Modulation of aldosterone-induced stimulation of ENaC synthesis by changing the rate of apical Na\(^+\) entry

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Dijkink, Lisette, Anita Hartog, Carel H. Van Os, and René J. M. Bindels. Modulation of aldosterone-induced stimulation of ENaC synthesis by changing the rate of apical Na\(^+\) entry. Am J Physiol Renal Physiol 281: F687–F692, 2001.—Primary cultures of immunodissected rabbit connecting tubule and cortical collecting duct cells were used to investigate the effect of apical Na\(^+\) entry rate on aldosterone-induced transepithelial Na\(^+\) transport, which was measured as benzamil-sensitive short-circuit current (I\(_{sc}\)). Stimulation of the apical Na\(^+\) entry, by long-term short-circuiting of the monolayers, suppressed the aldosterone-stimulated benzamil-sensitive I\(_{sc}\) from 320 ± 49 to 117 ± 14%, whereas in the presence of benzamil this inhibitory effect was not observed (335 ± 74%). Immunoprecipitation of \(^{[35}S\)methionine-labeled \(\beta\)-rabbit epithelial Na\(^+\) channel (rbENaC) revealed that the effects of modulation of apical Na\(^+\) entry on transepithelial Na\(^+\) transport are exactly mirrored by \(\beta\)-rbENaC protein levels, because short-circuiting the monolayers decreased aldosterone-induced \(\beta\)-rbENaC protein synthesis from 310 ± 51 to 56 ± 17%. Exposure to benzamil doubled the \(\beta\)-rbENaC protein level to 281 ± 68% in control cells but had no significant effect on aldosterone-stimulated \(\beta\)-rbENaC levels (282 ± 68%). In conclusion, stimulation of apical Na\(^+\) entry suppresses the aldosterone-induced increase in transepithelial Na\(^+\) transport. This negative-feedback inhibition is reflected in a decrease in \(\beta\)-rbENaC synthesis or in an increase in \(\beta\)-rbENaC degradation.

In addition to regulation of ENaC by aldosterone (5, 24, 25), long-term exposure to vasopressin also stimulates ENaC expression (8). Several other studies identified additional mechanisms involved in the regulation of ENaC activity, including changes in pH, ATP, Ca\(^{2+}\) concentrations, Na\(^+\) concentrations, and cell swelling (10, 13, 19). Notably, the role of these parameters in the short-term action of ENaC was studied. Furthermore, the role of these factors in aldosterone-induced stimulation of transepithelial Na\(^+\) transport has not been investigated. The aim of the present study was, therefore, to investigate the effect of the rate of apical Na\(^+\) entry on long-term effects of aldosterone. So far, the mechanisms by which ENaC synthesis is regulated in response to changes in the rate of apical Na\(^+\) entry are still poorly understood. Two forms of negative-feedback regulation by increased Na\(^+\) concentrations have been described (1, 33), namely, self-inhibition and feedback inhibition. Self-inhibition could be due to a direct interaction of extracellular Na\(^+\) with ENaC itself (28). In salivary duct cells it was found that Na\(^+\) channel activity does not change with increasing extracellular Na\(^+\) (19), whereas in frog skin Na\(^+\) channel activity is controlled by extracellular Na\(^+\) (11). On the other hand, feedback inhibition could also be mediated by an increase in intracellular Na\(^+\) concentration (19).

Primary cultures of rabbit CNT and CCD cells were used to study the effect of changes in the driving force for apical Na\(^+\) entry on aldosterone regulation of ENaC.

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activity. In one protocol, primary cultures were short-circuit to stimulate apical Na\(^{+}\) influx. In another protocol, monolayers were incubated overnight with benzamil to block apical Na\(^{+}\) influx. In both protocols, the benzamil-sensitive short-circuit current (I\(_{sc}\)) and β-ENaC (where rb is rabbit) protein levels were determined.

