Steroid hormone-dependent expression of blocker-sensitive ENaCs in apical membranes of A6 epithelia

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Baxendale-Cox, Lynn M., Randall L. Duncan, Xuehong Liu, Kieron Baldwin, Willem J. Els, and Sandy I. Helman. Steroid hormone-dependent expression of blocker-sensitive ENaCs in apical membranes of A6 epithelia. Am. J. Physiol. 273 (Cell Physiol. 42): C1650–C1656, 1997.—Weak channel blocker-induced noise analysis was used to determine the way in which the steroids aldosterone and corticosterone stimulated apical membrane Na+ entry into the cells of tissue-cultured A6 epithelia. Among groups of tissues grown on a variety of substrates, in a variety of growth media, and with cells at passages 73–112, the steroids stimulated both amiloride-sensitive and amiloride-insensitive Na+ transport as measured by short-circuit currents in chambers perfused with either growth medium or a Ringer solution. From baseline rates of blocker-sensitive short-circuit current between 2 and 7 μA/cm2, transport was stimulated about threefold in all groups of experiments. Single channel currents averaged near 0.3 pA (growth medium) and 0.5 pA (Ringer) and were decreased 6–20% from controls by steroid due to the expected decreases of fractional transcellular resistance. Irrespective of baseline transport rates, the steroids in all groups of tissues stimulated transport by increase of the density of blocker-sensitive epithelial Na+ channels (ENaCs). Channel open probability was the same in control and stimulated tissues, averaging 0.3 in all groups of tissues. Accordingly, steroid-mediated increases of open channel density responsible for stimulation of Na+ transport are due to increases of the apical membrane pool of functional channels and not their open probability.

MATERIALS AND METHODS

Cell culture. A6 cells from American Type Culture Collection (ATCC; Rockville, MD) at passage 69 were subcultured repeatedly in plastic flasks and used at passages 73–112. Three groups of experiments to be referred to as groups A, B, and C were done with differences not only in passage number but also the growth medium and the permeabilized substrates on which the tissues were grown. Cells in group A at passages 73–80 originated in Dr. R. L. Duncan’s laboratory (Renal Division, J. Leibovitz-Ham growth medium (16, 17), and will not be described previously (9). Confluent monolayers were brought to Urbana for the experimental part of the studies that were carried out in 1985–1986. Cells in group B were purchased from ATCC at passage 69, subcultured, used at passage 75 with tissue growth on Millipore HA substrates (Millipore, Bedford, MA), and studied in 1994 in Urbana. Cells in group C were obtained as a gift to Dr. W. J. Els from Dr. W. Van Driessche, used at passages 107–112 with tissue growth on Millipore HA substrates, and studied in 1995 in Cape Town. The results of other groups of experiments were the same, with tissues grown on Transwell-Clear (Costar, Cambridge, MA) and Anocell (Whatman, Clifton, N.J.) substrates and in a Leibovitz-Ham growth medium (16, 17), and will not be reported here.

Growth and perfusion media.

The growth medium for group A experiments was a glutamine-, glucose-, and pyruvate-supplemented Dulbecco’s modified Eagle’s medium (D5648, Sigma Chemical, St. Louis, MO) diluted 15% NaHCO3 (8 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), insulin (5 μU/ml), and 10% fetal bovine serum (FBS) were added to this medium. Cells and tissues were maintained in a humidified incubator at 37°C with air containing 4% CO2. Ten days after seeding of the cells on the collagen-coated Nuclepore membranes, the tissues were fed serum- and insulin-free medium. The tissues were studied on days 14–26 in their control, steroid-depleted states of transport.

WHEREAS STEROID HORMONES are known to stimulate Na+ transport in tight epithelia, the specific steps and mechanisms involved in steroid-mediated regulation of epithelial amiloride-sensitive apical membrane Na+ channels (ENaCs) are unknown. Both mineralocorticoids and glucocorticoids act over periods of hours to increase Na+ entry into the cells (19) via open channels (15). Because changes of open channel density may reflect changes of channel open probability (P0) and/or the density of functional channels (Nf), the fundamental question has been whether regulation of transport occurs by way of P0 and/or Nf.

