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FURTHER STUDIES ON THE EFFECT OF ALDOSTERONE ON ELECTRICAL RESISTANCE OF TOAD BLADDER

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SUMMARY

The fall in transepithelial electrical resistance which accompanies aldosterone stimulation of short-circuit current (I_{sc}) in toad urinary bladder has been studied further to evaluate the possible causal role of this response in hormonal stimulation of Na^+ transport. A steady-state change in tissue conductance was found to depend upon both the simultaneous stimulation of transport by the steroid and the metabolic state of the tissue. Changes in metabolic state alone did not alter resistance. A sustained increase in Na^+ transport, dependent on pretreatment with aldosterone and elicited by addition of glucose, could be obtained without a sustained decrease in resistance. Amiloride, an inhibitor of Na^+ uptake, produced changes in I_{sc} that were linearly correlated with its effects on tissue conductance. On the basis of the conductance-I_{sc} relationship with amiloride, the I_{sc} response to aldosterone was about twofold higher than would be predicted from its effects on conductance alone. Despite the apparent lack of a simple quantitative dependence of the change in I_{sc} on the change in conductance when the response is fully developed, the results suggest that conductance changes may mediate the initial or early stage of the response.

INTRODUCTION

Aldosterone regulation of active Na^+ transport in epithelia appears to be mediated either by facilitating the entry of Na^+ into the cells at the mucosal (apical) membrane [1], or by enhancing the energy-dependent Na^+ pump at the exist step, presumably located at the serosal (basal-lateral) membrane [2, 3]. These hypotheses are based on the two barrier model of transepithelial transport [4–7] which envisions the control mechanisms operative at both the "passive" entry and "active" exit

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barriers to be independent processes linked by the magnitude of the intracellular Na^+ transport pool.

Civan and Hoffman [8] predicted from an electrical analog of this model that there should be a coincident decrease in total transepithelial d.c. electrical resistance if aldosterone acts by increasing Na^+ conductance at the apical membrane. In short-circuited preparations of toad bladder they showed that aldosterone produced an 18 % decrease in resistance concomitantly with a 90 % increase in short-circuit current (I_{sc}) and concluded that the electrical response is consonant with enhancement of apical entry of Na^+. They recognized, however, that the significance of the finding was unclear, since changes in the resistance of the Na^+ pump or a basal element in series with the pump, or in a parallel Na^+ conductance pathway, could also yield changes in transepithelial resistance equivalent in magnitude to that specifically dependent on the apical Na^+ conductance. Moreover, hormonal effects on Cl^- permeability may also have to be considered [9].

The present study was designed to explore further the possible causal relationship between the steroid's effect on transepithelial resistance and its effect on I_{sc}. The results indicate that within the limitations imposed by the two-barrier model, and on the basis of the response to amiloride, the decrease in resistance evoked by aldosterone appears to be insufficient to account for the entire increase in Na^+ transport. Moreover, the stimulation of Na^+ transport by aldosterone could be elicited without a statistically significant steady-state change in resistance. The early stage of the I_{sc} response, however, correlates with the change in conductance.

MATERIALS AND METHODS

Colombian toads (*Bufo marinus*) purchased from Tarpon Zoo, Tarpon Springs, Florida, were stored moist without food prior to use. After double pithing, the urinary hemibladders were removed, rinsed, and mounted as diaphragms in double lucite chambers (area = 2.5 cm^2) for the determination of I_{sc} and potential difference (PD) on paired quarter bladders by the method of Ussing and Zerahn [10].

Hemibladders were incubated overnight with, or without, exogenous substrate in Ringer solution of the following composition: 90 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM KH_2PO_4, 0.5 mM $MgSO_4$ [11]. The following morning all media were replaced with fresh solutions and 1–2 h allowed for stabilization. Penicillin (800 units/ml), streptomycin (0.9 mg/ml) and chloramphicol (10 μg/ml), were present throughout and the media were gassed continuously with 97 % O_2 + 3 % CO_2. Temperature = 24–25 °C, pH = 7.5–7.7, and osmolarity = 220–230 mosM were maintained constant throughout the experiments.

