Aldosterone Action and Sodium- and Potassium-Activated Adenosine Triphosphatase in Toad Bladder

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ABSTRACT Urinary hemibladders obtained from toads soaked in water or saline were treated with aldosterone, 10^{-6} M, either $1\frac{1}{2}$ or 16 h after mounting. After $2\frac{1}{2}$ h exposure to the hormone, short-circuit current was increased by 110–192% and open-circuit potential by 20–44% as compared with untreated paired hemibladders. Mucosal cells were then assayed for sodium-potassium-stimulated adenosine triphosphatase (ATPase). No increase occurred in activity per milligram protein or in the portion of total activity dependent on sodium. Activity at low sodium concentrations was also measured and analyzed by means of the Hill equation in terms of K , the apparent dissociation constant of the enzyme-sodium complex, and n , a number that expresses the degree of interaction between binding sites. Neither K nor n was significantly altered by aldosterone. A few experiments were also carried out at low ATP concentrations (0.3 mM); again no change in sodium-dependent activity was noted. The results indicate that aldosterone does not stimulate sodium transport by increasing the quantity of sodium-potassium adenosine triphosphatase in mucosal cells or the dependence of this activity on sodium or ATP concentrations.

INTRODUCTION

The mechanism by which aldosterone stimulates sodium transport remains obscure. An increase in protein synthesis is evidently involved (1) but the nature of the

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protein synthesized has not been elucidated. One obvious possibility is sodium- and potassium-activated adenosine triphosphatase (Na-K-ATPase)¹ itself, since this enzyme appears to be responsible for energy-linked transport of sodium across epithelia (2).

Previous efforts to demonstrate an increase in the specific activity of Na-K-ATPase after aldosterone have met with conflicting results. In rat kidney, enzyme activity declines after adrenalectomy (3–7). Some (7, 8) but not all (3, 5) workers have found an increase after aldosterone administration, especially in outer medulla. However, glucocorticoids also restore renal Na-K-ATPase activity (3, 5, 7, 9), and attempts to correlate changes in electrolyte excretion with restoration of the activity of this enzyme have not been wholly successful (3, 6–9). In toad bladder, three reports indicate unchanged Na-K-ATPase after aldosterone (10–12), using a rather insensitive method; physiological responses to the hormone in these bladders were not demonstrated.

No studies apparently have been directed at the question of whether aldosterone may induce a qualitative change in Na-K-ATPase, such that greater reaction rates occur at the same substrate concentrations. The availability of a more sensitive assay for Na-K-ATPase in toad bladder (13) prompted an examination of this question.

METHODS

Preparation of the hemibladders. Large toads (*Bufo marinus*) were killed by decapitation and pithing as quickly as possible. The hemibladders were dissected out, rinsed several times with amphibian Ringer solution (111 mM NaCl, 3 mM NaHCO₃, 2.7 mM CaCl₂, 2 mM MgCl₂, 3.4 mM KCl), and mounted as sacs on glass cannulas as described previously (14, 15). Four groups of experiments were performed. In each experiment, two pairs of hemi-

¹ Abbreviations used in this paper: Na-K-ATPase, sodium- and potassium-activated adenosine triphosphatase; PD, potential difference; SCC, short-circuit current.

bladders were used; aldosterone was added to one of each pair. The two control bladders and the two treated bladders were then combined for enzyme assay. In group I the toads were kept in sphagnum moss with access to water. Group II toads were kept partially immersed in 0.6% NaCl for 48 h before killing. Group III toads were also placed in 0.6% NaCl for 48-96 h and the hemibladders incubated for 14½ h at room temperature, in beakers containing amphibian Ringer solution with penicillin G (50 mg/liter). The hemibladders were then rinsed, as before, and suspended in fresh Ringer solution containing glucose (5.5 mM). Group IV toads were not soaked in saline, but the hemibladders were incubated overnight as in group III.

Measurements of potential difference (PD) and short-circuit current (SCC) were made as previously described (14, 15) except that salt bridges contained 3 M NaCl instead of KCl. In the first and second groups of experiments, the hemibladders were maintained in the open-circuit state throughout except for 10-s intervals when SCC was measured. Hemibladders in groups III and IV were kept short-circuited except for 10-s intervals required for measurement of PD and were also maintained thermostatically at 25°C. The pairs of hemibladders were allowed to equilibrate for 90 min before aldosterone (10^{-6} M) was added to the serosal medium of one hemibladder. Methanol diluent was added to the other member of the pair.

