Energetics of sodium transport in the urinary bladder of the toad. Effect of aldosterone and sodium cyanide.

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Experiments were designed to determine whether the stimulatory effect of aldosterone on sodium transport involves an increase in tissue ATP. Urinary bladders that were removed from toads presoaked in 0.6% saline for 48-72 h, mounted as sacs, and maintained in open circuit except for brief observation of short circuit current every 30 min responded to 100 nM aldosterone added to the serosal bath with an increase in short circuit current to 170% of control hemibladders, which plateaus at 2-3 h. Tissue (ATP)/(ADP) X (Pi) measured in perchloric acid extracts increased to a maximum of 208% of controls (P less than 0.001) and ATP increased to 116% of controls (P less than 0.01) at 180 min. The short circuit current response to aldosterone paralleled the increase in ATP and (ATP)/(ADP) X (Pi) measured at 75, 120, 180, and 240 min. In bladders clamped at -150 mV, the short circuit current response to aldosterone was greater: 280% of controls (P less than 0.001) and tissue (ATP)/(ADP) X (Pi) increased to 191% of controls (P less than 0.001). In continuously short circuited bladders and bladders clamped at +75 mV, the short circuit current response to aldosterone and the change in ATP, ADP, or Pi were markedly diminished. 100 microM amiloride added to mucosal bath decreased the short circuit current to zero and inhibited […]

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Abstract. Experiments were designed to determine whether the stimulatory effect of aldosterone on sodium transport involves an increase in tissue ATP. Urinary bladders that were removed from toads presoaked in 0.6% saline for 48-72 h, mounted as sacs, and maintained in open circuit except for brief observation of short circuit current every 30 min responded to 100 nM aldosterone added to the serosal bath with an increase in short circuit current to 170% of control hemibladders, which plateaued at 2-3 h. Tissue (ATP)/(ADP) × (P) measured in perchloric acid extracts increased to a maximum of 208% of controls (P < 0.001) and ATP increased to 116% of controls (P < 0.01) at 180 min. The short circuit current response to aldosterone paralleled the increase in ATP and (ATP)/(ADP) × (P) measured at 75, 120, 180, and 240 min. In bladders clamped at -150 mV, the short circuit current response to aldosterone was greater: 280% of controls (P < 0.001) and tissue (ATP)/(ADP) × (P) increased to 191% of controls (P < 0.001). In continuously short circuited bladders and bladders clamped at +75 mV, the short circuit current response to aldosterone and the change in ATP, ADP, or P, were markedly diminished. 100 µM amiloride added to mucosal bath decreased the short circuit current to zero and inhibited the short circuit current response to aldosterone, whereas tissue ATP increased to 141% (P < 0.05). 100, 250, and 500 µM NaCN dropped the short circuit current to 59, 35, and 24% of control values, respectively. Concurrently, tissue ATP measured at 60 min after the addition of NaCN dropped to 79, 66, and 56% of control values, respectively, and tissue ATP/ADP dropped to 68, 50, and 40%, respectively. The data revealed significant correlation between the change in the rate of sodium transport produced by aldosterone or NaCN as measured by the short circuit current and the concentration of ATP (r = 0.96, P < 0.001), as well as ATP/ADP (r = 0.95, P < 0.001). In conclusion, these results support the view that the stimulatory effects of aldosterone on sodium transport involve an increase in ATP or (ATP)/(ADP) × (P).

Introduction

Aldosterone stimulates sodium transport across a variety of epithelial membranes including the toad bladder (1). The response requires protein synthesis and appears to be mediated by aldosterone-induced proteins (AIP) (2-5). The mechanism by which AIP facilitates sodium transport is not well understood. AIP may increase the permeability of the mucosal membrane to sodium, thereby indirectly facilitating the extrusion of sodium at the serosal surface of the epithelial cell (permease hypothesis) (6-8). The observed rise in intracellular sodium concentration (6, 7) and the fall in transepithelial electrical resistance (8) are consistent with the permease hypothesis. However, Lipton and Edelman (9) showed no change in intracellular sodium concentration in response to aldosterone. Goodman et al. (10) found that aldosterone potentiated the subsequent effect of vasopressin on sodium transport in the absence of aerobic metabolism and oxidative phosphorylation. They proposed that aldosterone

