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ALDOSTERONE STIMULATION OF SODIUM TRANSPORT*

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SUMMARY

On the basis of current concepts of the basic components of the Na^+ transport system across epithelial structures, we inferred that aldosterone could stimulate Na^+ transport in one of three ways: (1) by facilitating the entry of Na^+ into the effector epithelial cells, as vasopressin is believed to do, (2) by increasing the intrinsic activity of the Na^+ pump, or (3) by increasing the local concentration of the high-energy intermediate of the Na^+ pump. Three sets of experimental results were presented: (1) The response of the isolated toad bladder to vasopressin and to aldosterone under conditions of substrate depletion. (2) The effect of aldosterone on the response of the Na^+ transport system to saturating concentrations of vasopressin. (3) The effect of aldosterone on the Na^+ flux ratio during reversed net flow of Na^+ produced by a fixed serosal to mucosal electrical potential difference of 100 mV (serosa positive) and a 1:5 mucosal to serosal Na^+ concentration gradient. We found that in substrate-depleted hemibladders, aldosterone had no effect on Na^+ transport but vasopressin produced the usual rise. In substrate-enriched hemibladders, the absolute increase in active Na^+ transport in response to vasopressin was greater after maximum stimulation by aldosterone than in control hemibladders. During steadily maintained reversed net flow of Na^+, aldosterone produced a significant increase in the Na^+ flux ratio. These results support the concept that aldosterone increases the output of the Na^+ pump independently of an effect on the permeability of the mucosal surface epithelial cell.

INTRODUCTION

The evidence now available indicates that aldosterone regulates Na^+ transport by inducing synthesis of proteins *de novo via* stimulation of RNA synthesis[1-4]. To

Abbreviation: scc, short-circuit current.

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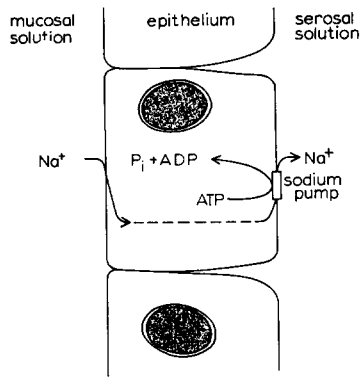

Fig. 1. Model of the Na^+ transport system in the toad bladder.

formulate the possible pathways from steroid-dependent synthesis of proteins to an increased rate of Na^+ transport requires some knowledge of the basic components of the Na^+ transport system. A plausible model of the Na^+ transport system of epithelial structures (such as anuran skin or urinary bladder) has been deduced from electrophysiological, isotope flux and biochemical studies and is shown in Fig. 1 (refs. 5–8). Entry of Na^+ into the epithelial cell from the external or lumenal solution is depicted as a passive process in series with active extrusion of Na^+ from the interior of the cell into the sub-epithelial space. The evidence also indicates that a principal component of the Na^+ pump located in the inner (serosal) face of the epithelial cell membrane probably is a (Na^+-K^+-Mg^{2+})-activated ATPase and that ATP is the proximate energy donor for active transport of Na^+ (ref. 9).

Based on this model, the steroid-induced proteins could accelerate the rate of Na^+ transport in three ways: (1) by increasing the permeability of the mucosal barrier to Na^+, thereby facilitating the entry of Na^+ into the epithelial cell, (2) by increasing the activity of the Na^+ pump directly (*e.g.*, increased synthesis or activation of the transport ATPase), and (3) by increasing the rate of synthesis of the energy donor, presumably ATP, and as a result the local concentration of ATP at the pump site. There is, of course, no *a priori* reason to assume that only one of these mechanisms is brought into play but we will concern ourselves with the question of which of these mechanisms may be shown to be involved in the process.

In the earlier studies, PORTER AND EDELMAN[10] found that aldosterone increased the ratio of unidirectional fluxes of Na^+ across the isolated urinary bladder of the toad, suggesting an action directly on the Na^+ pump or on the supply of high-energy intermediates to the pump. The latter possibility was also supported by the finding of a negligible response to aldosterone in substrate-depleted bladders and that subsequent addition of glucose or pyruvate elicited a full mineralocorticoid effect without an appreciable latent period[1]. Based on the radiosodium tissue-labeling method, however, SHARP AND LEAF[11,12] and CRABBÉ[13] concluded that aldosterone accelerates Na^+ transport by increasing the permeability to Na^+ of the mucosal surface of the effector epithelial cells. Thus, the conflicting evidence on the penultimate step (the ultimate effect, obviously, is to increase the output of the Na^+ pump) in the action of aldosterone on Na^+ transport led us to carry out the following additional experiments on

the isolated toad bladder, designed to give more information on this issue: (1) A comparison of the dependence of aldosterone- and vasopressin-mediated rises in Na^+ transport on an adequate supply of exogenous substrate. (2) The effect of aldosterone on the response of the Na^+ transport system to vasopressin. (3) The effect of aldosterone on the Na^+ flux ratio under voltage-clamp conditions.

METHODS

Urinary bladders of the toad, *Bufo marinus*, were excised, mounted in glass chambers filled with frog-Ringer solution, and monitored electrically, as described previously[10]. Active Na^+ transport was estimated by the short-circuit current (scc) method of USSING AND ZERAHN[14].