Occasional experiments in which SCC did not respond to aldosterone were omitted. 2½ h after the addition of aldosterone, homogenates of mucosal cells obtained from both pairs of hemibladders were prepared and assayed according to Cortas and Walser (13). Deoxycholate (0.1%) was used. Incubation was for 10 min. In group II additional tubes were included containing 2, 4, 8, or 16 mM NaCl. In group III, additional tubes were included containing 6, 9, 13, or

20 mM NaCl. In group IV, additional tubes were included containing 0.3 mM Tris-ATP as well as 1 mM Tris-ATP. A regenerating system was not used. No more than 20% of the total ATP was consumed during the 10-min incubation.

These results at low sodium concentrations were fitted to the Hill equation (16):

$$\ln[(V_{\max}/V) - 1] = -n \ln[Na] + \ln K,$$

where $V_{\max}$ is Na-dependent reaction rate measured at 100 mM Na, V is the sodium-dependent reaction rate at lower sodium concentrations, K is the apparent dissociation constant of the enzyme-sodium complex, and n is a number which is a function of cooperative interaction between sites. Least-squares regression was used to find K and n from measurements of $V_{\max}$ and V in each experiment. In order to find the mean $\ln K$ for each set, values for $\ln K$ for each experiment were averaged.

RESULTS

The electrical responses are shown in Fig. 1. Significant increases in SCC and PD were seen in all four groups of experiments. The increase in SCC exceeded the increase in PD, as others have noted (1). In bladders incubated overnight, a significant increment in mean SCC was apparent within 1 h. However, the greatest response, at 2½ h, was seen in group I. Mean values in untreated bladders for SCC and PD at 2½ h are shown in Table I. PD was lower in overnight-incubated bladders but SCC was not significantly reduced. In untreated bladders from

FIGURE 1 Electrical responses to aldosterone (10^{-6} M) (Aldo) administration in four groups of experiments, expressed as percentage change of treated hemibladder divided by percentage change in paired control hemibladder at the same time intervals. Significant increments in SCC and PD are seen in all four groups, in some cases as early as 1 h after hormone addition. The increase in SCC exceeds the increase in PD.

TABLE I
Effect of Aldosterone on Potential, Short-Circuit Current, Na-K-ATPase Specific Activity, Na-K-ATPase Activity at 0.3 mM ATP and the Kinetic Parameters of the Enzyme's Dependence on Sodium Concentration in Toad Bladders

Mean values at 2½ h in untreated hemibladders						Mean percent change in untreated hemibladders during 2½ h		Mean percent change at 2½ h after aldosterone as compared with untreated hemibladders					
PD	SCC	V _{max}	V _{0.3}	−ln K	n	PD	SCC	PD	SCC	V _{max}	V _{0.3}	−ln K	n
<i>mV</i>	<i>μA/mg wet wt</i>	<i>μmol/mg protein per h</i>	<i>μmol/mg protein per h</i>										
Group I: access to water (<i>N</i> = 11)													
99	2.06	26				+10	+85	+20	+192	+3			
±4	±0.41	±1				±6	±31	±6	±55	±4			
Group II: saline soaked (<i>N</i> = 4)													
93	1.53	19		8.7	2.0	+52	+224	+32	+134	+20		+1	−4
±7	±0.41	±1		±1.8	±0.3	±11	±51	±10	±26	±9		±28	±24
Group III: saline soaked and incubated overnight (<i>N</i> = 11)													
75	1.47	16		7.0	1.6	−7	+12	+44	+110	+3		+14	+15
±5	±0.14	±1		±0.3	±0.1	±5	±6	±13	±13	±5		±10	±10
Group IV: access to water and incubated overnight (<i>N</i> = 4)													
76	1.53	28	23			+12	+47	+28	+111	−17	0		
±10	±0.17	±4	±2			±6	±23	±8	±25	±7	±14		

* Abbreviations: PD, potential; SCC, short-circuit current; V_{max}, Na-K-ATPase specific activity; V_{0.3}, Na-K-ATPase activity at 0.3 mM ATP; K, apparent dissociation constant of the enzyme-sodium complex; n, a number which is a function of cooperative interaction between sites.

all four sets, SCC rose during this 2½-h period; PD rose only in group II.

