Spironolactone

AN ALDOSTERONE AGONIST IN THE STIMULATION OF H^+ SECRETION BY TURTLE URINARY BLADDER

ALLAN MUELLER and PHILIP R. STEINMETZ, *Department of Medicine, University of Iowa College of Medicine, Iowa City, Iowa 52242*

ABSTRACT In the isolated turtle bladder, spironolactone inhibits sodium transport in the presence of aldosterone or endogenous mineralocorticoid hormone. In contrast to this antagonism for the stimulation of sodium transport by aldosterone, the stimulation of hydrogen ion secretion by aldosterone is not inhibited by spironolactone. In hormone-depleted bladders, spironolactone stimulates hydrogen ion secretion. The extent of stimulation is similar to that of aldosterone. Spironolactone functions as an agonist for aldosterone for the stimulation of urinary acidification.

INTRODUCTION

The stimulation of sodium transport in many epithelia by the mineralocorticoid hormone, aldosterone, can be abolished by steroidal spirolactones (1–6). Crabbé (1–3), Porter (4), and Sakauye and Feldman (6) have shown that spironolactone (in a concentration of ~50–200 times that of aldosterone) antagonizes the action of aldosterone in the toad urinary bladder. This antagonism has been shown to occur by the binding of spironolactone to cytoplasmic and nuclear receptors with the displacement of aldosterone (7–11). This inhibitory effect of the spirolactones on sodium transport has also been shown in the distal nephron of the kidney and has become the basis for the use of spironolactone as a diuretic in patients with states of hyperaldosteronism and sodium retention (3, 5, 12).

In the kidney, aldosterone stimulates not only the reabsorption of sodium but also the secretion of hydrogen ion and potassium (5). Little is known, however, about the mechanisms by which potassium and hydrogen secretion are increased. The increases could be caused by the increased electrical potential difference (lumen negative) generated by increased sodium transport. Alternatively, aldosterone might stimulate these secretory processes by separate mechanisms.

Recent studies have shown that aldosterone can directly stimulate hydrogen secretion in two urinary epithelia; in the bladder of the Colombian toad (13) and the bladder of the freshwater turtle, *Pseudemys scripta* (14). The studies in turtle urinary bladder have further shown that the stimulation of hydrogen secretion precedes that of sodium transport and has different substrate requirements (14). In this tissue, the stimulation of both transport systems, however, appeared to be abolished by actinomycin D.

In the present study, the differences between the stimulation of sodium and hydrogen ion transport were further explored in the turtle bladder by means of spironolactone, SC 9420, which is a relatively pure antagonist for sodium transport in toad bladder (6). The study indicates that in turtle bladder, spironolactone has its usual inhibitory effect on sodium transport by antagonism to aldosterone, but that it functions as an agonist for aldosterone in the stimulation of urinary acidification.

METHODS

Paired hemibladders of the freshwater turtle, *P. scripta*, were mounted in lucite chambers as previously described (15). Control and experimental halves were obtained from the same bladder. H^+ secretion, J_H[1], was measured by pH stat titration at pH 7.0 and expressed as nanomoles per minute per 8 cm^2. Net sodium transport, J_{Na}, was obtained from the short-circuit current corrected for J_H and was expressed in microamperes per 8 cm^2.

The media contained (expressed in millimoles per liter) Na, 115; K, 3.5; Ca, 1.8; Cl, 121.5; HPO_4, 0.3; and dextrose, 5. The mucosal solution was gassed with CO_2-free air and the serosal solution was gassed with 1% CO_2 in air.

The bladders were depleted of endogenous mineralocorticoid hormone by soaking the turtles for 2–3 days in 0.7% NaCl and by depletion overnight in the lucite chambers (Results). D-

This study was presented at the 61st Annual Meeting of the Federation of American Societies for Experimental Biology, Chicago, Ill., 6 April, 1977.

Received for publication 25 October 1977 and in revised form 1 February 1978.

