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THE SIMILARITY OF EFFECTS OF VASOPRESSIN, ADENOSINE-3',5'-PHOSPHATE (CYCLIC AMP) AND THEOPHYLLINE ON THE TOAD BLADDER *

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The role of antidiuretic hormone in the mechanism of urinary concentration is well established (1–3). It is generally agreed that the hormone reduces urine flow by increasing the permeability of the distal nephron to water, thereby facilitating osmotic flow of water out of the tubule lumen into the surrounding interstitium. The increase in permeability is thought to be accomplished by enlargement of aqueous channels or pores in the luminal membrane. This view of the action of vasopressin is based on direct *in vitro* demonstrations of hormone-induced permeability changes in the epithelial structures, frog skin (4), and toad bladder (5, 6) and is supported by recent micropuncture studies in the vertebrate nephron (1, 3). Addition of vasopressin to the inner surface of either frog skin or toad bladder evokes two characteristic changes: *1*) an increase in the permeability of the membrane to water, manifested by an increase in the net flow of water along an osmotic gradient; *2*) an increase in sodium transport across the membrane, generally estimated as the so-called short circuit current.

The biochemical basis of vasopressin action is not known. Alterations in oxygen consumption and rate of glycolysis after addition of the hormone have been observed *in vitro* (6). However, these changes are independent of the effect on permeability and are related to the associated increase in sodium transport. Recently Sutherland and Rall (7) and Haynes and Berthet (8) have emphasized the role of adenosine-3',5'-phosphate (cyclic AMP) in the action of a variety of hormones. They have shown that ACTH and glucagon, to cite two examples, have in common the property of stimulating the production of cyclic AMP in their specific receptor tissues, and have proposed that cyclic AMP alters the activity of enzymes within the receptor tissue, finally yielding the physiologically recognizable effect of the hormone. Subsequent to these studies, Hilton and Bergen and their associates (9, 10) have demonstrated that the injection of vasopressin into the adrenal artery stimulates the release of cortisol, an effect analogous to that of ACTH, and furthermore, that injection of vasopressin into the portal vein causes the hepatic release of glucose, an effect analogous to that of glucagon. It is likely that the vasopressin effect in both the adrenal and liver involves the intermediacy of cyclic AMP. In view of these observations we considered the possibility that vasopressin may stimulate the production of cyclic AMP in both toad bladder and renal tissue, and that cyclic AMP in some unknown fashion is responsible for modifying the permeability of these structures to water. This is expressed graphically in Figure 1. Although the conversion of ATP to cyclic AMP occurs in the absence of vasopressin, it is assumed that a critical increase in the concentration of cyclic AMP may be effected either by vasopressin or by theophylline, which is known to interfere with the breakdown of cyclic AMP to inactive adenosine-5-phosphate (5'-AMP) in other tissues (11). The present studies were designed to test this hypothesis by examining the effects of cyclic AMP and theophylline on net water movement and short circuit current in the toad bladder. A preliminary report of some of the studies has been published elsewhere (12).

METHODS

The bladder of the toad, *Bufo marinus,* was selected for these studies. The effect of vasopressin on net water movement and short circuit current across this mem-

* A preliminary report of these findings was presented at the May 1961 meeting of the American Society for Clinical Investigation.

TABLE I

Effect of vasopressin on net water movement

Period	Side A		Side B	
	Serosal solution	Weight loss	Serosal solution	Weight loss
		mg/min		*mg/min*
1	Ringer's	0.3	Ringer's	0.3
2	Vasopressin (500 μU/ml)	11.8	Vasopressin (5 mU/ml)	24.8
3	Ringer's	0.5	Ringer's	0.3

