Regulation of Na Channels of the Rat Cortical Collecting Tubule by Aldosterone

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ABSTRACT The activity of apical membrane Na channels in the rat cortical collecting tubule was studied during manipulation of the animals' mineralocorticoid status in vivo using a low-Na diet or the diuretic furosemide. Tubules were isolated and split open to expose the luminal membrane surface. Induction of Na channel activity was studied in cell-attached patches of the split tubules. No activity was observed with control animals on a normal diet. Channel activity could be induced by putting the animals on the low-Na diet for at least 48 h. The mean number of open channels per patch (NP_o) was maximal after 1 wk on low Na. Channels were also induced within 3 h after injection of furosemide (20 mg/kg body wt per d). NP_o was maximal 48 h after the first injection. In both cases, increases in NP_o were primarily due to increases in the number of channels per patch (N) at a constant open probability (P_o). With salt depletion or furosemide injection NP_o is a saturable function of aldosterone concentration with half-maximal activity at ~8 nM. When animals were salt repleted after 1–2 wk of salt depletion, both plasma aldosterone and NP_o fell markedly within 6 h. NP_o continued to decrease over the next 14 h, while plasma aldosterone rebounded partially. Channel activity may be dissociated from aldosterone concentrations under conditions of salt repletion.

INTRODUCTION

The adrenal steroid aldosterone is a major regulator of salt and water balance in vertebrates (Garty, 1986; Rossier and Palmer, 1992). The most important target organs for the hormone are the electrically tight, Na-reabsorbing epithelia, particularly in the kidney and the gut. A primary action of the hormone is the increase in Na permeability of the apical membrane of these cells, leading to increased Na reabsorption. This has been demonstrated both with electrical measurements on intact epithelia (Nagel and Crabbé, 1980; Palmer, Li, Lindemann, and Edelman, 1982; Lewis and Wills, 1983; Sansom and O'Neil, 1985) and with Na fluxes into isolated membrane vesicles (Asher and Garty, 1988). There is, however, relatively

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little known about the action of aldosterone at the single channel level. Palmer et al. (1982) used noise analysis to show that the increase in permeability in the toad bladder was the result of an increase in the apparent number of conducting channels, with no change in the single channel current. More recently, Kemendy et al. (Ling, Kemendy, Kokko, Hinton, Marunaka, and Eaton, 1990; Kemendy, Kleyman, and Eaton, 1992) have shown in A6 cells that a major effect of the hormone is to increase Na channel open probability (P_o).

In this study, we have correlated the Na channel activity in rat cortical collecting tubules (CCTs) with the levels of circulating aldosterone under various degrees of salt deprivation and diuretic treatment. We have used the patch-clamp technique to estimate channel density, conductance, and P_o under these conditions.

MATERIALS AND METHODS

Biological Preparations

Sprague-Dawley rats of either sex (100–150 g) raised free of viral infections (Charles River Laboratories, Wilmington, MA) were fed either a normal rat chow (Purina Formulab 5008; Na content 2.8 g/kg, K content 11 g/kg) or a low-Na diet (#902902; Na content 3.8 mg/kg, K content 8.6 g/kg; ICN Biochemicals, Cleveland, OH). Furosemide was injected intraperitoneally at a dose of 20 mg/kg body wt. For salt repletion, animals were given normal chow and saline drinking water (0.9% NaCl) ad lib. In one series of experiments the animals were implanted subcutaneously with osmotic minipumps (model 2002; Alza Corp., Palo Alto, CA). The pumps were filled with aldosterone (Sigma Chemical Co., St. Louis, MO) dissolved in polyethylene glycol 300 at concentrations designed to provide rates of infusion of 250 (μg/kg body wt)/d.

Animals were killed by cervical dislocation, the kidneys removed, and the CCTs dissected free and opened manually to expose the luminal surface. The split tubules were attached to a small plastic rectangle coated with Cell-Tak (Collaborative Research, Bedford, MA) and placed in a perfusion chamber mounted on an inverted microscope. Measurements of single channel activity were carried out at room temperature (23–25°C), or in some cases at 37°C. Tubules were maintained at room temperature in NaCl superfusate (see below) for up to 3 h before measurements were made. Throughout this period there was no obvious effect of time on the number or properties of the channels. Principal cells of the tubule were identified visually as described previously (Pácha, Frindt, Sackin, and Palmer, 1991; Silver, Frindt, and Palmer, 1992).

Solutions

Tubules were superfused with solution consisting of (mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 2 glucose, and 10 HEPES, adjusted to pH 7.4 with NaOH. In one set of experiments (Fig. 4) the superfusing solution contained Kgluconate 140 mM instead of NaCl. The difference in superfusing solutions did not greatly affect channel density or properties. Comparing maximally stimulated animals, mean values in NaCl and Kgluconate superfusates were: N = 4.5 ± 0.6 vs. 3.8 ± 0.5; P_o = 0.50 ± 0.04 vs. 0.52 ± 0.04; g = 7.9 ± 0.2 vs. 7.7 ± 0.2 pS. Thus in some of the summary figures data using the two perfusates are combined. The patch-clamp pipettes were filled with solution containing (mM): 140 LiCl, 3 $MgCl_2$, and 10 HEPES, adjusted to pH 7.5 with NaOH. This solution was used rather than a Na-containing solution because the single channel currents are larger when Li is the conducted ion. This facilitates the analysis of the patch-clamp records.

Electrical

Basic patch-clamp methods were as described previously (Palmer and Frindt, 1986, 1988). Recording of currents and analysis of data were carried out with an Atari 1040 ST computer equipped with interface and data acquisition software (Instrutech, Mineola, NY). Current records were stored on video tape using a pulse-code modulator. Computation of the mean number of open channels (NP_o) was carried out either by hand or using the TAC program (Instrutech). The number of channels in the patch (N) was determined from the number of discrete current levels observed. This may in some cases underestimate N. Justification for using this estimate is discussed below.

Fluorescence

The basic apparatus for the measurements of fluorescence was described previously (Silver et al., 1992). This consisted of a model IM35 (Carl Zeiss, Inc., Thornwood, NY) inverted microscope equipped for epifluorescence with quartz interior optical components. The exciting and emitted light passed through a 40× water immersion objective (N.A. 0.75; Carl Zeiss, Inc.). The microscope was coupled to an alternating wavelength illumination system (Delta Scan; Photon Technology International, New Brunswick, NJ).

