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Aldosterone Control of the Density of Sodium Channels in the Toad Urinary Bladder

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Summary. Near-instantaneous current-voltage relationships and shot-noise analysis of amiloride-induced current fluctuations were used to estimate apical membrane permeability to Na (P_{Na}), intracellular Na activity (Na_c), single-channel Na currents (i) and the number of open (conducting) apical Na channels (N_0), in the urinary bladder of the toad (*Bufo marinus*). To facilitate voltage-clamping of the apical membrane, the serosal plasma membranes were depolarized by substitution of a high KCl (85 mM) sucrose (50 mM) medium for the conventional Na-Ringer's solution on the serosal side.

Aldosterone (5×10^{-7} M, serosal side only) elicited proportionate increases in the Na-specific current (I_{Na} and in P_{Na}, with no significant change in the dependence of P_{Na} on mucosal Na (Na_o). P_{Na} and the control of P_{Na} by aldosterone were substrate-dependent: In substrate-depleted bladders, pretreatment with aldosterone markedly augmented the response to pyruvate (7.5×10^{-3} M) which evoked coordinate and equivalent increases in I_{Na} and P_{Na}.

The aldosterone-dependent increase in P_{Na} was a result of an equivalent increase in the area density of conducting apical Na channels. The computed single-channel current did not change. We propose that, following aldosterone-induced protein synthesis, there is a reversible metabolically-dependent recruitment of pre-existing Na channels from a reservoir of electrically undetectable channels. The results do not exclude the possibility of a complementary induction of Na-channel synthesis.

Key words transepithelial Na transport · apical Na permeability · Na-channel density · aldosterone

Introduction

Aldosterone is a potent regulator of transcellular Na transport in many epithelia, including the urinary bladder and ventral skin of amphibia and the distal segments of the mammalian nephron (Crabbé, 1963*a*). Although the magnitude of the effects have been well characterized in a number of systems, the mediating pathways in mineralocorticoid action have not been defined clearly. Consideration has been given to regulation of energy metabolism (induction of mitochondrial enzymes), modulation of apical Na permeability (e.g., induction of a permease), and to stimulation of the basal-lateral Na pump (induction of the Na/K-ATPase) as the primary mineralocorticoid mediators (Edelman, Bogoroch & Porter, 1963; Sharp & Leaf, 1966; Kirsten, Kirsten, Leaf & Sharp, 1968; Civan & Hoffman, 1971; Schmidt, Schmid & Dubach, 1973; Lewis, Eaton & Diamond, 1976; Frizzell & Schultz, 1978; Law & Edelman, 1978; Nagel & Crabbé, 1980).

In addition to the possibility that aldosterone acts only on a single step in the regulation of I_{Na}[1], consideration has also been given to the possibility of compound or multiple actions (e.g., on apical Na permeability and on the basal-lateral Na pump). On the basis of invariance in the measured intracellular Na content at the height of the mineralocorticoid response, Lipton and Edelman (1971) proposed a bipolar mechanism involving coordinate increases in P_{Na} and the output of the Na pump both effects are mediated by augmentation of energy metabolism.

In this study we have used near-instantaneous I_{Na}–V curves and current shot noise induced by amiloride, in epithelia depolarized by a high serosal K-concentration, the analyze the effects of aldosterone on the Na current, the apical Na permeability, the single-channel Na current, and the area density of conducting Na channels (Fuchs, Hviid Larsen & Lindemann, 1977; Lindemann & Van Driessche, 1977; Palmer, Edelman & Lindemann, 1980). The results indicate that K-depolarized epithelia respond to aldosterone with proportionate increases in I_{Na} and P_{Na}, which are dependent on the presence of cellular substrates. The increase in P_{Na} is based on an increase in N_0 with no significant change in i. The results are discussed in terms of reversible recruitment of apical Na channels from a reservoir of inactive (electrically

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[1] The abbreviations and notations used in this paper are defined in the accompanying paper, Li, Palmer, Edelman & Lindeman, 1982

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undetectable) channels. A complementary stimulation of the Na pump may also be present but was not assessed in the present study.

Materials and Methods

Bufo marinus (Mexican origin, obtained from Lemberger, Wisconsin) were maintained in tanks with access to tap water *ad lib.* To minimize the background aldosterone concentration, all toads were Na-loaded by partial immersion in 60 mM NaCl for 48 to 72 hr before use. The urinary bladders were removed, mounted as flat sheets in Ussing-type chambers (3 cm^2 exposed area) for measurement of current-voltage relationships, current fluctuations, and membrane area, as described in the preceding papers (Palmer et al., 1980; Li et al., 1982).

Paired hemibladders were incubated in serosal solutions consisting of (in mM): KCl, 85; sucrose, 50; $CaCl_2$, 1.0: $MgSO_4$, 0.5; K-phosphate, 3.5 (adjusted to pH 7.5), and glucose, 5. All of the mucosal solutions contained varying concentrations of Na_2SO_4 plus K_2SO_4 at a combined constant activity of 60 mM, 1 mM Ca gluconate, and 5 mM Tris-H_2SO_4 (pH = 7.5). The mucosal solution used in a particular experiment will be identified by the activity of Na in that solution. When exogenous substrate was present throughout, paired hemibladders were preincubated for 1 to 4 hr in high K-sucrose serosal medium and either 20 or 60 mM Na on the mucosal side. Either aldosterone (final concentration = 5×10^{-7} M) or the diluent (high K-sucrose) was then added to the serosal medium. In the substrate depletion-repletion experiments, the paired hemibladders were preincubated in substrate-free Na-Ringer's for 15 to 20 hr, and the serosal medium of one of each pair contained aldosterone (5×10^{-7} M). The serosal solutions were then replaced with substrate-free high K-sucrose Ringer's (with or without aldosterone, experimental *vs.* control) and challenged by addition of a small volume of isoosmolar Na pyruvate (final concentration = 7.5 mM) 1 hr later.

Near-instantaneous current-voltage (I–V) curves were recorded as described previously (Fuchs et al., 1977; Palmer et al., 1980; Li et al., 1982). The hemibladders were continuously short circuited for 1 hr prior to measurement. The "shunt" I–V curves were obtained after replacing all of the mucosal Na with K and waiting for decay of the resulting outward-current transient. The I_{Na}–V curve was obtained by subtraction of the shunt currents (at a given V) from those obtained in the presence of mucosal Na (at the same V). P_{Na} and Na_c were calculated by fitting the I_{Na}–V curve with the constant-field equation.

In the substrate replete experiments the I_{Na}–V measurements were repeated at various times after addition of aldosterone or the diluent to the serosal media (i.e., 1 to 6 hr). In the substrate depletion-repletion experiments, these measurements were made just before and serially (i.e., 10 to 120 min) after addition of Na pyruvate (as an isoosmotic stock solution) to the serosal media (final concentration = 7.5 mM).

To study the effects of aldosterone on the single-channel properties of the apical membrane, recording and analysis of amiloride-induced current fluctuations (shot-noise) was carried out as described by Li et al. (1982). The basal-lateral membranes were depolarized with the high K-sucrose medium and a mucosal Na activity of 60 mM was used throughout. The measurements were made just before addition of aldosterone and at the maximum of the response, which took 4 to 6 hr. The control hemibladders (from the same toads) were mounted and treated identically, as the experimental hemibladders, but did not receive hormone. The time-course of I_{sc} was followed in parallel to that of the experimental hemibladders, but noise measurements were not made on the control tissues. The area density (N) of all electrically detectable Na channels was estimated by:

$$N = N_0(1 + Na_o/K_N) \quad (1)$$

where $(1 + Na_o/K_N)^{-1}$ is the steady-state probability of a channel's being in the open state in the absence of amiloride. K_N was estimated from Na-dependence of P_{Na} as described below.

