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The Effect of Aldosterone on the Accumulation of Adenosine 3′:5′-Cyclic Monophosphate in Toad Bladder Epithelial Cells in Response to Vasopressin and Theophylline

(steroid permissive effect/permeability changes/cAMP degradation)

JEFFREY S. STOFF, JOSEPH S. HANDLER, AND JACK ORLOFF

Laboratory of Kidney and Electrolyte Metabolism, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

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ABSTRACT **Vasopressin and theophylline both increase the content of adenosine 3′:5′-cyclic monophosphate (cAMP) in epithelial cells of the urinary bladder of toads (*Bufo marinus*). Incubation of the tissue with 0.2 μM aldosterone markedly increases this response to the two agents; incubation for a similar time without steroid reduces the response. The permeability responses (sodium transport and water flow) of the intact tissue to vasopressin, theophylline, and exogenous cAMP are also considerably greater in bladders incubated with aldosterone than without. The results are interpreted as indicating that the foregoing permissive effects of aldosterone on the permeability responses to vasopressin and theophylline are mediated by a steroid-dependent increase in the accumulation of cAMP in the pertinent epithelial cells, probably as a consequence of a diminution in the rate of degradation of the intracellular nucleotide.**

Aldosterone, in addition to stimulating sodium transport across the urinary bladder of the toad (1), amplifies the physiologic response of the tissue to vasopressin (2, 3). The peptide hormone increases sodium transport and osmotic water flow across the bladder by a mechanism involving the intermediacy of adenosine 3′:5′-cyclic monophosphate (cAMP) (4, 5). These responses to vasopressin are considerably greater in bladders incubated with aldosterone for several hours than in those depleted of aldosterone by incubation in steroid-free medium for a comparable time (3). This "permissive" effect of aldosterone is also evident with respect to exogenous cAMP and theophylline (an inhibitor of the degradation of the nucleotide), in that the permeability responses to these agents are also greater in steroid-treated tissue. These observations were interpreted (3) as indicating that the steroid affected a step in the permeability process subsequent to the vasopressin-induced accumulation of cAMP, conceivably by altering the responsiveness of the tissue to the nucleotide. As reported below, however, the elevation in the concentration of cAMP produced by vasopressin or theophylline, or a combination of the two, is considerably greater in epithelial cells of steroid-treated bladders than in those of steroid-depleted bladders. The data are consistent with the view that the effect of aldosterone on the permeability responses to vasopressin is mediated by an increase in the concentration of cAMP in the pertinent epithelial pool, rather than by a change in the sensitivity of the tissue to the nucleotide. It is probable that this is a consequence of steroid-dependent inhibition of the degradation of cAMP. Whether the direct stimulatory effect of aldosterone on sodium transport that occurs in the absence of vasopressin also involves cAMP remains to be determined.

METHODS

Toads (*Bufo marinus*) were obtained from National Reagents, Bridgeport, Conn., and were kept on San-I-Cell moistened with tap water.

The cAMP content of the epithelial cells of toad bladders was determined under various experimental conditions. Bladders were excised from doubly-pithed toads, and were divided into quarters. After incubation in Ringer solution (3) at 23–25° for 2 hr, one-quarter bladder from each of two toads was transferred to a separate erlenmeyer flask containing Ringer solution, with or without aldosterone and/or other agents, and incubated for various lengths of time. "Fresh" tissue was removed for analysis of the cAMP content of the cells at the end of 2 hr of the second incubation. Bladder segments were depleted of steroid before analysis by incubation in Ringer solution containing 20 mM glucose for 18–25 hr. "Steroid-treated" bladders remained in a similar Ringer solution containing aldosterone for a comparable time before analysis. The effect of vasopressin on the cAMP content of cells was assessed after incubation with the peptide hormone for the final 15 min of the experimental period, that of theophylline was determined after incubation for the final 30 min. The details of other studies are described elsewhere in the paper.

All experiments were ended by placing the bladder on a glass surface, mucosal-side up. The epithelial cells were rapidly scraped off the bladder with the edge of a glass microscope slide, and the sheet of epithelial cells was quickly frozen in liquid nitrogen. About 1 min elapsed from the time an intact bladder was removed from an erlenmeyer flask to the time the separated cells were frozen.