brane has been characterized in detail by others (5, 6). The method adopted for estimation of net water movement is similar to that described by Bentley (5). The bilobed bladder was dissected free from the pithed animal and divided into two separate sacs. In most studies one bladder sac served as the simultaneous control for the other. Each of the sacs was tied securely to a small glass tube, filled with 2 ml of dilute Ringer's solution (40 milliosmolar) and suspended in a chamber containing 20 ml of aerated Ringer's solution (200 milliosmolar). Mixing of each solution was achieved by bubbling a fine jet of gas through the outer bathing medium. Test substances were added to either or both bathing media, depending upon the experimental procedure. Net movement of water along the osmotic gradient (from mucosal to serosal surface of bladder sac) was estimated by weighing the sac in air at 30-minute intervals and noting the weight loss in this period. After each 30-minute period, fresh solutions were reintroduced into both chamber and sac, in order to minimize the effect of changes in osmolality on the results. Two to three washings of the bladder were generally sufficient to eliminate the effect of test substances when these were reversible. Short circuit current and potential difference were measured according to the technique of Ussing and Zerahn (13). In these studies either a 1 or 4.5 square centimeter segment of bladder served as a membrane separating the two halves of a Lucite chamber. A modified Boyle-Conway Ringer's solution was used in studies of net water movement, whereas Bentley's medium was used in the short circuit current and potential studies. The composition of the Boyle-Conway solution was as follows: NaCl, 72.5 mM; $NaHCO_3$, 25; Na_2HPO_4, 2.5; KH_2PO_4, 0.5; KCl, 1.98; $MgSO_4$, 1.2; Na_2SO_4, 0.65; $CaCl_2$, 1.6; dextrose, 5.5. Three per cent CO_2 in oxygen was used as the gas phase and the final pH was 7.6 to 7.7. The composition of the Bentley Ringer's solution used in the electrical studies was as follows: NaCl, 111.0 mM; $CaCl_2$, 2.7; KCl, 3.35; $NaHCO_3$, 2.38; dextrose, 5.5. The final pH was 8.0. After the addition of test substances to either of the Ringer's solutions, the pH was readjusted to its initial value. Air was used as the gas phase.

The cyclic AMP used in these studies was purchased from Schwarz Bio-Research and Sigma Chemical Company and was chromatographically pure. Commercial Pitressin was purchased from Parke, Davis and Company and is referred to as vasopressin in the text.

The statistical analysis of the data is expressed in terms of the standard error of the mean.

RESULTS

The effect of vasopressin, cyclic AMP, and theophylline on the permeability to water of the toad bladder. The effect of the addition of vasopressin to the solution bathing the serosal surface (outer surface of the bladder sac) is illustrated in Table I. It should be noted that net water movement in the control period was negligible. This rarely exceeded 1 mg per minute in any of the studies. The marked dose-dependent effect of vasopressin on net water movement, as well as its reversal after washing and reintroduction of fresh Ringer's solution, is similar to that observed previously (5). Not illustrated is the absence of an effect of the hormone when placed in the mucosal bathing solution (6).

The addition of 1 μmole per ml of cyclic AMP to the serosal bathing solution resulted in a prompt increase in net water movement along the osmotic gradient (Table II, side A) indistinguishable from that due to vasopressin. This effect was also reversible and in eight studies was not provided by

TABLE II

Effect of cyclic AMP on net water movement

Period	Side A		Side B	
	Serosal solution	Weight loss	Serosal solution	Weight loss
		mg/min		*mg/min*
1	Ringer's	0.5	Ringer's	0.7
2	Cyclic AMP (1 μmole/ml)	9.7	5'-AMP (1 μmole/ml)	0.5
3	Ringer's	0.8	Cyclic AMP (1 μmole/ml)	8.3

TABLE III

Effect of cyclic AMP on net water movement

Period	Side A Serosal solution	Side A Weight loss	Side B Serosal solution	Side B Weight loss
		mg/min		*mg/min*
1	Ringer's	0.6	Ringer's	1.0
2	Cyclic AMP (1 μmole/ml)	3.2	Cyclic AMP (10 μmoles/ml)	26.7
3	Vasopressin (200 mU/ml)	30.8	Ringer's	0.6

the addition of cyclic AMP to the mucosal surface. On the other hand, in eight other studies, equimolar 5'-AMP, the degradation product of cyclic AMP, was entirely without effect on water movement (Table II, side B). Equimolar ATP was also ineffective in a similar number of studies, indicative of the specificity of the 3',5'-AMP response.