Results

Time Course

The time-course of action of aldosterone on I_{Na} and P_{Na} in the presence of exogenous substrate is shown in Fig. 1. As described previously for I_{sc}, after a latent period of ~1 hr, aldosterone elicited a curvilinear increase in I_{Na} to a new steady-state value that was twice that of the control hemibladder after 6 hr (Porter & Edelman, 1964). The time-course of the change in P_{Na}, obtained from I_{Na}–V curves, was indistinguishable from that of I_{Na}, implying a close correlation between apical Na permeability and transepithelial Na transport in the K-depolarized epithelia.

Steady-State Analysis

In 13 pairs of hemibladders, I_{Na}–V analysis performed before and 6 hr after addition of aldosterone or the diluent, revealed increases of 126, 126, and 91%, in I_{Na}, P_{Na}, and Na_c, respectively (Table 1). In the control hemibladders, I_{Na} and P_{Na} were unchanged, although Na_c increased from 1.7 to 2.23 mM (31%). Linear regression analysis of the plot of the fractional change in I_{Na} *vs.* P_{Na}, 6 hr after addition of aldosterone or diluent to the serosal medium, yielded a slope of 0.97, a zero intercept, and a correlation coefficient (r) of 0.99 (Fig. 2). These results imply that changes in apical Na permeability play a significant and perhaps even a decisive role in the I_{Na} response to aldosterone.

Effect of Mucosal Na

Additional experiments were carried out to evaluate the effects of aldosterone on the decrease in P_{Na} elicited by increasing the concentration of Na in the mucosal solution (Na_o). The I_{Na}–V curves were obtained before and 6 hr after the addition of the steroid or the diluent to the serosal media. The changes in mucosal Na were made sequentially by replacement with K at constant ionic strength. While there was no clear effect on the shunt I–V curve, aldosterone affected the I_{Na}–V curves at all of the concentrations of mucosal Na that were tested (Fig. 3*a, b*). To assess the effect of aldosterone on the dependence of P_{Na} on Na_o, we used:

$$P_{Na} = \frac{P_{max} \cdot K_N}{K_N + Na_o} \quad (2)$$

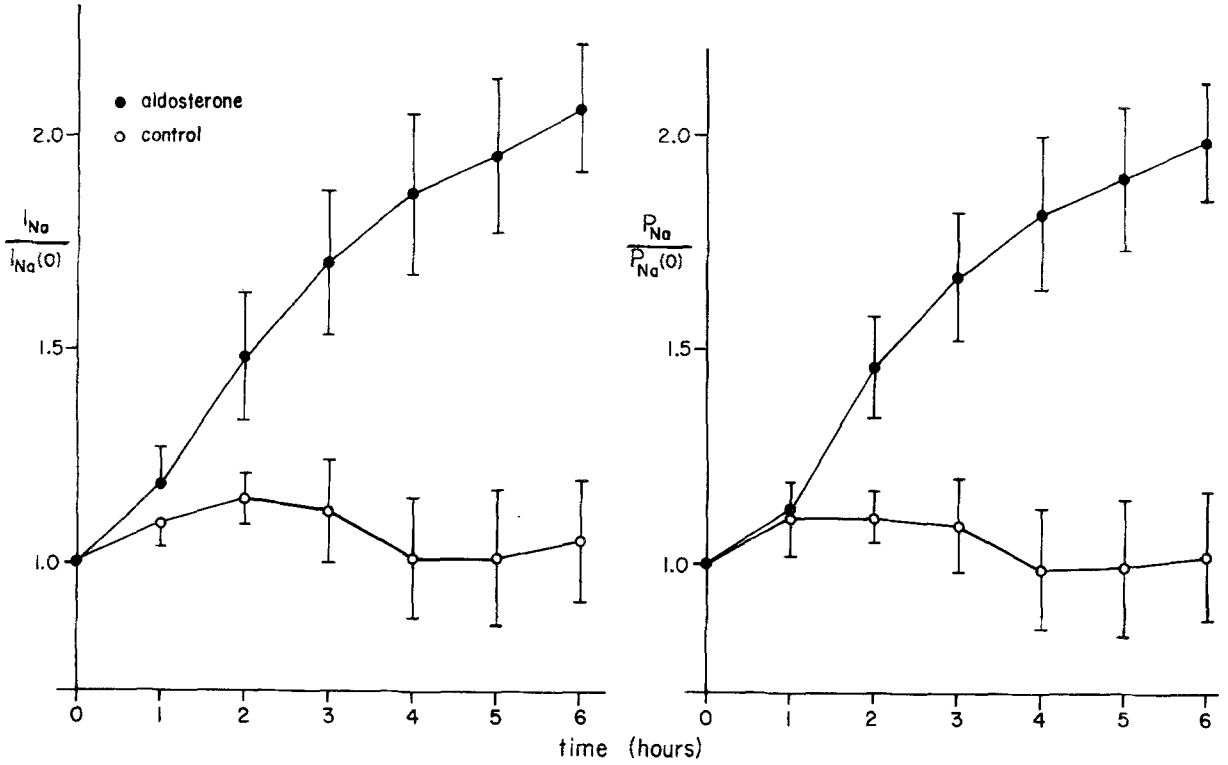

Fig. 1. Time course of the effects of aldosterone on I_{Na} and P_{Na}. Paired hemibladders were pre-incubated in the high K-sucrose serosal solution (containing 5 mM glucose) for 1 to 4 hr. I_{Na} and P_{Na} (estimated by I_{Na}–V analysis) were measured just before the addition of aldosterone (5×10^{-7} M) or diluent (control) to the serosal side (with 20 mM Na in the mucosal solution). The same parameters were remeasured at 60-min intervals for 6 hr. -●-=aldosterone-treated hemibladders; -o-=control hemibladders. Data are presented as the values of I_{Na} or P_{Na} divided by the values at the time of aldosterone addition. Initial values were: $I_{Na}=7.3\pm2.2$ μA/μF (control) and 7.7 ± 2.4 μA/μF (aldosterone): $P_{Na}=0.41\pm0.12\times10^{-5}$ cm/sec (control) and $0.43\pm0.13\times10^{-5}$ cm/sec (aldosterone). The data represent means ±SEM (4 hemibladder pairs)

Table 1. Effects of aldosterone on I_{Na}–V parameters[a]

	Initial values			Fractional change (aldosterone)[b]		
	I_{Na} (μA/μF)	P_{Na} (10^{-5} cm/sec)	Na_c (mM)	I_{Na}	P_{Na}	Na_c
Aldosterone	6.47 ± 0.97	0.36 ± 0.06	1.61 ± 0.17	2.26 ± 0.37[c]	2.26 ± 0.32[c]	1.91 ± 0.36[c]
Control	7.15 ± 1.70	0.41 ± 0.10	1.70 ± 0.18	0.91 ± 0.16	0.92 ± 0.16	1.31 ± 0.10[c]

[a] Paired hemibladders from Na-loaded toads were incubated in high K-sucrose on the serosal side for 1–4 hr. Just before the addition of aldosterone or diluent, the I–V relationships were measured with and without mucosal Na (20 mM) (initial values). Aldosterone was added to the serosal side only (final concentration $=5\times10^{-7}$ M). The I–V curves were remeasured 4–6 hr later, after a new steady state had been attained. All results are given as means ±SEM, (13 hemibladder pairs).

[b] Fractional changes were estimated as the ratio of the value 4–6 hr after addition of aldosterone or diluent to the value just before addition.