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Energetics of Sodium Transport in the Urinary Bladder of the Toad

Effect of Aldosterone and Sodium Cyanide

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1. Abbreviations used in this paper: AIP, aldosterone-induced protein; PC, phosphocreatine; PD; potential difference; PDte/PDoe, ratio of PD at time t to beginning of experiment for experimental groups; PDte/PDoe, ratio of PD at time t to beginning of experiment for control groups; SCC, short circuit current; SCCte/SCCo, ratio of SCC at time t to beginning of experiment for experimental groups; SCCte/SCCo, ratio of SCC at time t to beginning of experiment for control groups; TCA, tricarboxylic acid.

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stimulates sodium transport by increasing the activity and/or the number of Na-K pumps. However, in vitro measurement of Na-K-ATPase in toad bladders that responded to aldosterone did not show a change in maximum velocity (V_{max}) or in Michaelis constant (K_{m}) for sodium and ATP (11, 12). ATP^{32}(P \gamma) sodium-dependent binding to mucosal homogenates of the toad bladder also failed to respond to aldosterone (13). Experiments in which triarboxylic acid (TCA) cycle intermediates and inhibitors of mitochondrial NADH dehydrogenase were added to substrate-depleted bladders suggested that aldosterone-induced ATP generation underlies the mechanism of aldosterone action (13-16).

Aldosterone increased the activity of several TCA cycle enzymes, notably citrate synthase activity in toad bladder (17), accompanied by an increase in NADH/NAD^{+} ratio with a time course parallel to the change in urinary Na^{+}/K^{+} (18, 19). This increase in NADH/NAD^{+} enhances the formation of ATP by phosphorylation of ADP. Handler et al. (20) observed no significant change in the levels of ATP, ADP, and AMP, in bladders incubated with aldosterone for 15 h, but phosphocreatine (PC) levels decreased and creatine levels increased significantly. The long incubation followed by mucosal cell scraping before freezing may alter the nucleotide levels.

We have reported elsewhere that both ATP and (ATP)/(ADP) \times (P) are increased in turtle bladder mucosal sacs exposed to aldosterone (21, 22). In the present study, ATP, ADP, and P, were measured in freshly mounted hemibladders at different times after the onset of aldosterone action in open circuit and also at 180 min while clamped at different potentials. In addition, ATP and ADP were measured 60 min after the addition of 100 \mu M, 250 \mu M, and 500 \mu M NaCN and 180 min after addition of 100 nM aldosterone in amiloride-treated hemibladders. The nucleotide levels were correlated with the short circuit current.