Substrate dependence of aldosterone and vasopressin

Five sets of experiments were completed. In the first set of experiments, vasopressin (final concentration = 100 mU/ml) was added to the serosal media of 78 hemibladders, approx. 5 h after incubation in standard frog-Ringer solution enriched with glucose (final concentration = 0.01 M). The scc was recorded continuously. The baseline scc at the time of addition of the vasopressin to the medium (scc_B) and the scc at the point of maximum increase (scc_M) were taken as the index of the response of the Na^+ transport system to vasopressin. In the second set of experiments, 13 hemibladders were incubated in substrate-free frog-Ringer solution for 16 h, the media were then exchanged for fresh substrate-free frog-Ringer solution, and vasopressin was added to the serosal media (final concentration = 100 mU/ml). The response was estimated from a comparison of scc_B and scc_M. After scc_M was identified, the media were exchanged for fresh frog-Ringer solution enriched with glucose (final concentration = 0.01 M). After 2 h of incubation in the presence of glucose, the hemibladders were again challenged with 100 mU/ml vasopressin. In the third set of experiments, 18 hemibladders were incubated in frog-Ringer solution that contained glucose (final concentration = 0.01 M), penicillin (final concentration = 0.1 mg/ml), and streptomycin (0.1 mg/ml) for 16 h. The media were then exchanged for fresh glucose-enriched, frog-Ringer solution and (+)-aldosterone was added to the serosal media (final concentration = 0.7 μM). 6 h after the addition of aldosterone, vasopressin was added to the serosal media (final concentration = 100 mU/ml) and scc_B and scc_M were recorded. In the fourth set of experiments 7 pairs of hemibladders were incubated in glucose-enriched frog-Ringer solution overnight and the following morning the media were exchanged for fresh, glucose-enriched media, as in the third set of experiments. Aldosterone was added to the serosal media (final concentration = 0.7 μM) of one of each pair of hemibladders and the scc recorded for an additional 3 h in both chambers. The fifth set of experiments, using 8 pairs of hemibladders, was like the fourth set except that neither the preincubation nor the incubation medium contained substrate.

Effect of aldosterone on the response to vasopressin

Twelve pairs of hemibladders were incubated overnight in frog-Ringer solution that contained glucose (0.01 M), penicillin (0.1 mg/ml) and streptomycin (0.1 mg/ml). The following morning the media were exchanged for fresh glucose–frog-Ringer solutions and (+)-aldosterone was added to the serosal medium (final concentration = 0.7

μM) of one of each pair and an equal amount of the diluent to the serosal medium of the other member of the pair. 6 h after the addition of either steroid or diluent, vasopressin was added to the serosal media (final concentration = 100 mU/ml) of both members of each pair. The scc was recorded continuously for 7 h after the addition of aldosterone or the diluent.

Voltage clamp and Na^+ fluxes

Pairs of hemibladders were incubated overnight in frog-Ringer solution containing glucose (0.01 M), penicillin (0.1 mg/ml), and streptomycin (0.1 mg/ml). The following morning the media were exchanged for fresh glucose–Ringer solution. The serosal solutions were of standard composition (*i.e.*, Na^+ = 114 mequiv/l) but the mucosal solutions were modified by replacing four-fifths of the NaCl with choline chloride (*i.e.*, Na^+ = 23 mequiv/l and choline$^+$ = 91 mequiv/l). Aldosterone was added to the serosal solutions (final concentration = 0.7 μM) of one of each pair of hemibladders and at the same time the trans-epithelial electrical potential difference was clamped at 100 mV, serosal surface positive, by reversing the current flow of the short-circuiting system. Hyperpolarization at 100 mV was maintained by adjusting the current flow in the external circuit at 30-min intervals.

Simultaneous bidirectional flux of Na^+ was determined by adding ^{22}Na (0.3 μC/ml) to the serosal solutions and ^{24}Na (1.5 μC/ml) to the mucosal solutions, and sampling both solutions at hourly intervals. The tracers were added to the media at the time of addition of aldosterone (arbitrarily taken as time zero). Aliquots of the media were assayed for ^{22}Na and ^{24}Na by differential counting in a two-channel Autogamma system (Packard Instrument Co.) both before and after total decay of ^{24}Na (*i.e.*, 23 half-lives). All counts were corrected for decay and background.

A comparison was made of the volume of dilution available for inulin and Na^+ from the serosal side of the toad bladder (serosal inulin space *versus* serosal Na^+ space). Hemibladders were preincubated and exposed to the conditions of the voltage-clamp experiments described above. [^{3}H]Inulin (final concentration = 2.5 μC/ml) and ^{22}Na (final concentration = 0.3 μC/ml) were added simultaneously to the serosal solutions. After 15, 60, or 120 min, pairs of hemibladders were removed from their chambers, blotted briefly, weighed, dried overnight at 105°, and reweighed. The dried tissues were homogenized in 0.5 M perchloric acid and an aliquot of the supernatant was assayed with a liquid-scintillation spectrometer (Packard Instrument Co.). The appropriate corrections for quenching were made with ^3H and ^{22}Na internal standards. The volumes of dilution were calculated in the conventional way.