**MATERIALS AND METHODS**

Primary cultures of rabbit kidney “cortical collecting system.” Rabbit kidney CNT and CCD cells, hereafter referred to as the cortical collecting system, were immunodissected from kidney cortex of New Zealand White rabbits (±0.5 kg body wt) with the monoclonal antibody R2G9 and set in primary culture on permeable filters (0.33 cm\(^2\); Costar, Cambridge, MA) as previously described in detail (2). All experiments were performed with confluent monolayers between 5 and 8 days after the cells were seeded. Sixteen hours before the experiments, the monolayers were short-circuited by flooding the monolayers with culture medium incubated with aldosterone (10\(^{-7}\) M, both sides) or benzamil (10\(^{-5}\) M, apical) and combinations of these treatments.

**Ussing chamber experiments.** For the measurement of transepithelial I\(_{sc}\), filter cups (area 0.33 cm\(^2\)) were routinely washed three times with incubation medium containing (in mM) 140 NaCl, 2 KCl, 1 K\(_2\)HPO\(_4\), 1 KH\(_2\)PO\(_4\), 1 MgCl\(_2\), 1 CaCl\(_2\), 5 glucose, 5 l-alanine, and 10 HEPES-Tris (pH 7.4) and then mounted between two half-chambers and bathed at 37°C with incubation medium. The solutions bathing the monolayers were connected via agar bridges and Ag-AgCl electrodes to a voltage-clamp current amplifier (Physiological Instruments, San Diego, CA), and the I\(_{sc}\) was recorded before and after the addition of 10\(^{-5}\) M benzamil (apical side) and the benzamil-sensitive component of the I\(_{sc}\) was used as an estimate of transcellular sodium transport.

**Measurements of extracellular ion concentrations.** Confluent monolayers (0.33 cm\(^2\)) were treated as described in the text. After 16 h of incubation, extracellular Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\) concentrations were determined by removing duplicate 20-μL samples from the apical and basolateral compartments. The Na\(^{+}\) and K\(^{+}\) contents of the samples were measured by flame photometry (Eppendorf FCM 6343, Hamburg, Germany). The Ca\(^{2+}\) concentration was measured using a colorimetric test kit (Boehringer, Mannheim, Germany).

**Immunoprecipitation of β-ENaC.** Confluent monolayers (1.33 cm\(^2\)) were treated as described in the text. Two hours before the end of the incubation period, filter cups were washed three times for 5 min with DMEM without methionine and subsequently labeled at 37°C for 2 h by apical addition of 0.2 μCi/filter [\(^{35}\)S]methionine (ICN Pharmaceuticals, Irvine, CA). After incubation, the cells were washed, scraped, and immunoprecipitated by incubation with affinity-purified β-ENaC antisera as previous described in detail (5). The immunoprecipitated proteins were resuspended in 25 μL of Laemmli sample buffer and denatured for 5 min at 95°C. Next, 20 μL of the samples were loaded on a 7% (wt/vol) SDS-polyacrylamide gel and electrophoresed. The gel was stained for 10 min at 65°C with 0.25% (wt/vol) Coomassie brilliant blue, 10% (vol/vol) acetic acid, and 50% (vol/vol) methanol; destained twice for 10 min at 65°C with 7% (vol/vol) acetic acid and 25% (vol/vol) methanol; rinsed in water; and incubated twice for 10 min with DMSO and twice for 15 min with 20% (wt/vol) 2,5-diphenyloxazole (Sigma Chemical, St. Louis, MO) in DMSO. After two 5-min rinses in water, the gel was dried and exposed to a film with an intensifying screen at −80°C. The relative amount of [\(^{35}\)S] incorporation was determined with Molecular Analyst (Bio-Rad, Hercules, CA).