[1] *Abbreviations used in this paper*: J_H, H^+ secretion; J_{Na}, net sodium transport.

Aldosterone (Sigma Chemical Co., St. Louis, Mo.) was added in 50 μl of methanol to give a final concentration of 0.5 μM. This concentration is half the concentration used by LeFevre (16) in the turtle bladder and has been effective in the stimulation of J_H and J_{Na} in our previous study (14). Spironolactone (Sigma Chemical Co.), 17-hydroxy-7α-mercapto-3-oxo-17α-pregn-4-ene-21-carboxylic acid γ-lactone 7-acetate, was added to 50 μl of methanol to give a final concentration of 50 μM. The same volume of methanol was added to the control halves. The addition of hormone or diluent was made only to the serosal solution.

RESULTS

Aldosterone action in turtle bladder: experimental considerations. Three practical aspects of these studies are emphasized before the presentation of the results obtained with spironolactone. (*a*) It is virtually impossible to obtain fresh turtle bladders that are depleted of endogenous mineralocorticoid hormone. In the absence of a satisfactory method to maintain adrenalectomized turtles, we have used the bladders of saline-soaked turtles which are only partially depleted of the hormone (pithing and bladder removal probably cause secretion of the hormone). To get near complete depletion of endogenous mineralocorticoid, the bladders were incubated for 20 h in Lucite chambers. After a 20-h period of depletion in vitro (this study and 17), the halves exposed to aldosterone showed increases in J_H and J_{Na} compared to control halves of the same bladders in all 14 bladders studied in our laboratory. The results were considerably less predictable (17) when comparisons were made during the first 6 h after mounting, presumably because of the presence of endogenous hormone. (*b*) For an investigation of the interactions between aldosterone and its antagonist, spironolactone, it would be ideal to compare four identical quarters of the same bladder: one control, one exposed to aldosterone alone, one exposed to spironolactone alone, and one treated with spironolactone before exposure to aldosterone. Unfortunately the requirement to have four segments with comparable initial rates of J_H measurable by pH stat titration could not be met in any of the eight bladders so studied. To overcome this limitation, three sets of separate comparisons were made between halves of bladders instead of the four-way comparisons in the quarters of bladders. First, exposure to aldosterone alone caused consistent stimulation of J_H and J_{Na} compared to control halves (14, 17). Second, aldosterone-exposed halves were compared with and without pretreatment with spironolactone. Finally, exposure to spironolactone alone was compared to control halves. (*c*) As in previous studies (14) the term "stimulation" refers to the increased transport by experimental halves over the control halves of the same bladders. Most bladders have higher rates at the time of mounting than after 20 h in the chambers even when exogenous aldosterone and glucose are present. The initial rates were measured to ensure that both bladder halves had adequate and comparable transport rates for H^+ and Na^+. As pointed out above, the experimental bladder halves exposed for 20 h to 0.5 μM aldosterone consistently exhibited higher transport rates than the control halves, although the rates were often less than the rates observed in the fresh state. The survival time of epithelial cell layers in vitro is ~48 h. Little is known about the mechanism of this decline in function, it is likely that various nutrient and poorly understood humoral factors are involved. For the purpose of our study, however, it is important that the presence of aldosterone plays a major role in sustaining the transport rates in vitro.

Effects of pretreatment with spironolactone on the action of aldosterone. Table I shows that J_{Na} was inhibited in the bladder halves pretreated with spironolactone and subsequently exposed for 20 h to exogenous aldosterone. In contrast, J_H was unaffected by spironolactone. The transport rates at the time of mounting of the bladder halves before hormone addition were the same for the control and experimental halves. J_{Na} was 183±23 μA and J_H was initially 12.1±0.9 nmol/min, 20 h before the values of Table I were obtained.

Fig. 1 shows in six other experiments how J_H was unaffected by spironolactone pretreatment, whereas J_{Na} was inhibited by 25±5%. In these experiments, both halves were again depleted of hormone in the chamber for 20 h. Both halves were exposed to aldosterone for 4 h.