Transepithelial electrical potentials were measured with a Keithley 610B electrometer in series with calomel electrodes. I_{sc} was applied intermittently at the indicated intervals with Ag/AgCl electrodes and measured with a Daystrom Weston Standard ammeter. Electrodes were connected to chamber solutions via KCl-agar bridges. The distance between the bridges from the calomel half-cells was adjusted to achieve a 10 Ω resistance in each chamber without bladders in place. Electrode assemblies were replaced if potential assymetry was greater than 0.5 mV.

An approximately linear current-voltage relationship has been found in these preparations within the range of values encountered in this study [6, 9, 12]. Conse-

quently, total tissue resistance was calculated simply as the ratio of open circuit PD to I_{sc}. In all aldosterone and substrate addition experiments, however, resistances were also measured as the steady-state $\Delta PD/\Delta I_{sc}$ ratio resulting from a 10 mV deflection from the resting level [8]. The steady-state current was achieved in $\approx$ 30 sec or less. As the pattern of responses and absolute resistance values obtained were indistinguishable with both methods, only the results of the former calculation are reported.

Aldosterone (chromatographically homogeneous) was obtained from CalBiochem, and amiloride (*N*-amidino-3,5,diamino-6-chloropyrazine-carboxamide) was a gift from Merck, Sharp and Dohme. All conventional reagents (Baker and Adams) were, at minimum, analytical grade.

RESULTS

The time-course of the effect of aldosterone on I_{sc} and transepithelial resistance was determined with intermittent rather than continuous [8], short-circuiting as shown in Fig. 1. The effects of aldosterone on I_{sc} were statistically significant when the results were analyzed either by absolute differences (i.e., mean differences $[\bar{x}\Delta(A-C)]$) or by the differences in the time-normalized ratios [i.e. $I_{sc(t)}/I_{sc(0)}$ (aldo)$-I_{sc(t)}/I_{sc(0)}$ (control). The smaller resistance changes, however, yielded significant differences in resistances only when the results were expressed in terms of the time-normalized ratios. On the basis of the time-normalized ratios, aldosterone produced a 275 % increase in I_{sc} ratio and a 29 % decrease in the resistance ratio (Fig. 1).

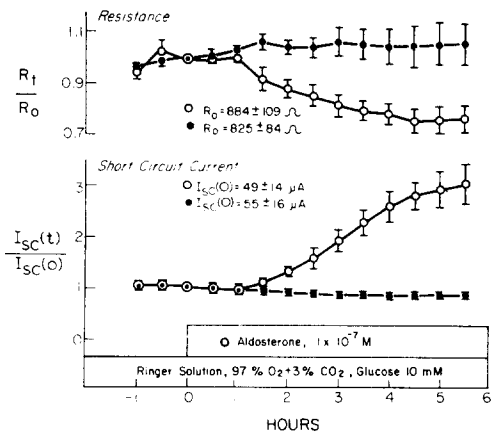

Fig. 1. Time-course of the effect of aldosterone on transepithelial resistance (upper graph) and short circuit current (lower graph). R_t/R_0 denotes the transepithelial electrical resistance at time "t" divided by that at time zero in each quarter-bladder. $I_{sc}(t)/I_{sc}(0)$ denotes the short circuit current ratio computed similarly. The data are given as the mean ± 1 SE. The bladders were preincubated in 10 mM glucose/Ringer's solution for 16 to 18 h. At $t = 0$, aldosterone (final concentration $= 10^{-7}$ M) was added to the serosal and mucosal solutions of one quarter bladder and diluent Ringer's solution to the other. The resistance was calculated as the ratio of the spontaneous PD/I_{sc}. At $t = 0$ and 5.5 h, the absolute mean differences in I_{sc} $[\bar{x}\Delta(A-C)]$ between aldosterone and control populations were -6 ± 9 and 73 ± 14 ($P < 0.01$) $\mu A/2.54$ cm^2, respectively. At the same time points, the mean differences in resistance $[\bar{x}\Delta(A-C)]$ were 59 ± 113 and $-184\pm124\,\Omega/2.5$ cm^2, respectively. 'P' value was calculated by the paired "t" test. $N = 10$ pairs of quarter bladders.