For measurements of cell pH, the open tubules were loaded with 2′,7′-bis (carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Inc., Eugene, OR) for 60 min at room temperature and superfused at 37°C with the same solution used for the patch-clamp experiments. Single principal cells or two adjacent principal cells were illuminated at 440 and 490 nm and the emission was collected with a high-pass filter at 520 nm. Intracellular calibration of the dye was performed according to the method of Thomas, Buchsbaum, Zimniak, and Racker (1979) at the end of each experiment. The tubule was superfused with a KCl solution containing the K/H exchanger nigericin (10 μM) at two to three different pH values between 6.5 and 7.8. The response of the dye, measured as the ratio of emissions at 440 and 490 nm, was linear over this range.

Plasma Steroids

A sample of blood for determination of circulating levels of steroids was obtained at the time that the animals were killed. Corticosterone and aldosterone were measured in 0.2–1 ml of plasma. Tritiated corticosterone and aldosterone were added as internal standards to each plasma sample. The plasma was extracted twice with 8 ml fresh ether, dried under a stream of filtered air, and resolubilized in 1 ml of a mixture of ethyl acetate:iso-octane (9:1). This mixture had previously been saturated with a solution of ethylene glycol:water (8:2). The resolubilized extract was chromatographed on Abraham's system III columns (3 g celite with a stationary phase of 8:2 ethylene glycol:water). The columns were first washed with 1 ml of iso-octane to remove nonpolar lipids and steroids. Desoxycorticosterone, corticosterone, cortisol, and aldosterone were eluted sequentially from the columns with the following mixtures of iso-octane:ethyl acetate: 3.5 ml of 80:20, 3.5 ml of 70:30, 5 ml of 60:40, and 5 ml of 50:50. The second and fourth fractions containing corticosterone and aldosterone, respectively, were dried under vacuum and resolubilized in 1 ml of 0.5 M phosphate buffer for radioimmunoassays.

Corticosterone and aldosterone were measured by radioimmunoassay. The antibody for aldosterone was obtained from the National Hormone and Pituitary Program at the University of Maryland School of Medicine (Baltimore, MD). It had $<1\%$ cross-reactivity with most steroids. The antibody for corticosterone was a gift from Dr. Paul Vecsei at the University of Heidelberg (Heidelberg, Germany). This antibody had 5% cross-reactivity with deoxycorticosterone. Cross-reactivity with other steroids was $<1\%$. Cross-reactivity of the antibodies for corticosterone and aldosterone was avoided since the plasma steroids were separated by celite

chromatography. The sensitivities of the corticosterone and aldosterone radioimmunoassays were 0.03 μg/dl and 3 ng/dl, respectively.

RESULTS

Single Channel Measurements

Na channels were recognized in cell-attached patches according to criteria described previously (Palmer and Frindt, 1986, 1988). The currents were inward except at large negative pipette potentials and the single channel conductance was in the range of

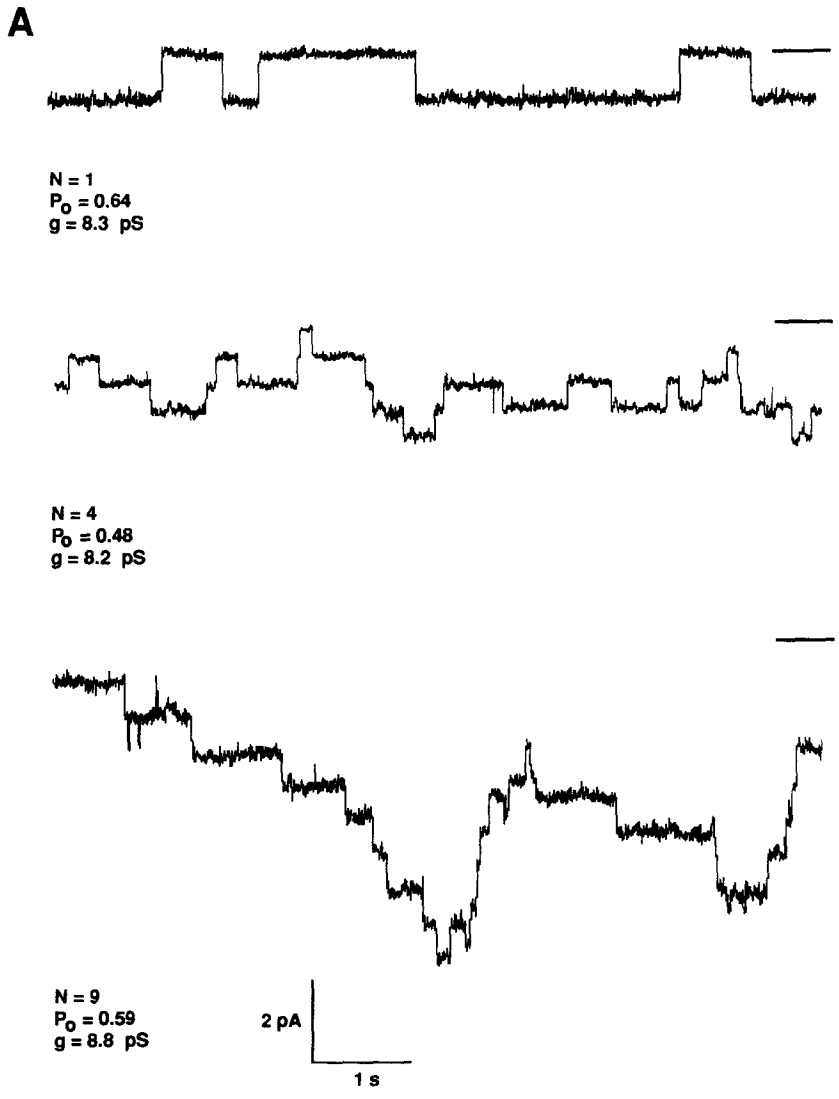

FIGURE 1

7–10 pS with 140 mM Li in the pipette. The number of channels in the patch was highly variable.