**Chemicals.** Benzamil was obtained from Research Biochemical International (Natick, MA). All other chemicals were obtained from Sigma. Benzamil and aldosterone were dissolved in ethanol, the final concentration of which never exceeded 0.1% (vol/vol). Aldosterone was added to the apical and basolateral sides, whereas benzamil was added to the apical side only.

**Statistics.** Results are given as means ± SE. For all experiments, statistical significance was determined by ANOVA; in the case of significance, individual groups were compared by contrast analysis according to Fisher. The level of statistical significance was set at P < 0.05.

**RESULTS**

**Effect of the rate of apical Na\(^{+}\) influx on the aldosterone-stimulated, benzamil-sensitive I\(_{sc}\).** Primary cultures of rabbit CNT and CCD cells were used to determine whether changes in the rate of apical Na\(^{+}\) influx have an effect on transcellular Na\(^{+}\) transport, measured as the benzamil-sensitive I\(_{sc}\). Stimulation of the rate of apical Na\(^{+}\) influx was accomplished by short-circuiting the monolayers for 16 h by flooding the apical and basolateral compartments with culture medium to establish electrical contact. As a result, the apical membrane of the CCD and CNT cells would be hyperpolarized, which would stimulate the apical Na\(^{+}\) entry. Reduction of apical Na\(^{+}\) entry was accomplished by apical exposure of the monolayers to benzamil for 16 h.

Incubation of the monolayers with aldosterone (10\(^{-7}\) M, both sides) for 16 h significantly (P < 0.05) increased the benzamil-sensitive I\(_{sc}\) by 320 ± 49%, whereas in chronically short-circuit-monolayers no increase of aldosterone was observed (117 ± 14% of control benzamil-sensitive I\(_{sc}\), P > 0.1) (Fig. 1). After short-circuiting was terminated for 1 h, there was no recovery of the aldosterone-stimulated, benzamil-sensitive I\(_{sc}\), whereas benzamil-sensitive I\(_{sc}\) (data not shown). Furthermore, short-circuiting the monolayers had no effect on basal benzamil-sensitive I\(_{sc}\) (72 ± 10% of control levels, P > 0.1). When the Na\(^{+}\) influx was blocked by exposure to benzamil for 16 h, the benzamil-sensitive I\(_{sc}\) was doubled compared with the control level. However, no significant effect on the aldosterone-induced, benzamil-sensitive I\(_{sc}\) was apparent (335 ± 74 and 320 ± 49% for benzamil and aldosterone exposure and aldosterone exposure alone, respectively). In addition, the doubling of the benzamil-sensitive I\(_{sc}\) by benzamil also occurred under short-circuit conditions. After 16 h of incubation, the pH of the apical compartment was reduced to 5.6 in untreated monolayers, whereas during short-circuiting of the monolayers the extracellular pH remained at 7.4. To exclude acidification of the apical compartment as the major regulator in the stimulatory effect of aldosterone, we combined chronic short-circuiting with chronic benzamil treatment. In this latter condition, blockage of the apical Na\(^{+}\) entry at pH 7.4, the short-circuiting-induced inhibition of aldosterone-stimulated transcellular Na\(^{+}\) transport was also not realized.
Figure 1. Effect of apical Na\textsuperscript{+} entry on the benzamil-sensitive short-circuit current (I_{sc}) across primary cultures of rabbit connecting tubule (CNT) and cortical collecting duct (CCD) cells. Unstimulated and aldosterone (Aldo; 10\textsuperscript{-7} M, both sides)-stimulated monolayers were short-circuited (SC) for 16 h, incubated with benzamil (Benz; 10\textsuperscript{-5} M, apical side), or both short-circuited and incubated with benzamil. After 16 h of exposure, each filter was washed 3 times with normal medium and I_{sc} was measured in an Ussing chamber. Values were normalized by those obtained for the control cells (13 ± 2 μA/cm\textsuperscript{2}). Values are means ± SE of at least 6 filters. *P < 0.05, significantly different from control.