[c] Denotes statistical significance; $P<0.05$, for the fractional change.

where P_{max} is the maximum Na permeability of the apical membrane and K_N the concentration of mucosal Na required to reduce P_{Na} to $0.5\times P_{max}$ (Fuchs et al., 1977). The results were analyzed by plotting $1/P_{Na}$ against Na_o and least squares regression; the intercepts on the ordinate and abscissa gave estimates of P_{max} and K_N, respectively (Fig. 4). In five experiments, P_{max} values were $0.70\pm0.18\times10^{-5}$ cm/sec before, and $1.93\pm0.47\times10^{-5}$ cm/sec after aldosterone. K_N decreased from 22 ± 6 to 17 ± 4 mM. The mean fractional increase in P_{max} ($181\pm26\%$) was statistically significant ($P<0.005$). The mean fractional decrease in K_N was $21\pm14\%$ and was not statistically significant. These results indicate that the aldosterone-dependent increase in P_{Na} is primarily a consequence of the increase in P_{max}, and that K_N is not

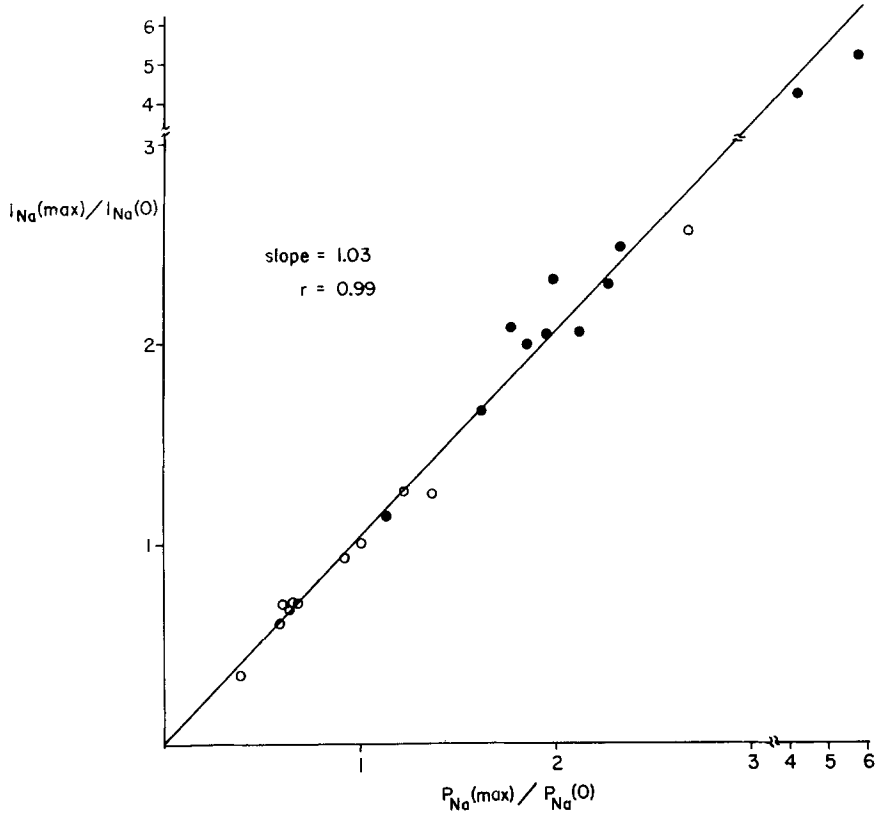

Fig. 2. Correlation of the effects of aldosterone on I_{Na} and P_{Na}. Paired hemibladders were pre-incubated in high K-sucrose serosal solution for 1 to 4 hr. I_{Na} and P_{Na} were measured just before the addition of aldosterone (5×10^{-7} M) or diluent to the serosal side. The same parameters were measured 4 to 6 hr later, when a new steady state was attained. The fractional change in I_{Na}, defined as the ratio of the final value of I_{Na} to the value just before addition of the hormone, is plotted *vs.* the fractional change in P_{Na}. ○ = control hemibladders; ● = aldosterone-treated hemibladders. The regression line was obtained by least squares analysis and does not include the point on the extreme right [●]. If this point is included, the slope = 1.08; r = 0.99. The means ± SEM of these results are given in Table 1

significantly altered. The magnitude of the increase in P_{max} essentially accounts for the increase in I_{Na} listed in Table 1.

Substrate Depletion-Repletion

Successive I_{Na}–V curves were obtained from paired hemibladders preincubated for 15–20 hr in substrate-free media with or without aldosterone (5×10^{-7} M) and before and after addition of 7.5 mM Na pyruvate. The fidelity of the fit of the I_{Na}–V curves with the constant field equation was maintained throughout the period of measurement. This is illustrated in Fig. 5*a* by typical curves obtained at 0 time and 90 min after the addition of Na pyruvate to an aldosterone-treated hemibladder. The time-course of the changes in I_{Na}, P_{Na}, and Na_c, in this experiment is shown in Fig. 5*b* and the results obtained in five pairs are summarized in Table 2. On pyruvate repletion I_{Na} and P_{Na} increased in concert over a 90-min period, with a latent period of less than 20 min. Ninety minutes after the addition of pyruvate, the aldosterone-dependent differential increases were 102 and 89%, in I_{Na} and P_{Na}, respectively. In contrast, Na_c fell to about the same extent in both groups. It is of interest that in substrate-depleted hemibladders not treated with aldosterone, pyruvate did not elicit statistically significant increases in I_{Na} or P_{Na}, despite the significant fall in Na_c from 2.13 to 1.64 mM. The small drop in Na_c presumably is a consequence of stimulation of the basal-lateral Na pump in response to increased availability of substrate and was about equal in both groups.

The available information implies that the effect of aldosterone can be divided into two sequential events: induction of protein synthesis followed by an increase in P_{Na}. The results in Table 2 indicate that the second event, the increase in P_{Na}, has an absolute requirement for available substrate.

Current Fluctuation Analysis

Current fluctuations were recorded from serosally K depolarized epithelia with 60 mM Na activity in the mucosal solution. Under these conditions I_{Na} increased in 19 of 22 pairs of hemibladders from Na-loaded toads by at least 50% relative to the controls in response to aldosterone. In the other three pairs no difference was detectable. For the purpose of fluctuation analysis, only those hemibladders which showed a significant absolute increase in I_{Na}, as well as an increase relative to controls, were used. In these 10 hemibladders the fractional increase in the ratio (I_{Na} (6 hr)/I_{Na} (0 hr)) was 2.4 ± 0.2. When this ratio is divided by the same ratio in the control bladders, the relative increase in I_{Na} was 3.5 ± 0.5 (mean ± SEM).

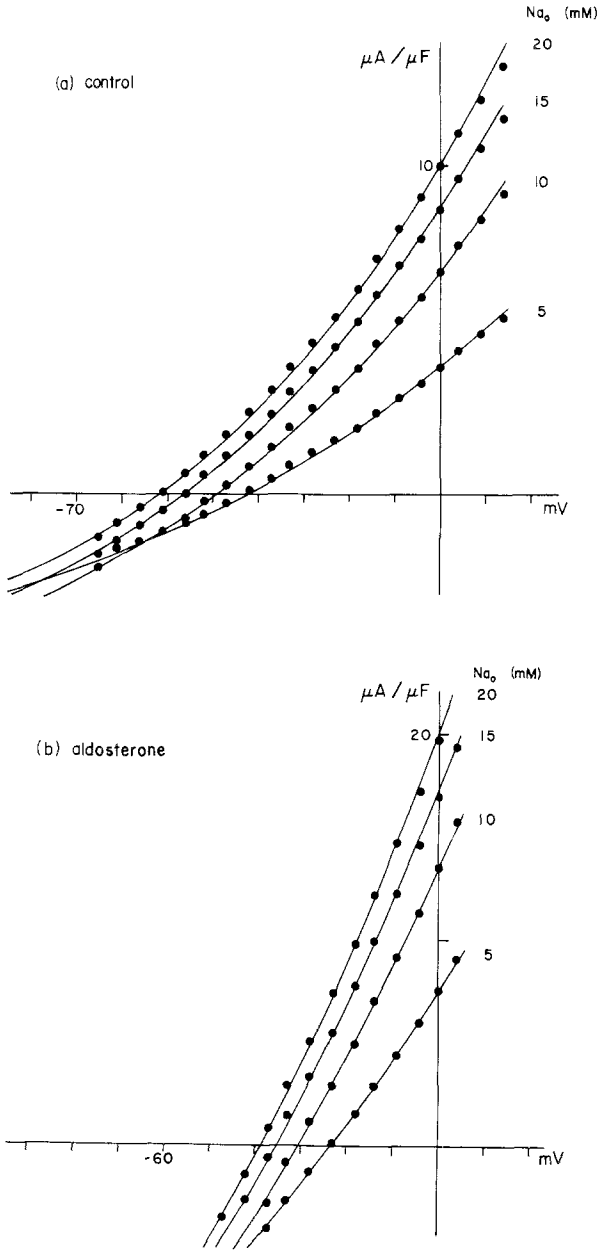

Fig. 3. Effects of aldosterone and mucosal Na and I_{Na}–V curves. A single hemibladder was pre-incubated for 1 hr in high K-sucrose solution on the serosal side. I–V curves were first obtained with 20, 15, 10 and 5 mM Na in the mucosal solution, and with Na-free solution to measure the shunt I–V curve. Aldosterone (5×10^{-7} M) was then added to the serosal side, and the I–V curves remeasured 6 hr later. I_{Na}–V curves were obtained by substraction of the shunt I–V curves from those in the presence of each mucosal Na activity, and were fitted with the constant field equation (solid lines). (*a*) I_{Na}–V curves before, (*b*) I_{Na} curves after, aldosterone addition