Nucleotides were extracted at 1° by allowing the frozen cells to thaw, with thorough mixing, in 8% Cl_3CCOOH containing tracer [^{3}H]cAMP to monitor recovery (50–65%). After centrifugation at 700 × *g* for 15 min, the supernatant fluid was transferred to 5.5 × 0.5 cm cation-exchange (BioRad AG 50W-X8, 100–200 mesh) columns that had been equilibrated with 0.1 N HCl. The cAMP was eluted with distilled water. Samples were lyophilized and dissolved in an appropriate volume of double-glass-distilled water, and were assayed at two dilutions, each in duplicate, by a modification of the method of Gilman (6). Each value for cAMP represents the mean of duplicate determinations performed at two dilutions, corrected for recovery of [^{3}H]cAMP, and expressed per mg of tissue protein (7). The assay permitted detection of as little as 0.5 pmol. All duplicates agreed within ±10%. Incu-

TABLE 1. *Effect of vasopressin and theophylline on cellular cAMP content of "fresh" tissue*

Vasopressin (mU/ml)	Theophylline (mM)	cAMP, (pmol/mg of protein)
—	—	8.9 ± 1.03 (6)
3	—	12.1 ± 0.83 (4)
25	—	26.3 ± 2.87 (14)
—	10	15.0 ± 0.85 (12)
—	30	36.1 ± 1.81 (4)
3	10	50.3 ± 1.31 (4)
25	10	81.5 ± 13.2 (6)

Quarter bladders were incubated for a total of 4 hr (see *text*). Vasopressin was added 15 min before and theophylline 30 min before the end of the final incubation period. Values for cAMP are the mean ±S.E., for the number of samples indicated in parentheses. All responses to vasopressin and to theophylline were significantly ($P < 0.05$) greater than the control.

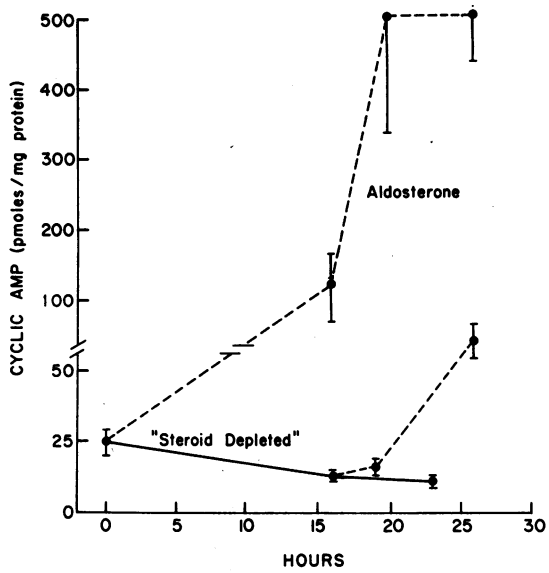

FIG. 1. Time course of aldosterone effect on response to vasopressin. Paired tissues were incubated in the presence (*upper curve*) and absence (*lower curve*) of aldosterone. Steroid-depleted tissues were divided into two groups at 16 hr. One group was placed in medium containing 0.2 μM aldosterone (*dashed line*), the other in steroid-free medium (*solid line*). All tissues received vasopressin (25 mU/ml) for the final 15 min. Values for cAMP are the mean ±SE for $n = 6$.

bation of tissue extracts with bovine-heart cyclic nucleotide phosphodiesterase reduced the cAMP content to undetectable concentrations.

The vasopressin used was Pitressin powder without chlorbutanol, a gift of Parke-Davis. Dexamethasone was a gift of Merck, Sharp & Dohme. Aldosterone and adenosine 3′:5′-cyclic monophosphate were purchased from Calbiochem, theophylline and cycloheximide from Nutritional Biochemicals, and cyclic 3′:5′-nucleotide phosphodiesterase from Sigma. [^{3}H]cAMP was obtained from Schwartz BioResearch (16.3 Ci/mmol) or from New England Nuclear Corp. (24.1 Ci/mmol).