**Methods**

Large female toads (Bufo marinus), obtained from Mogul Ed Co., Oshkosh, WI, were kept for a maximum of 5 wk, being force-fed once per week with dog food. For 2–3 d before the experiment, they were soaked in 0.6% NaCl solution to decrease endogenous aldosterone secretion (1). The abdomen was opened and the blood replaced by amphibian Ringer's solution (111 mM NaCl, 3 mM NaHCO_{3}, 2.7 mM CaCl_{2}, 3.4 mM KCl, and 2 mM MgCl_{2}, pH 7.6) perfused through the aorta and drained from a cut in the liver. When all the blood was cleared from the hemibladders, these were dissected out, rinsed, and immediately mounted as sacs on glass cannulae as described previously (23, 24). Potential difference (PD) and short circuit current were measured as described by Usting and Zerhan (25). Bladders that failed to develop a PD of 60 mV or more were discarded. 10 groups of experiments were performed. In Group I, bladders were maintained in open circuit, except for 5-s intervals every 30 min during which the short circuit current was recorded. Once the PD and short circuit current were stable for 30 min, aldosterone was added to a final concentration of 100 nM in the serosal bath of one hemibladder. To the other hemibladder, methanol, the diluent for aldosterone, was added in the same amount. PD and short circuit current, recorded just before the additions, were called PD_{oe} and short circuit current (SCC)_{oe} for aldosterone-treated hemibladders and PD_{oc} and SCC_{oc} for control hemibladders. The experiment was terminated at 75 min (i.e., after the onset of stimulation was definitely observed), 120, 180, and 240 min in different subgroups of experiments. The PD and short circuit current at different times are called PD_{te} and SCC_{te} for hemibladders treated with aldosterone and PD_{tc} and SCC_{tc} for control hemibladders. In group II, the hemibladders were clamped continuously at −150 mV (mucosa negative). In group III, the hemibladders were clamped continuously at 0 mV. In Group IV, the hemibladders were clamped continuously at +75 mV (mucosa positive). In group V, the hemibladders were clamped at their own spontaneous potential. In groups II, III, IV, and V the spontaneous PD and short circuit current were measured for periods of 5–10 s every 30 min. In Group VI, amiloride in a final concentration of 100 \mu M was added to both hemibladders, the short circuit current dropped to zero, 100 nM aldosterone was added to the serosal side of one hemibladders and methanol to the other. All experiments in group II to VI were terminated at 180 min. In groups VII, VIII, and IX, 100 \mu M, 250 \mu M, and 500 \mu M NaCN was added, respectively, to the serosal bath. The three concentrations of NaCN were chosen to drop the short circuit current by ~25, 50, and 75%, respectively. In group X, 100 nM aldosterone and 100 nM prednisolone was added to the serosal bath of each hemibladder. In Groups VII to IX, the experiments were terminated 60 min after the addition of NaCN, and in group X, 150 min after the addition of aldosterone and prednisolone. In all groups the hemibladders were then quickly emptied and freeze-clamped between two aluminum blocks previously soaked in liquid nitrogen. Extraction by perchloric acid and measurement of ATP, ADP, and P, were done as described by Fawaz and Fawaz (26); P, was measured colorimetrically (26, 27). The protein precipitated by the perchloric acid was measured by the biuret method (28).

**Results**

The short circuit current and PD responses to 100 nM aldosterone in the groups I to IV are shown in Figs. 1 and 2, respectively. Each point reflects the mean±SEM. The short circuit current responses in group V did not differ from Group I. The observed short circuit current and PD response to aldosterone was largest in the bladders clamped at −150 mV. The mean rise of short circuit current relative to control ([SCC_{te}/SCC_{oe}] / [SCC_{tc}/SCC_{oc}]) at 180 min was 2.85±0.30 (P < 0.01), and that of (PD_{te}/PD_{oe})/(PD_{tc}/PD_{oc}) was 1.40±0.05 (P < 0.01).

In the open circuit mode, the response to short circuit current followed the same pattern as the response at −150 mV, but the peak value was significantly lower, with (SCC_{te}/SCC_{oe})/ (SCC_{tc}/SCC_{oc}) ratio of 1.72±0.008 (P < 0.01); and (PD_{te}/PD_{oe})/(PD_{tc}/PD_{oc}) ratio of 1.20±0.03 (P < 0.01).

The response was markedly diminished and not significant up to 180 min for either short circuit current or PD when bladders were clamped at 0 or +75 mV (Figs. 1 and 2).

In bladders kept at their spontaneous PD, (ATP)/(ADP) \times (P) was observed to increase to 138% of controls at 75 min (P < 0.05) reaching 208% of controls at 180 min (P < 0.001) and then declined to 142% of controls at 240 min (Fig. 3). ATP increased while ADP and P, decreased as shown in Table I. ATP/ADP increased to 113, 123, 143, and 129% of controls at
were spontaneous PD, control hemibladders are shown potentials clamped at +75 mV (△) (n = 6); mean±SEM.

75, 120, 180, and 240 min, respectively. The various changes of the phosphate compound levels under conditions of different clamped potentials at 180 min after the addition of aldosterone are shown in Table II. The hemibladders clamped at −150 mV had significant increase in the ATP as well as (ATP)/(ADP) × (P) in the aldosterone treated as compared with the control hemibladders with a mean percentage increase to 117% of controls for ATP (P < 0.01) and 191% of controls for (ATP)/(ADP) × (P) (P < 0.001).