To estimate the intracellular concentration of Na^+, it was necessary to make independent corrections for the extracellular fluid Na^+ content of the mucosal and serosal sides of the hemibladders. Thus, [^{14}C]inulin was used to estimate the volume of extracellular fluid of the mucosal side and [^{3}H]inulin the volume of extracellular fluid of the serosal side. The protocol was the same as in the voltage-clamp experiments, except that 90 min before the application of the voltage clamp, [^{14}C]inulin (final concentration = 5.0 μC/ml) was added to the serosal solutions, and ^{22}Na (specific activity = 2000 counts/min · μequiv) was present in both mucosal and serosal solutions. The voltage clamp (100 mV, serosa positive) was applied to one of the hemibladders while the control hemibladder operated at its spontaneous potential difference. 90 min after the application of the voltage clamp the hemibladders were removed

from the chambers, blotted briefly, dried, and extracted as described above. The extracts were assayed for ^{3}H, ^{14}C, and ^{22}Na activity by counting at two voltage settings in a two-channel liquid-scintillation spectrometer. Appropriate corrections for quenching were made with separate internal standards of each of the isotopes. Intracellular Na^+ content was calculated from total tissue water corrected for extracellular water and total Na^+ (assuming that tissue specific activity was the same as that of the medium after 180 min of equilibration) corrected for extracellular Na^+.

RESULTS

Response of substrate-depleted hemibladders to aldosterone and vasopressin

In a previous study[10] it was found that aldosterone had little or no effect on active Na^+ transport in substrate-depleted hemibladders but produced significant increases in both the net transport of Na^+ and in the flux ratio of Na^+ in hemibladders bathed in substrate-rich media. A similar experiment was performed in the present study in order to provide a quantitative estimate of the dependence of the action of aldosterone on the supply of substrate. The results of this experiment are shown in Table I. In glucose-rich media, aldosterone (0.7 μM) induced a 44% rise in Na^+ transport in 3 h, whereas in glucose-free media, the rate of Na^+ transport fell 6%. Thus, the response to aldosterone is nil in hemibladders deprived of substrate. The absolute dependence of mineralocorticoid action on an adequate supply of substrate is not likely to be a result of a failure of steroid-induced protein synthesis in the substrate-depleted state as it has been shown that introduction of either glucose, pyruvate, oxaloacetate, or acetoacetate to the media 3 h after aldosterone evokes the response of the Na^+ transport system with no appreciable latent period[1,15]. It is reasonable to suppose that substrate dependence is a consequence either of an action on the energy supply to the Na^+ pump (*i.e.*, that aldosterone regulates the production of a high-energy intermediate) or that the Na^+ pump is incapable of increasing its output because the energy supply is rate-limiting in the substrate-deprived hemibladders regardless of the site of action of the steroid. As a test of the latter possibility, we compared the response of substrate-depleted toad bladders to vasopressin

TABLE I

THE RESPONSE OF SUBSTRATE-DEPRIVED BLADDERS TO ALDOSTERONE

The serosal and mucosal media either contained glucose (+) or were substrate-free (o). The results are given as mean ± standard error of the mean and were computed for the change in scc recorded 3 h after the addition of either aldosterone (0.7 μM) or the diluent to the serosal media.

No. of pairs	*Glucose (0.01 M)*		*Aldosterone (μM)*	*Change in scc (%)*	
	Pre-incubation	*Incubation*			
7	+	+	o	1 ± 8	$P < 0.05$
	+	+	0.7	44 ± 16	
8	o	o	o	−17 ± 12	$P > 0.10$
	o	o	0.7	−6 ± 8	

and aldosterone. The available evidence indicates that vasopressin stimulates Na^+ transport by increasing the passive conductance of the mucosal membrane barrier thus raising the concentration of Na^+ at the pump site and thereby increasing the output of the pump[16–18]. If aldosterone also acts to increase the permeability of the mucosal membrane barrier to Na^+, then the action of vasopressin in substrate-depleted hemibladders should similarly depend on exogenous substrate.

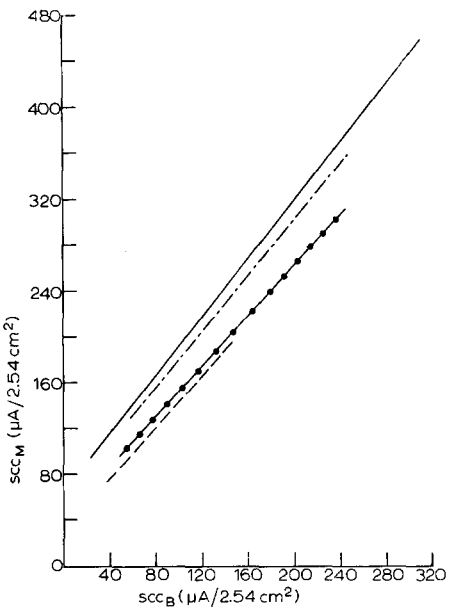

Fig. 2. The dependence of the vasopressin-induced rise in scc on the availability of glucose. The response to vasopressin (100 mU/ml) in the serosal medium is indicated by the dependence of the maximum rise (scc_M) on the baseline (scc_B). Set 1 (—) consisted of the control hemibladders incubated in 0.01 M glucose, Set 2 (— —) of the substrate-depleted hemibladders, Set 3 (●—●) of the substrate-replenished hemibladders, and Set 4 (— — — —) of the aldosterone-treated, substrate-replenished hemibladders. There was no statistically significant difference between the four populations.