Using methods of blocker-induced noise analysis, Baxendale et al. (2) first reported that aldosterone and corticosterone stimulated transport by increase of Nf in tissue-cultured A6 epithelia. In native tissues of rat renal cortical collecting ducts, patch clamp revealed that stimulation of transport by diet and aldosterone occurred by increase of Nf (14). Similarly, A6 epithelia grown on rat tail collagen films respond to aldosterone by increase of Nf (12). Because the ways in which cells regulate their channels may depend on the substrate on which A6 cells are grown (3, 11), we were led in scope of the experiments reported here to study a variety of passages of A6 cells (passages 73–112) originating in different laboratories on substrates other than collagen-coated Nuclepore membranes that were used in our original experiments (2) of steroid effects on P0 and Nf.

We report that aldosterone and corticosterone stimulate transport by increase of Nf with no change of P0 of amiloride-sensitive Na+ channels, regardless of substrate, serum, growth medium, or baseline expression of transport.

electrophysiology; sodium channels; tissue culture; cortical collecting ducts; kidney; noise analysis

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The growth medium for group B and C experiments was a Dulbecco’s modified Eagle’s medium (84–5022EC, Gibco, Grand Island, NY) with N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; 4 mM), penicillin (25 U/ml) and streptomycin (25 µg/ml) (17-719R, BioWhittaker, Walkersville, MD), and 10% FBS (Hydene, Logan, UT). Cells and tissues were grown in the presence of humidified air containing 1% CO2 in an incubator at 28°C. Removal of the FBS before treatment with steroids was found to be unnecessary and was not done in these groups of experiments.

We report the results of experiments in which control and steroid-treated tissues originated from the same lots of tissues. Before the day of an experiment, tissues were fed either growth medium (control) or the same medium containing exogenous steroid (0.27 µM aldosterone or corticosterone). Both control and steroid-treated tissues were studied at intervals between ~4 and 50 h postfeeding and handled in the same ways during transfer of the tissues from the inserts to continuous perfusion chambers designed for noise analysis (1). Group A tissues were perfused with a Ringer solution consisting of 100 mM NaCl, 2.4 mM KHCO3, 1.0 mM CaCl2, and 5 mM glucose. Group B and C tissues were perfused with growth medium minus the FBS and antibiotics, thereby ensuring that the tissues were being studied under essentially the same conditions under which they were grown.

Electrical measurements. The methods of study with blocker-induced noise analysis were identical to those described in detail previously (6, 7, 10). After transfer to the chambers, the tissues were short-circuited continuously for at least 1 h to allow the macroscopic short-circuit currents (Isc) to stabilize. Thereafter, during periods of ~30 min, the apical membranes of the cells were exposed in steps to increasing concentrations (5–50 µM) of the weak Na+ channel blocker 6-chloro-3,5-diaminopyrazine-2-carboxamide (CDPC; Aldrich Chemical, Milwaukee, WI). Current noise at each blocker concentration (B) was amplified, digitized, and Fourier transformed to yield power density spectra from which the low-frequency plateaus (S0) and corner frequencies (f0) of the induced Lorentzians were determined by nonlinear curve fitting of the combined Lorentzians. "1/f." noise at the lower frequencies and amplifier noise at the higher frequencies. Blocker on- and off-rate coefficients (k0 and k∞, respectively) were calculated from the slopes and intercepts of B = αf0 plots, respectively, yielding the blocker equilibrium constant KB = k∞/k0.

After complete washout of CDPC from the apical solution and stabilization of the Isc (~10–15 min), the channels were completely (~99.8%) blocked with 100 µM amiloride to yield the amiloride-insensitive current (IscAmil). When all Na+ was removed from the apical solution (1:1 substitution with impermeant cations), the IscAmil fell to zero, indicating that the IscAmil was due to amiloride-insensitive Na+ conductive channels at the apical membranes of the cells.

Defining the blocker-sensitive macroscopic currents IscB = Isc − IscAmil, the single channel currents at any B are

\[ I_{scB} = \frac{S_0(2f_0)^2}{4f_0Bk_∞B} \]

Extrapolation of the IscB to zero blocker concentration provided the single channel currents in the absence of blocker (Isc0) before any autoregulatory changes that could have influenced their values. Blocker-sensitive open channel density in the absence of blocker is Nsc = Isc0/Isc0, expressed in units of open channels per planar square centimeter or per 100 µm², where the latter approximates the area per cell.

