ON THE MECHANISM OF ACTION OF ALDOSTERONE*

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Abstract.—Studies to elucidate the mode of action of aldosterone have been carried out in the amphibian urinary bladder. The following previously reported hypotheses were evaluated: (1) aldosterone stimulates sodium transport by increasing the amount of sodium available to the sodium pump; (2) aldosterone enhances energy production for the sodium pump; and (3) aldosterone-stimulated sodium transport is obligatorily coupled to aerobic metabolism. In the present experiments, aldosterone potentiated the effect of vasopressin on sodium transport in the absence of aerobic metabolism or oxidative phosphorylation. This effect was not due to enhanced energy supply. Thus both hypotheses 2 and 3 appear not to be valid. In addition, aldosterone-stimulated sodium transport exhibited increased sensitivity to the specific inhibitor, ouabain, and this inhibition was readily reversed by K+. These findings, as well as previously reported work, have led us to propose that aldosterone stimulates sodium transport by inducing a change either in the sodium pump itself, i.e., synthesis or activation, or in its environment in the serosal plasma membrane of the responsive cells.

Aldosterone, the most potent of the mammalian mineralocorticoids, has been shown to stimulate the active transport of sodium across a variety of epithelia. The amphibian urinary bladder, a tissue consisting of a single layer of epithelial cells supported by a submucoosa of connective tissue and smooth muscle, responds in vitro to the addition of physiological doses of aldosterone by augmented sodium transport. This response is characterized by a latent period of 45–120 minutes and is thought to involve de novo RNA and protein synthesis, as the response is prevented by preincubation of the tissue with either the antibiotics actinomycin D or puromycin.

Three different hypotheses have been considered as possible explanations for the mode of action of aldosterone: (1) it enhances the passive entry of sodium across the mucosal surface of the epithelial cells; (2) it enhances the supply of energy to a sodium pump located on the serosal surface of these cells; or (3) it directly enhances the activity of this serosal sodium pump. Most recent consideration has been directed to the first two of these alternatives. Sharp and Leaf have favored the view that aldosterone enhances the mucosal entry of sodium, but Edelman concludes that the hormone acts by increasing the rate of synthesis of ATP and thereby drives the sodium pump or Na+/K+-activated ATPase on the serosal membrane. In addition, both laboratories have reported that hormone-stimulated Na+ transport is absolutely dependent upon energy supplied by oxidative metabolism and oxidative phosphorylation.
Other laboratories have reported data which are difficult to reconcile with the theory of mucosal entry (see below). The purpose of this report is to present evidence against the view that aldosterone promotes Na\(^+\) transport by a primary effect on energy production. The present results are more easily interpreted in terms of a direct effect of aldosterone either on the activity or synthesis of the serosal membrane Na\(^+\)/K\(^+\)-activated ATPase.

Methods.—Female toads (Bufo marinus) were obtained from National Reagents Co., Bridgeport, Conn. All animals were placed in 0.1 \(M\) NaCl for 4–6 hr prior to a particular experiment. The animals were then pithed and the two hemibladders removed and placed overnight at room temperature in substrate-free aerated Ling-Ringer phosphate solution\(^{11}\) containing 50 mg per liter of both penicillin-G and streptomycin sulfate. The following morning, the hemibladders were mounted in an Ussing-type voltage clamp as described by Sharp and Leaf\(^{6}\) in which each hemibladder is clamped in a double chamber, allowing independent measurement of short-circuit current from two halves of each hemibladder. The chambers of the clamp were filled with Ling-Ringer phosphate solution, containing 4 mM glucose, and short-circuit current was recorded continuously. Aldosterone (10\(^{-7}\) \(M\)) or, in control experiments, methanol carrier was added to both the mucosal and serosal chambers of the individual bladder sections. Anaerobiosis was induced by covering the buffer in the chambers with mineral oil and then gassing the solution with oxygen-free nitrogen, or by adding 2 mM NaCN. Either vasopressin (Pitressin; Parke, Davis & Co.) or dibutyryl 3',5'-AMP (Schwarz BioResearch) was added at the indicated concentrations to the serosal solutions. For determination of adenine nucleotides, the tissues were quick-frozen in liquid nitrogen and then extracted with perchloric acid, and the nucleotides were determined by standard fluorometric assays.\(^{12}\) The values obtained in these assays were referred to concentrations per milligram of dried PCA-precipitable material.