To provide a quantitative index of the response to vasopressin, the maximum scc (scc_M) after the addition of vasopressin to the serosal media (final concentration = 100 mU/ml) was plotted against the baseline scc (scc_B) recorded just before the addition of vasopressin. The regression lines obtained by the least-squares method are shown in Fig. 2 for the following populations: (1) Control hemibladders in substrate-rich media. (2) Substrate-depleted hemibladders (16 h of preincubation in substrate-free media). (3) Substrate-replenished hemibladders (16 h of preincubation in substrate-free media followed by 2 h of incubation in 0.01 M glucose–Ringer solution). (4) Aldosterone-stimulated, substrate-replenished hemibladders (6 h after treatment with 0.7 μM aldosterone in 0.01 M glucose–Ringer solution). Statistical evaluation of the regression relationship shown in Fig. 2 indicates there are no significant differences in the response of the Na^+ transport system to vasopressin in these four populations of toad bladders. Thus, neither the availability of exogenous substrate nor prior stimulation with aldosterone altered the Na^+ transport response to vasopressin. These results

indicate that the Na^+ transport system is not solely limited by the supply of substrate even in substrate-depleted hemibladders and supports the inference that aldosterone acts specifically to increase the energy supply to the pump rather than to make more Na^+ available to the pump for transport.

Effect of aldosterone on the response to vasopressin

If the inference that aldosterone acts to increase the energy supply to the Na^+ transport system is correct, then the addition of vasopressin to the steroid-stimulated hemibladders should result in a greater absolute increase in active Na^+ transport than in the control hemibladder. The point here is the expectation that vasopressin-mediated facilitation of the entry of Na^+ into the epithelial cells in the presence of an enriched supply of high-energy intermediates should result in a greater output of the Na^+ pump. If, on the other hand, both aldosterone and vasopressin act at the same site, *e.g.*, to increase the permeability of the mucosal barrier, the absolute response to vasopressin should be attenuated by pretreatment with aldosterone since either the potentiality for the permeability change or the response of the Na^+ pump to an increased local concentration of Na^+ should be limited.

TABLE II

EFFECT OF ALDOSTERONE ON THE RESPONSE TO VASOPRESSIN

scc_0 denotes the scc recorded at the time of addition of aldosterone or the diluent to the serosal media. scc_B denotes the scc 6 h after the addition of aldosterone or diluent to the media and just before adding vasopressin to the serosal media. scc_M are the currents recorded at the peak of the response to vasopressin (100 mU/ml). $\triangle scc$ is the difference between scc_M and scc_B. All results are given as mean $\pm$ standard error of the mean for 13 pairs of hemibladders. The short-circuit currents are in $\mu A/2.54\ cm^2$.

Aldosterone (μM)	scc_0	scc_B	scc_M	$\triangle scc$	$\frac{\triangle scc}{scc_0} \times 100$
0	95 ± 13	78 ± 11	120 ± 16	42 ± 7.5	49 ± 8.5
0.7	90 ± 10	154 ± 13	221 ± 19	67 ± 8.8	83 ± 15
P	Nil	<0.01	<0.01	0.05	0.005

The results of the effect of pretreatment with aldosterone on the response to vasopressin are shown in Table II. It can be seen that the vasopressin-mediated rise in scc was greater in the aldosterone-treated than in the control hemibladders. The absolute increase in Na^+ transport, after vasopressin was $67 \pm 8.8\ \mu A/2.54\ cm^2$ in the steroid-treated group, compared to an increase of $42 \pm 7.5\ \mu A/2.54\ cm^2$ in the control group (Table II). These data, therefore, do not support the conclusion that aldosterone and vasopressin have the same penultimate site of action. Although the evidence derived from the effects of combinations of substrates, vasopressin and aldosterone is self-consistent, we next sought an independent physiological test of the hypothesis that aldosterone acts by increasing the output of the Na^+ pump rather than on the facilitation of Na^+ entry into the epithelial cells.

Voltage clamp and Na^+ flux ratios

USSING AND ZERAHN[14] proposed that in the absence of an external electrochemical gradient for Na^+, the magnitude of the driving force for active Na^+ transport (E_{Na^+}) could be inferred from the ratio of unidirectional fluxes. They also concluded that if an agent increased net flux of Na^+ at a constant flux ratio, the action could be assigned to a decrease in a passive resistance boundary. It is very likely, however, that in the toad bladder system under short-circuited conditions (*i.e.*, where the external electrochemical gradient for Na^+ is nil) an increase in the flux ratio can not be assumed to represent a direct action on the driving force of the Na^+ pump. The reason why this is so may be seen from the model depicted in Fig. 1. If an agent were to facilitate the entry of Na^+ across the mucosal boundary, the initial effect would be an increase in intracellular concentration of Na^+. SKOU[9] has summarized the evidence that the transduction of chemical bond energy to Na^+ transport work involves a (Na^+ - K^+ - Mg^{2+})-activated ATPase. A key property of this enzyme system is that within a limited range, the rate of hydrolysis of ATP is dependent on the local concentration of Na^+. It is to be expected, therefore, that an increase in intracellular Na^+ concentration would stimulate an increase in the flux ratio. Thus, in an earlier study from our laboratory it was concluded that the possibility of an action of aldosterone on the conductance of the mucosal barrier was not excluded by the finding of a significant increase in the Na^+ flux ratio after aldosterone[10]. If the flux ratio experiments, however, could be carried out under conditions where a change in the permeability of the mucosal membrane barrier would not lead to an increase in intracellular Na^+ concentration, then a rise in the flux ratio should be indicative of an increase in the driving force of the Na^+ pump.