The changes in sodium-dependent portion of Na-K-ATPase, as measured at [Na] = 100 mM, are shown in the column labeled "V_{max}." No significant increase occurred in any group. Furthermore, in groups I and III, an increase as small as 10% would have been detectable, judging from the standard errors of these means. The percentage of total ATPase activity dependent upon sodium averaged 32% and was unaffected by aldosterone.

In K averaged -8.7±1.8 in group II and -7.0±0.3 in group III. Aldosterone did not change it. The constant n averaged 2.0±0.3 in group II and 1.6±0.1 in group IV; again, aldosterone had no significant effect. These results are also illustrated in Fig. 2 in which the mean values from group III are plotted in accordance with the Hill equation.

Results of the four experiments designed to assess enzyme activity at reduced ATP concentration are summarized in Table I. The electrical responses were similar to those seen in the previous experiments. The sodium-dependent activity at 0.3 mM ATP was about 20% lower than at 1 mM ATP, suggesting a K_m for ATP of approximately 0.15 mM. This is in accordance with

a previous estimate (17). Aldosterone did not affect sodium-dependent activity at either 1 mM ATP or 0.3 mM ATP.

DISCUSSION

These results appear to establish that the stimulation of sodium transport in toad bladder is not attributable to an increase in total sodium- and potassium-stimulated enzyme activity in mucosal cells or in the dependence of the rate of this reaction on sodium concentration. In addition, preliminary data suggest that the dependence of reaction rate on ATP concentration is unaltered.

Before dismissing the possibility that aldosterone acts by inducing the synthesis of Na-K-ATPase, several possible interpretations should be considered. First, it is conceivable that small changes in total Na-K-ATPase, undetectable by our methods, could be responsible for large changes in the rate of sodium transport. While this hypothesis may appear logical, it is difficult to defend. Some of the measured enzyme may be involved in cell homeostasis rather than in transcellular movement of sodium. Consequently a twofold increase in the rate of sodium transport might require an increase of less

FIGURE 2 Hill plot of Na-K-ATPase activity at low sodium concentrations (V_{max}), expressed in relation to activity at 100 mM Na (V_{max}), as a function of sodium concentration (in moles per liter), in control and aldosterone (Aldo)-stimulated hemibladders. No effect of the hormone is apparent.

than twofold in the total quantity of enzyme present, but there is no apparent way in which a small change in the amount of enzyme could, in itself, cause a large change in the rate of transport.

Secondly, the enzyme activity as measured *in vitro* may not accurately reflect the enzyme activity *in situ*. This could occur if activity were partially destroyed in preparing the tissue for enzyme assay. On the other hand, the preparative techniques employed, including NaI and deoxycholate, may have removed an inhibitory influence on enzyme activity normally present *in situ*. Accurate knowledge of the stoichiometry of the Na-K-ATPase reaction would be helpful in evaluating these possibilities. However, as Essig and Caplan (18) have pointed out, a fixed stoichiometry is highly unlikely. Since not only the stoichiometry of reaction (19), but also the overall transport capacity of the reaction (15), is increased by depolarization, no valid comparison between *in vitro* enzyme reaction rate and *in situ* transport rate can presently be made. Aldosterone could act by increasing the potential dependence of transport capacity in ways as yet poorly understood.

Thirdly, we have not explored in detail the dependence of reaction rate on ATP, adenosine diphosphate (ADP),

and magnesium concentrations. This is a technically difficult problem that requires further investigation. We have also not examined the dependence of reaction rate on potassium concentration.

Previous attempts to demonstrate an effect of aldosterone on Na-K-ATPase in toad bladder have been negative (10-12). However, the assay system employed in these earlier experiments showed only 10-15% stimulation by sodium and potassium, rendering quantitative conclusions difficult. Furthermore, no physiological response of the tissue was demonstrated in these experiments before assay.

The hypothesis that aldosterone acts by increasing the supply of energy to the pump, originally proposed by Edelman (1), has not been examined here. However, ATP concentration in aldosterone-stimulated toad bladder is not increased (20-22).

Crabbé (23) and Sharp and Leaf (24) have suggested that aldosterone acts by increasing the access of sodium to the enzyme. Although our data have no direct bearing on this hypothesis, they are consistent with it.

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