The response to 1 μmole per ml of cyclic AMP was qualitatively the same in all studies. Net water movement uniformly increased; however, the magnitude of the response varied and was frequently less than that provided by a maximal dose of vasopressin. In 47 studies the net water movement rose to a mean value of 7.0 ± 0.64 mg per minute. In contrast, 10 μmoles per ml of cyclic AMP uniformly elicited an increase in net water movement which approximated that produced by a maximal dose of vasopressin. A representative study is illustrated in Table III. In 5 other experiments the rates of net water movement were 19.6, 21.8, 23.7, 26.4, and 34.5 mg per minute.

Since methyl xanthines are known to interfere with the degradation of cyclic AMP to its inactive 5'-AMP form (11), the response of the toad bladder to theophylline was examined. Within the context of the thesis presented in Figure 1, it was predicted that theophylline would also accelerate net water movement. That this prediction was correct is illustrated in Table IV. In this study 40 μmoles per ml of theophylline added to the serosal bathing medium elicited a response in net water movement similar to that provided by 250 mU per ml of vasopressin. Theophylline, like cyclic AMP and vasopressin, is ineffective when applied to the mucosal surface of the toad bladder. This was demonstrated in four studies. The magnitude of the response to 10 μmoles per ml of theophylline added to the serosal surface was generally greater than that due to 1 μmole per ml of cyclic AMP. The mean response to 10 μmoles per ml of theophylline in 30 experiments was 13.4 ± 0.24 mg per minute.

TABLE IV

Effect of theophylline on net water movement

Period	Side A Solution	Side A Weight loss	Side B Solution	Side B Weight loss
		mg/min		*mg/min*
1	Ringer's	0.7	Ringer's	0.7
2	Theophylline (40 μmoles/ml)	18.2	Vasopressin (250 mU/ml)	24.3
3	Ringer's	1.7	Ringer's	0.7

The effect of theophylline on water movement is additive to that of a submaximal dose of vasopressin. In the experiments illustrated in Table V the response to 500 μU per ml of vasopressin alone was first compared with that to 10 μmoles per ml of theophylline. The additive effect of the two agents is apparent in the third 30-minute period. Theophylline and cyclic AMP are also additive (Table VI) as are cyclic AMP and a submaximal dose of vasopressin (Table VII). This last was noted in 7 of 8 lobes examined.

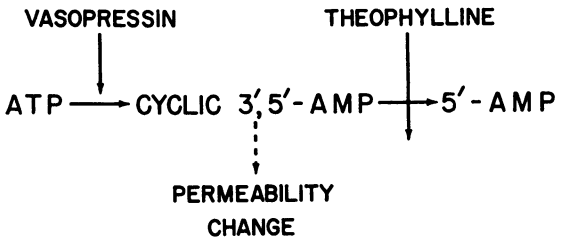

FIG. 1. SCHEMATIC REPRESENTATION OF VASOPRESSIN ACTION.

Having established that cyclic AMP and theophylline both mimic vasopressin in the toad bladder insofar as permeability to water is concerned, the effect of known inhibitors of vasopressin action on the response to cyclic AMP and theophylline was examined. N-ethylmaleimide (NEM) and certain other sulfhydryl blocking agents have been shown by Rasmussen, Schwartz, Schoessler and Hochster (14) to interfere with the action of vasopressin. Table VIII illustrates the effect of

pretreatment of the bladder with 1 μmole per ml of NEM on the subsequent response to cyclic AMP, theophylline, and vasopressin. The table is a composite of three separate studies on individual bladders and does not include the control data. NEM alone may augment water movement to a slight degree (14). The slight acceleration of water movement noted in the cyclic AMP and vasopressin studies (with NEM) was observed in the periods prior to addition of these agents and