Examples of recordings from three different patches are shown in Fig. 1. These patches appeared to contain one, four, and nine channels, estimated from the number of discrete current levels observed. Distribution of these current levels is shown in Fig. 1 *B*. The distribution for the first patch with one channel is straightfor-

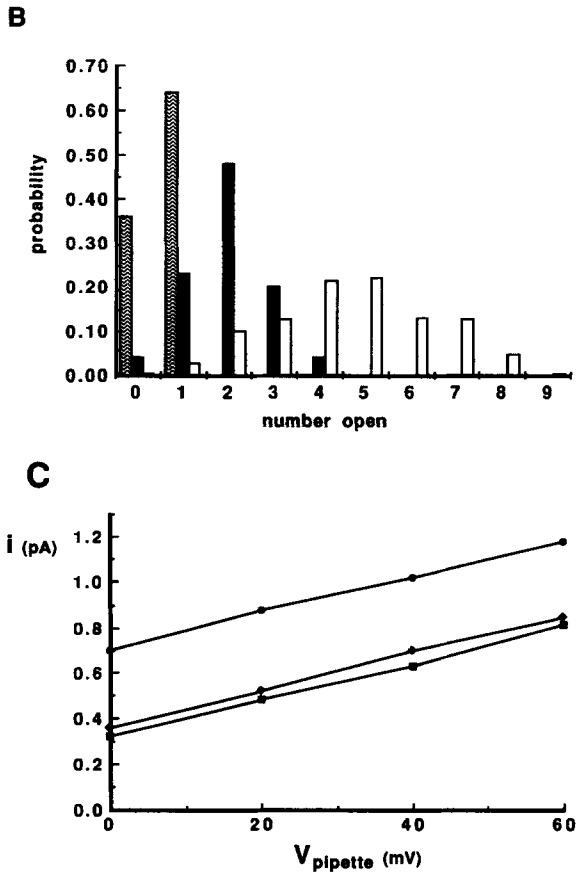

FIGURE 1. Cell-attached recordings from patches with different levels of Na channel activity. (*A*) The top trace shows a patch with one Na channel. The tubule was from a rat that was salt depleted for 15 d and subsequently salt repleted for 20 h. The pipette potential was +40 mV. The middle trace shows a patch where there were four active channels. The tubule was from a rat that was salt depleted for 9 d and salt repleted for 12 h. Pipette potential was +40 mV. The bottom trace shows a patch with at least nine channels. The level with all channels closed is not visible in this segment. The tubule was from a rat that was salt depleted for 8 d. Pipette potential was +60 mV. Channel openings give downward deflections. Current levels with all channels closed are indicated by solid lines on the right-hand side of the traces. (*B*) Histograms show the probability of observing *n* channels open for the top (*wavy bars*), middle (*filled bars*), and bottom traces (*open bars*) of *A*. (*C*) Open channel *i-V* relationships for the three patches in *A*. Data from the top trace are shown as circles, from the middle trace as squares, and from the bottom trace as diamonds.

ward and shows that the channel spent 64% of the time in the open state. In the second patch there were apparently four active channels, with a mean P_o of 0.48. The distribution of the states was reasonably close to that expected for four independent channels with identical P_o values. The third patch apparently had nine channels, with a mean P_o of 0.59. Here the distribution of states was approximately normal, and from the symmetry of the distribution the P_o is close to 0.5.

The current–voltage (i-V) relationships for these channels are shown in Fig. 1 *C*. In all three patches the slopes of the i-V curves were similar, indicating a single channel conductance of ~8.5 pS for $V_p > 0$. The value of g is higher than that observed when Na is the major conducted ion (5 pS [Palmer and Frindt, 1986]), but is similar to that reported previously when the pipette contained 140 mM Li (Palmer and Frindt, 1988). The value of i at $V_p = 0$ is larger for the patch with one channel. This probably indicates a larger negative resting membrane potential in this cell, which was from a salt-repleted animal.

In the patches with a large number of channels it was difficult to be sure that all possible states had indeed been visited and therefore the value of N is a lower estimate. For example, in the patch with $N = 9$ shown in Fig. 1 the states with zero and nine channels open were observed once and twice, respectively, over a recording period of 82 s. To see if we were substantially underestimating N with this method, we analyzed the kinetics of channel opening and closing. We had previously found that these kinetics could be described by a model with a single open and a single closed state, with similar mean lifetimes and a P_o of ~0.5 (Palmer and Frindt, 1986). Under

TABLE I

Estimates of Channel Kinetics for Patches with Few Channels and Many Channels

	N	P_o	$\langle\Delta t\rangle$	$N\langle\Delta t\rangle$
			s	s
Group 1 (low N) $n = 16$	2.3 ± 0.2	0.47 ± 0.03	1.18 ± 0.21	2.26 ± 0.24
Group 2 (high N) $n = 16$	8.8 ± 0.3	0.53 ± 0.02	0.25 ± 0.03	2.09 ± 0.23

Groups were selected as described in the text. The mean dwell time $\langle\Delta t\rangle$ depends on both the kinetics of the channels and the number of channels in the patch. The parameter $N\langle\Delta t\rangle$ is an estimate of the mean open and closed times and should be independent of N.

these conditions the mean lifetime of the states with 0, 1, . . . N channels open should be approximately equal, and inversely proportional to the number of channels actually in the patch. Accordingly, we calculated the mean residence time for all states, $\langle\Delta t\rangle$. The true mean open/closed state lifetimes (assumed to be equal) of an individual channel $\langle\Delta t^o\rangle$ should be given as: $\langle\Delta t^o\rangle = N\langle\Delta t\rangle$. If our estimate of N is too low, then the calculated value of $\langle\Delta t^o\rangle$ for these patches should also be underestimated.

We first calculated $\langle\Delta t^o\rangle$ for 16 patches, obtained under a variety of experimental conditions, in which we could define N with confidence (Table I). In these patches there were one to three channels, and the states with 0 and N channels open were observed at least 10 times each. This provides a high level of confidence that the total number of channels can be estimated correctly. Values of $\langle\Delta t^o\rangle$ were 2.26 ± 0.24 s. This is then an estimate of the average of the mean open and mean closed time for these channels, somewhat lower than the values of 3.4–3.9 s obtained in an earlier study (Palmer and Frindt, 1986). We then analyzed 16 records of patches with N = 7–12. Although N could not be estimated with certainty, the mean value of $\langle\Delta t^o\rangle$ was

2.09 ± 0.23 s, very close to that calculated for the group with small N. This indicates that we are not *grossly* underestimating N in these experiments. If this were the case the calculated $\langle \Delta t^o \rangle$ values would be much lower. Obviously we could be underestimating N by 10–20% in these cases, but this would not affect the conclusions of the study in any way.