RESULTS

The effect of vasopressin and theophylline on the cAMP content of cells isolated from "fresh" tissue is summarized in Table 1. Three mU/ml of vasopressin or 10 mM theophylline produced significant increases in the cAMP content of the epithelial cells. The effects of 25 mU/ml of vasopressin or 30 mM theophylline were considerably greater. In addition, as expected, the two agents together had a synergistic effect. These changes in cAMP content are similar to, but relatively greater than, those reported (8) in studies in which the cAMP content of the entire bladder was measured. The results are consistent with the thesis that vasopressin exerts its physiologic effects in this tissue via cAMP.

The cAMP content of cells obtained from bladders after 18 hr of steroid depletion and 18 hr of incubation with 0.2 μM aldosterone is summarized in Table 2. The basal concentration of cAMP was not altered by steroid treatment, as has been reported (9). The addition of vasopressin (25 mU/ml) for the final 15 min of incubation increased the concentration of cAMP in cells from both sets of tissue. The increment in steroid-treated tissue, however, was considerably greater than that in tissue incubated for the same period without aldosterone. We conclude that the increased accumulation of cAMP in steroid-treated tissue accounts for the "permissive" effect of aldosterone on the permeability responses to vasopressin. It appears unnecessary to postulate that the steroid

TABLE 2. *Effect of vasopressin and theophylline on cellular cAMP content of steroid-depleted and treated tissues*

Vasopressin (mU/ml)	Theophylline (mM)	n*	Steroid-depleted (pmol cAMP/mg of protein)	0.2 μM Aldosterone (pmol cAMP/mg of protein)	P†
—	—	18	7.2 ± 0.39	7.7 ± 0.49	<0.2
25	—	6	12.2 ± 0.84	125. ± 41.2	<0.025
—	10	6	16.8 ± 1.59	18.3 ± 1.86	<0.4
—	30	4	31.9 ± 1.94	45.2 ± 3.96	<0.05
3	10	4	41.7 ± 3.30	53.7 ± 3.77	<0.05
25	10	6	105. ± 13.8	527. ± 91.7	<0.005

Paired quarter-bladders were incubated for 18 hr (*lines 1 and 2*), 24 hr *line 3*, or 22 hr (*lines 4–6*), with or without aldosterone. Vasopressin and theophylline were added as described in Table 1. 20 mM glucose was present in Ringer solution until the last 2 hr of incubation, at which time 5 mM Ringer solution was used.

* n = Number of pairs.

† P = Significance of the mean of differences between paired samples with and without aldosterone.

TABLE 3. *Effect of steroids on response to vasopressin*

Steroid	cAMP content (pmol/mg of protein)
None	16.3 ± 1.62 (8)
Testosterone	11.8 ± 2.48 (4)
Aldosterone	472. ± 81.1 (8)
Dexamethasone	534. ± 121. (4)

Paired quarter-bladders were incubated for 25 hr with 0.2 μM steroid as indicated. Vasopressin, 25 mU/ml, was added to all flasks for the last 15 min. Values presented are the mean ±SE for the number of samples indicated in the parentheses.

must increase the sensitivity of the tissue to endogenous nucleotide.

The time course of the steroid effect is illustrated in Fig. 1. Note that the peak increase in accumulation of cyclic nucleotide in cells of steroid-treated bladders after treatment with vasopressin occurred at 25 hr. In other studies, designed to examine the first 7 hr of the response of "fresh" tissue with and without aldosterone, 1–3 hr of incubation in steroid was necessary before a detectable difference in the cAMP content in response to vasopressin could be observed between the two sets of tissues. The response to vasopressin in steroid-depleted tissue (*lower curve*, Fig. 1) declined progressively. At the end of 16 hr of depletion, aldosterone increased the responsiveness to vasopressin, but only after several hours of incubation in steroid.

Fig. 2 illustrates the effect of different concentrations of vasopressin on the cAMP content of cells from steroid-treated and depleted tissues. Although all concentrations of vasopressin (1–25 mU/ml) produce greater permeability responses in steroid-treated bladders (unpublished observations), a marked difference in the cAMP content of the two sets of cells was noted only when 10 and 25 mU/ml of vasopressin were tested. Despite our inability to detect a difference in the cAMP content of steroid-treated and depleted cells with 1 and 3 mU/ml of the polypeptide, we consider it likely that these concentrations of vasopressin do, in fact, elevate the concentration of cAMP to a greater extent in steroid-treated cells. The elevation may be localized to a tissue compartment responsible for the physiologic effects, but that represents only a small fraction of the total cellular pool of the nucleotide under these circumstances. Analogous considerations apply to the results with theophylline summarized in Table 2. No detectable effect of the steroid on the concentration of cAMP is seen with 10 mM theophylline, despite amplification of the physiologic effects (unpublished observations). In contrast, 30 mM theophylline, as well as a combination of 10 mM theophylline and 3 mU/ml of vasopressin (neither of which alone was sufficient), clearly increased the accumulation of cAMP in the presence of aldosterone.