Changes of Nsc arise from changes of Po and/or Nt, where Nsc = PoNt. If the blocker interacts only with the open state of ENaCs (10), Po can be estimated from the fractional blocker-dependent decreases of Nsc according to

\[ P_o = \lim_{B \to 0} \left( \frac{1 - N_{sc}^0N_o}{N_{sc}^0N_o} \right) \frac{K_B}{B} \]

Because CDPC interacts rapidly with open channels, channels redistribute among open, blocked, and closed states with a time constant limited by the very slow spontaneous gating kinetics of the channels (τ ~1–2 s with mean open and closed times of several seconds). At the equilibrium redistribution of channels, the fractional inhibition of open channel density and thus Na+ entry into the cells is dependent on the Po. Extrapolation of the apparent values of Po at various B values to zero B circumvents the autoregulatory increases of Nsc that lead to underestimates of the Po at any B (10; see RESULTS). As will be evident in RESULTS, the Po values of blocker-sensitive currents estimated by noise analysis as indicated above are virtually the same as those of the long open time channel measured by patch clamp of A6 epithelia, underscoring the validity of the assumption that blocker interacts only with the open state of the channel in A6 epithelium as in those of frog skin.

The channel densities determined this way are a measure of the pool of apical membrane channels directly involved in Na+ transport. Hence, Isc0 = Isc0P0Nt, where the functional Nt = Nsc/Po. Values are means ± SE.

RESULTS

Blocker-sensitive and blocker-insensitive Isc. Compared with previous published reports in studies of frog skin (6, 7, 10), A6 epithelia behaved in identical ways in response to CDPC inhibition of apical membrane Na+ channels, except the control baseline rates of transport were generally considerably less than those in native tissues (e.g., frog skin, toad urinary bladder, cortical collecting duct, and others). As indicated in Fig. 1, the strip chart recordings of the changes of Isc in response to staircase increases of blocker concentration showed the typical scalloped appearance, indicative of autoregulatory increases of channel density (1, 10), and the
and 
f to highly selective Na
ative channels. 
I currents relaxed toward the original control 
CDPC from the apical solution. From peak values, the 
baseline rates of transport, which ranged from 2.33–
tfold in all groups of experiments, regardless of their 
1 blocker-sensitive Na 
less than 1 µA/cm² at 
I 
ments is presented in Table 1. In general, the 
I 
Ringer solution perfusing the tissues (group A) caused marked inhibition of the 
I 
CDPC. For all groups of experiments and as indicated 
I 
frequencies (Sf), originating at input 
I 
frequencies (Sf), and noise at higher 
I 
frequencies (Sf), 

C1652 ALDOSTERONE AND CORTICOSTERONE EXPRESSION OF ENaCs

Table 1. Stimulation of Na⁺ transport by aldosterone and corticosterone

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Time, h</th>
<th>I_sc, µA/cm²</th>
<th>I_{Amil}^o, µA/cm²</th>
<th>I_{Amil}^i, µA/cm²</th>
<th>I_{Na}, µA</th>
<th>N_o, channels/100 µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>34</td>
<td>5.27 ± 0.35</td>
<td>0.39 ± 0.12</td>
<td>0.50 ± 0.01</td>
<td>9.9 ± 0.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aldosterone or corticosterone</td>
<td>27</td>
<td>14.15 ± 0.66</td>
<td>0.93 ± 0.37</td>
<td>0.47 ± 0.01</td>
<td>28.8 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Control</td>
<td>16</td>
<td>2.33 ± 0.23</td>
<td>0.13 ± 0.02</td>
<td>0.30 ± 0.01</td>
<td>7.3 ± 0.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aldosterone</td>
<td>15</td>
<td>7.77 ± 0.30</td>
<td>0.23 ± 0.05</td>
<td>0.27 ± 0.01</td>
<td>29.0 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>Control</td>
<td>11</td>
<td>7.36 ± 0.64</td>
<td>0.13 ± 0.11</td>
<td>0.36 ± 0.02</td>
<td>20.3 ± 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aldosterone</td>
<td>11</td>
<td>20.41 ± 1.27</td>
<td>0.37 ± 0.21</td>
<td>0.29 ± 0.02</td>
<td>71.5 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>Control</td>
<td>8</td>
<td>4.43 ± 0.63</td>
<td>0.21 ± 0.14</td>
<td>0.29 ± 0.01</td>
<td>14.4 ± 2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aldosterone</td>
<td>8</td>
<td>11.80 ± 1.44</td>
<td>0.14 ± 0.21</td>
<td>0.23 ± 0.01</td>
<td>52.2 ± 7.9</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of observations. I_sc, short-circuit current; I_{Amil}^o, amiloride-insensitive current; I_{Na}, single channel current in the absence of blocker; N_o, blocker-sensitive open channel density.

typical overshoot of the I_sc after complete washout of 
CDPC from the apical solution. From peak values, the 
currents relaxed toward the original control I_sc within 
~10 min. Addition of 100 µM amiloride to the apical solution caused marked inhibition of the I_sc but not to 
zero, leaving an I_{Amil}^o. Removal of all Na⁺ from the 
Ringer solution perfusing the tissues (group A) caused 
the I_{Amil}^o to fall to zero, indicating that the I_{Amil}^o was due 
to highly selective Na⁺-conductive but blocker-insensitive 
channels.