The time course of the evolution of channel activity after placing the rats on a low-Na diet is shown in Fig. 2. Channels such as those shown in Fig. 1 were not observed when the animals were on a normal diet or after 1 d on the low-Na diet. Na

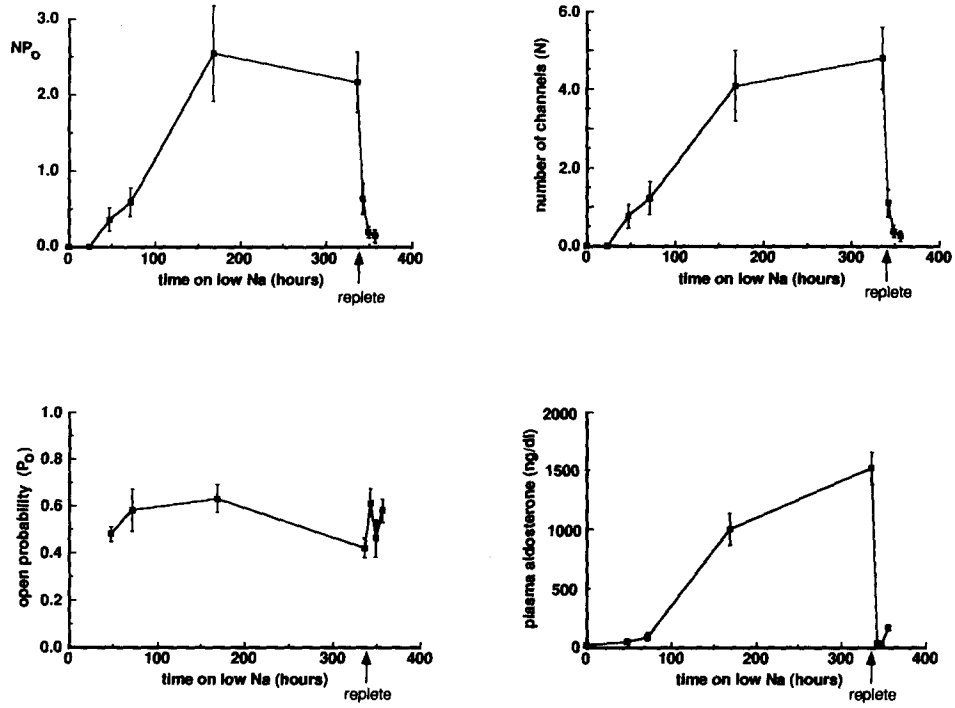

FIGURE 2. Time course of the evolution of Na channel activity in rats on a low-Na diet. The animals were placed on the low-Na diet at time 0. Values at 168 h are pooled from rats on low Na for 6–8 d. Values at 336 h are pooled from rats on low-Na for 13–16 d. For Na repletion the diet was switched back to normal chow and 0.9% NaCl was added to the drinking water. Mean values of NP_o, N, P_o, and plasma aldosterone are shown. Each point represents the mean ± SEM for 10–41 patches.

channel activity, measured as NP_o, first appeared 2 d after the animals were put on the low-Na regimen, increased rapidly over the next several days, and appeared to reach a maximum level at day 7. NP_o remained at a maximal level through day 14. When the animals were Na repleted by giving them normal chow and including 0.9% NaCl in their drinking water, the channels disappeared rapidly. 12 h after repletion, NP_o had fallen to $<10\%$ of the initial values. The major change during the time courses of Na depletion and repletion appeared to be in the number of channels, N, with the P_o remaining at ~ 0.5 throughout.

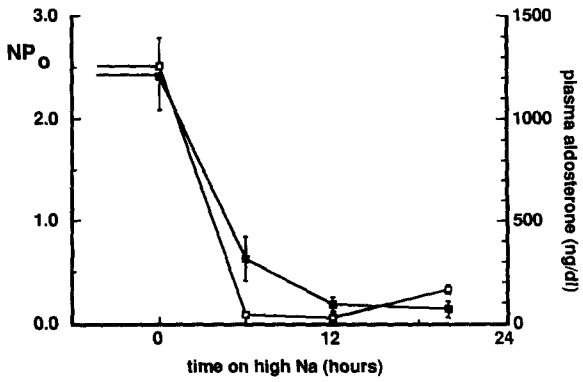

FIGURE 3. Time course of NP_o and plasma aldosterone after salt repletion. Data from Fig. 2 are replotted to compare the time courses of changes in channel activity and circulating hormone levels.

Also shown in Fig. 2 is the time course of the increase in plasma aldosterone. Like the channel activity, aldosterone increased slowly over the first 3 d and then rapidly over the next 4 d. Hormone levels continued to increase during the second week of Na depletion, reaching mean levels of 1,500 ng/dl, an increase of 75-fold over control levels. Upon salt repletion, aldosterone levels fell very quickly, approaching basal levels within 6 h. A curious finding was that after 20 h of salt repletion, aldosterone levels increased again, to 170 ng/dl (Fig. 3). We have no explanation for this effect, which was not accompanied by an increase in NP_o. Thus, in this instance NP_o and aldosterone levels are clearly dissociated.