Results and Discussion.—The results of a typical experiment are shown in Figure 1. An initial addition of aldosterone to one half of the double chamber led to a rise in short-circuit current (sec) after the expected delay of 90 minutes. Then when vasopressin was added to both halves of the chamber, the aldosterone-treated and the control section of bladder showed the rapid rise in short-circuit current that is typically induced by this hormone.\(^{13}\) Vasopressin was then washed out by several complete rinsings of the chambers, and the current from the two halves returned to their respective baseline values. Anaerobiosis was then induced with nitrogen, and the short-circuit current fell in both halves to new baselines at which there was no longer any noticeable difference between the two currents. Vasopressin was again added to both sides, and both responded with an increase in short-circuit current; however, the response to vasopressin on the side pretreated with aldosterone was consistently greater than that seen on the control side. The data were reported as the ratio of the maximal change in short-circuit current after vasopressin divided by the current before vasopressin in order to compare the data from different bladders with different rates of basal transport, because it was found that the absolute magnitude of the response to vasopressin varied directly with the magnitude of the basal short-circuit current. The results in Table 1 show that under anaerobic conditions, whether produced by \(N_2\) or by NaCN, vasopressin always produced a significantly greater increase in short-circuit current in bladders pretreated with aldosterone than in the controls. In order to observe this effect of aldosterone, it was necessary to pretreat the bladder with the hormone. If both aldosterone and vasopressin were added
Fig. 1.—Effect of vasopressin (AVP) on short-circuit current (scc) under aerobic and anaerobic conditions in aldosterone-treated tissue and control. The upper trace is that obtained in the control bladder, and the lower is that from the aldosterone-treated tissue. Note that in this particular experiment the basal scc differed in the two hemibladders. In other experiments, the situation was reversed with the basal current of the control side being higher than that eventually treated with aldosterone. In some instances the basal short-circuit current was equal on the two hemibladders. Regardless of the relationship of the basal levels of this current, responses similar to those illustrated were always observed.

simultaneously or if aldosterone was added, 15 minutes before vasopressin, to bladder sections incubated under N₂, there was no potentiation of the vasopressin response. Although in this study no clear difference could be seen between baseline anaerobic transport in aldosterone-treated and control tissue, Handler et al. have reported that under anaerobic conditions aldosterone-stimulated transport was still greater than control.¹⁴ The aldosterone potentiation of the vasopressin response under anaerobic conditions, as well as the latter finding, clearly demonstrate an effect of aldosterone on sodium transport in the absence of aerobic metabolism.

One obvious possibility which might account for a potentiation of the vasopressin response by aldosterone would be that of enhancing the rate of synthesis of 3',5'-AMP or decreasing its rate of degradation, because this cyclic nucleotide is considered the primary intermediate in the response of this tissue to vasopressin.¹⁵ If aldosterone acted in this manner, then one should be able to prove this in two ways: (1) the rise in 3',5'-AMP after vasopressin should be greater in the aldosterone-treated tissue than in control tissue; and (2) prior treatment with
aldosterone should not potentiate the response of the bladder to either 3',5'-AMP or its dibutyryl analog. However, as shown in Table 1, the response to a standard dose of dibutyryl 3',5'-AMP was greater in aldosterone-treated bladder sections than in appropriate control sections when both were incubated under cyanide. Thus, aldosterone pretreatment potentiated the effect of equal doses of either vasopressin or dibutyryl 3',5'-AMP. Conversely, preliminary measurements of 3',5'-AMP concentrations in the tissue under anaerobic conditions five minutes after vasopressin treatment showed that there was no difference in tissues pretreated with aldosterone as compared with controls.

**Table 1.** Relative responses, in terms of short-circuit currents, of bladder to vasopressin under anaerobic (ΔN₂ or ΔCN) and aerobic (ΔO₂) conditions normalized to equal basal level of short-circuit currents.

<table>
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<tr>
<th>Expt.</th>
<th>Cont</th>
<th>Aldo</th>
<th>ΔCont</th>
<th>Expt.</th>
<th>Cont</th>
<th>Aldo</th>
<th>ΔCont</th>
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<td>2.7</td>
<td>2</td>
<td>2.6</td>
<td>11.3</td>
<td>4.3</td>
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<tr>
<td>3</td>
<td>0.6</td>
<td>3.3</td>
<td>2.7</td>
<td>3</td>
<td>1.4</td>
<td>2.0</td>
<td>1.4</td>
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ΔCN/ΔO₂—Dibutyryl C-AMP 5.5 mM Added

<table>
<thead>
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<th>Cont</th>
<th>Aldo</th>
<th>ΔAldo/ΔCont</th>
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\[
\frac{\Delta N}{\Delta O} = \frac{\text{peak sec after vasopressin} - \text{sec before vasopressin}}{\text{sec before vasopressin}}
\]

An alternative possibility to account for this effect of aldosterone is to postulate that it somehow enhances the rate of ATP production from glycolysis and thus drives the sodium pump by supplying more ATP. If this were the case, one would expect to see a greater increase in the ATP concentrations in aldosterone-treated as compared with control bladders. However, as shown in Figure 2, the level of ATP and the ATP/AMP ratio were lower in the aldosterone-treated bladders under anaerobic conditions than in controls. The concentration of ATP and the ATP/AMP ratio did not change significantly for the first six minutes after vasopressin addition to either the aldosterone-treated tissue or the control tissue, although there was a tendency for an initial fall on the control side. The importance of these data is that, as shown in Figure 1, the peak response to vasopressin occurred at approximately six minutes. After this, when the short-circuit current fell, ATP concentrations rose on both sides. At no time was the ATP concentration or ATP/AMP higher in the aldosterone-pretreated section than in the control, and at most times the ATP level was significantly lower. This result agrees with a previous report of glycolytic intermediate and adenine nucleotide content of aldosterone-treated tissue under aerobic conditions; this indicated that the rate-limiting enzymes of glycolysis were not directly activated by aldosterone but were activated secondarily by
increased sodium transport. Thus some explanation, other than enhanced energy production, is necessary to account for the effect of aldosterone.