Current power densities in the presence of 60 mM apical Na but in the absence of amiloride decreased with increasing frequency with a slope of approximately -2. The relationship was less steep at low frequencies (near 0.1 Hz). After addition of aldosterone the shape of the spectrum did not show clear changes but power densities were generally larger (Fig. 6*a*). Spectra obtained in the absence of Na or in the presence of apical Na together with maximal concentrations of amiloride were not clearly altered by aldosterone (Fig. 6*b*), an observation which is compatible with the fact that the shunt conductance was not significantly changed by the hormone.

On addition of submaximal concentrations of amiloride to apical solutions containing 60 mM Na activity, Lorentzian components appeared in the spectra. Plateaus decreased and corner-frequencies increased when the amiloride concentration was raised (Fig. 6*c*) as expected from the reversible blocking action of amiloride (Lindemann & Van Driessche, 1977). After treatment with aldosterone, the plateau power densities at the same amiloride concentration were larger (Fig. 6*d*) but changes in corner frequency were small (Fig. 7*a*).

The apparent microscopic rate constants of the blocking effect of amiloride were evaluated from slope and ordinate intercepts of plots of $2\pi f_c^A$ *vs.* the amiloride concentration (Fig. 7*a*). The on-rate constant k'_{02} (the slope), which can be estimated precisely, was not changed significantly by aldosterone. This result (Fig. 7*b*) was obtained when rate constants from individual experiments were evaluated and then averaged, and also when rate constants were obtained from the regression lines in Fig. 7*a* (i.e., the means of the corner frequencies of all experiments taken at each amiloride concentrations *vs.* the amiloride concentration). The apparent off-rate constant k'_{20} (ordinate intercept) showed more variation, and a small increase in response to aldosterone was obtained with both methods of evaluation (Fig. 7c). The increase, however, was not statistically significant. In consequence, the apparent microscopic inhibition constant ($K'_A = k'_{20}/k'_{02}$) of the blocking effect of amiloride shows a small but statistically insignificant increase in response to aldosterone (Fig. 7*d*). For comparison, the macroscopic inhibition constant K_A^{ma}, was evaluated from linearized dose-response curves, i.e., from regression lines in plots of Na_o/I_{Na} amiloride concentration. The mean K_A^{ma} of 10 experiments decreased slightly, from 0.38 ± 0.04 before, to 0.32 ± 0.03 μM after, exposure to aldosterone.

The computed shot-current values (i) for single Na channels randomly blocked by amiloride were close to 0.15 pA at 60 mM mucosal Na activity in K-depolarized short-circuited hemibladders. The corresponding translocation rate is about 10^6 Na ions per second through open channels. These currents were not significantly different in the control- and stimulated-states (Fig. 8*a*). Furthermore, the i-values did not correlate with the corresponding I_{Na}, mea-

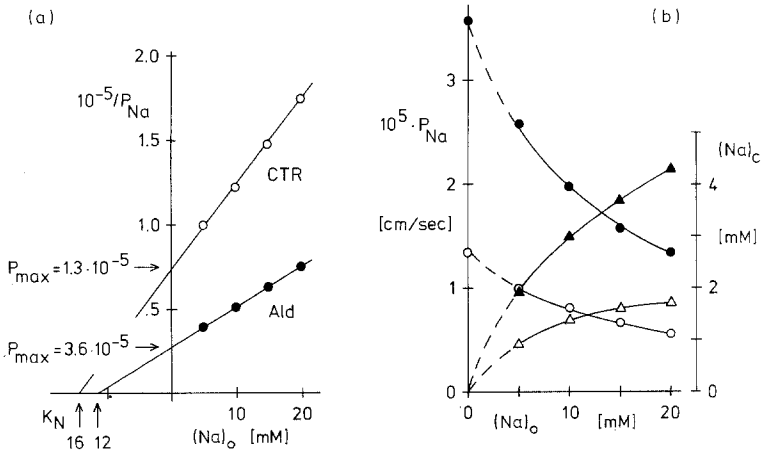

Fig. 4. Effect of aldosterone on the response to mucosal Na in a single hemibladder. (*a*): Steady-state values of P_{Na}, obtained from fitting the I_{Na}–V curves in Fig. 3 with the constant field equation, are plotted as $1/P_{Na}$ *vs.* Na_o, before (*CTR*) and after addition of aldosterone (*Ald*). Lines are least-squares fits to the data points. The resultant values were: $P_{max}=1.3\times10^{-5}$ cm/sec (control), 3.6×10^{-5} cm/sec (aldosterone), $K_N=16$ mM (control), 12 mM (aldosterone). (*b*): Decrease of P_{Na} (○, ●) and increase of Na_c (○, ▲) with increasing Na_o (steady state). P_{max} values, indicated at zero Na_o, were obtained by linear regression analysis as shown in *a*. ○, △=control; ●, ▲=aldosterone