The relatively rapid fall in NP_o observed during the first 12 h of salt repletion could be accounted for by the fall in plasma aldosterone, or could reflect in part increased salt intake per se. To test this point, we performed a second repletion experiment in which aldosterone levels were maintained at maximal levels (>500 ng/dl) using osmotic minipumps. Under these circumstances channel activity was intermediate (Table II). The mean value of NP_o was significantly lower than that for the Na-depleted, high-aldosterone animals, but significantly higher than that for Na-repleted, low-aldosterone animals. Thus the decline in channel activity after salt

TABLE II

Effect of Na Repletion on NP_o and Plasma Aldosterone in the Presence and Absence of Exogenous Aldosterone

	NP_o	Plasma aldosterone
		ng/dl
Na depleted	2.41 ± 0.33	1,260 ± 132
	(n = 37)	(n = 9)
Na repleted (12 h)	0.19 ± 0.07	26 ± 10
	(n = 50)	(n = 3)
Na repleted (12 h) + aldosterone	1.28 ± 0.24	750 ± 150
	(n = 38)	(n = 4)

Na-depleted rats were maintained on a low-Na diet for 7–14 d. Na-repleted rats were given normal chow and saline drinking water for 12 h. Na-repleted + aldosterone rats were given exogenous aldosterone via osmotic minipumps implanted 4–7 d before repletion. n refers to the number of patches in the case of NP_o and to the number of animals in the case of the plasma aldosterone.

repletion may reflect a combination of reduced aldosterone levels and increased salt intake.

The induction of channel activity under these circumstances is quite slow compared with the time course of the known effects of aldosterone on epithelial transport, presumably because the increases in circulating aldosterone are slow. To study more rapid effects of the hormone, we first injected the animals with aldosterone and studied the CCT 3–5 h later. While channels were observed in preliminary experiments, the results were not very consistent, perhaps due to the rapid metabolism and excretion of the hormone. Better results were obtained by injection of the diuretic

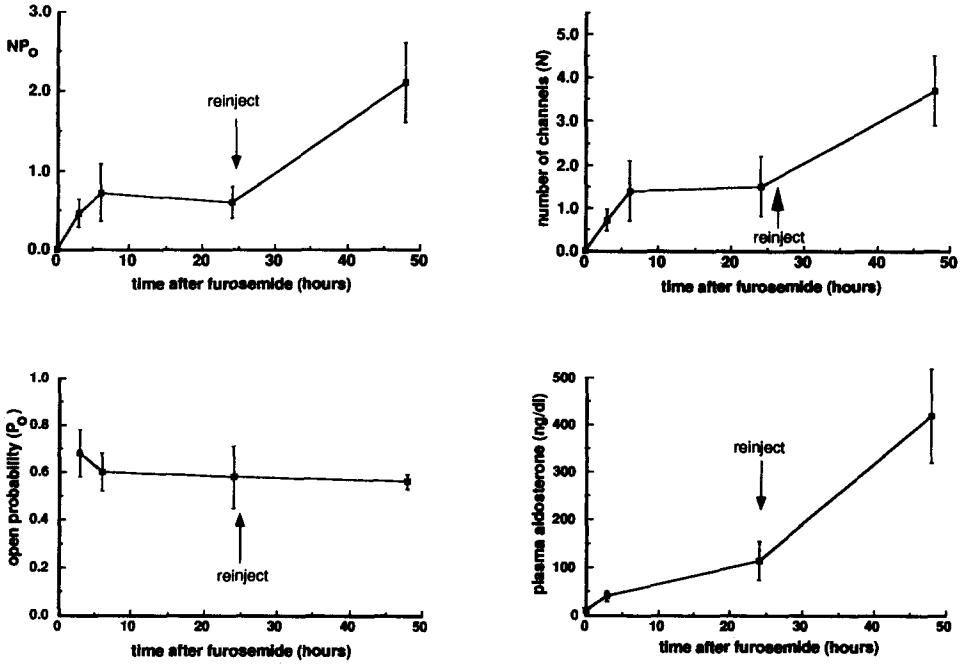

FIGURE 4. Time course of the evolution of Na channel activity in rats injected with furosemide. The animals were given one injection at time 0. Animals studied after 48 h were given a second injection 24 h after the first. Tubules were superfused with Kgluconate solution. Mean values of NP_o, N, P_o, and plasma aldosterone are shown. Each point represents the mean ± SEM for 12–22 patches.

furosemide. This drug causes volume depletion and endogenous aldosterone secretion. Animals receiving furosemide were also given the low-Na diet. Indeed, channels could be reproducibly observed 3 h after injection of furosemide (Fig. 4). Injection of a similar amount of saline did not result in the appearance of Na channels. Channel activity increased further 6 h after furosemide injection, remaining approximately constant up to 24 h. When a second injection of the drug was given after 24 h, a higher level of channel activity was observed 24 h later. The value of NP_o was similar to that attained after 1–2 wk of Na depletion (Fig. 2). Again, the increases in NP_o

appeared to be attributable mainly to increases in N. P_o did not change significantly throughout the time course of diuretic administration.

Histograms of N are shown in Fig. 5. The tubules were arbitrarily divided into fully stimulated states (7–14 d on the low-Na diet or 2 d on furosemide) and partially stimulated states (2–7 days on the low-Na diet, 3–6 h on furosemide, 6–12 h of Na repletion). As can be seen in these histograms, the fully stimulated tubules were characterized by many more patches with more than five channels and many fewer patches with no channels. The distribution is broader than that of a normal or binomial distribution and may be bimodal, although we do not have enough data to

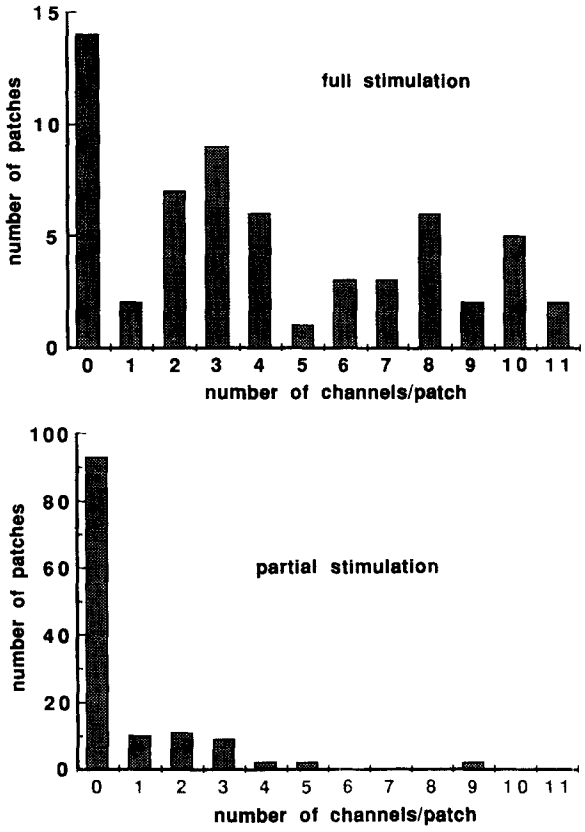

FIGURE 5. Histograms for the number of channels per patch. Patches were divided into two groups. The full stimulation group (*top*) included animals on a low-Na diet for 1 wk or more and those given furosemide for 48 h. The partial stimulation group (*bottom*) included animals on a low-Na diet for 2–4 d, Na repleted for 6 h, and treated with furosemide for 3–6 h.

establish this point. The distribution for the partially stimulated tubules is closer to the binomial, but there were two observations with $N = 9$. This would be very rare if the channels were distributed randomly in the membrane and suggests the possibility of clustering.