TABLE V

Additive effect of theophylline plus vasopressin *

	Side A					Side B				
		Weight loss					Weight loss			
Period	Solution	I	II	III	IV	Solution	I	II	III	IV
		mg/min		*mg/min*			*mg/min*		*mg/min*	
1	Ringer's	0.4	0.6	0.7	1.0	Ringer's	0.5	0.6	0.6	0.8
2	Vasopressin (500 μU/ml)	14.0	10.7	15.5	12.5	Theophylline (10 μmoles/ml)	1.8	17.1	13.8	21.5
3	Vasopressin (500 μU/ml) + theophylline (10 μmoles/ml)	28.1	36.9	35.8	19.6	Theophylline (10 μmoles/ml) + vasopressin (500 μU/ml)	22.5	35.6	26.8	22.0

* Roman numerals I through IV refer to separate experiments.

TABLE VI

Additive effect of theophylline plus cyclic AMP *

	Side A					Side B				
		Weight loss					Weight loss			
Period	Solution	I	II	III	IV	Solution	I	II	III	IV
		mg/min		*mg/min*			*mg/min*		*mg/min*	
1	Ringer's	0.5	0.6	1.3		Ringer's	0.5	0.6	0.3	1.0
2	Theophylline (10 μmoles/ml)	12.4	9.0	1.7		Cyclic AMP (1 μmole/ml)	1.3	11.5	18.3	4.2
3	Theophylline (10 μmoles/ml) + cyclic AMP (1 μmole/ml)	32.2	19.2	19.9		Cyclic AMP (1 μmole/ml) + theophylline (10 μmoles/ml)	20.9	33.7	28.4	21.6

* Roman numerals I through IV refer to separate experiments.

TABLE VII

Additive effect of vasopressin plus cyclic AMP *

	Side A					Side B				
		Weight loss					Weight loss			
Period	Solution	I	II	III	IV	Solution	I	II	III	IV
		mg/min		*mg/min*			*mg/min*		*mg/min*	
1	Ringer's	1.0	0.9	0.6	0.8	Ringer's	1.0	0.9	0.7	0.9
2	Vasopressin (500 μU/ml)	3.0	2.4	12.5	16.1	Cyclic AMP (1 μmole/ml)	9.5	13.4	4.2	8.3
3	Vasopressin (500 μU/ml) + cyclic AMP (1 μmole/ml)	12.6	24.0	18.5	3.9	Cyclic AMP (1 μmole/ml) + vasopressin (500 μU/ml)	17.4	23.0	17.1	26.6

* Roman numerals I through IV refer to separate experiments.

TABLE VIII

Effect of pretreatment of bladder with N-ethylmaleimide

With NEM		Without NEM	
Solution	Weight loss	Solution	Weight loss
	mg/min		*mg/min*
Cyclic AMP (1 μmole/ml)	2.5	Cyclic AMP (1 μmole/ml)	7.8
Theophylline (10 μmoles/ml)	0.5	Theophylline (10 μmoles/ml)	27.0
Vasopressin (5 mU/ml)	1.2	Vasopressin (5 mU/ml)	22.8

is ascribable to the NEM alone. It is apparent that NEM prevents the increase in net water movement otherwise produced by cyclic AMP, theophylline, and vasopressin. This was observed in a total of four studies. Similar results have been obtained using *p*-chlormercuriphenylsulfonate, another sulfhydryl blocking agent.

Acidification of the bladder bathing solution has been shown to inhibit the action of vasopressin (5, 14, 15). As can be seen in Table IX, reduction of the pH of the bathing medium from 7.6 to 6.5 diminishes the response to theophylline. The mean response to theophylline in an acid medium in eight studies was 1.6 ± 0.6 mg per minute. In contrast, the response of the tissue to cyclic AMP is not appreciably altered (mean 5.7 ± 0.6 mg per minute).