In bladders kept in open circuit or clamped at their spontaneous PD, similar but smaller changes were seen. In depolarized and reversed polarized bladders, no significant changes were seen (Table II and Fig. 4).

The short circuit current decreased to 10% after the addition of 100 μM amiloride to the mucosal bath. There was no change in short circuit current after 100 nM aldosterone in amiloride-treated hemibladders. However, ATP increased to 141% in these hemibladders. ATP decreased to 79, 66, and 56% of controls after the addition of 100, 250, and 500 μM NaCN, respectively, while ATP/ADP decreased to 67, 50, and 40%, respectively. These were associated with similar changes in short circuit current and PD as shown in Figs. 5 and 6, respectively. Figs. 7 and 8 show the variation in the short circuit current concomitant with the changes in ATP and ATP/ADP. In open circuit whether after inhibition by NaCN or stimulation by aldosterone, the changes in ATP or ATP/ADP are proportional to the changes in the short circuit current (r = 0.96, P < 0.001 for ATP and r = 0.95, P < 0.001 for ATP/ADP).

**Discussion**

The increase in short circuit current after aldosterone administration was observed at 75 min and reached a peak level at 180 min in open circuit bladders. The increase was correlated with changes in ATP and (ATP)/(ADP) × (P). In bladders clamped at −150 mV, the magnitude of the change in the nucleotide levels and short circuit current in response to aldosterone was more than in open circuit bladders at 180 min, whereas the changes in nucleotide levels and short circuit current in response to aldosterone were markedly diminished at 0 and +75 mV and not significant up to 180 min. This may seem at variance with results of other investigators (29, 30). Porter and Edelman (29), under similar conditions using continuously short-circuited freshly mounted diaphragms, showed that the response to aldosterone was markedly diminished, if not absent. However, under different experimental conditions, short circuit current response to aldosterone varied significantly. Overnight
incubation in aldosterone-free media followed by substrate repletion produced a highly responsive and reproducible system (28). This could be the result of ionic changes or an increase in the amino acid pool available for ATP formation. Soaking the toads in different concentrations of saline and for variable periods of time results in varying responsiveness to aldosterone (1, 31, 32). Whether this is due to variable concentrations of aldosterone in the serum and tissues or changes in ionic concentrations remains controversial. Our results show that the response of the short circuit current and PD to 100 nM aldosterone is potential dependent. Estimated sodium flux through the active transport pathway has been reported to be decreased by hyperpolarization and increased by depolarization or reversed polarization going from 0.14 μA/mg at −150 mV to 2.66 μA/mg at +75 mV (24). Furthermore, depolarization increased the “transport pool” of sodium defined as the quantity of sodium within the epithelium labeled by radioactive sodium from the mucosal medium (33). Toad bladder epithelial cell swelling indicating increased cellular sodium was observed when the potential was reversed to +50 mV (34). All the above evidence indicates that the rate of transport is increased by depolarization, which would imply that the rate of ATP hydrolysis required for such transport would also increase, and this implication is strongly supported by the observation that oxygen consumption is also increased by depolarization (35). Yet, despite the fact that there may be pronounced changes in the rate of transport and ATP hydrolysis with shifts in the voltage, we observed no change in the level of ATP, or in the (ATP)/(ADP) × Pi ratio. This would mean that the ATP-utilizing system (transport) is so tightly coupled to the ATP-generating system (respiration-oxidative phosphorylation) that scarcely detectable changes are registered in the steady state levels of ATP, ADP, and Pi. How then, does one explain the fact that aldosterone will affect these parameters in open circuit or hyperpolarized bladders, but not in depolarized or reversed-polarized bladders? A reasonable answer may lie in the greatly different magnitudes of the total fluxes through the systems. In a low-flux situation (open circuit or hyperpolarization) a small increment in the rate of ATP generation would easily have a detectable effect on the steady state nucleotide levels, whereas in a high-flux situation (depolarization) the same increment in rate would represent a small increase in an already large number and might have no detectable effect.