Effect of extracellular ion concentrations on transepithelial Na\textsuperscript{+} transport. Next, we determined the Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} concentrations in the extracellular medium of control and short-circuited monolayers untreated or treated with aldosterone. Table 1 shows that after 16 h of incubation, the apical Na\textsuperscript{+} concentration of untreated and aldosterone-stimulated monolayers decreased from 140 to 82 and 59 mM, respectively, whereas apical K\textsuperscript{+} concentration increased from 5 to 32 and 38 mM, respectively. The apical Ca\textsuperscript{2+} concentration in unstimulated and stimulated conditions decreased from 1.0 to 0.36 and 0.47 mM, respectively. The influence of these extracellular ion concentrations on the modulation of the aldosterone-induced, benzamil-sensitive I_{sc} was examined by mimicking the described circumstances. Na\textsuperscript{+} (80 mM) or 30 mM K\textsuperscript{+} in the medium during short-circuiting and aldosterone treatment had no stimulating effect on benzamil-sensitive I_{sc} (50 ± 5 and 106 ± 27%, respectively). Moreover, when the extracellular Ca\textsuperscript{2+} concentration was reduced by the Ca\textsuperscript{2+} chelator EGTA (0.8 mM) or the intracellular Ca\textsuperscript{2+} concentration by the Ca\textsuperscript{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid acetoxymethyl ester (10 μM) for 16 h, the Na\textsuperscript{+} channel activity did not change in aldosterone-stimulated, short-circuited monolayers (117 ± 25 and 54 ± 31%, respectively). Taken together, the extracellular Na\textsuperscript{+}, K\textsuperscript{+}, or Ca\textsuperscript{2+} concentrations did not influence the aldosterone-induced ENaC activity of rabbit CNT and CCD cells.

Effect of the rate of apical Na\textsuperscript{+} influx on aldosterone-stimulated β-rbENaC protein levels. The aldosterone-stimulated rbENaC proteins were measured after the driving force for apical Na\textsuperscript{+} entry was changed. The β-rbENaC protein levels were determined by radioactive immunoprecipitation of this 97-kDa protein. The results of a representative immunoblot of immunoprecipitated β-rbENaC from \textsuperscript{35}S-methionine-labeled primary cultures of rabbit CNT and CCD are shown in Fig. 2. Aldosterone treatment for 16 h increased the β-rbENaC protein level by 310 ± 51% (Fig. 3). After 16-h short-circuiting, the β-rbENaC protein level remained unaffected. The inhibitory effect of chronically short-circuiting the monolayers on the aldosterone-induced benzamil-sensitive I_{sc}, as shown above, was accompanied by a significant decrease in aldosterone-stimulated β-rbENaC protein synthesis from 310 ± 51 to 115 ± 12% of control levels. Furthermore, in monolayers exposed to benzamil for 16 h, the β-rbENaC protein synthesis was increased to a protein level (282 ± 68%) comparable to that found in aldosterone-induced monolayers. In monolayers in the combined condition of short-circuiting, benzamil exposure, and aldosterone exposure, the β-rbENaC protein synthesis was stimulated to the same level (286 ± 45%). Thus, also on the protein level, benzamil treatment overcame the inhibitory effect on aldosterone stimulation of short-circuiting the monolayers.

**DISCUSSION**

This study examines the influence of the rate of apical Na\textsuperscript{+} entry on aldosterone regulation of transepithelial Na\textsuperscript{+} transport. In primary cultures of rabbit kidney cortical collecting system, aldosterone exposure stimulates an increase in benzamil-sensitive Na\textsuperscript{+} transport, resulting from an increase in α- and β-ENaC protein synthesis (5). In the present study, we have demonstrated that the aldosterone-stimulated rbENaC transcription or translation process is blocked by a long-term short-circuiting of the monolayers. It is likely that short-circuiting the