A summary of I_sc and I_{Amil}^o in all groups of experiments is presented in Table 1. In general, the I_{Amil}^o 
represented a relatively small fraction of the I_sc (1.2– 
7.4%) in the control and steroid-stimulated states of 
transport. I_sc were maximally stimulated about three-
fold in all groups of experiments, regardless of their 
baseline rates of transport, which ranged from 2.33– 
7.36 µA/cm² to as high as 20.41 µA/cm² in stimulated 
tissues. Subtraction of the I_{Amil}^o from I_sc yielded the 
blocker-sensitive Na⁺ currents I_{Na} in the absence of 
blocker and I_{Amil}^o at any blocker concentration.

Single-channel currents and open channel densities. 
Despite the low baseline values of transport (compared 
with native tissues like frog skin and toad urinary 
bladder), noise analysis was possible at I_sc somewhat 
less than 1 µA/cm² at B = 5 µM CDPC. As indicated in 
Fig. 2, Lorentzians were easily resolved at 5–50 µM 
CDPC. For all groups of experiments and as indicated 
for group A experiments summarized in Fig. 3, the f_c 
varied linearly with B in both control and steroid- 
treated tissues. S_o and I_{Amil}^o, of control and steroid-
treated tissues changed in accordance with a kinetic scheme of 
closed = open = blocked states, where the blocker 
interacts only with the open state of the channel. As 
indicated in Fig. 3 for group A tissues and summarized 
Table 1 for all groups, the I_{Na} were less in steroid-
treated tissues than in controls due at least in part to 
depolarization of apical membrane voltage in their 
stimulated states of transport (3, 7).

Because single channel currents changed little com-
pared with I_{Na}, open channel density, N_o, calculated 
from the quotient I_{Na}/I_{Amil}^o must increase with steroid 
stimulation of transport. Plotted in Fig. 4 are the 
individual values of N_o against I_{Na} for all groups of 
experiments, with means of control and steroid-
treated tissues summarized in Table 1. In maximally stimu-
lated tissues, N_o is ~50 channels/100 µm² or ~50 
channels/cell. At 1 µA/cm², there can only be about two 
to three open channels per cell on average, which 
explains the difficulty in finding channels by patch 
clamp in steroid-depleted A6 epithelia. In this regard, 
noise analysis permitted study of the blocker-sensitive 
channels without difficulty at this extreme lower bound 
of transport.

Blocker on- and off-rates. The k_{bo} and k_{ob} measured in 
A6 epithelia, summarized in Table 2, were virtually the
same as those reported for Na\textsuperscript{+} channels in frog skin. Accordingly, the blocker site appears to be conserved between these tissues. Both the access time to the blocker site and the residency time at the site were essentially the same among all passages of cells and conditions of growth, yielding $K_B$ that averaged $\sim 30$ µM. It was apparent that the channels recruited by steroid possessed virtually the same kinetic interactions with CDPC.

$P_o$ and $N_T$. It is evident from the data summarized in Fig. 5 that steroid stimulation of transport does not occur by increase of $P_o$. $P_o$ averaged between $\sim 0.25$ and $0.35$ in all groups of tissues and was not changed by steroid treatment of the tissues. The mean values of $P_o$ measured here by noise analysis are virtually the same as those of the long open time channel described by Kemendy et al. (12) in their patch-clamp studies of A6 epithelia.

The density of functional channels undergoing spontaneous fluctuations between closed and open states, $N_T$, was calculated from the quotient $N_o/P_o$ and summarized in Fig. 6. In all groups, steroid treatment resulted in increase of $N_T$ and no change of $P_o$. Variation of transport between $\sim 2$ and $20$ µA/cm² among all control and steroid-treated tissues could be ascribed simply to increase of $N_T$, as illustrated in Fig. 7.