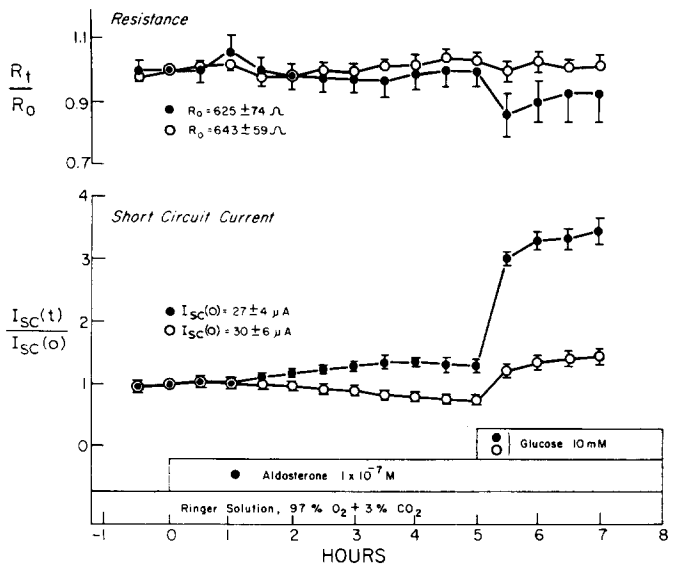

Fig. 2. Effect of aldosterone on transepithelial resistance (upper graph) and I_{sc} (lower) in substrate-depleted hemibladders and after addition of glucose. Paired bladders were incubated overnight (16–18 h) in Ringer's solution without substrate. Aldosterone was added at $t = 0$, to experimental quarter-bladders. At $t = 5$ h, an isosmotic (220 mosM) glucose solution was added to mucosal and serosal bathing solutions (final concentration = 10 mM glucose) for both experimental and control tissues. Paired student "t" test comparison of the resistance ratio $R_{5.5}/R_{5.0}$ obtained for aldosterone treated bladders (0.85 ± 0.05) with the corresponding value for control bladders (0.98 ± 0.01) indicates a significant fall in resistance at $t = 5.5$ h ($P < 0.02$). Comparison of the ratios $R_{7.5}/R_0$ for both quarter-bladders (aldosterone = 0.92 ± 0.09, control = 1.02 ± 0.03) indicates that final resistances were not significantly different from $t = 0$ values ($P > 0.10$). Data are expressed as indicated in the legend of Fig. 1. $N = 10$ pairs of quarter-bladders.

In Colombian toads there is little increase in I_{sc} in response to aldosterone in substrate-depleted bladders but, an immediate differential increase in I_{sc} of the hormone-treated hemibladder occurs after substrate repletion [13, 14]. To test whether the aldosterone-induced rise in Na^+ transport and the decrease in resistance are coupled even under varying metabolic conditions, bladders incubated overnight in substrate-free media were exposed to hormone, and the I_{sc} and resistance levels monitored before and after a subsequent addition of glucose. The results in Fig. 2 indicate that during the energy-limited period there was a small increase in I_{sc} and no statistically significant decrease in resistance. After substrate repletion, R_t/R_0 fell transiently, about 10%, in the aldosterone-treated bladders concurrent with the rise in I_{sc}. 1 h after substrate addition, the R_t/R_0 ratios tended to converge and thereafter the differences in either absolute or ratio values were not statistically significant. Thus, in substrate-repleted bladders the steady-state, steroid-dependent effect on Na^+ transport was obtained (i.e., I_{sc} was more than doubled by hormone), without a significant effect on total resistance. A less than 10% decrease in R_t/R_0, however, might not have been detectable.