Although there was no systematic trend of changes in P_o with mineralocorticoid status, there was a very large variation in P_o from patch to patch, similar to variations reported previously by us (Palmer and Frindt, 1988). These variations could be seen in patches from different cells from the same animal or even the same tubule. We do not know if different patches from the same cell would have less variability. A scattergram of P_o vs. N is shown in Fig. 6. There was no significant correlation

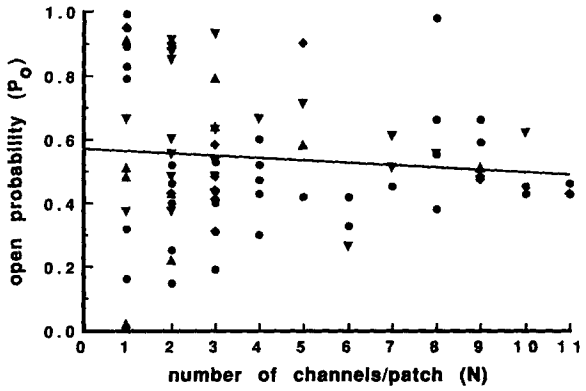

FIGURE 6. Scatter plot of P_o vs. N for all patches studied. The line represents the least-squares linear correlation. Different symbols represent data from Na-depleted animals (*circles*), furosemide-treated animals (*inverted triangles*) and Na-repleted animals (*upright triangles*).

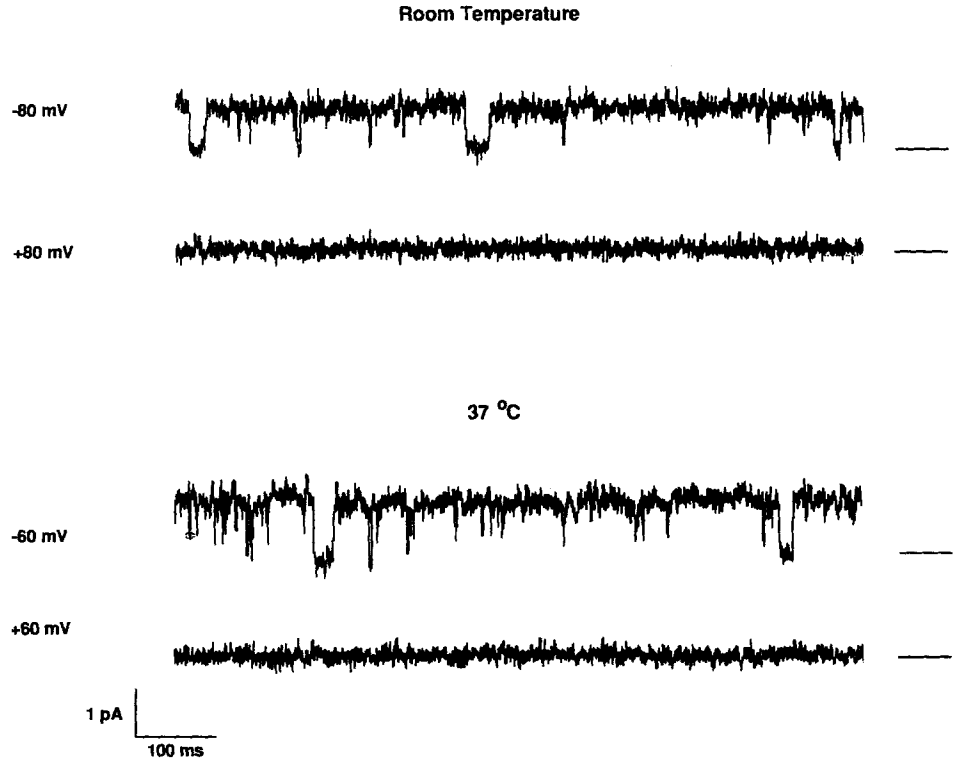

FIGURE 7. Absence of Na channel activity in Na-repleted animals. Recordings from two patches are shown, one at room temperature and one at 37°C. Pipette potentials are shown to the left of each trace. Levels at which channels are closed are indicated by lines to the right of each trace. At positive pipette potentials no channel activity could be seen. At negative potentials outward currents attributed to the activity of low-conductance, high P_o K channels are easily distinguished.

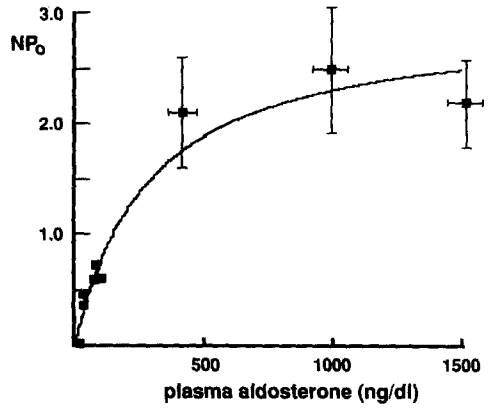

FIGURE 8. Plot of mean NP_o vs. aldosterone. Mean values are plotted for rats Na depleted for 3, 4, 7, or 14 d, and furosemide treated for 3, 6, 24, and 48 h. The solid line represents a least-squares fit to the equation: $NP_o = (NP_o)_{max}/[1 + K_d/\ (aldo)]$, with $(NP_o)_{max} = 3.0$ and $K_d = 280$ ng/dl.

between the two parameters. The larger scatter at low N compared with high N may reflect the fact that at high N only the average values of P_o for more than one channel are plotted.