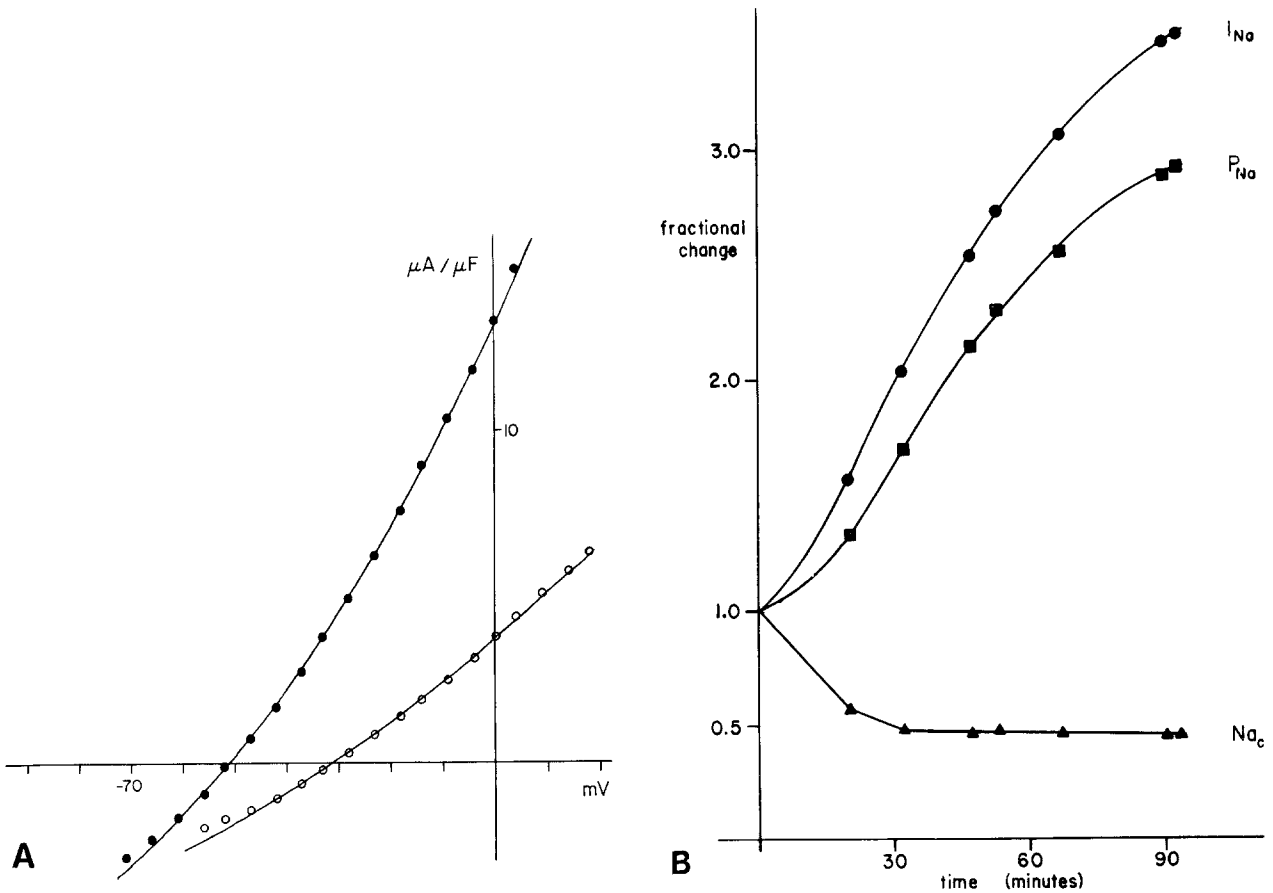

Fig. 5. (*a*): Effect of pyruvate on I_{Na}–V parameters in a single hemibladder. A hemibladder was pre-incubated for 16 hr in substrate free Na-Ringer's solution with aldosterone (5×10^{-7} M) on the serosal side. The serosal medium was then changed to substrate-free high K-sucrose solution with aldosterone and the tissue incubated for an additional hr. *I–V* curves were measured in the absence of mucosal Na, and in the presence of 20 mM mucosal Na. Na pyruvate (7.5 mM) was then added to the serosal medium. The *I–V* curves were remeasured after 90 min. I_{Na}–V curves obtained before (-○-) and 90 min after the addition of pyruvate (●) are shown. The solid lines represent best fits with the constant field equation. (*b*): The time course of I_{Na}, P_{Na} and Na_c after addition of pyruvate in a single substrate depleted aldosterone-treated hemibladder (same hemibladder as in *a*). Fractional changes were estimated as the values of each parameter to the corresponding values just before pyruvate addition. Initial values were $I_{Na}=3.76$ μA/μF; $P_{Na}=0.27\times10^{-5}$ cm/sec and $Na_c=5.8$ mM

Table 2. Effects of aldosterone and pyruvate on I_{Na}–V parameters (substrate depletion-repletion)[a]

	Initial values			Fractional change (pyruvate)[b]		
	I_{Na} (μA/μF)	P_{Na} (10^{-5} cm/sec)	Na_c (mM)	I_{Na}	P_{Na}	Na_c
Aldosterone	6.5±1.3	0.41±0.08	3.72±0.64	2.13±0.38[c]	2.01±0.29[c]	0.85±0.12
Control	1.9±0.5	0.11±0.04	2.13±0.59	1.11±0.15	1.12±0.12	0.77±0.02[c]

[a] Paired hemibladders from Na-loaded toads were incubated in substrate-free Na-Ringer's solution for 15–20 hr with or without aldosterone (5×10^{-7} M, serosal side only). The serosal medium was then changed to high K-sucrose solution and the tissues incubated for an additional hr, after which the I–V relationships were measured with and without 20 mM Na_o (initial values). Na pyruvate (7.5 mM) was added to the serosal medium of both hemibladders, and the I–V relationships remeasured 60–90 min later, when the response of I_{sc} to the substrate was complete. All results are given as means±SEM (5 hemibladder pairs).

[b] Fractional changes were estimated as the ratio of the values at the peak of the response to pyruvate to the value just before pyruvate addition.

[c] Denotes statistical significance: $P<0.05$, for the fractional change

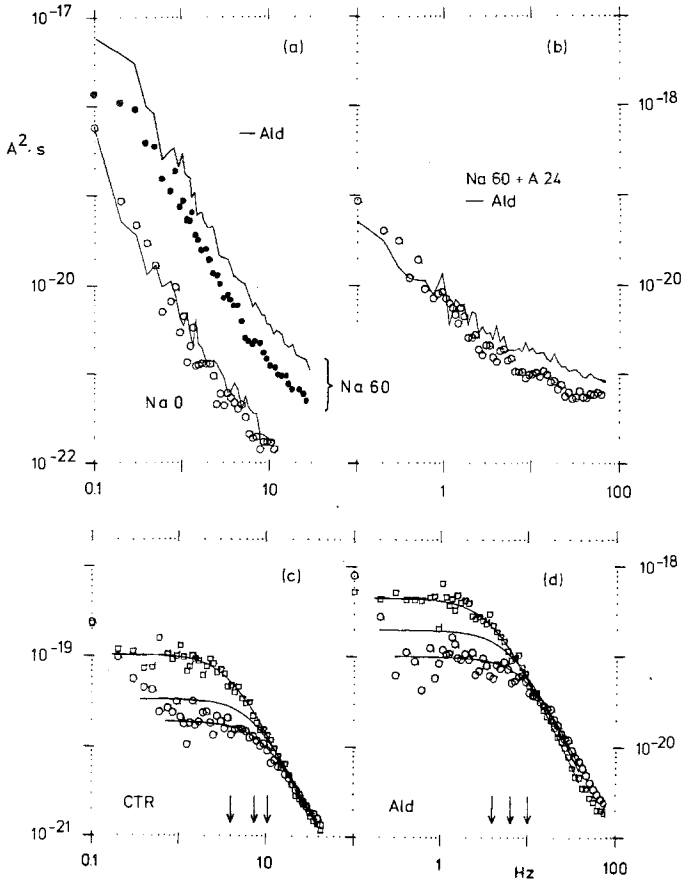

Fig. 6. Current power density spectra obtained from a toad bladder epithelium depolarized with high serosal K and clamped to zero mV. Tissue area corresponded to 6 μF of apical capacitance. (*a*): Spectra obtained in the absence of amiloride with 0 and 60 mM Na_o before (○, ●) and 5 hr after (–) aldosterone addition. (*b*): Spectra obtained with Na transport fully blocked by 24 μM amiloride in the presence of 60 mM Na_o before (○) and 5 hr after (—) aldosterone addition. (*c*): Amiloride-induced Lorentzian spectral components obtained with 1.2, 2.4 and 3.6 μM amiloride in the presence of 60 mM Na_o. The data points of 2.4 μM amiloride are not shown. The smooth curves are Lorentzian fits, each limited to the frequency range covered by the curve. Corner frequencies are indicated by arrows. (*d*): Same experiment as *c*, 5 hr after addition of aldosterone. Plateau power densities have increased but corner frequencies did not change significantly

sured in the absence of amiloride ($r<0.10$). In contrast, the mean area density of conducting channels increased 2.2-fold in response to aldosterone, and the increase in N_0 correlated linearly and closely with the increase in I_{Na} ($r=0.95$) as shown in Fig. 8*b* and *c*. These results show that the aldosterone-regulated increase in P_{Na} is accounted for by a corresponding increase in N_0 (*cf.* Figs. 2 and 8*c*).