In the presence of only endogenous mineralocorticoid hormone, spironolactone appeared to increase J_H in eight fresh bladders, but the increase was of borderline significance (Fig. 2). J_{Na}, however, was again inhibited by spironolactone.

These results show that spironolactone does not act

TABLE I
Effect of Spironolactone on J_{Na} and J_H in Bladders Subsequently Exposed to Aldosterone

J_{Na}			J_H		
Control + ALDO*	SPIRO + ALDO	ΔJ_{Na} (Sp-c)	Control + ALDO	SPIRO + ALDO	ΔJ_H (Sp-c)
μA			nmol/min		
114 ±35	53 ±19	−61‡ ±23	9.5 ±3.0	9.6 ±2.2	+0.1 ±1.9

* ALDO, aldosterone; Sp-C, spironolactone-control; SPIRO, spironolactone.

‡ Significant by paired analysis ($P < 0.05$). If the ratio J_{Na}/J_{Na0} were compared, the P value was <0.005. SPIRO caused no significant change in either J_H/J_{H0} or ΔJ_H. Mean values±SEM are given for six paired hemibladders. Bladders were exposed for 20 h to 0.5 μM ALDO. Experimental halves were pretreated with 50 μM SPIRO 1 h before the addition of ALDO. The serosal solution contained 5 mM glucose and 1% CO_2 in air.

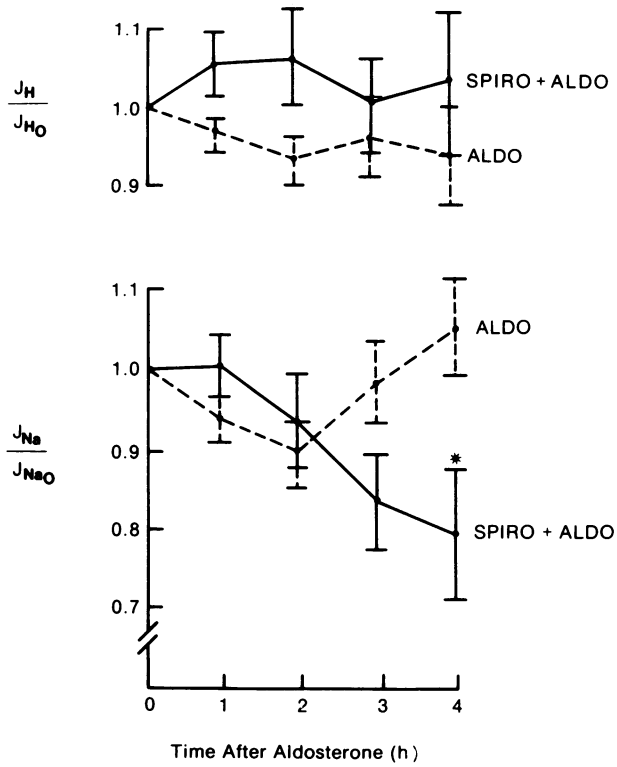

FIGURE 1 The effect of spironolactone (SPIRO) on the responses of J_H and J_{Na} to aldosterone (ALDO) in hormone-depleted bladders. Ratios are graphed for six paired hemibladders. Experimental halves were pretreated with 50 μM spironolactone. At time zero, 0.5 μM aldosterone was added to SPIRO and control halves. The serosal solution contained 5 mM glucose and 1% CO_2 in air. *Significant by paired analysis ($P < 0.05$).

as an antagonist for the aldosterone stimulation of J_H and suggest that spironolactone may act as an agonist for aldosterone in the stimulation of J_H.