**Table 1. Changes in ion concentrations in the apical medium after influencing of apical Na\textsuperscript{+} entry**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>Aldosterone</th>
<th>Aldosterone + SC</th>
<th>Aldosterone + SC + Benzamil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical [Na\textsuperscript{+}]</td>
<td>81.8 ± 3.9</td>
<td>59.4 ± 3.6\textsuperscript{*}</td>
<td>149.2 ± 2.3\textsuperscript{*}</td>
<td>154.9 ± 4.0\textsuperscript{*}</td>
</tr>
<tr>
<td>Apical [K\textsuperscript{+}]</td>
<td>32.2 ± 2.2</td>
<td>37.8 ± 5.1</td>
<td>6.3 ± 0.1\textsuperscript{*}</td>
<td>4.8 ± 0.1\textsuperscript{*}</td>
</tr>
<tr>
<td>Apical [Ca\textsuperscript{2+}]</td>
<td>0.36 ± 0.02</td>
<td>0.47 ± 0.04</td>
<td>1.02 ± 0.04\textsuperscript{*}</td>
<td>1.03 ± 0.03\textsuperscript{*}</td>
</tr>
</tbody>
</table>

Values are means ± SE of at least 6 experiments. Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} concentrations (in mM) in the apical medium of primary cultures of rabbit connecting tubule (CNT) and cortical collecting duct (CCD) cells are shown. Control and aldosterone (10\textsuperscript{-7} M, both sides)-stimulated monolayers were short-circuited (SC) and exposed to benzamil (10\textsuperscript{-5} M, apical side). After 16 h of exposure, ion concentrations in the apical medium were measured. Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} concentrations in the medium before incubation were 140, 4, and 1 mM, respectively. *Significantly different from corresponding control, P < 0.05.
monolayers increases the intracellular Na\(^+\) concentration due to an increase in the apical Na\(^+\) influx by hyperpolarization of the apical membrane (6, 9, 17). Our study further shows that the rate of apical Na\(^+\) entry has a significant effect on ENaC expression, because benzamil treatment overrides the inhibitory effect of short-circuiting on aldosterone-stimulated Na\(^+\) transport. This feedback inhibition is mediated either by a decrease in β-ENaC synthesis or by an increase in β-ENaC degradation.

The mechanisms by which intracellular Na\(^+\) concentration might affect ENaC activity are unclear. Chronically short-circuiting the monolayers, and thereby increasing intracellular Na\(^+\) concentration, may inhibit the aldosterone-induced ENaC expression via regulatory elements on the α-ENaC, β-ENaC, and γ-ENaC gene(s), for example, via an as-yet unidentified Na\(^+\)-responsive element. Many regulatory pathways, controlled by cytosolic Na\(^+\) concentration, are potentially involved in the feedback regulation of ENaC. In salivary duct cells, Komwatana et al. (20, 21) have identified a G0 protein as the mediator of a Na\(^+\)-feedback system. This described model for feedback regulation is as follows: cytosolic Na\(^+\) binds to an intracellular Na\(^+\)-responsive receptor, activating the G0 protein, and the α-subunit of the G0 protein causes the ubiquitine-protein ligase Nedd4 to ubiquinate and inactivate ENaC (16). In Xenopus laevis oocytes, Na\(^+\) feedback inhibition is present together with Nedd4-dependent regulation of ENaC but does not require G protein function (15). Nedd4 proteins contain WW domains, which can bind to the PY motifs of β-ENaC subunits and ubiquinate the ENaC COOH termini, leading to endocytosis and degradation of ENaC. This feedback-inhibition model could be applied to the present study in the rabbit cortical collecting system. Short-circuiting of the monolayers will result in a higher cytosolic Na\(^+\) concentration, and Na\(^+\) binds in a concentration-dependent manner to an intracellular Na\(^+\)-responsive receptor, which activates Nedd4 and leads to degradation of ENaC subunits. Mutations in the PY motifs associated with Liddle syndrome, an inherited form of salt-sensitive hypertension, also interfere with the feedback regulation by intracellular Na\(^+\) (7, 13, 18).