Discussion

Response of P_{Na} and Na_c

The earlier studies of Crabbé (1963*b*) and Sharp and Leaf (1964) directed attention to the possible role of increases in P_{Na} in the action of aldosterone. This proposal was supported by the findings that in the isolated toad bladder aldosterone increased the total

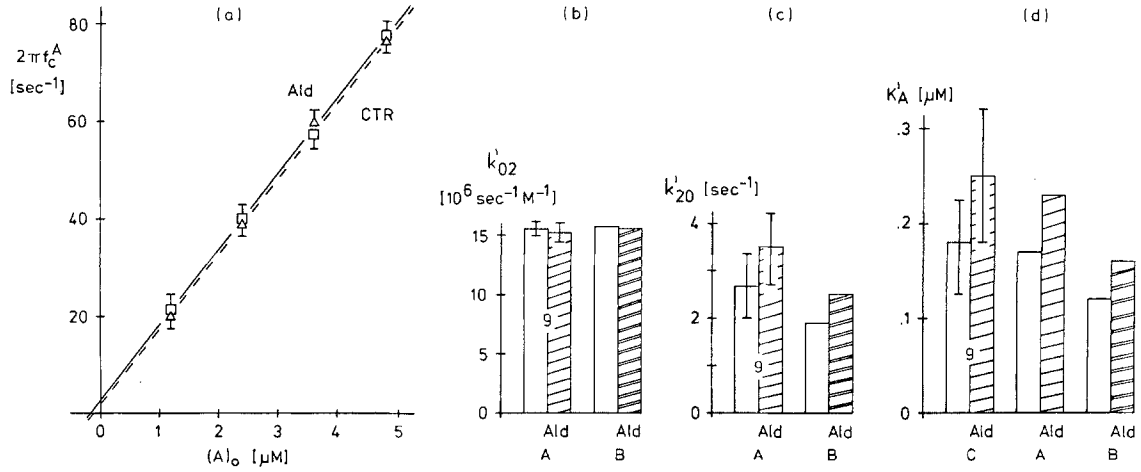

Fig. 7. (*a*): Corner frequency (f_c^A) as a function of amiloride concentration in a rate-concentration plot. Power density spectra were obtained just before and 4–6 hr after addition of aldosterone (5×10^{-7} M) to the serosal side, in the presence of 1.2, 2.4, 3.6, and 4.8 μM amiloride in the mucosal solution (added serially). The spectral data were fitted with Lorentzian curves (Fig. 6) and $2\pi f_c^A$ was plotted *vs.* the amiloride concentration. △ = before aldosterone; □ = after aldosterone. The lines represent least-squares fits to the data points. --- before aldosterone; —— after aldosterone. Data represent means ± SEM for 10 hemibladders. (*b*): Apparent microscopic on-rate constant k'_{02} of the block by amiloride. *A*: k'_{02} evaluated individually with rate-concentration plots (mean ± SEM of 9 experiments). *B*: k'_{02} from mean rates of *a* (slopes of regression lines). (*c*): Apparent microscopic off-rate constant, k'_{20}. *A*: k'_{20} evaluated individually with rate-concentration plots (mean ± SEM of 9 experiments). *B*: k'_{20} from mean rates of *a* (intercept of regression line with ordinate). (*d*): Microscopic apparent inhibition constant of the amiloride block. *A*: K'_A determined from mean values of k'_{02} and k'_{20} in *b* (*A*) and *c* (*A*). *B*: K'_A determined from mean rate-concentration plot in 7(*a*). *C*: K'_A determined from individual rate-concentration plots (mean ± SEM of 9 experiments)

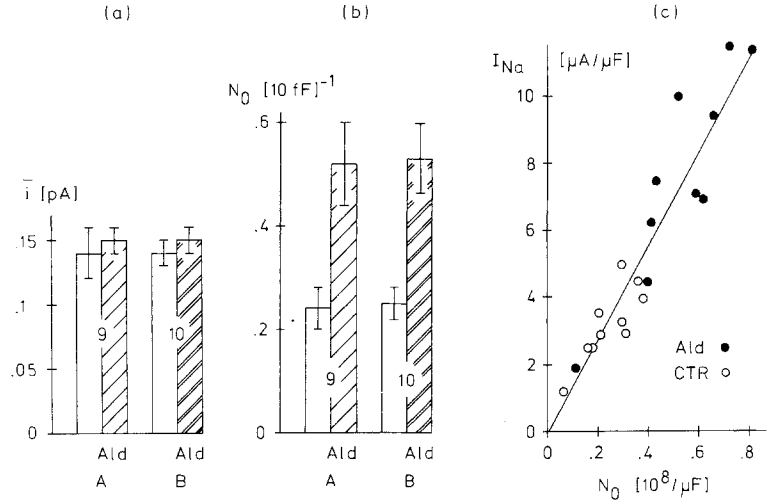

Fig. 8. Effect of aldosterone on single-channel currents and on the area density of conducting Na channels. Individual hemibladders were pre-incubated for 1 to 4 hr with high K-sucrose solution on the serosal side. Control values of I_{Na} and the single-channel parameters i and N_0 were obtained for the period just before the addition of aldosterone (5×10^{-7} M to the serosal side only) and 4 to 6 hr later, when the increase in I_{sc} in response to the hormone was complete (from 3.2 ± 0.36 to 7.63 ± 1.03 μA/μF, 10 experiments). (*a*): Mean channel current i. *A*: i ± SEM (9 experiments) for which the amiloride rate constants from each individual experiment were used to calculate the single-channel current for that same experiment. *B*: i calculated using the mean rate constants obtained in Fig. 7*a* for all 10 experiments. (*b*): Area density N_o of conducting Na channels (Na_0 = 60 mM, A_0 = 0). *A*: N_0 calculated using individual i values from *a* (*A*). *B*: N_0 calculated using individual i values from *a* (*B*). (*c*): Correlation of N_0 and I_{Na} for all 10 experiments. (○) before, (●) after aldosterone. The least squares regression line of all data points shown (r = 0.93) has a slope of 0.14 pA per open channel

transepithelial electrical conductance and that this increase was restricted to the transcellular pathway; i.e., no effect on paracellular conductance was evident (Civan & Hoffman, 1971; Saito & Essig, 1973). In addition, analysis of the time-course of the change in I_{sc} as a function of the change in epithelial conductance revealed parallel effects for the first 3–4 hr, followed by a disproportionate increase in I_{sc} during the next 2–3 hr of observation (Spooner & Edelman, 1975). While these studies did not distinguish between

hormonal effects on the apical and basal-lateral membrane resistances, microelectrode recordings from toad skin recently showed a predominant decrease of the apical membrane resistance after aldosterone stimulation, which presumably reflects an increase in P_{Na} (Nagel & Crabbé, 1980). An effect on P_{Na}, either direct or indirect, was also inferred by Lewis et al. (1976) in studies on the effects of aldosterone on transepithelial electrical conductance across the rabbit urinary bladder, and by Frizzell and Schultz (1978) based on the use of amphotericin B to evaluate the effects of aldosterone in rabbit colon.

The present results, obtained with K-depolarized epithelia, confirm the increase of apical P_{Na} in response to aldosterone. With exogenous substrate present continuously, the differential (experimental *vs.* control hemibladder) increase in I_{Na} (+148%) was matched by an equivalent increase in P_{Na} (+146%). The increase in P_{Na} reflects a corresponding increase in P_{max}, as the Na-sensitivity of P_{Na} was essentially unchanged. The differential increases in I_{Na} and P_{Na} were accompanied by a differential increase in Na_c of 46% (Table 1). It should be noted that this respresents an absolute increase in Na_c of only about 0.9 mM (at 20 mM Na_o). Lipton and Edelman (1971) found no change in the average intraepithelial Na concentration, measured chemically, after addition of aldosterone. In other studies, however, radiosodium uptake into a kinetically defined labile pool of Na, as well as the size of this pool was increased (Crabbé, 1963*b*; Sharp & Leaf, 1964; Crabbé, 1974). The latter results, which are supported by our data, were taken to indicate that aldosterone stimulates apical Na uptake. Our results indicate a lesser differential increase in Na_c as compared to I_{Na} and P_{Na}. This raises the possibility of a coordinate action on apical Na entry and basal-lateral Na exit.

Lewis et al. (1976), and Frizzell and Schultz (1978) discussed the possiblity that aldosterone may act primarily by increasing the rate of extrusion of Na from the cell, thus lowering intracellular Na activity. This, in turn, might increase P_{Na} by release from a negative feedback mechanism. In our experiments, with K-depolarized bladder the observed increase in Na_c after addition of aldosterone is not compatible with this mechanism. Moreover, in our previous study, P_{Na} of K-depolarized toad bladders was insensitive even to substantial changes in Na_c (Palmer et al., 1980).