One possibility which has been considered is that aldosterone promotes the passive entry of Na\(^+\) at the mucosal surface of the cell. The present experiments cannot rule out this possibility, but the results of Fanestil, et al., who used voltage clamp techniques, and the recent experiments of Cuthbert and Painter, who used current clamp methods both argue strongly against the hypothesis that aldosterone promotes the passive entry of sodium at the mucosal surface.

Because of these results, we were led to reconsider more seriously the possibility that aldosterone has a direct effect upon the sodium pump or Na\(^+\)/K\(^+\)-activated ATPase on the serosal surface of the epithelial cells. Sharp and Leaf had noted previously that aldosterone-treated sodium transport appeared unusually sensitive to inhibition by ouabain, but offered no explanation for this observation. We undertook to reinvestigate this phenomenon. As shown in Figure 3a, ouabain at 10\(^{-6}\) M caused a significant inhibition of sodium transport in aldosterone-treated bladder sections, but had no effect in comparable control sections. Addition of 10\(^{-4}\) M ouabain caused further inhibition of sodium transport in the aldosterone-treated segment, but only slight inhibition of transport in the control segment. At equal rates of sodium transport, induced by either washing the ouabain from the aldosterone-treated side or adding vasopressin to the control side, the aldosterone-stimulated transport still exhibited a tenfold
greater sensitivity to ouabain inhibition than did the control side (Fig. 3b). Furthermore, the ouabain inhibition of sodium transport in aldosterone-treated bladders was readily reversed by the addition of potassium chloride to a concentration of 4 mM, but ouabain inhibition of sodium transport in control bladders was not reversed by an increase to 12 mM potassium (Fig. 4). These results indicate that the pump or ATPase in the aldosterone-treated tissue is different from that in the control tissue, both in its sensitivity to inhibition by ouabain and in the ability of K⁺ to reverse this inhibition.

These results become of greater interest when considered in the light of the recent studies of Gachelin and Bastide. They reported that there are two membrane-bound Na⁺/K⁺-activated adenosine triphosphatases in the frog urinary bladder. In one, half-maximal activation of ATPase activity by Na⁺ is achieved at a concentration of 25 mM and by K⁺ at 1 mM. These constants are similar to those commonly seen with Na⁺/K⁺ adenosine triphosphatases from other tissues. However, the same values for the second enzyme were: Na⁺, 5 mM; K⁺, 0.1 mM. These values are significantly lower than those of the first enzyme. Gachelin and Bastide proposed that this second enzyme might be involved in the active entry of Na⁺ at the mucosal surface of the cell. However, in the light of the present results, a more attractive alternative is that both these enzymes are on the serosal surface of the cells, either of the same or different cell
types. The characteristics of the responses to ouabain and K⁺ of the sodium transport system in aldosterone-treated bladders observed in the present study are consistent with aldosterone causing the synthesis or activation of the second ATPase described by Gachelin and Bastide. However, further experiments are required to test this possibility.

An alternative possibility is that aldosterone does not induce the synthesis of a new ATPase, but changes the environment of the ATPase in the membrane and thereby alters its properties. If changes in membrane and lipid synthesis did underline the action of aldosterone, they could account for a number of the unusual metabolic effects of aldosterone upon the bladder such as a stimulation of acetoacetate oxidation⁹ and an inhibition of glucose-1-C¹⁴ oxidation.¹⁹ A similar proposal regarding the mechanism of action of cortisol has been made by Nordlie et al.²⁰ in the case of the membrane-bound enzyme glucose-6-phosphatase in liver microsomes.

Either alternative could equally well account for the fact that, either in the presence¹⁶, ²¹ or absence of oxygen, the response of the toad bladder to vasopres-

sin, in terms of sodium transport, is enhanced if this organ has been pretreated with aldosterone. However, difficulty is encountered in explaining simply the observation that under either aerobic or anaerobic conditions aldosterone pre-
treatment enhances the change in water permeability of the bladder induced by a standard dose of vasopressin.²¹ ²² This permeability effect has been attributed to a different hormone-tissue interaction than the one responsible for adrenal steroid stimulation of Na⁺ transport.²¹ This permeability enhancement could, however, be the result of a generalized change in cell membranes brought about by steroid hormone treatment. Whatever the mechanism for the aldosterone enhancement of permeability, the accumulated evidence indicates that the effect of aldosterone upon sodium transport cannot be simply accounted for by a single effect of enhancing the supply of available energy to the sodium pump or pumps. Aldosterone has direct and possibly complex effects upon the cell mem-

branes and/or membrane-bound enzymes of epithelial cells of the urinary bladder.

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† Pennsylvania Plan Scholar and postdoctoral fellow, Pennsylvania Heart Association.
22 Eggens, P., R. Walter, and I. L. Schwartz, unpublished observations.