Electrophysiological data indicate that active transport of Na^+ is a property of the epithelial cell layer of the toad bladder[7]. In addition, the epithelial cell membranes merge at the mucosal surfaces to form tight junctions and it has been inferred that the tight junctions restrict the intercellular movement of water and ions between the lumen of the bladder and the sub-epithelial space[19,20]. If this model is correct, then in the steady state there should be a reversal of the driving force for the flow of Na^+ across the mucosal epithelial cell membrane surface when the electrochemical gradient for Na^+, oriented serosa to mucosa, exceeds the driving force of the Na^+ pump. Thus if the net flux of Na^+ (Φ) through the effector epithelial cells is reversed under voltage-clamp conditions, an increase in the permeability of the mucosal membrane barrier will facilitate the passage of Na^+ from the intracellular compartment into the lumen of the bladder and no increase in intracellular Na^+ concentration will occur. Under these circumstances the Na^+ pump will not increase in activity and the flux ratio (f_{Na^+}) will either be unchanged or fall. If, on the other hand, the output of the Na^+ pump is enhanced either by a direct action of the steroid-induced proteins or *via* an increase in the local concentration of ATP, then the Na^+ flux ratio should increase despite the reversed net flow of Na^+.

To obtain a fixed electrochemical gradient sufficient to produce a steady net flow of Na^+ oriented from serosa to mucosa, the concentration of Na^+ in the musocal solution was reduced to one-fifth that of the serosal solution and at the same time the serosal side was made 100 mV positive with respect to the mucosal side with a voltage-clamp circuit. Under these experimental conditions net Na^+ flux was reversed and the magnitude of flow in the reversed direction was reasonably steady throughout the 6 h

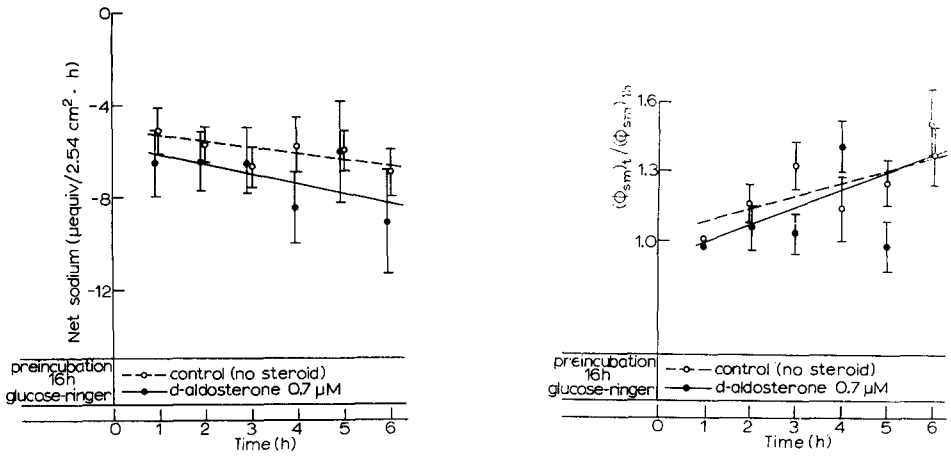

Fig. 3. Net Na^+ flux under voltage-clamp conditions as a function of time. The negative sign denotes net movement in the serosal to mucosal direction. Time zero is time of addition of aldosterone (final concentration = 0.7 μM) to the serosal medium of one of each pair of hemibladders (●) and of the diluent to the control (○). Vertical lines are ± 1 standard error of the mean. The regression line was obtained by the method of least squares. The regressions do not differ significantly.

Fig. 4. The normalized unidirectional flux in the serosa to mucosa direction as a function of time. $(\Phi_{sm})_t$ and $(\Phi_{sm})_{1h}$ denote the fluxes for time t, and for the first hour of the experiment, respectively. At 1 h, mean Φ_{sm} was 5.9 for the control and 7.3 μequiv/2.54 cm²·h for the aldosterone-treated hemibladders. Vertical lines are ± 1 standard error of the mean. The regression lines, obtained by the method of least squares, do not differ significantly.

