perfusion with blood) is not immediate to approach preexperimental capillary perfusion basal levels (during intact capillary perfusion with blood), suggesting the maintenance of residual effects of aldosterone. In addition, Fig. 2A suggests that during capillary perfusion with control solution the J_{HCO3} shows some decrease, but without reaching significance. These findings taken together support the view that in basal conditions a portion of the normally observed proximal bicarbonate reabsorption may be dependent on endogenous aldosterone. Another additional possibility is that after capillary perfusion with aldosterone a portion of the observed proximal J_{HCO3} (during the recovery period in the presence of blood in the capillary) may be dependent on delayed aldosterone effects (Fig. 2, B and D). This behavior is compatible with our present data indicating that after ~6 min of hormonal addition to the bath the [Ca^{2+}]_i levels begin to increase and at 10 min they were significantly higher than controls (Fig. 5 and Table 3). This behavior also agrees with our recent results (19) showing that in isolated proximal S3 segments of rat kidney aldosterone (10^{-12}, 10^{-10}, or 10^{-8} M) has a dose-dependent nongenomic (2 or 15 min) and genomic (1 h) action on [Ca^{2+}]_i and on the Na^+/H^+ exchanger.

In conclusion, we have undertaken in vivo stationary microperfusion studies in the cortical proximal tubule (S2 segment) of the rat to evaluate the effects of aldosterone (10^{-12}–10^{-5} M) on HCO_3 reabsorption. Our results indicate, for the first time, that luminal or peritubular aldosterone (10^{-12} M) has a direct nongenomic stimulatory effect on net J_{HCO3} and on [Ca^{2+}]_i, and that probably GRs participate in this process. The data also indicate that endogenous aldosterone stimulates net J_{HCO3} in the in vivo cortical proximal tubule.

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