The equivalent electrical circuit analog [15] used in the analysis of the response to aldosterone [8, 12] is composed of a "passive" leak resistance, R^p, in parallel with

an "active" pathway. The latter consists of a voltage source "E_{Na}", in series with a resistance, R^a, representing elements within the pump and in the apical and basal membranes. To determine if the complex resistance responses shown in Figs 1 and 2 could be interpreted in terms of individual elements, in the active pathway, further studies were done under varying metabolic conditions, and with the pyrazine diuretic, amiloride.

Substrate addition to aldosterone- and substrate- depleted bladders of Colombian, but not Dominican toads [16] results in an increase in Na^+ transport, presumably due to an increase in ATP concentration or the ATP/ADP ratio at the Na^+ pump site. Civan and Hoffman [8] studied the effects of substrate on I_{sc} and resistance in aldosterone-pretreated, substrate-depleted hemibladders of Dominican toads. Since in Colombian toads, substrates stimulate Na^+ transport even in steroid-depleted bladders, the resistance response to substrate repletion was tested in steroid-depleted bladders of Colombian origin. The data in Table I indicate that on addition of 10 mM glucose, there was no significant change in resistance despite a 2.5-fold increase in I_{sc} ratios. Thus, the initial increase in conductance on substrate repletion in the presence of aldosterone (Fig. 2) cannot be explained as a purely substrate-dependent effect.

There is considerable evidence that amiloride inhibits active Na^+ transport in a variety of epithelia by blocking the entry of Na^+ across the apical plasma membrane [17–22]. The effects of varying concentrations of amiloride on I_{sc} and resistance were determined both on application of the inhibitor to the mucosal surface and on the "rebound" after its removal. Paired control bladders were treated identically except that amiloride was omitted from the addition solution in order to control for time and procedure dependent variations. A representative experiment using $5 \cdot 10^{-5}$ M amiloride is shown in Fig. 3. The general pattern of the response was the same at concentrations of $5 \cdot 10^{-6}$, $5 \cdot 10^{-7}$, and $5 \cdot 10^{-8}$ M. The data for the entire series are summarized in Table II. After removal of the inhibitor, a transient "overshoot" in

TABLE I

EFFECT OF SUBSTRATE REPLETION ON TRANSEPITHELIAL RESISTANCE AND I_{sc}

Results are given as means ±SE at various times after glucose addition compared to their values at time zero. Paired hemibladders were incubated overnight in Ringer's solution without substrate. After changing to fresh Ringer's solution the following morning, glucose (final concentration 10 mM) was added at zero time to mucosal and serosal bathing solutions of the experimental quarter-bladders. $N = 10$ pairs. At time zero the absolute values were $I_{sc} = 20 \pm 9\,\mu A$ and $R_0 = 869 \pm 98\,\Omega$ in the controls, and $I_{sc}(0) = 21 \pm 5\,\mu A$ and $R_0 = 792 \pm 89\,\Omega$ in the experimental quarter-bladders (exposed area = 2.5 cm^2). The ratios were constructed as described in the legend of Fig. 1.

Time (min)	Control		Experimental	
	R_t/R_0	$I_{sc}(t)/I_{sc}(0)$	R_t/R_0	$I_{sc}(t)/I_{sc}(0)$
15	1.01±0.04	1.04±0.03	1.04±0.04	1.32±0.07*
30	0.99±0.02	1.01±0.02	0.99±0.05	1.89±0.13*
60	0.99±0.03	1.01±0.03	0.94±0.03	2.38±0.21*
120	0.96±0.03	0.98±0.05	0.97±0.06	2.59±0.32*

* $P < 0.01$ for paired Student "t" comparison between ratios obtained for control and experimental bladders.