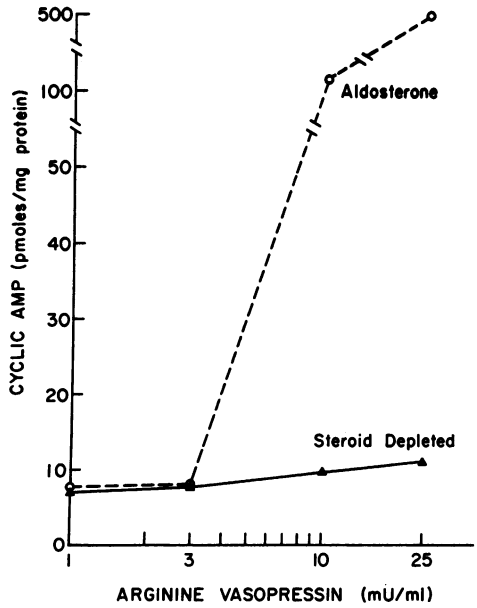

FIG. 2. cAMP content as a function of the concentration of vasopressin in tissues incubated with (O) and without (Δ) 0.2 μM aldosterone for 24 hr. $n = 4$.

The specificity of the steroid response is illustrated in Table 3. Cyclic AMP concentrations in response to vasopressin were unaltered by testosterone, which does not affect the permeability response to vasopressin (unpublished observations). In contrast, dexamethasone, which amplifies the physiologic response to vasopressin, as does aldosterone (3), had an equivalent effect on the accumulation of cAMP in response to the peptide. In other studies, the minimal concentration of aldosterone necessary to elicit a change in cAMP accumulation was 4 nM; 0.2 μM produced a maximal effect.

Finally, as illustrated in Table 4, 0.5 mg/ml of cycloheximide, an inhibitor of protein synthesis, markedly reduced the effect of aldosterone on cAMP accumulation. Inhibition of the vasopressin-induced accumulation of cAMP was also noted in depleted tissue. The latter observation may be indicative of

TABLE 4. *Effect of vasopressin on cellular cAMP content of steroid-treated and depleted tissues, with and without cycloheximide*

Aldosterone (0.2 μM)	Cycloheximide (0.5 μg/ml)	cAMP content (pmol/mg of protein)	Difference due to cycloheximide	n	P
0	0	21.8			
			−5.06 ± 1.48	7	<0.025
0	+	16.7			
+	0	601.			
			−470. ± 103	4	<0.025
+	+	131.			

Where indicated, cycloheximide was present in the incubation medium for 25 hr, aldosterone was present for the final 23 hr. Vasopressin (25 mU/ml) was present for the final 15 min of incubation.

incomplete disappearance of residual endogenous steroid or, alternatively, may merely reflect inhibition of the synthesis of proteins involved in the accumulation of cAMP in response to vasopressin.

DISCUSSION

It is apparent that the permissive effect of aldosterone on the permeability responses of the toad bladder to vasopressin is explicable on the basis of increased accumulation of cAMP under these circumstances. Since the steroid also potentiates the physiologic effect of exogenous cAMP, and yet does not detectably alter the intracellular concentration of the nucleotide in the basal state, direct stimulation of adenylate cyclase activity by aldosterone is an unlikely cause either for the amplification of the physiologic response or for the increased accumulation of cAMP after vasopressin or theophylline administration. The most likely explanation for the remarkable increase in the cAMP content of steroid-treated tissue after treatment with vasopressin or theophylline, and consequent amplification of the physiologic response to these agents and exogenous cAMP, is a reduced rate of degradation of the nucleotide. Hydrolysis of the nucleotide by cyclic nucleotide phosphodiesterase (10) is the only known intracellular mechanism for the degradation of cAMP. Though the steroid, in our view, must in effect reduce the activity of the phosphodiesterase, it does not do so by direct interaction with the enzyme (as does theophylline). Theophylline, in contrast to aldosterone, acts rapidly, elevates the cAMP content of the cells of itself, and also elicits an increase in both osmotic water flow and sodium transport. The relatively flat dose-response curve of steroid depleted tissue towards vasopressin (Fig. 2) is consistent with our hypothesis in that it may reflect release of inhibition of phosphodiesterase activity. Under these circumstances, despite increased adenylate cyclase activity with higher concentrations of vasopressin, the intracellular concentration of the nucleotide may be predominantly regulated by phosphodiesterase no longer under steroid control. Whether the well-known effect of aldosterone alone on sodium transport also may ultimately involve a change in phosphodiesterase activity in a cAMP pool responsible for sodium transport is not known.