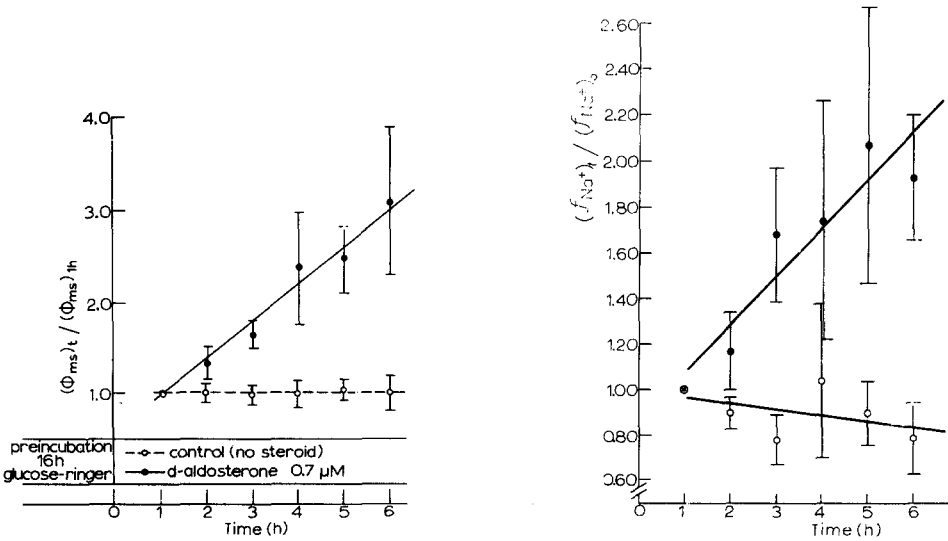

Fig. 5. The normalized unidirectional flux in the mucosa to serosa direction as a function of time. $(\Phi_{ms})_t$ and $(\Phi_{ms})_{1h}$ are the fluxes for time t, and for the first hour of the experiment, respectively. At 1 h, mean Φ_{sm} was 0.84 for the control and 0.82 μequiv/2.54 cm²·h for the aldosterone-treated hemibladders. Vertical lines are ± 1 standard error of the mean. The regression lines, obtained by the method of least squares, differ significantly at the 0.1% level.

Fig. 6. The effect of aldosterone on the Na^+ flux ratio. Time zero is time of addition of aldosterone (final concentration = 0.7 μM) to the serosal medium of one of each pair of hemibladders (●) and of the diluent to the control (○). n = 6, vertical lines are ± 1 standard error of the mean.

of study (Fig. 3). In both the control and aldosterone-treated hemibladders the magnitude of the net flux (Φ) decreased approximately linearly at about the same rate. The regression lines in Fig. 3 do not differ significantly.

As shown in Fig. 4, the unidirectional flux of Na^+ from serosa to mucosa (Φ_{sm}) increased gradually to the same extent in both the control and steroid-treated hemibladders. There was no differential effect on the movement of Na^+ in the passive direction. The magnitude of the spontaneous increase in Φ_{sm} accounts for the time-dependent decrease in net flow shown in Fig. 3.

In the control hemibladders unidirectional flux of Na^+ from mucosa to serosa (Φ_{ms}) remained constant throughout the 6 h of study but rose linearly to three times the initial value in the aldosterone-treated hemibladders (Fig. 5). From the data shown in Figs. 4 and 5, we computed the time-dependent changes in the Na^+ flux ratio which are shown in Fig. 6. It is noteworthy that in the control hemibladders the Na^+ flux ratio remained reasonably steady, tending to fall about 20% over the 5-h period of study, and in the aldosterone-treated group there is a progressive and significant increase in the Na^+ flux ratio. Moreover, the time-course of the increase in the Na^+ flux ratio in response to aldosterone is virtually the same as the time-course of increase in net Na^+ transport[10]. These results are given in absolute values in Table III and indicate that aldosterone induced a significant increase in the Na^+ flux ratio at

TABLE III

EFFECT OF ALDOSTERONE ON THE Na^+ FLUX RATIO

Data are given as the mean ± standard error of the mean for 6 pairs of hemibladders.

Aldosterone (μM)	*Na^+ flux ratio*			*P*
	1st hour	*6th hour*	△	
0	0.164 ± 0.05	0.117 ± 0.03	−0.046 ± 0.03	Not significant
0.7	0.159 ± 0.09	0.263 ± 0.13	+0.104 ± 0.04	0.05

ratios less than 1 (*i.e.*, during reversed net flow). This increase can not be attributed to an increase in intracellular Na^+ concentration, provided that the reversed net flux of Na^+ was through the effector epithelial cells rather than between these cells. Since the interpretation of the flux ratio under voltage-clamp conditions depends critically on the path for reversed flow, we tested the assumption that measurable quantities of Na^+ moved through, rather than between epithelial cells in two ways: (1) A comparison was made of the serosal Na^+ space and the serosal inulin space. (2) The effect of the 100-mV voltage clamp on the intracellular concentration of Na^+ was measured.

If all of Φ_{sm} is *via* intercellular paths, then the serosal Na^+ space will approximate the serosal extracellular fluid volume (*i.e.*, inulin space). The magnitude of intracellular penetration of Na^+ will be proportional to the difference between the Na^+ space and the inulin space. As shown in Fig. 7, during the application of the 100-mV voltage clamp and a serosal to mucosal Na^+ gradient of 5:1, the serosal Na^+ space exceeded the serosal inulin space by a factor of 1.9, 1.7, and 1.5, at 15, 60, and 120 min, respectively. These results indicate that the serosal surfaces of a significant

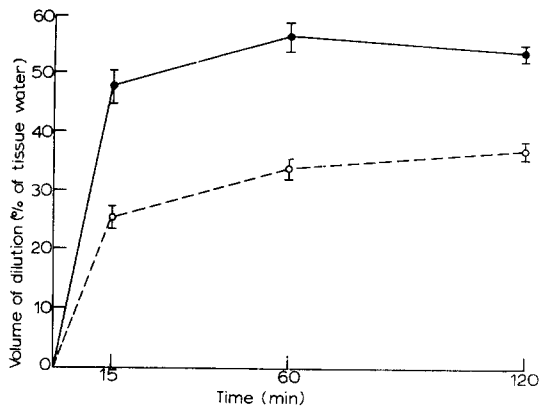

Fig. 7. The volume of dilution of ^{22}Na (●) and inulin (○) as a function of time. The tracers were added to the serosal medium at time zero. The serosal to mucosal chemical gradient for Na^+ was 5:1 and the electrical gradient was 100 mV, serosal side positive. The chemical and electrical gradients were maintained at a constant level throughout the period of equilibration. Vertical lines are ± 1 standard error of the mean. There were 8 pairs of hemibladders at 15 min, 6 at 60 min, and 8 at 120 min.

fraction of the cells of the toad bladder are permeable to Na^+ and that steady-state distribution of Na^+ is complete in 15 min.