TABLE II

COMPARISON OF THE EFFECTS OF AMILORIDE ON I_{sc} AND TRANSEPITHELIAL RESISTANCE

Values are means ±SE. I_{sc} and R_0 values are given as μA and Ω per 2.54 cm^2 respectively. Ratios compare values at numerator time points (subscripts) with those obtained at time zero. I_{sc} and resistance ratios $t = 10/t = 0$ record the normalized change after 10 min of amiloride treatment. Amiloride was removed after 40 min. Comparisons after 40 min are given as the ratio $t = 40/t = 0$. Ratios of $t = 65/t = 0$ compare final and initial values obtained. Experimental conditions and procedures as given in Fig. 3.

	($n = 8$)		($n = 7$)		($n = 7$)		($n = 6$)	
	Control	$5 \cdot 10^{-8}$ M	Control	$5 \cdot 10^{-7}$ M	Control	$5 \cdot 10^{-6}$ M	Control	$5 \cdot 10^{-5}$ M
$I_{sc}(0)$	62±13	64±8	67±11	65±9	67±9	73±10	92±17	91±15
R_0	716±100	795±66	593±71	552±77	732±58	633±65	584±49	523±67
$I_{sc}(10)/I_{sc}(0)$	0.99±0.01	0.88±0.01	1.02±0.01	0.41±0.03	0.99±0.01	0.23±0.02	0.99±0.01	0.10±0.03
R_{10}/R_0	1.01±0.01	1.07±0.01	1.00±0.01	1.27±0.06	1.00±0.01	1.51±0.16	1.00±0.01	1.63±0.16
$I_{sc}(40)/I_{sc}(0)$	0.96±0.01	0.89±0.01	1.08±0.03	0.57±0.02	0.96±0.01	0.25±0.01	0.97±0.01	0.12±0.02
R_{40}/R_0	1.03±0.01	0.95±0.01	0.95±0.05	1.17±0.05	1.01±0.01	1.49±0.15	1.00±0.01	1.58±0.16
$I_{sc}(65)/I_{sc}(0)$	0.88±0.04	0.87±0.02	1.02±0.05	1.16±0.03	0.82±0.02	0.96±0.03	0.86±0.01	0.94±0.04
R_{65}/R_0	0.98±0.03	0.96±0.02	0.92±0.02	0.80±0.07	0.91±0.05	0.97±0.03	0.99±0.05	1.05±0.05