Although we have been unable to demonstrate a difference in the activity of cyclic nucleotide phosphodiesterase in steroid-treated and depleted tissues, two observations are pertinent in this regard: (*a*) the elevated liver cyclic nucleotide phosphodiesterase activity of adrenalectomized rats is reduced by *in vivo* administration of 6α-methylprednisolone (11) and (*b*) cyclic nucleotide phosphodiesterase activity of rat hepatoma cells in tissue culture is reduced by incubation with dexamethasone (Manganiello, V. & Vaughan, M., personal communication). It is conceivable that steroid regulation of cyclic nucleotide phosphodiesterase may be mediated by changes in the rate of synthesis or activity of a "protein activator" of the enzyme, analogous to that recently reported (12).

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1. Crabbé, J. (1961) "Stimulation of active sodium transport by the isolated toad bladder with aldosterone *in vitro*," *J. Clin. Invest.* **40**, 2103–2110.
2. Fanestil, D. C., Porter, G. A. & Edelman, I. S. (1967) "Aldosterone stimulation of sodium transport," *Biochim. Biophys. Acta* **135**, 74–88.
3. Handler, J. S., Preston, A. S. & Orloff, J. (1969) "Effect of adrenal steroid hormones on the response of the toad urinary bladder to vasopressin," *J. Clin. Invest.* **48**, 823–833.
4. Orloff, J., Handler, J. S. & Preston, A. S. (1962) "The similarity of the effects of vasopressin, adenosine 3′,5′-phosphate (cyclic AMP) and theophylline on the toad bladder," *J. Clin. Invest.* **41**, 702–709.
5. Orloff, J. & Handler, J. S. (1967) "The role of adenosine 3′,5′-phosphate in the action of antidiuretic hormone," *Amer. J. Med.* **42**, 757–768.
6. Gilman, A. G. (1970) "A protein binding assay for adenosine 3′:5′-cyclic monophosphate," *Proc. Nat. Acad. Sci. USA* **67**, 305–312.
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) "Protein measurement with the Folin phenol reagent," *J. Biol. Chem.* **193**, 265–275.
8. Handler, J. S., Butcher, R. W., Sutherland, E. W. & Orloff, J. (1965) "The effect of vasopressin and of theophylline on the concentration of adenosine 3′,5′-phosphate in the urinary bladder of the toad," *J. Biol. Chem.* **240**, 4524–4526.
9. Kirchberger, M. A., Witkum, P. & Sharp, G. W. G. (1971) "On the similarity of the effects of aldosterone and adenosine 3′,5′-phosphate on Na^+ transport and glucose metabolism in toad bladder," *Biochim. Biophys. Acta* **241**, 876–883.
10. Butcher, R. W. & Sutherland, E. W. (1962) "Adenosine 3′,5′-phosphate in biological materials. I. Purification and properties of cyclic 3′,5′-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3′,5′-phosphate in human urine," *J. Biol. Chem.* **237**, 1244–1250.
11. Senft, G., Schultz, G., Munske, K. & Hoffman, M. (1968) "Effects of glucocorticoids and insulin on 3′,5′ AMP phosphodiesterase activity in adrenalectomized rats," *Diabetologia* **4**, 330–335.
12. Cheung, W. Y. (1971) "Cyclic 3′,5′-nucleotide phosphodiesterase, evidence for and properties of a protein activator," *J. Biol. Chem.* **246**, 2859–2869.