The orientation of the voltage was such as to drive Na^+ through cells that are permeable to Na^+ and are also a part of the resistance barrier (*e.g.*, smooth muscle cells should be shunted by the surrounding, extracellular fluid and Na^+ should move around rather than through these cells). It is apparent from microelectrode studies that only the epithelial cell layer of the toad bladder forms a high-resistance pathway for Na^+ conductance[7]. As shown in Table IV, the application of the voltage clamp

TABLE IV

INTRACELLULAR Na^+ CONCENTRATION

The results are given as μequiv/g cell water for 6 pairs of hemibladders.

Open circuit	*100-mV clamp*	*Mean difference*
40.5 ± 5.07	46.0 ± 3.89	5.57 ± 1.60 $t = 3.47$ $P < 0.025$

resulted in an absolute increase in intracellular concentration of Na^+ of 5.6 μequiv/g of cell water. The significant increase in cell Na^+ indicates that Na^+ is being driven through and not just between the epithelial cells by the imposition of the voltage clamp.

DISCUSSION

In 1951, USSING AND ZERAHN[14] proposed a method for estimating the driving force of the Na^+ pump from unidirectional fluxes of Na^+ across an isolated epithelial

cell boundary in the special case of a vanishingly small electrochemical gradient of Na^+ across the boundary. They found that the addition of neurohypophyseal extract to the isolated frog skin increased the rate of active Na^+ transport and had no significant effect on the Na^+ flux ratio and inferred that this hormone acts by lowering the resistance to the flow of Na^+. With the isolated toad bladder system, LEAF AND DEMPSEY[21] observed that vasopressin increased the mean Na^+ flux ratio from 7.9 to 10.9 but drew no inferences from these observations because of large spontaneous variations in the serosal to mucosal fluxes. FRAZIER and co-workers[17,18] concluded that vasopressin acts only on the mucosal permeability barrier on the basis of the increase in the rate of radiosodium labeling of the toad bladder from the mucosal solution and an increased rate of radiosodium elution from the bladder into the mucosal solution under the influence of vasopressin. If the sole action of vasopressin is to increase the permeability of the mucosal surface of the epithelial cell membranes, then the evidence presented here indicates that aldosterone does not act at this site. In substrate-depleted hemibladders, aldosterone has no measurable effect on Na^+ transport whereas the response to vasopressin is not significantly attenuated (*cf.* Table I and Fig. 2). Moreover, anaerobiosis completely eliminates the response to aldosterone but not to vasopressin[15,22]. It is important to note that vasopressin stimulates an increase in O_2 consumption and glycogenolysis only in the presence of Na^+-enriched media[21]. Thus it appears that vasopressin facilitates Na^+ entry into the epithelial cells and the resultant rise in intracellular Na^+ concentration serves as the stimulus to active Na^+ transport. The increase in energy metabolism induced by vasopressin can be attributed to its action on Na^+ transport which acts as a pacemaker for metabolic activity. Thus neither substrate depletion nor anaerobiosis obliterates the response to vasopressin. It is reasonable to conclude, therefore, that the failure of substrate-depleted or O_2-deprived hemibladders to respond to aldosterone is not a consequence of a paralysis of the Na^+ pump. The possibility that the dependence of mineralocorticoid action on substrate and O_2 is a result of metabolic requirements for steroid-induced protein synthesis is probably eliminated by the findings that addition of substrate to the media 3 h after aldosterone or of O_2 4 h after aldosterone evokes the steroid response with no discernible latent period indicating that the steroid-induced proteins have already been formed and await only an adequate supply of substrate and O_2 to exert their effect on the Na^+ transport system[1,15].

If aldosterone-induced proteins acted primarily to increase Na^+ conductance of the mucosal surface membrane, all substrates providing energy for Na^+ transport in steroid-free hemibladders should also provide energy for aldosterone-dependent Na^+ transport. Alternatively, the new proteins may accelerate the production of high-energy intermediates in which case substrates that enter the metabolic pathway after the enzymatic steps stimulated by aldosterone would not elicit the mineralocorticoid effect but would, nevertheless, support Na^+ transport in steroid-free hemibladders. FIMOGNARI, PORTER AND EDELMAN[15] found that in substrate-depleted hemibladders the precursors of citrate (glucose, pyruvate, oxaloacetate and acetoacetate) were synergistic with aldosterone in the regulation of Na^+ transport, and FANESTIL found that propionate, a precursor of succinyl-CoA, supported Na^+ transport fully in the absence of aldosterone but failed to elicit the mineralocorticoid effect in the presence of aldosterone. These results support the concept that aldosterone regulates Na^+ transport by stimulating enzymatic steps involved in the production of high-energy intermediates.