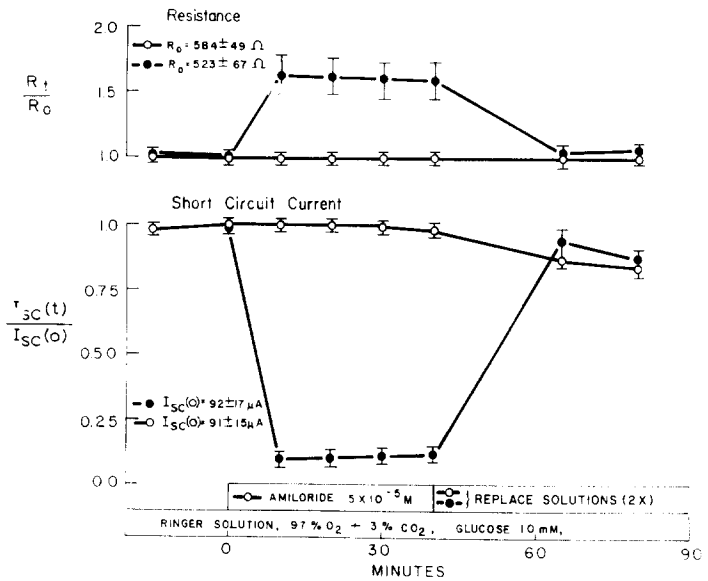

Fig 3. Effect of amiloride on transepithelial resistance (upper graph) and I_{sc} (lower graph). Paired hemibladders were incubated overnight in 10 mM glucose Ringer's solution. At $t = 0$, amiloride was added to mucosal solutions of test tissues (final concentration $= 5 \cdot 10^{-5}$ M). Controls received only diluent. At $t = 40$ min, amiloride was removed by replacing mucosal and serosal solutions of both test and control chambers with fresh glucose-Ringer's medium. The washout procedure was repeated after a further 5 min. Data are expressed as indicated in the legend to Fig. 1. $N = 6$ pairs of quarter bladders.

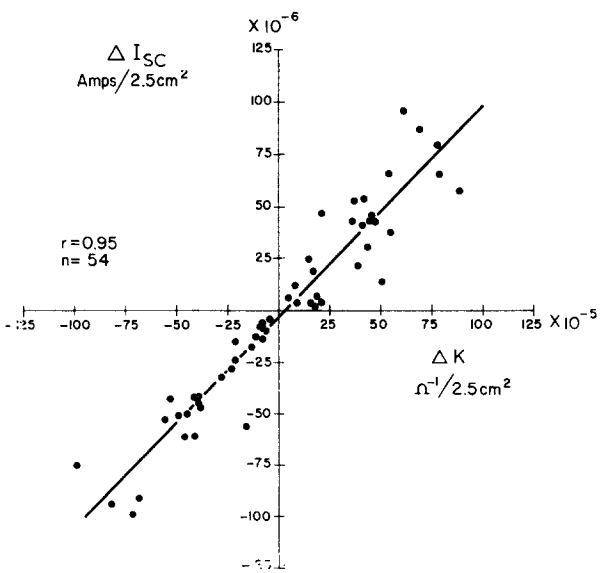

Fig. 4. Relationship between the amiloride-induced change in Na^+ transport (ΔI_{sc}) and in transepithelial conductance (ΔK). Differences in SCC and conductance values obtained on the addition (t_0-t_{10}) and the removal ($t_{40}-t_{65}$) of amiloride were taken from each of the experiments in Table II. Transepithelial conductances were calculated as the ratio I_{sc}/PD. The line of best fit (solid line) was determined by least squares regression. The regression equation for this line is: $\Delta I_{sc} = 1.02 \pm 0.04$ $(\Delta K) - 3.17 \pm 1.98$. The intercept is not statistically significantly different from a value of zero. The correlation coefficient of the regression is 0.95 and the number of observations is 54.

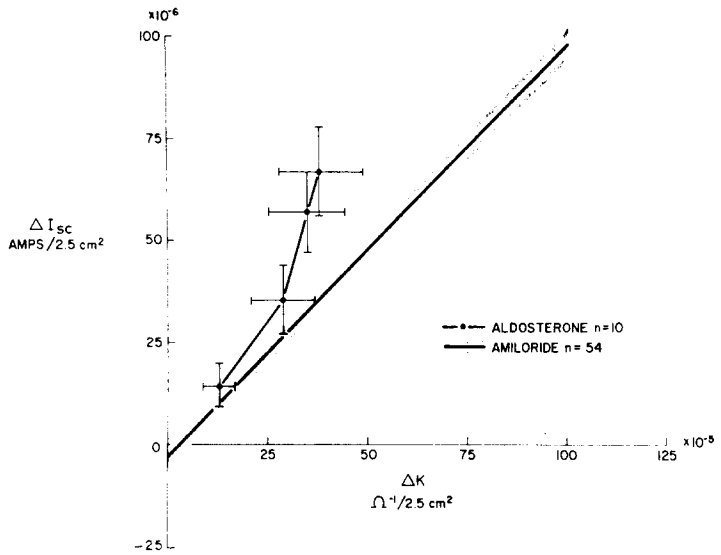

Fig. 5. Comparison of the I_{sc}-conductance relationship associated with addition of aldosterone (–•–) compared to that on removal of amiloride (—). Values plotted for aldosterone were calculated from the results of the experiment shown in Fig. 1. Points plotted are for 2, 3, 4, and 5h of hormone treatment. The amiloride results from Fig. 4 are shown as the calculated regression line ±1 SE.