Channel Currents and Area-Densities

The amiloride-sensitive, Na-specific apical transport pathway of anuran epithelia has been classified as channels ("pores") rather than carriers, on the basis of the high single unit ion translocation rate. In frog skin epithelium, with the serosal membrane depolarized and 60 mM Na-activity in the outside solution, the calculated translocation rate was more than 10^6 Na ions/sec (Lindemann & Van Driessche, 1977). Our results for toad bladder, also indicate a translocation rate in the order of 10^6 Na ions/sec, which is too high for a shuttle-type carrier (Armstrong, 1975). On the basis of a channel model, the aldosterone-dependent increase in P_{Na} could reflect either an increase in the number of conducting channels in the apical membrane or an increase in Na conductance of each channel. The results of our shot-noise analysis indicate that aldosterone increased the density of conducting channels more than two-fold but did not change the single-channel Na current (Fig. 8). The channels can be characterized further in terms of binding affinity for amiloride. The apparent on- and off-rate constants (k'_{02} and k'_{20}) for Na-channel blockade by amiloride were essentially unchanged by aldosterone (Fig. 7). Cuthbert and Shum (1975) also reported that aldosterone does not alter the binding affinity or the dose-response curve of I_{sc} to amiloride. In contrast, Park and Edelman (C.S. Park & I.S. Edelman, *personal communication*) found a two- to threefold decrease in the sensitivity of the toad bladder to amiloride after treatment with aldosterone. Crabbé (1980) found a decrease in sensitivity to the drug after overnight incubation with the hormone, but not with acute hormone treatment.

Na Self-Inhibition

In frog skin epithelium, external Na inactivates Na channels reversibly, possibly by binding to a "modifier site" (Fuchs et al., 1977; Aceves & Cuthberg, 1979; Van Driessche & Lindemann, 1979). This effect is also evident in the toad bladder (Fig. 4). Without the use of fast-flow techniques it is difficult to decide whether Na_o itself or a Na_o-dependent increase in Na_c is responsible for the decrease in P_{Na} in toad bladder (Fuchs et al., 1977). We favor a predominant direct role of Na_o since Na_c was less than 5 mM in our experiments, and P_{Na} was insensitive to Na_c in this range (Palmer et al., 1980).

A change in the poise of the equilibrium between N_0 and N_1 (the area density of channels blocked by Na) could offer an explanation for the hormonally induced increase in N_0. Our observations suggest that this is not the mechanism by which aldosterone increase N_0 for the following reasons: (i) the fractional increase in P_{Na} induced by aldosterone was about the same at 5 and 20 mM Na_o. (ii) When analyzed in terms of first-order inhibition kinetics [Eq. (2)]. P_{Max} was increased by aldosterone while K_N decreased slightly rather than increased (Fig. 4). Thus, impair-

ment of the self-inhibition of Na entry does not mediate the increase in N_0 induced by aldosterone.

Channel Recruitment

Since aldosterone increases N_0 but does not decrease K_N it must increase both N_1 and N_0, i.e., the area density N of all channels which are electrically detectable. The occupancy equation [Eq. (1)], in the absence of amiloride, can be used to estimate N. The measured mean K_N's of 22 mM (control) and 17 mM (aldosterone treated), yielded values of N of 1.0 Na-channels per 10 fF (1 μm^2) for the control and 2.6/10 fF for the aldosterone-treated epithelia. Arguments are presented below in support of the inference that the increase in N draws from a preexisting reservoir of electrically undetectable channels (N_x). We shall call the conversion of N_x to N "recruitment". In the previous paper (Li et al., 1982), we showed that the natriferic response to neuropeptides can also be described as recruitment. It is presently unknown, however, whether the fast recruitment induced by oxytocin and the slower recruitment induced by aldosterone involve the same reservoir of electrically silent channels.

An alternative method for estimating N is provided by the binding of amiloride to the Na channels. Aldosterone increased the density of amiloride binding sites (by inference the density of Na channels) in both isolated toad bladder epithelial cells and frog skin epithelium (Cuthberg, Okpako & Shum, 1974; Cuthbert & Shum, 1975). In the former study, however, site densities of 1,400/μm^2 were found in the absence of aldosterone, a value more than 1,000-fold greater than the channel densities obtained with shot-noise analysis in the present study. Even if amiloride should in addition bind to the nonconducting fraction of Na channels N_x, the discrepancy in channel density remains unresolved. Furthermore, by fluctuation analysis aldosterone and oxytocin had the same effect on N, except for differences in time-course (Li et al., 1982). If amiloride binding provides a valid method for estimating N, there should be a corresponding increase in bound amiloride in the presence of this peptide. Amiloride binding studies, however, did not detect the peptide-dependent increase in Na channel density (Cuthbert & Shum, 1975). It is possible that amiloride binds to the nonconducting channels recruitable by oxytocin, but not to those recruitable by aldosterone.

Metabolic Dependence of Channel Recruitment

Abundant evidence indicates that stimulation of Na transport by aldosterone is mediated by augmentation of protein synthesis (Edelman et al., 1963; Law & Edelman, 1978). An increase in channel density could, of course, result from *de novo* synthesis of Na channel proteins. Indeed, Scott, Reich, Brown and Yang (1978) and Scott, Reich and Goodman (1979) recently reported that some of the aldosterone-induced proteins are associated with a membrane-rich fraction. The available data are also compatible with the view that aldosterone activates pre-existing channels (N_x) by a mechanism involving aerobic metabolism: (i) The rapid increase in channel number after addition of oxytocin to the serosal medium (Li et al., 1982) demonstrates the existence of inactive but readily recruitable Na channels in, or accessible to, the apical membrane. Aldosterone might recruit channels from the same pool, although there is no direct evidence for this. (ii) The decrease in P_{Na} after 2-deoxyglucose treatment (Palmer et al., 1980) provides evidence that the maintenance of P_{Na} requires unimpaired energy metabolism. (iii) The substrate depletion-repletion experiments, in the present study, show that P_{Na} is augmented by the synergistic action of aldosterone and pyruvate with a latent period of less than 30 min (Fig. 5, Table 2). Similarly, Spooner and Edelman (1975) found that aldosterone stimulation of both I_{sc} and transepithelial electrical conductance depended on the metabolic state. (iv) The differential response to substrate repletion 3 to 4 hr after the addition of aldosterone is insensitive to inhibitors of RNA and protein synthesis added at the time of substrate repletion (Fanestil, Herman, Fimognari & Edelman, 1968; Chu & Edelman, 1972). Taken together these results imply that the increase in P_{Na} is not a sole consequence of synthesis of new channel proteins. Rather, the recruitment of Na channels appears to be a reversible process, facilitated by or somehow requiring metabolic input, as shown schematically in Fig. 9.

Other evidence implicating enhanced energy metabolism in the action of aldosterone was obtained in a variety of studies. In substrate-depleted toad bladders, the I_{sc} response to aldosterone is sharply reduced, and all precursors of citrate, e.g., glucose, pyruvate, lactate, acetoacetate, and oxaloacetate, act synergistically with the steroid, in enhancement of I_{sc} (Fimognari, Porter & Edelman, 1967). In the isolated frog skin, thermodynamic analysis yielded proportionate increases in I_{sc} and in the metabolic driving force, in response to aldosterone (Saito, Essig & Caplan, 1973). In addition, Spires and Weiner (1980) measured I_{sc} and QCO_2, with and without uncoupling of oxidative phosphorylation, and concluded that vasopressin acts primarily on the transport apparatus but that aldosterone stimulates availability of energy for Na transport.