If the inferences that vasopressin acts by increasing Na^+ conductance and that aldosterone acts by increasing the energy supply to the Na^+ pump are correct, then in concert these regulators should have additive or enhancing effects on Na^+ transport. If vasopressin and aldosterone both acted at the same site to facilitate Na^+ entry into the effector epithelial cells, aldosterone should tend to reduce the response to vasopressin and *vice versa*. As shown in Table II, the absolute increase in active Na^+ transport stimulated by vasopressin is greater in aldosterone-treated hemibladders than in the controls.

All of the studies on substrate dependence, O_2 dependence, and the comparative effects of vasopressin and aldosterone support the conclusion that the primary action of aldosterone is to increase the output of the Na^+ pump not by facilitating the entry of Na^+ across the mucosal boundary but by increasing the supply of a high-energy intermediate, presumably ATP. The converse conclusion, that the site of action of aldosterone is on the barrier to Na^+ movement at the mucosal surface of the bladder epithelial cells, was reached by SHARP AND LEAF[11,12], and by CRABBÉ[13] on the basis of radiosodium studies.* The radiosodium method, however, is liable to misinterpretation as no information is available on the distribution of the additional radiosodium among the diverse cell types or the inter-epithelial spaces of the toad bladder[19,20]. Thus, we made use of the flux ratio technique as an alternative means of testing the hypothesis of steroid action on the permeability barrier.

KEDEM AND ESSIG[23] gave the general form of the flux ratio (f_{Na^+}) equation. Their Eqn. 19 may be rewritten for the Na^+ flux ratio as:

$$RT \ln f_{Na^+} = -X_{ec} + \sum_i X_i + X_m \qquad (1)$$

where X_{ec} denotes the electrochemical potential difference for Na^+ of the bathing media, X_i the contribution of all trans-epithelial flows to oriented Na^+ flow (*e.g.*, solvent drag, solute–solute interactions) and X_m the direct contribution of metabolism to oriented Na^+ flow. When the electrochemical potential difference is kept constant at any arbitrary level:

$$RT \frac{df}{f} = d\sum_i X_i + dX_m \qquad (2)$$

Thus the fractional change in the flux ratio is an index of the change in the contribution of active transport to Na^+ transport provided that there is no significant change in coupled trans-epithelial flows of other species (*i.e.*, $d\sum_i X_i = 0$). PORTER AND EDELMAN[10] found that the isolated toad bladder bathed in identical solutions and short-circuited (*i.e.*, $X_{ec} = 0$) responded to 0.7 μM (+)-aldosterone with an increase in the Na^+ flux ratio of from 2.9 ± 0.4 in the controls to 4.4 ± 0.6 in the steroid-treated group ($P < 0.05$). Assuming that $d\sum_i X_i = 0$, this increase in the flux ratio is indicative of an increase in the activity of the Na^+ pump. As mentioned above, however, this effect could result from an action on the mucosal barrier to the entry of Na^+ into the effector epithelial cell. If the driving force for Na^+ transport across the mucosal membrane surface were reversed, then under these conditions an action of aldosterone on the permeability to Na^+ of the mucosal membrane surface could not evoke an increase

* See also NOTE ADDED IN PROOF in ref 15.

in X_m. Under a constant driving force that was sufficient to maintain steady reversed net flows of Na^+ (*i.e.*, from serosa to mucosa) (+)-aldosterone produced a significant increase in f_{Na^+} (Fig. 6 and Table III). The time-course of the increase in f_{Na^+} was about the same as that of the increase in net Na^+ transport measured by the scc technique (*cf.* Fig. 6 and PORTER AND EDELMAN[10]). If the net flow of Na^+ across the mucosal face of the epithelial cell membranes is also reversed under these conditions, these results indicate that steroid action involves an effect on the output of the Na^+ pump independent of an effect on mucosal membrane permeability. Reversal of net flow of Na^+ across the mucosal face of the epithelial cell membranes during the application of the voltage clamp is assured only if a sufficient quantity of Φ_{sm} (*i.e.*, an amount greater than Φ_{ms}) passes through rather than between the epithelial cells.

That an intercellular path for Φ_{sm} may exist in the toad bladder is suggested by the work of USSING AND WINDHAGER[24] who found that a significant fraction of Na^+ outflux across the frog skin was intercellular when the inside surface was bathed with hypertonic urea. In the toad bladder system, however, the complementary statement can be made with some assurance, namely that a significant fraction of Φ_{sm} passes through, rather than between epithelial cells as the Na^+ space is larger than the inulin space and intracellular Na^+ concentration is increased by imposing a 100-mV fixed potential difference (Fig. 7 and Table IV). Thus the effect of aldosterone on f_{Na^+} during reversed net flow of Na^+ probably signifies an action on the output of the Na^+ pump independent of an effect on Na^+ conductance of the mucosal surface cell membranes. This action could result from a direct influence on the structural components of the Na^+ pump or from stimulation of the biosynthesis of a high-energy intermediate. The main component of the Na^+ pump probably is a $(Na^+ - K^+ - Mg^{2+})$-activated ATPase[8,9]. Aldosterone did not activate a membrane-bound partially purified preparation of this enzyme, nor did it increase the content or activity of this ATPase in the isolated toad bladder or in the kidney of the adrenalectomized rat[25,26]. In addition, the results of studies on the specificity of substrate–aldosterone synergism support the postulate of aldosterone induction of a protein(s) that is active in the biosynthesis of a high-energy intermediate of the Na^+ pump[15].

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