I_{sc} was often observed (Fig. 3 and Table II) which closely resembles the "overshoot" seen on readdition of Na^+ to Na^+-free mucosal solutions [22]. As reported previously [19–22], there was a completely reversible increase in resistance and decrease in I_{sc} with increasing inhibitor concentrations. As shown in Fig. 4, there was a linear relationship between the absolute change in I_{sc} ($\Delta\mu A/2.5\ cm^2$) and the absolute change in transepithelial conductance ($\Delta\Omega^{-1}/2.5\ cm^2$). Regression analysis of the raw data indicates that the line of best-fit is not significantly different from a line of identity intersecting the origin. The homogeneity of the regression ($r = 0.95$) also suggests that the absolute changes observed are relatively independent of initial basal values since inspection of Table II reveals that the basal I_{sc} and resistances varied by more than 50 %.

If the "rebound" increase in I_{sc} and conductance after the washout of amiloride are taken as equivalent to stimulation of Na^+ transport by enhanced apical conductance to Na^+, the regression line in Fig. 4 may be used as a basis for quantitative estimates of the role of conductance changes in the response to mineralocorticoids. The change in I_{sc} produced by aldosterone in Fig. 1 was consistently greater than predicted by the change in conductance alone (Fig. 5).

DISCUSSION

The results of the present study confirm the findings of Civan and Hoffman [8] in that in substrate-repleted toad bladder, aldosterone produced a concurrent fall in transepithelial electrical resistance and rise in I_{sc} (i.e., active Na^+ transport) and in substrate-depleted bladders, substrate alone evoked a significant increase in I_{sc}, yet had

no effect on resistance. The decrease in resistance after addition of aldosterone may reflect a change in apical Na^+ conductance but, current uncertainties in the definition of the determinants of total resistance limits a straight-forward interpretation of these results.

The metabolic state, however, has a definite effect on the quantitative relationships between I_{sc} and conductance in response to aldosterone. As shown in Fig. 2, addition of substrate elicited a transient fall in resistance about 1 h in duration, only in the aldosterone treated population. The steroid dependent rise in I_{sc} on the other hand, was sustained throughout. It is of interest that the statistically significant change in R_t/R_0 noted in the first hour after glucose addition to aldosterone pre-treated bladders in our preparations (from Colombian toads) was not seen in the experiments of Civan and Hoffman [8] who used pyruvate as the substrate in hemibladders from Dominican toads. The central point, however, is our finding of a normal I_{sc} response to aldosterone without a significant change in total transepithelial resistance (Fig. 2). Thus, the nature of the steroid effect on total resistance and the role of this effect in the action on Na^+ transport is clearly not established.

Multiple determinants of total transepithelial resistance have been identified in previous studies. Civan and Frazier [23] estimated that the apical and basal-lateral plasma membranes each contributed about 50 % of the total transepithelial resistance. In addition to the series barriers, passive diffusion pathways in parallel with the conductance of the active "channel" have also been recognized [8, 9, 24], Saito and Essig [25] analyzed the contribution of the parallel passive pathway to aldosterone-dependent changes in conductance by measuring tracer ion fluxes under voltage clamp conditions and concluded that aldosterone had no effect on parallel passive conductance nor, on the magnitude of the electromotive force E_{Na} estimated as the clamped voltage needed to nullify active Na^+ transport. These findings are consistent with an increase in ion conductance via the active pathway (K^a). This inference, however, does not negate the possible participation of energetic factors in the regulation of apical conductance or of basal extrusion (Na^+ pump) pathways in the response to aldosterone. Saito et al. [26] recently provided evidence that aldosterone increased the free energy of a critical oxidative reaction which "drives" active Na^+ transport in the frog skin. The rise in K^a (or the fall in total resistance) noted in their studies on toad bladder may or may not reflect changes restricted to apical Na^+ conductance. An important unresolved problem is how the energetic and permeability factors interrelate in the overall process.