The effect of cyclic AMP, theophylline, and vasopressin on sodium transport. As indicated earlier, in addition to an effect on the permeability of the membrane to water, vasopressin also increases the short circuit current (sodium transport) across the toad bladder (6, 16). The current rise in potential difference which is observed is a reflection of the increase in sodium transport. It is significant that an increase in short circuit current and potential difference was also produced by both cyclic AMP and theophylline in the present studies. The peak changes in short circuit current in 23 studies after the addition of cyclic AMP, 5′-AMP, theophylline, and vasopressin are summarized in Table X. With the exception of three of the four studies in which 5′-AMP was added, all of the agents increased short circuit current. The response to cyclic AMP was generally not as sustained as that due to large doses of vasopressin. This is illustrated in Figure 2 in which a continuous recording of short circuit current after addition of 13 μmoles per ml of cyclic AMP is compared with that after addi-

TABLE IX

Effect of theophylline, cyclic AMP, and pH

	Side A		Side B	
Period	Solution	Weight loss	Solution	Weight loss
		mg/min		*mg/min*
1	Ringer's (pH 7.6)	0.9	Ringer's (pH 7.6)	0.5
2	Ringer's (pH 7.6)	1.2	Ringer's (pH 6.5)	0.8
3	Theophylline (10 μmoles/ml)	11.1	Theophylline (10 μmoles/ml)	1.7
4	Ringer's (pH 7.6)	1.1	Ringer's (pH 6.5)	1.3
5	Cyclic AMP (1 μmole/ml)	10.2	Cyclic AMP (1 μmole/ml)	8.6

TABLE X

Effect of cyclic AMP, 5′-AMP, theophylline, and vasopressin on short circuit current

Agent	Short circuit current: Control	Short circuit current: Experimental*	Duration†
	μa/cm²		*min*
Cyclic AMP:			
13 μmoles/ml	25	60	10
8 μmoles/ml	13	28	16
3 μmoles/ml	35	48	8
	43	57	8
1 μmole/ml	15	32	30
	12	16	4
	10	18	7
	12	23	14
	9	18	18
5′-AMP,	8	7	
1 μmole/ml	12	13	
	18	25	5
	12	13	
Theophylline,	13	22	>30‡
10 *μmoles/ml*	11	26	> 8‡
	14	28	>15‡
	9	15	20
	12	22	>30‡
Vasopressin,	11	22	>30‡
250 *μU/ml*	8	14	14
	5	14	10
	5	13	40
	11	22	10

* Each datum represents the peak current reached. The peak response was attained 5 to 10 minutes after addition of the agent.

† The duration of the response was arbitrarily measured as the length of time the current was maintained at 75% of the peak increase over the control period.

‡ The experiment was terminated before an appreciable fall in current had occurred.

tion of 10 mU per ml of vasopressin. Simultaneous measurements of short circuit current on a contiguous although isolated half of the bladder after addition of 5'-AMP (13 μmoles per ml) and vasopressin are also noted. The difference in response between cyclic AMP and 5'-AMP is striking. The response of the toad bladder to 10 μmoles per ml of theophylline is illustrated in Figure 3. It is noteworthy that theophylline stimulates sodium transport as do cyclic AMP and vasopressin. Ussing has reported a similar effect on frog skin with theophylline (17). Simultaneous measurements of potential difference are also noted in this figure. As would be expected, potential difference also increased after addition of cyclic AMP in other studies.