Other feedback loops possibly involved in the down-regulation of ENaC have also been studied in the past. Chalfant et al. (4) showed that in lipid bilayers rENaC currents were dependent on intracellular pH and were not influenced by changes in extracellular pH. In A6 cells (36) as well as in rat cortical collecting tubule (27), changes in intracellular pH correlate positively with changes in Na\(^+\) current and transepithelial conductance. An important finding in our study was that an extracellular pH between 7.4 and 5.6 is not a dominant factor in aldosterone-stimulated ENaC synthesis, because benzamil overcomes the inhibitory effect of short-circuiting on aldosterone-stimulated ENaC expression. In the short-circuiting situation, aldosterone-induced ENaC expression can also be decreased under the control of reduced ATP levels. The rise in intracellular Na\(^+\) will increase the energy used by the Na-K pump, leading to a decrease in ATP levels. Downregulation of ENaC as a result of increased demand for ATP by the Na-K pump is described by Frindt et al. (10). In addition, during benzamil exposure a rise in ATP can be involved in the stimulatory effect on ENaC expression. Another proposed factor involved in feedback inhibition is intracellular Ca\(^{2+}\). In the primary cultures of CNT and CCD there is no effect on the benzamil-sensitive \(I_{\text{Na}}\) after buffering of the intracellular or extracellular Ca\(^{2+}\) concentrations of the short-circuit and aldosterone-exposed monolayers. The literature on experiments with increased cytoplasmic Ca\(^{2+}\) concentrations reports several discrepancies. In rat kidney, there is no direct effect of Ca\(^{2+}\) measured in inside-out patches, whereas in studies with vesicles of toad bladder and in rabbit cortical collecting tubules an
increase in Ca\(^{2+}\) leads to a reduced amiloride-sensitive Na\(^+\) influx (9, 12).

One conclusion of our data is that an increase in cell volume, induced by increased Na\(^+\) influx, prevents the cell from reacting properly to aldosterone. Apparently, cell volume control has a higher priority than a response to aldosterone. Recently, the role of the cell volume-sensitive kinase sgk (serine-threonine kinase) in ENaC regulation has also been described (22, 26, 29). Sgk is rapidly and strongly upregulated by aldosterone in rat cortical collecting duct. In addition, coexpression of sgk with ENaC in X. laevis oocytes stimulated ENaC activity about sevenfold. The sgk transcription level correlates negatively with cell volume (35). Cell swelling-associated degradation of sgk also provides a possible explanation for the inhibition of aldosterone-induced Na\(^+\) transport, which we observed in short-circuited cells. Blocking of Na\(^+\) entry by benzamil may lead to cell shrinkage, and this could stimulate the accumulation of sgk and thereby ENaC activity. It is of interest to note that modulation of Na\(^+\) influx by short-circuiting A6 cells in the absence of aldosterone has an effect opposite to what we report here for rabbit primary cultures of CNT and CCD cells in the presence of aldosterone. Rokaw et al. (31) showed that decreasing Na\(^+\) influx decreases \(I_{sc}\) and increasing Na\(^+\) influx increases \(I_{sc}\). These observations were made in cultures in the absence of aldosterone. Therefore, intracellular Na\(^+\) concentration may have dual effects depending on the absence or presence of aldosterone.

In conclusion, in the present study we have demonstrated that in primary cultures of the rabbit cortical collecting system the aldosterone transcription-translation process can be inhibited by chronically short-circuiting the monolayers. Chronic benzamil treatment can overcome this inhibitory effect. The obvious explanation is feedback inhibition by an increase in the intracellular Na\(^+\) concentration as a result of hyperpolarization of the apical membrane. Further experiments are needed to delineate the molecular mechanism behind this feedback inhibition by intracellular Na\(^+\).

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