DISCUSSION

Regardless of baseline rates of Na\textsuperscript{+} transport, age and origin of the cells, substrates on which the cells were grown, growth media, and serum, our results demonstrate that the steroid hormones aldosterone and corticosterone increase Na\textsuperscript{+} transport by increase of the density of functional channels at the apical membranes of the cells. The channels are the same, as judged by the similarity in the kinetics of blocker interaction of the channels and by the magnitudes of the single channel currents that are in the same range as those measured by patch clamp and those expected for single channel conductance near 5 pS (3). In comparison with frog skin, the channels appear to be identical, possessing extremely high selectivity for Na\textsuperscript{+} where the predominant population of channels is amiloride inhibitable. Because the $P_o$ is not changed by aldosterone or corticosterone, stimulation of transport must be due to increase in the apical membrane pool size of functional channels involved in Na\textsuperscript{+} transport. It is widely appreciated that channels may be recruited from pools of nonfunctional channels present in the apical membranes and/or trafficked to the apical membrane from the cytosol. Our experiments do not address this issue or the mechanism whereby steroids increase $N_T$. Using
Regardless of the origin of the channels, Na\(^{+}\) to a long open time state in A6 cells. Accordingly, reported that aldosterone shifted channels from a short time channels to the macroscopic rates of Na\(^{+}\) transport in the same range, then the contribution of short open time channels observed by patch clamp of A6 epithelia two populations of Na\(^{+}\) currents are unknown.

Because blocker-induced noise analysis measures only those channels that are blocker sensitive and because blocker-insensitive Na\(^{+}\) currents represent a very small fraction of Na\(^{+}\) transport, increases of transport must be attributable to increases of blocker-sensitive Na\(^{+}\). Nevertheless, blocker-insensitive currents are also increased by steroids but represent a comparatively small fraction of Na\(^{+}\) transport in the presence and absence of exogenous steroids. The gating kinetics and single channel conductance of blocker-insensitive Na\(^{+}\) channels are unknown.

In this regard, Kemendy et al. (12) observed by patch clamp of A6 epithelia two populations of Na\(^{+}\) channels distinguished by large differences in their mean open times but otherwise exhibiting the same single channel currents and, hence, single channel conductance. Short mean open time channels (\(\tau_o \sim 40\) ms) coexisted with long open time channels (\(\tau_o \sim 1,600\) ms) in the same patches. It is unknown whether the short open time channels observed by patch clamp are blocker sensitive or insensitive. Thus the role and function of short open time channels remain unknown but could perhaps be related to blocker-insensitive Na\(^{+}\) channels.

The densities of short and long open time channels are in the same range, then the contribution of short open time channels to the macroscopic rates of Na\(^{+}\) transport would be in the range of a few percent of the I\(_{\text{sc}}\). With identical channel densities and closed times and with \(\tau_o\) of 40 and 1,600 ms, the contribution of short open time channels to the I\(_{\text{sc}}\) is 2.5%.

Origin of channels. It is of particular interest to know the origin of the long open time channels, the density of which is increased by steroids. The hypothesis by Kemendy et al. (12) that steroids change open probabil-

### Table 2. Kinetics of CDPC blocker interaction with ENaCs

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>(n)</th>
<th>Time, h</th>
<th>(k_{\text{on}}), rad/s (\mu)M</th>
<th>(k_{\text{off}}), rad/s</th>
<th>(K_b), (\mu)M</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Control</td>
<td>34</td>
<td>4–50</td>
<td>8.13 ± 0.11</td>
<td>244.9 ± 5.2</td>
<td>30.2 ± 0.57</td>
</tr>
<tr>
<td></td>
<td>Aldosterone</td>
<td>27</td>
<td>4–50</td>
<td>8.34 ± 0.11</td>
<td>243.4 ± 4.3</td>
<td>29.2 ± 0.36</td>
</tr>
<tr>
<td>B</td>
<td>Control</td>
<td>16</td>
<td>18–24</td>
<td>7.19 ± 0.09</td>
<td>215.1 ± 3.9</td>
<td>30.1 ± 0.86</td>
</tr>
<tr>
<td></td>
<td>Aldosterone</td>
<td>15</td>
<td>18–24</td>
<td>6.77 ± 0.09</td>
<td>220.2 ± 4.6</td>
<td>32.7 ± 0.99</td>
</tr>
<tr>
<td>C1</td>
<td>Control</td>
<td>11</td>
<td>11–24</td>
<td>7.94 ± 0.25</td>
<td>276.9 ± 17.9</td>
<td>34.8 ± 1.82</td>
</tr>
<tr>
<td></td>
<td>Aldosterone</td>
<td>11</td>
<td>11–24</td>
<td>7.91 ± 0.25</td>
<td>334.7 ± 20.3</td>
<td>42.5 ± 2.50</td>
</tr>
<tr>
<td>C2</td>
<td>Control</td>
<td>8</td>
<td>8–48</td>
<td>7.24 ± 0.42</td>
<td>191.5 ± 12.7</td>
<td>26.7 ± 1.69</td>
</tr>
<tr>
<td></td>
<td>Aldosterone</td>
<td>8</td>
<td>8–48</td>
<td>7.18 ± 0.33</td>
<td>211.0 ± 18.5</td>
<td>29.7 ± 2.65</td>
</tr>
</tbody>
</table>