Effects of spironolactone alone on J_{Na} and J_H in hormone-depleted bladders. To explore this possibility the effects of spironolactone alone were examined in 11 bladders depleted for 20 h of endogenous mineralocorticoid hormone and compared with control halves of the same bladders. As shown in Table II, spironolactone caused no inhibition of J_{Na} in these hormone-depleted bladders, a result consistent with the interpretation that endogenous mineralocorticoid is required for the inhibition by spironolactone. On the contrary, there was an increase in the mean J_{Na} in the spironolactone-treated halves. The increase, however, was of borderline significance because of variability of the results. J_H, on the other hand, was consistently and significantly greater in spironolactone-treated halves than in the control halves. The difference of 8.1±2.2 μmol/min was comparable to that observed previously in experiments with aldosterone alone (14) as shown in Table III. Both spironolactone and aldosterone caused significant increases in J_H.

DISCUSSION

These results indicate that spironolactone acts as an antagonist for aldosterone in the stimulation of sodium transport in the turtle bladder as in other urinary epithelia. This antagonism was observed when endogenous mineralocorticoid hormone or aldosterone were present. In the absence of aldosterone in bladders depleted of endogenous hormone for 20 h in the chamber, the effects of spironolactone were rather variable. In 2 of the 11 bladders (Table II), sodium transport was inhibited, in the others it was stimulated. It is possible that the two bladders had residual mineralocorticoid activity and that the others were so depleted that spironolactone served as a partial agonist. Sakauye and Feldman (6) reported that spironolactone had no agonistic properties by itself in toad bladder after a shorter period (6 h)

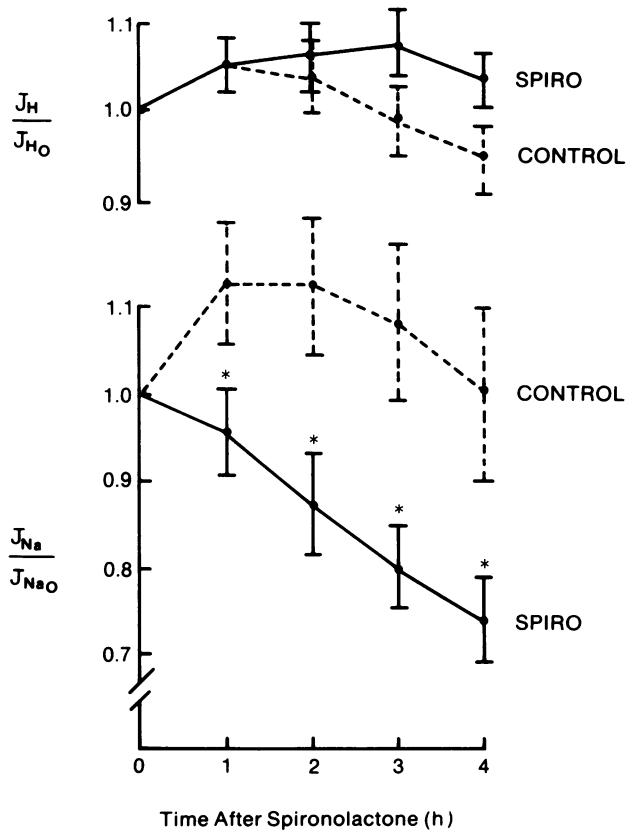

FIGURE 2 The effect of SPIRO on J_H and J_{Na} in fresh turtle bladders. Ratios are graphed for eight paired hemibladders. Experimental halves received 50 μM spironolactone at time zero (~4 h after mounting). The serosal solution contained 5 mM glucose and 1% CO_2 in air. *Significant by paired analysis ($P < 0.05$).

of hormone depletion. Several other spirolactones, however, had agonistic properties for sodium transport in the absence of aldosterone.

Our results suggest that the stimulation of J_H is not inhibited by spironolactone at any level of endogenous mineralocorticoid activity or in the presence of aldosterone. After 20 h of hormone depletion, J_H was stimulated in all bladders and the magnitude of stimulation was comparable to that observed with aldosterone alone. Spironolactone, therefore, functioned as a physiological agonist for aldosterone without any antagonistic properties.