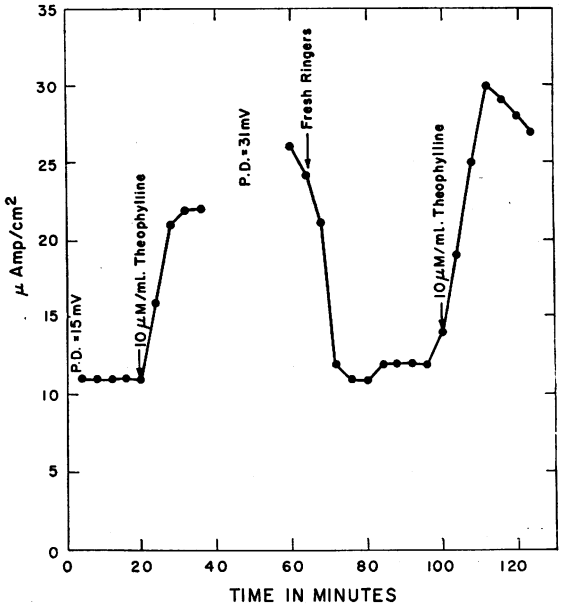

FIG. 3. EFFECT OF THEOPHYLLINE ON SHORT CIRCUIT CURRENT AND POTENTIAL. Short circuit current was not measured during the 20-minute period beginning 40 minutes after the start of the experiment. The potential difference in this interval was 31 mv.

DISCUSSION

The striking similarity of the effects of cyclic AMP, theophylline, and vasopressin on the toad bladder is consistent with the view that vasopressin induces its effect on permeability by stimulating the production and accumulation of cyclic AMP in this tissue (Figure 1). It is pertinent that vasopressin has been shown to increase the activity of toad bladder glycogen phosphorylase in preliminary studies (18). Since the amount of active phosphorylase is increased by cyclic AMP in liver (7), muscle (19), and adrenal cortex (20), this may be viewed as presumptive evidence favoring the present hypothesis. On the other hand, it has not been possible thus far to demonstrate a vasopressin-induced increase in cyclic AMP production in bladder tissue.

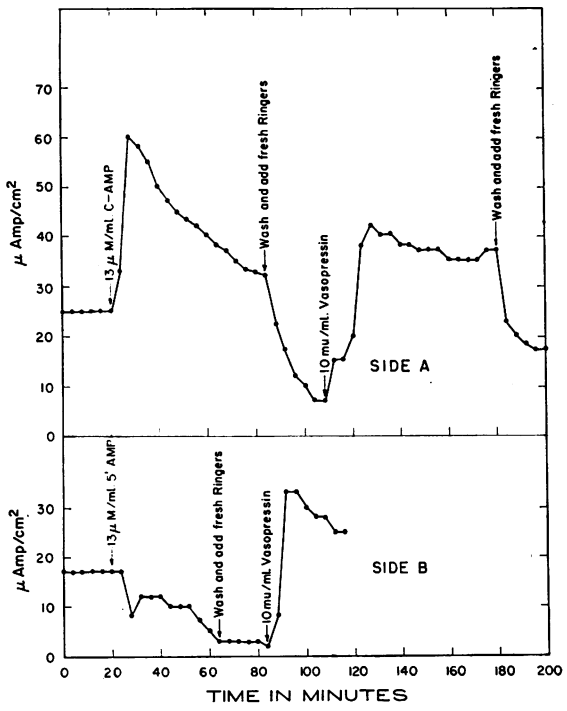

FIG. 2. EFFECT OF CYCLIC AMP, 5'-AMP, AND VASOPRESSIN ON SHORT CIRCUIT CURRENT.

The precise role of cyclic AMP in the permeability process is unknown. It is known to influence a variety of enzymes, including phosphorylase, in other tissues (7), but how these enzymes and the reactions they catalyze may affect permeability is unclear. In view of the multiplicity of effects ascribed to cyclic AMP thus far (7), it is probable that it influences many reactions and that its known catalytic effects are not those involved in the induction of the permeability changes. With respect to renal tissue, it is significant that many of the enzymes known to be involved in the cyclic AMP system in other tissues are present in the kidney as well; thus the cyclyzing enzyme responsible for the conversion of ATP to cyclic

AMP (7), the diesterase involved in the degradation of cyclic AMP to 5′-AMP (21), the entire phosphorylase system (7, 18), all have been demonstrated in renal tissue. It seems reasonable to conclude that vasopressin may exert its effect in the kidney in a manner analogous to that proposed for the toad bladder.