The observation that CCTs from salt-repleted animals had fewer observable channels differs from that of Kemendy et al. (1992), who reported that aldosterone-depleted A6 cells contained channels with a reduced P_o. The lack of Na channel activity, within the resolution limits of our system, is illustrated in Fig. 7. At positive (hyperpolarizing) pipette potentials there were no observable transitions. At negative (depolarizing) potentials outward currents were seen that could be attributed to small-conductance K channels (Frindt and Palmer, 1989). To ascertain whether the lack of channel activity might be the result of unphysiological temperature (23–25°C), another set of experiments was carried out at 37°C. In 20 good seals from three salt-repleted animals no Na channel activity was evident. An example of a patch that

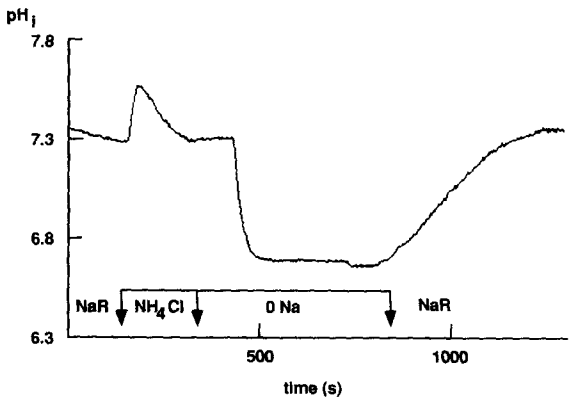

FIGURE 9. Measurement of cell pH and Na-H exchange activity in principal cells. Tubules were initially bathed in normal NaCl Ringer's solution and the fluorescence of BCECF was monitored. After measuring the basal pH the cell was given an acid load using a pulse of NH_4Cl. Removal of NH_4Cl was with a Na-free perfusate, resulting in a decreased pH_i with little recovery. Readdition of Na to the perfusate elicited an alkalinization of the cell to basal levels. The Na-dependent recovery rate was calculated as the slope of the alkalinization phase.

lacked Na channel activity, but which contained small-conductance K channels, is shown in Fig. 7. Under identical conditions in salt-depleted animals Na channels with P_o's of ~0.5 are readily observed (Silver, Frindt, Windhager, and Palmer, 1993).

To illustrate the relationship between channel activity and mineralocorticoid status, NP_o is plotted as a function of plasma aldosterone levels for salt-depleted and furosemide-treated animals (Fig. 8). The relationship appears to be a saturating one, as would be expected for a receptor-mediated system. The region of greatest sensitivity to aldosterone is from 0 to 200 ng/dl, and saturation is evident above 500 ng/dl. The data could be fitted reasonably well with the simple relationship: $NP_o = (NP_o)_{max}/[1 + K_d/\text{(aldo)}]$, with $(NP_o)_{max} = 3.0$ and $K_d = 280$ ng/dl. This value for half-maximal activity corresponds to ~8 nM.

Effects on Cell pH

We have investigated the extent to which changes in intracellular pH might mediate the increase in activity of Na channels. Fig. 9 shows a typical trace in which pH is monitored in a principal cell using BCECF fluorescence. The baseline pH was 7.32.

TABLE III

Effect of Aldosterone on pH_i and Na/H Exchange in Rat CCT

	pH_i	Na-dependent acid extrusion*
Control ($n = 5$)	7.39 ± 0.07	2.7 ± 0.3
Na depleted (7–14 d) ($n = 14$)	7.41 ± 0.03	2.6 ± 0.3

Data are given as means and SEM for 5 rats on a normal diet and 14 rats on a low-Na diet for 1–2 wk.

*$d(pH_i)/dt$, pH units/second × 10^3.

The cell was then acid loaded using the NH_4 pulse technique. In the absence of Na there was little recovery of pH. Readdition of Na resulted in prompt recovery of pH to the control level. The baseline pH and the rate of acid extrusion after an NH_4 pulse is summarized in Table III for rats on normal and low-Na diets. There was no effect of the diet on either the basal pH or the apparent activity of the Na-H exchanger.

DISCUSSION

Dependence of Na Channel Activity on Diet

We confirm in this report the finding that normal, adrenal-intact rats on normal chow do not express functional Na channels measured either as single channel events or as amiloride-sensitive, whole-cell conductance (Palmer and Frindt, 1986; Frindt, Sackin, and Palmer, 1990). This finding is consistent with previous reports that isolated, perfused rat CCTs do not exhibit electrogenic, amiloride-sensitive Na reabsorption unless the mineralocorticoid levels are raised (Tomita, Pisano, and Knepper, 1985;

Reif, Troutman, and Schafer, 1986). The rat colon shows a similar pattern of transepithelial transport. Although the colon of a normal rat will reabsorb Na, this reabsorption is not amiloride sensitive unless the rat is Na depleted or infused with aldosterone or glucocorticoids (Martin, Jones, and Hayslett, 1983; Will, Cortright, DeLisle, Douglas, and Hopfer, 1985; Pácha, Popp, and Capek, 1988). Thus the rat CCT and colon differ from the corresponding rabbit tissues, as well as most amphibian model systems, in which there is normally a basal rate of Na transport even with aldosterone-depleted or adrenalectomized animals.

Role of Aldosterone in Induction of Channel Activity

We have reported previously that Na depletion greatly enhanced Na channel activity in the rat CCT (Palmer and Frindt, 1986; Frindt et al., 1990). The data presented here suggest that this enhancement can be accounted for by increased levels of circulating aldosterone. First, the increase in activity was reasonably well correlated with increases in plasma aldosterone levels measured by radioimmunoassay. Second, increasing aldosterone levels by furosemide injection produced similar results but on a faster time scale. Third, during salt repletion both plasma aldosterone and channel activity fell rapidly.

From a comparison of channel activities and plasma aldosterone levels, it appears that most of the upregulation of channel activity occurs between control levels of 20 and 200 ng/dl. These levels correspond to concentrations of 0.6–6 nM. Higher concentrations of up to 1,000 ng/dl, achieved with Na depletion, do not appear to be much more effective. The data shown in Fig. 8 can be described by a simple dose–response relationship between aldosterone concentration and channel activity, with an apparent K_d for activation of ~8 nM. However, results in the companion paper (Palmer, Antonian, and Frindt, 1993) suggest that the relationship between aldosterone concentration and channel activity may be steeper than that predicted from this simple hyperbola. In either case the data are consistent with the operation of a high-affinity receptor system with a K_d in the nanomolar range, corresponding to the type I or mineralocorticoid receptor (Rossier, Geering, Atkinson, and Roch-Ramel, 1985).