How should the different effects exerted by spironolactone on J_H and Na^+ absorption be interpreted? Previous studies (18–20) have shown that the two transport systems are not coupled directly. They affect each other only indirectly via the electrical potential difference and the availability of CO_2 for J_H (20). In the present study these indirect forms of coupling were excluded since all experiments were carried out in the short-circuited state and in the presence of exogenous CO_2.

Several descriptive explanations for our results must be considered. First, the different behavior of spironolactone in the stimulation of the two transport systems is in accord with the previously reported differences in the substrate requirements and time-course between the stimulation of H^+ and Na^+ transport (14). It is possible that both spironolactone and aldosterone stimulate J_H by some direct mechanism altering the lipid composition of the cell membrane (21) and thereby the conductance in the active transport pathway for urinary acidification. This possibility, however, is not supported by the observation that actinomycin D abolished both the stimulation of H^+ and Na^+ transport by aldosterone (14); neither can it be considered excluded. The second set of explanations invokes separate pathways for the stimulation of the two transport systems. The separation could take place at the level of the cytoplasmic hormone receptors or at subsequent sites in the pathways leading to the synthesis of effector proteins. Our physiologic studies provide no information on whether there are separate hormone receptors, separate gene sites that respond differently to the same receptor complexes or whether the stimulatable transport processes for Na^+ and H^+ are contained in different cell types.

TABLE II

Effect of Spironolactone on J_{Na} and J_H in Hormone-Depleted Bladders

J_{Na}			J_H		
Control	SPIRO*	ΔJ_{Na} (Sp-c)	Control	SPIRO	ΔJ_H (Sp-c)
	μA			*nmol/min*	
100 ±24	206 ±40	+106 ±48 ($0.05 < P < 0.10$)	13.4 ±1.8	21.4 ±2.7	+8.1‡ ±2.2 ($P < 0.005$)

* Sp-C, spironolactone-control; SPIRO, spironolactone.

‡ Significant by paired analysis. The P values were the same if instead of Δ values, the ratios of J_{Na}/J_{Na0} were compared. Mean values±SEM are given for 11 paired hemibladders. The experimental halves were exposed for 20 h to 50 μM SPIRO. The transport rates in the control and experimental halves were the same at the time of mounting, 20 h before the results were presented. J_{Na0} was 447±26 μA and J_{H0} was 24.9±1.6 nmol/min. These initial values were about twice the initial values of the experiments of Table I (see text). The experiments of this table were done in September and October, whereas those of Table I were done in March and April. The serosal solutions contained 5 mM glucose and 1% CO_2 in air.

TABLE III

Comparison of the Effects of Spironolactone and Aldosterone on J_H in Hormone-Depleted Bladders

	J_H			
	Control	Steroid	ΔJ_H (St-c)	
		nmol/min		
SPIRO*	13.4 ±1.8	21.4 ±2.7	+8.1‡ ±2.2	$n = 11$
ALDO	10.1 ±1.5	16.0 ±3.2	+6.0‡ ±2.2	$n = 6$

* SPIRO, spironolactone; St-C, steroid-control.

‡ Significant by paired analysis ($P < 0.05$). Mean values ±SEM are given for two different groups of paired hemibladders. The experimental halves were exposed for 20 h to either 50 μM SPIRO or 0.5 μM ALDO. The serosal solution contained 5 mM glucose and 1% CO_2 in air.

ACKNOWLEDGMENT

This research was supported by grant AM 17568 from the National Institutes of Health.

REFERENCES

1. Crabbé, J. 1964. Decreased effectiveness of aldosterone on active sodium transport by the isolated toad bladder in the presence of other steroids. *Acta Endocrinol.* **47:** 419–432.
2. Crabbé, J. 1971. Inhibition by spirolactone of the effect of aldosterone on transepithelial sodium transport in extrarenal activity of aldosterone and its antagonists. Proc. Symp. Nice, 7–10. Exerpta Medica, Amsterdam.
3. Crabbé, J. 1963. The sodium-retaining action of aldosterone. Editions Arscia S. A., Presses Acad. Eur. S. C. Brussels (thesis).