Although the evidence implicates aerobic metabolism in both the maintenance of the basal area density

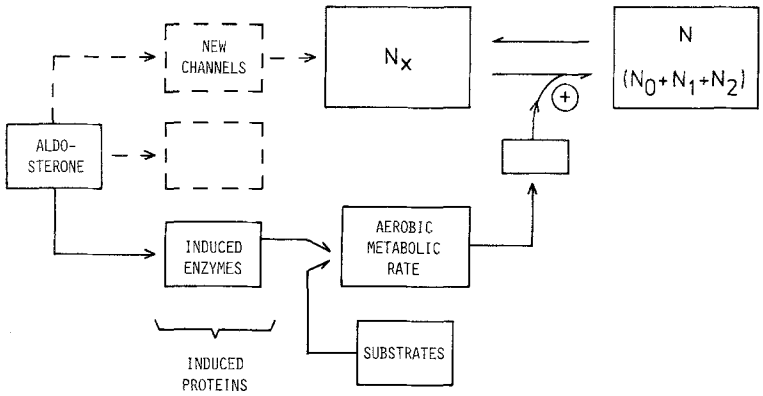

Fig. 9. Possible pathways of aldosterone action on apical Na channels. In this scheme, aldosterone promotes the reversible recruitment of Na channels from the reservoir N_x indirectly by inducing the synthesis of new enzymes (e.g., citrate synthase) involved in aerobic metabolism. Changes in metabolism possible coupled to the induction of other regulatory macromolecules, then lead to the increase in N, at the expense of N_x, through an unknown mechanism (empty box on the right). In addition, the steroid may induce new channels, which would be added to N_x and become available for recruitment

of open Na channels and in agumentation of channel density by aldosterone, induction of the synthesis of new channels and a consequent increase in N_x, could contribute to the response (Fig. 9). Newly induced channels would also require metabolism-dependent reversible activation (recruitment) to become electrically detectable.

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References

Aceves, J., Cuthbert, A.W. 1979. Uptake of [^{3}H] bezamil at different sodium concentrations. Inferences regarding the regulation of sodium permeability. *J. Physiol. (London)* **295**:491–504

Armstrong, C.M. 1975. Evidence for ionic pores in excitable membranes. *Biophys. J.* **15**:932–933

Chu, L.L.H., Edelman, I.S. 1972. Cordycepin and α-amanitin: Inhibitors of transcription as probes of aldosterone action. *J. Membrane Biol.* **10**:291–310

Civan, M.M., Hoffman, R.E. 1971. Effect of aldosterone on electrical resistance of toad bladder. *Am. J. Physiol.* **220**:324–328

Crabbé, J. 1963a. The Sodium-Retaining Action of Aldosterone. *Presses Academiques Europeennes S.C.* Brussels

Crabbé, J. 1963*b*. Site of action of aldosterone on the bladder of the toad. *Nature (London)* **200**:787–788

Crabbé, J. 1974. Influence of aldosterone on active sodium transport by the toad bladder: A kinetic approach. *Acta Physiol. Scand.* **90**:417–426

Crabbé, J. 1980. Decreased sensitivity to amiloride of amphibian epithelia treated with aldosterone. *Pfluegers Arch.* **383**:151–158

Cuthbert, A.W., Okpako, D., Shum, W.K. 1974. Aldosterone, moulting and the number of sodium channels in frog skin. *Brit. J. Pharmacol.* **51**:128 *P*

Cuthbert, A.W., Shum, W.K. 1975. Effects of vasopressin and aldosterone on amiloride binding in toad bladder epithelial cells. *Proc. R. Soc. London* **189**:543–575

Edelman, I.S., Bogoroch, R., Porter, G.A. 1963. On the mechanism of action of aldosterone on sodium transport: The role of protein synthesis. *Proc. Natl. Acad. Sci. USA* **50**:1169–1177

Fanestil, D.D., Herman, T.S., Fimognari, G.M., Edelman, I.S. 1968. Oxidative metabolism and aldosterone regulation of sodium transport. *In:* Regulatory Functions of Biological Membranes. J. Järnefelt, editor, p. 117. Elsevier, Amsterdam

Fimognari, G.M., Porter, G.A., Edelman, I.S. 1967. The role of the tricarboxylic acid cycle in the action of aldosterone on sodium transport. *Biochim. Biophys. Acta* **145**:89–99

Frizzell, R.A., Schultz, S.G. 1978. Effect of aldosterone on ion transport by rabbit colon *in vitro*. *J. Membrane Biol.* **39**:1–26

Fuchs, W., Hviid Larsen, E., Lindemann, B. 1977. Current-voltage curve of sodium channels and concentration dependence of sodium permeability in frog skin. *J. Physiol. (London)* **267**:137–166

Kirsten, E., Kirsten R., Leaf, A., Sharp, G.W.G. 1968. Increased activity of enzymes of the tricarboxylic acid cycle in response to aldosterone in the toad bladder. *Pfluegers Arch.* **300**:213–225

Law, P.Y., Edelman, I.S. 1978. Induction of citrate synthase by aldosterone in the rat kidney. *J. Membrane Biol.* **41**:41–64

Lewis, S.A., Eaton, D.C., Diamond, J.M. 1976. The mechanism of Na^+ transport by rabbit urinary bladder. *J. Membrane Biol.* **28**:41–70

Li, J.H.-Y., Palmer, L.G., Edelman, I.S., Lindemann, B. 1982. The role of sodium-channel density in the natriferic response of the toad urinary bladder to an antidiuretic hormone. *J. Membrane Biol.* **64**:77–89

Lindemann, B., Van Driessche, W. 1977. Sodium-specific membrane channels of frog skin are pores: Current fluctuations reveal high turnover. *Science* **195**:292–294

Lipton, P., Edelman, I.S. 1971. Effects of aldosterone and vasopressin on electrolytes of toad bladder epithelial cells. *Am. J. Physiol.* **221**:733–741

Nagel, W., Crabbé, J. 1980. Mechanism of action of aldosterone on active sodium transport across toad skin. *Pfluegers Arch.* **385**:181–187

Palmer, L.G., Edelman, I.S., Lindemann, B. 1980. Current-voltage analysis of apical sodium transport in toad urinary bladder: Effects of inhibitors of transport and metabolism. *J. Membrane Biol.* **57**:59–71

Porter, G.A., Edelman I.S. 1964. The action of aldosterone and related corticosteroids on sodium transport across the toad bladder. *J. Clin. Invest.* **43**:611–620

Saito, T., Essig, A. 1973. Effect of aldosterone on active and passive conductance and E_{Na} in the toad bladder. *J. Membrane Biol.* **13**:1–18

Saito, T., Essig, A., Caplan, S.R. 1973. The effect of aldosterone on the energetics of sodium transport in the frog skin. *Biochim. Biophys. Acta* **318**:371–382

Schmidt, U., Schmid, H., Dubach, U.C. 1973. Rapid Na-K-ATPase changes in the nephron of adrenalectomized rats after aldosterone application. *Clin. Res.* **21**:705

Scott, W.N., Reich, I.M., Brown, J.A., Jr., Yang, C.-P.H. 1978. Comparison of toad bladder aldosterone-induced proteins and proteins synthesized *in vitro* using aldosterone-induced messenger RNA as template. *J. Membrane Biol.* **Special Issue**:213–220

Scott, W.N., Reich, I.M., Goodman, D.B.P. 1979. Inhibition of fatty acid synthesis prevents incorporation of aldosterone-induced proteins into membranes. *J. Biol. Chem.* **254**:4957–4959

Sharp, G.W.G., Leaf A. 1964. Biological action of aldosterone *in vitro*. *Nature (London)* **202**:1185–1188

Sharp, G.W.G., Leaf A. 1966. Mechanism of action of aldosterone. *Physiol. Rev.* **46**:593–633

Spires, D., Weiner, M.W. 1980. Use of an uncoupling agent to distinguish between direct stimulation of metabolism and direct stimulation of transport: Investigation of antidiuretic hormone and aldosterone. *J. Pharmacol. Exp. Therap.* **214**:507–515

Spooner, P.M., Edelman, I.S. 1975. Further studies on the effect of aldosterone on electrical resistance of toad bladder. *Biochim. Biophys. Acta* **406**:304–314

Van Driessche, W., Lindemann, B. 1979. Concentration dependence of currents through single sodium-selective pores in frog skin. *Nature (London)* **282**:519–520

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