In the present study, NaCN reduced ATP and ATP/ADP of bladders in open circuit. This correlated with a proportional decrease in short circuit current. Such a proportional correlation occurred irrespective of whether the ATP and ATP/ADP was increased by aldosterone or decreased by different concentrations of NaCN (Figs. 7 and 8).
Table II. Mean Values for Adenine Nucleotides and P\textsubscript{i} of Control (C) and Aldosterone-treated (E) Hemibladders at Different Voltage Clamping

<table>
<thead>
<tr>
<th></th>
<th>ATP\textsuperscript{*}</th>
<th>ADP\textsuperscript{*}</th>
<th>P\textsubscript{i}*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>E</td>
<td>C</td>
</tr>
<tr>
<td>+75 mV</td>
<td>1.00±0.10</td>
<td>0.97±0.06</td>
<td>0.53±0.05</td>
</tr>
<tr>
<td>0 mV</td>
<td>1.01±0.07</td>
<td>1.04±0.04</td>
<td>0.52±0.02</td>
</tr>
<tr>
<td>Open circuit</td>
<td>0.87±0.03</td>
<td>1.03±0.03</td>
<td>0.51±0.03</td>
</tr>
<tr>
<td>-90 mV</td>
<td>0.96±0.02</td>
<td>1.11±0.03</td>
<td>0.53±0.03</td>
</tr>
<tr>
<td>-150 mV</td>
<td></td>
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* Values are expressed as micromoles per 100 mg proteins.
† Significantly different from control values \( P < 0.01 \).
‡ Significantly different from control values \( P < 0.05 \).

Prednisolone had no effect on sodium transport nor on the adenine nucleotide levels. Amiloride, known to inhibit sodium entry at the apical membrane, was associated with larger increase in ATP in response to aldosterone. These results demonstrate clearly that there is some relationship between the total cellular ATP level or the cellular ATP/ADP ratio and the rate of sodium transport: When cyanide is present, they both decline; when aldosterone is present, they both increase. The exact definition of the mechanism responsible for this relationship will not be possible until more is known about the intracellular distribution and binding of the nucleotides. The finding that much of intracellular ADP may be in a bound form, not readily available for reaction (36), might also apply to ATP to some degree, and the fact that the cellular ATP concentration greatly exceeds the reported \( K_m \) for the transport ATP-ase system implies that any dependence of the system on the ATP level results from some kind of compartmentalization. Saito et al. (37), approaching this question through the formalism of linear nonequilibrium thermodynamics and using the formula \( A = -\text{short circuit current}/(Jr/dPD) \), where \( A \) is the affinity of the metabolic reaction that is equivalent to the negative Gibbs free energy change \(-(\Delta G)\) and \( J_r \) is the rate of oxygen consumption, found that \( A \) increased 2.5-fold after aldosterone. A further increase of 50% above control level was obtained after amiloride. Our results using a biochemical approach confirm both these changes. The dependence of oxygen consumption on PD, which was abolished by ouabain in the presence or absence of aldosterone reflects that the changes are a function of the active transport system rather than those of other aspects of tissue metabolism (37). Deweer (38) proposed that a change in ATP/ADP produced by a decrease in ADP will favor Na-K exchange through the pump in preference to Na-Na exchange. Park and Edelman (39) showed that aldosterone irrespective of sodium concentration increased the inhibition constant \( K_i \) for amiloride but decreased the \( K_i \) for ouabain. Since the response of sodium transport to a gradual increase in mucosal sodium concentrations was enhanced by aldosterone while Na-K-ATPase remained constant (12, 13) they concluded that an increase in ATP through a variety of mechanisms that involve phosphorylation was responsible for the change in per-

Figure 6. Potential difference response to NaCN 100 \( \mu \text{M} \) (●) \( n = 6 \), 250 \( \mu \text{M} \) (○) \( n = 6 \), and 500 \( \mu \text{M} \) (△) \( n = 6 \) in time. The points represent the mean±SEM.