Values are means ± SE; \(n\), number of observations. ENaCs, epithelial Na\(^{+}\) channels; CDPC, 6-chloro-3,5-diaminopyrazine-2-carboxamide; \(k_{\text{on}}\) and \(k_{\text{off}}\), on- and off-rate coefficients, respectively; \(K_b\), blocker equilibrium constant.

identical methods of blocker-induced noise analysis, Granitizer et al. (8) reported that the glucocorticoid dexamethasone stimulates N\(_{\text{sc}}\) in A6 epithelia with no change of P\(_o\). Pácha et al. (14) reported that P\(_o\) was not influenced by mineralocorticoid status in the rat cortical collecting duct; in contrast, Kemendy et al. (12) reported that aldosterone shifted channels from a short to a long open time state in A6 cells. Accordingly, regardless of the origin of the channels, Na\(^{+}\) transport must be carried out principally by blocker-sensitive channels with long open and closed times and by channels in which P\(_o\) is the same at various states of mineralocorticoid and glucocorticoid status.

Blocker-sensitive and blocker-insensitive I\(_{\text{sc}}\). Since discovery of amiloride as a potent inhibitor of epithelial Na\(^{+}\) transport, it has been widely acknowledged that diuretic inhibits most, but not all, of the I\(_{\text{sc}}\). Because blocker-induced noise analysis measures only those channels that are blocker sensitive and because blocker-insensitive Na\(^{+}\) currents represent a very small fraction of Na\(^{+}\) transport, increases of transport must be attributable to increases of blocker-sensitive Na\(^{+}\). Nevertheless, blocker-insensitive currents are also increased by steroids but represent a comparatively small fraction of Na\(^{+}\) transport in the presence and absence of exogenous steroids. The gating kinetics and single channel conductance of blocker-insensitive Na\(^{+}\) channels are unknown.
ity of preexisting short open time channels into long open time channels by an all-or-none mechanism rests on critical observations. First, short open time channels must exist before exposure of the tissues to steroids. Second, assuming that the density of short open time channels is constant and that steroids stimulate transport by an all-or-none increase of the mean open time of these channels, decreases of short open time channels must be accompanied by identical increases of long open time channels if this is the only source of channels.

It is appreciated that interpretation of patch-clamp data is exacerbated, especially for the particular case where channel open times are small compared with their closed times (4), as is the case for the short open time channels. With 40-ms mean open time and 3,000-ms mean closed time of the short open time channels, there would, at a 95% level of confidence, have to be at least 13 channels in a patch to observe a double opening of this channel within 20 min of continuous recording (4, 12). The probability of observing multiple openings of these channels is even more remote, so estimates of short open time channel number in a patch are practically impossible. Accordingly, it would be impossible practically to know whether steroids cause changes of short open time channel densities and, hence, whether long open time channels originated from short open time channels as has been suggested (12).

The source of the apical pool of functional channels is unknown and remains a topic of particular interest. Our own experiments reported here shed no light on this issue. Our experiments do not rule out sources of nonfunctional channels within the apical membrane or sources originating from channel-containing intracellular vesicles. Aldosterone is known to induce a variety of proteins, among which may be channel subunits and other proteins involved in sorting and trafficking the channels to the apical membranes. Subunits are expressed in steroid-depleted tissues, and aldosterone does not change the levels of subunit RNA in the same way in all tissues, if at all (13). cDNA-injected oocytes express long open time channels (18) with no reports of short open time channels like those observed in A6 (12). Because apical membrane capacitance is increased by aldosterone in tissues treated overnight (13a) and in a time-dependent way during exposure to aldosterone for 6 h (11a), it is possible that steroids stimulate transport by trafficking of channels to the plasma membrane. Accordingly, steroids stimulate Na\textsuperscript{+} transport by increase of apical membrane N\textsubscript{T} with no change of P\textsubscript{o}. The question of origin of the channels remains open.

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