It is difficult to reconcile the present results with the current thesis of vasopressin action formulated by Fong and Schwartz and their co-workers (22, 23). These authors have demonstrated that vasopressin binds to kidney and bladder tissue and have suggested that this is accomplished by a linkage involving the disulfide (S–S) bridge of vasopressin and the free sulfhydryl (SH) groups on the membrane. The increase in permeability is thought to be effected by a subsequent series of disulfide-sulfhydryl (SS–SH) exchange reactions which reorient the membrane structure, opening aqueous channels in the process. In support of their thesis are the observations of Rasmussen and associates (14) that NEM, a sulfhydryl inhibitor, as well as acidification, which limits the dissociation of SH groups, interfere with both binding and action of the hormone. Both of these manipulations may do so for other reasons. NEM, as well as *p*-chlormercuriphenylsulfonate, as noted earlier, also abolish the response to cyclic AMP and theophylline. Neither of the latter compounds possesses the S–S bridge essential for the covalent linkage postulated by the authors. Furthermore, NEM alone exerts a deleterious effect on the toad bladder. It increases permeability in an irreversible fashion when applied to either surface of the bladder (14) and lowers the oxygen consumption of the tissue (14). Leaf (24) has reported that short circuit current is markedly reduced when the bladder is exposed to NEM and this has been confirmed in our laboratory (18). In view of these considerations, it is likely that the NEM effect is nonspecific and the results of the studies may not bear directly on the elucidation of the mechanism of action of the hormone.

The response to acidification is more complicated. As noted above, a reduction in the pH of the bathing solution to 6.5 prevents the permeability changes induced by both theophylline and vasopressin, but does not limit that due to cyclic AMP. The results are consistent with the *in vitro* observation that acidification interferes with the enzymatic conversion of ATP to cyclic AMP (25). Within the context of the present hypothesis (Figure 1) vasopressin would be unable to exert its permeability effect in an acid medium, nor would theophylline, whereas exogenous cyclic AMP would still be capable of evoking its usual response. It should be noted that these considerations do not account for the prevention of the binding of vasopressin to renal and bladder tissue produced by acidification. Thus, despite these objections to the SS–SH interchange hypothesis, it is probable that attachment of vasopressin to its receptor tissue, in the manner suggested by the authors, may constitute the first step in the chain of reactions ultimately leading to an increase in permeability.

SUMMARY

The effect of adenosine-3′,5′-phosphate, theophylline, and vasopressin on the permeability to water and the short circuit current (sodium transport) of toad bladder has been investigated. The addition of each of these substances to the serosal surface of the isolated toad bladder sac uniformly increases the osmotic flow of water across the membrane. The increase in permeability provided by cyclic AMP and theophylline resembles in all respects that due to vasopressin. The change in permeability is reversible, is not produced by the addition of the test agent to the mucosal surface of the bladder, and is associated with a rise in both short circuit current and potential difference across the membrane. N-ethylmaleimide, a sulfhydryl inhibitor, prevents the effect of cyclic AMP, theophylline, and vasopressin. Acidification of the bathing solutions, on the other hand, interferes with the action of vasopressin and theophylline, but not with that of cyclic AMP.

In view of the known role of cyclic AMP in the action of other hormones, the marked similarity of effects of cyclic AMP, theophylline, and vasopressin in toad bladder is consistent with the view that vasopressin exerts its effect in toad bladder, and by analogy in kidney, by stimulating the production and accumulation of cyclic AMP in the receptor tissue. It is not known what physical and biochemical events are initiated by cyclic AMP which ultimately result in an alteration in membrane structure that permits the accelerated flow of water along an osmotic gradient.

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