Figure 7. Short circuit current ratio (mean±SEM) as a function of change in (ATP\textsubscript{e}/ATP\textsubscript{c}) (mean±SEM), following 100 nM aldosterone at 180 min (●) \( n = 15 \), 240 min (○) \( n = 10 \), 120 min (△) \( n = 11 \), 75 min (◆) \( n = 11 \), and 150 min (◇) \( n = 6 \); following diluent (control) (●) \( n = 5 \); following NaCN 100 \( \mu \text{M} \) (■) \( n = 6 \), 250 \( \mu \text{M} \) (○) \( n = 6 \), and 500 \( \mu \text{M} \) (△) \( n = 6 \). The NaCN and 150 min aldosterone experiments were done in a different batch of toads 12-18 mo later. The 150 min aldosterone experiments had prednisolone as control. \( r = 0.96, P < 0.001 \).
Figure 8. Short circuit ratio (mean±SEM) as a function of change in (ATP/ADP)/(ATP/ADP), (mean±SEM), following 100 nM aldosterone at 180 min (●) (n = 15), 240 min (◇) (n = 10), 120 min (△) (n = 11), 74 min (▲) (n = 6), and 150 min (◇) (n = 6); following diluent (control) (○) (n = 5); following NaCN 100 µM (■) (n = 6), 250 µM (◇) (n = 6), and 500 µM (○) (n = 6). The NaCN and 155 min aldosterone experiments were done in a different batch of toads 12-18 mo later. The 150-min aldosterone experiments had prednisolone as control, r = 0.95, P < 0.001.

meability in addition to its effect on the turnover rate of the pump.

Recently, Palmer et al. (40), using the technique of near instantaneous current voltage relationships (41, 42), extended this observation and showed that aldosterone, after ATP synthesis, produced an ATP-dependent recruitment of preexisting sodium channels into the apical membrane. The K_m of this process for ATP is not determined and may be high, which makes the assumption of compartmentalization unnecessary. Despite these uncertainties, some deductions may be made from the fact that changes in ATP and ATP/ADP produced by NaCN or aldosterone correlated with changes in the rate of transport. First, the action of cyanide is well known. It inhibits respiration directly and thereby slows the rate of ATP generation, which effect is obvious from the decline in the measured ATP. The correlated decline in sodium transport experiments that cyanide is resulting in decreased ATP availability to the transport system, whatever the mechanism. A primary inhibition of the transport system by cyanide would cause the opposite effect, i.e., an increase in ATP as is shown in amiloride-treated bladders. Second, the action of aldosterone is not well known, but its general effect may be deduced, since it does not only increase both ATP and transport, it increases them in a ratio which is precisely related to the decrease caused by cyanide (Figs. 7 and 8). This implies that aldosterone is doing the reverse, namely increasing the distribution of ATP to the transport system. Again, a primary effect of aldosterone on the transport system would have been reflected by decrease in ATP resulting from a stimulated hydrolysis.

It is apparent that we are dealing here with effects mediated through the energy-supplying side of the cycle, and that the transporting side of the cycle is responding passively. Effects mediated through the transport side, brought about by directly manipulating the rate of transport with variation in the ionic gradients have been extensively analyzed (43) and not unexpectedly show the opposite kind of correlation with nucleotide levels. Thus, a primary stimulation of transport will decrease the -ΔGATP by accelerating hydrolysis of ATP, where as an inhibition of generation would also decrease -ΔGATP but would inhibit transport.

To summarize our views, which are admittedly speculative, we believe that the primary effect of aldosterone is on ATP generation, leading to increased utilization. An increase in steady state ATP levels in association with this change can only be observed when the pump is "idling" or nearly so, i.e., when flux through the pump pathway is low or nil, because utilization keeps pace with generation when flux is high. Amiloride, by contrast, uncouples ATP generation from ATP utilization, thereby leading to a higher steady state level of ATP. Cyanide primarily reduces ATP generation, which secondarily reduces transport. The slope of these decrements (Fig. 8) is the same as the slope of the aldosterone-induced increments.

In conclusion, these results support the view that the stimulatory effects of aldosterone on sodium transport involve an increase in ATP and/or (ATP)/(ADP) X (P). The mechanisms involved are still under investigation.

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References


Effects of Aldosterone and NaCN on Na Transport