The complexities in the interpretation of data on total resistance prompted us to compare the resistance-I_{sc} relationship of two specific procedures designed to affect changes in both E_{Na} and Na^+ conductance. Substrate repletion in steroid-depleted bladders was used to evaluate the effects of changes in E_{Na} on resistance-I_{sc} relationships. As shown in Table I, this maneuver, i.e., addition of substrate, elevated I_{sc} without altering conductance. In contrast, amiloride produced rapid and reversible effects on both I_{sc} and total conductance. The linear I_{sc}-conductance response obtained with this reagent over the entire range of values encountered in this study (from -100 to $+100$ $\mu A/2.5$ cm^2), thus provides one basis for evaluating the response to aldosterone. The four data points for aldosterone in Fig. 5 represent the absolute ΔI_{sc}-Δconductance values at 2, 3, 4, and 5 h after aldosterone, taken from the experiment in Fig. 1. Based on the amiloride "standard", the increase in I_{sc}

obtained with the hormone was consistently greater than that attributable to a simple change in conductance alone. In the early stage of the response, the aldosterone values (i.e., $\Delta I_{sc}/\Delta K$) increased in parallel with the amiloride "standard". After 5 h of hormone, however, the rise in I_{sc} is almost twice that predicted on the basis of the amiloride "standard". Thus, a simple increase in Na^+ conductance may not be sufficient to account for the effect of the hormone on Na^+ transport. This inference rests on the assumption that changes in I_{sc} above the basal level induced by aldosterone would be quantitatively similar to the depression below, and return to, basal levels produced by amiloride, if the mechanism of hormone action were restricted to the apical boundary (i.e., that linearity between ΔI_{sc} and ΔK would exist throughout). Although her results were analyzed only in terms of effects on I_{sc}, Robbie [22] noted a close correspondence in the amiloride dose-response curves in the presence and absence of aldosterone, suggesting that this assumption is valid. Recent experiments in this laboratory in which a similar amiloride I_{sc}-conductance relationship was obtained with intact "sac" preparations of bladder both before, and after stimulation by aldosterone, also support this assumption [Rossier and Edelman, unpublished studies]. Moreover, in these studies the slope of the I_{sc}-conductance regression line was significantly greater in the presence than in the absence of aldosterone.

Saito and Essig [25] applied the formulation of Ussing [27] to an analysis of the response to aldosterone: The I_{sc} is expressed as a function of the "electromotive force" of the transport system, E_{Na}:

$$I_{sc} = K^a E_{Na} \tag{1}$$

where K^a is the conductance of the active pathway and is related to the total (measured) conductance (K) by:

$$K = K^a + K^p \tag{2}$$

In Fig. 5, the results are plotted as ΔI_{sc} vs ΔK. If K^p is invariant, ΔK is equal to ΔK^a and the deviation of the later aldosterone points from the amiloride line implies a significant increase in E_{Na}. Saito and Essig [25], however, did not detect a rise in E_{Na} after aldosterone by the isotope flux method for evaluation of K^a and K^p. To reconcile these findings with ours would require further studies on differences between the two methods and experiments employed.

Although disparate results have been obtained in studies on the effects of aldosterone on toad bladder electrolyte composition [28–30], Lipton and Edelman [30] suggested that aldosterone may facilitate both apical entry of Na^+ and its active extrusion across the basal-lateral membrane. Our results (Figs 2 and 5) and those of Saito et al. [25, 26] are consistent with this interpretation of a "bipolar" control mechanism with the inference that energetic factors may be important in both.

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