We have not specifically tested the possibility that glucocorticoids can substitute for aldosterone. We presume that when glucocorticoid concentrations in the CCT itself are sufficiently high they can elicit a response by activating either mineralocorticoid receptors (Edelman, 1981) or glucocorticoid receptors (Naray-Fejes-Toth and Fejes-Toth, 1990). However, levels of corticosterone, the major circulating glucocorticoid in the rat, did not increase significantly with Na depletion (Table IV). Mean values rose by <50%, from 23 to 33 μg/dl, while aldosterone increased by more than 50-fold. This concentration of corticosterone is not sufficient to induce channel activity, and this hormone does not appear to be involved in an important way in the physiological response to Na deprivation.

During salt repletion, levels of both aldosterone and NP_o fell relatively quickly, again demonstrating a good correlation between hormone concentration and channel activity. One observation, however, suggests that the correlation may not be absolute. After 20 h of salt repletion, aldosterone levels rebounded slightly while channel activity remained low (Fig. 3). This suggests either that the channels can be suppressed by other factors under these conditions, or that the aldosterone was not

reelevated for a long enough time to reactivate the channels. One possibility is that during salt reloading there is a large increase in Na influx into the cells of the CCT which could trigger a negative feedback response (Silver et al., 1993). As a preliminary test of this idea, aldosterone levels were kept at levels presumed to be maximum during Na repletion using osmotic minipumps (Table II). NP_o fell to ~50% of Na-depleted levels. This suggests that the decline in channel activity observed during salt repletion is due to a combination of the reversal of the effects of aldosterone and of the salt loading itself. This is consistent with the hypothesis that the channels may undergo feedback inhibition under these conditions.

Effects of Aldosterone on the Na Channel

Earlier studies on the toad bladder using noise analysis have indicated that aldosterone increased the number of conducting channels rather than the current through individual channels. Since the spectrum of the amiloride-induced noise was a higher frequencies than that of spontaneous channel opening and closures, this implies an increase in the mean number of open channels (NP_o) and does not distinguish increases in N and P_o.

TABLE IV

Effect of Aldosterone on Plasma Levels of Adrenal Corticosteroids

	Aldosterone	Corticosterone
	ng/dl	*μg/dl*
Control	20 ± 5	23 ± 5
Na depleted (7–14 d)	1,260 ± 130	33 ± 10

Data are given as means and SEM for seven rats on a normal diet and eight rats on a low-Na diet for 1–2 wk.

Our results indicate that the primary effect of aldosterone is on the conducting channel density N. This conclusion is subject to the caveat, mentioned in Results, that estimates of P_o, and hence of N, are most reliable when N is small and P_o is close to 0.5. This was not always the case; however, these conditions do pertain to the situation in which there is minimal stimulation of the channels. We can therefore assert with a high level of confidence that under these conditions P_o is, on the average, ~0.5. If this is the case, then increases in P_o could account for at most a factor of 2 between the minimal and maximal degrees of stimulation. In fact, there is no indication that the average P_o is any higher than 0.5 even when NP_o is very large. Values much greater than 0.5 (or much less) would cause a skewing of the open level histogram as shown in Fig. 1 *B* toward high (or low) values.

Although there is a large scatter in the estimated values of P_o, particularly when N is low, there was no correlation between the values of P_o and the mineralocorticoid status (Figs. 2 and 4) or with the number of channels per patch (Fig. 6). The simplest interpretation of this finding is that aldosterone either increases the actual number of channels in the membrane or converts channels from a state where P_o is unmeasurably small to one where P_o is on the average 0.5, albeit variable. The variation may be the result of other regulatory factors.

A recent study in A6 cells (Kemendy et al., 1992) showed that aldosterone increased the P_o of Na channels. The major difference in their findings and ours is the presence in aldosterone-depleted A6 cells of channels with short open times and low P_o. Under presumably comparable steroid-depleted conditions, we see no activity at all in the rat CCT (Fig. 7). This difference could in fact be a quantitative rather than a qualitative one. Under our normal recording conditions we could have observed channel openings whose duration was 0.5 ms or longer. If channels in the aldosterone-depleted rat CCT had open times considerably shorter, or closed times considerably longer than those seen with A6 cells, then the channels might not be observed at all under our conditions. Thus in both cases the effect of the hormone would be to move the channels from a low P_o state (which in the rat CCT is not observable) to a high P_o state (in the rat CCT ~ 0.5). The all-or-none character of the transition from low P_o to high P_o reported by Kemendy et al. (1992) is consistent with this interpretation.

Mechanism of Aldosterone Stimulation of Na Channels

If aldosterone activates preexisting channels, then there is likely to be a cascade, or several cascades, of cellular events that lead to this activation. One interesting pathway that has been proposed to mediate aldosterone action in frog skin (Harvey and Ehrenfeld, 1988) and in the amphibian early distal tubule (Oberleithner, Weigt, Westphale, and Wang, 1987) involves the activation of Na-H exchange and the alkalinization of the cytoplasm. We were particularly interested in this possibility since previous results had shown that the Na channels of the rat CCT were quite sensitive to the pH of the cytoplasmic side of the membrane in excised patches (Palmer and Frindt, 1987). To test this idea we measured pH in the principal cells using BCECF fluorescence. These experiments were carried out in the absence of bicarbonate. This may elevate cell pH somewhat (Silver et al., 1992), which would be expected to increase channel P_o. The absence of bicarbonate is expected to exaggerate any effect of activation of Na-H exchange due to a lower cytoplasmic buffer capacity and the removal of bicarbonate-dependent, pH-regulatory systems. Even under these conditions we could find no evidence for an activation of Na/H exchange or for a change in cytoplasmic pH. This does not preclude the possibility that alkalization mediates much earlier effects of the steroid, but if this is the case such early effects must disappear in the chronically stimulated state.

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