4. Porter, G. A. 1968. In vitro inhibition of aldosterone-stimulated sodium transport by steroidal spironolactones. *Mol. Pharmacol.* **4:** 224–237.
5. Sharp, G. W. G., and A. Leaf. 1966. Mechanism of action of aldosterone. *Physiol. Rev.* **46:** 593–633.
6. Sakauye, C., and D. Feldman. 1976. Agonist and antimineralocorticoid activities of spirolactones. *Am. J. Physiol.* **231:** 93–97.
7. Sharp, G. W. G., C. W. Komack, and A. Leaf. 1966. Studies on the binding of aldosterone in the toad bladder. *J. Clin. Invest.* **47:** 450–459.
8. Feldman, D., J. W. Funder, and I. S. Edelman. 1972. Subcellular mechanisms in the action of adrenal steroids. *Am. J. Med.* **53:** 545–560.
9. Marver, D., J. Stewart, J. W. Funder, D. Feldman, and I. S. Edelman. 1974. Renal aldosterone receptors: studies with (^{3}H) aldosterone and the antimineralocorticoid (^{3}H) spirolactone (SC-26304). *Proc. Natl. Acad. Sci. U. S. A.* **71:** 1431–1435.
10. Funder, J. W., D. Feldman, E. Highland, and I. S. Edelman. 1974. Molecular modifications of anti-aldosterone compounds: effects on affinity of spironolactones for renal aldosterone receptors. *Biochem. Pharmacol.* **23:** 1493–1501.
11. Rossier, B. C., P. A. Wilce, and I. S. Edelman. 1977. Spironolactone antagonism of aldosterone action on Na^+ transport and RNA metabolism in toad bladder epithelium. *Membrane Biol.* **32:** 177–194.
12. Kagawa, C. M., J. A. Cella, and C. G. VanArman. 1957. Action of new steroids in blocking effects of aldosterone and deoxycorticosterone on salt. *Science (Wash. D. C.).* **126:** 1015–1016.
13. Ludens, J. H., and D. D. Fanestil. 1974. Aldosterone stimulation of acidification of urine by isolated urinary bladder of colombian toad. *Am. J. Physiol.* **226:** 1321–1326.
14. Al-Awqati, Q., L. H. Norby, A. L. Mueller, and P. R. Steinmetz. 1976. Characteristics of stimulation of H^+ transport by aldosterone in turtle urinary bladder. *J. Clin. Invest.* **58:** 351–358.
15. Steinmetz, P. R. 1967. Characteristics of hydrogen ion transport in urinary bladder of water turtle. *J. Clin. Invest.* **46:** 1531–1540.
16. LeFevre, M. E. 1973. Effects of aldosterone on the isolated substrate-depleted turtle bladder. *Am. J. Physiol.* **225:** 1252–1256.
17. Al-Awqati, Q. 1977. Effect of aldosterone on the coupling between H^+ transport and glucose oxidation. *J. Clin. Invest.* **60:** 1240–1247.
18. Steinmetz, P. R., R. S. Omachi, and H. S. Frazier. 1967. Independence of hydrogen ion secretion and transport of other electrolytes in turtle bladder. *J. Clin. Invest.* **46:** 1541–1548.
19. Schwartz, J. H., and P. R. Steinmetz. 1971. CO_2 requirements for H^+ secretion in the isolated turtle bladder. *Am. J. Physiol.* **220:** 2051–2057.
20. Steinmetz, P. R. 1974. Cellular mechanisms of urinary acidification. *Physiol. Rev.* **54:** 890–956.
21. Goodman, D. B. P., J. E. Allen, and H. Rasmussen. 1971. Studies on the mechanisms of action of aldosterone: hormone-induced changes in lipid metabolism. *Biochemistry.* **10:** 3825–3831.