BBA 75734

ON THE SIMILARITY OF EFFECTS OF ALDOSTERONE AND ADENOSINE 3′,5′-PHOSPHATE ON Na^+ TRANSPORT AND GLUCOSE METABOLISM IN TOAD BLADDER

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(Received May 4th, 1971)

SUMMARY

The stimulation of Na^+ transport in the toad bladder by aldosterone is associated with an increased utilization of oxygen and an increase in the evolution of $^{14}CO_2$ from [6-^{14}C]glucose in response to the increased energy requirement of stimulated Na^+ transport. These are secondary effects of aldosterone and are abolished by the removal of Na^+ from the mucosal bathing medium and consequent absence of Na^+ transport. At the same time, however, aldosterone decreases the evolution of $^{14}CO_2$ from [1-^{14}C]glucose. This effect persists even in the absence of Na^+ and has similar characteristics of time of onset, concentration response, steroid specificity, and sensitivity to actinomycin D and spirolactone.

Both the effect of aldosterone to decrease the evolution of $^{14}CO_2$ from [1-^{14}C]-glucose and its ability to stimulate Na^+ transport can be reproduced by adenosine 3′,5′-phosphate (cyclic AMP). This has been shown for endogenous cyclic AMP produced by the action of theophylline or vasopressin in combination with theophylline and for exogenous cyclic AMP as seen with N^6-2′-*O*-dibutyryl adenosine 3′,5′-phosphate (dibutyryl cyclic AMP) or with cyclic AMP in the presence of low concentrations of theophylline.

Although these results suggest that cyclic AMP might mediate the action of aldosterone, no change in tissue concentration of cyclic AMP was found even after prolonged incubation with the hormone.

INTRODUCTION

Aldosterone stimulates Na^+ transport in the toad bladder and, secondarily, oxidative metabolism[1,2]. During a study of glucose metabolism it was noted that the evolution of $^{14}CO_2$ from [6-^{14}C]glucose was increased as a consequence of the stimulation of Na^+ transport by aldosterone. Simultaneously, however, an effect of this hormone to decrease the $^{14}CO_2$ from [1-^{14}C]glucose was detected[3]. This effect, unlike

Abbreviations: cyclic AMP, adenosine 3′,5′-phosphate; dibutyryl cyclic AMP, N^6-2′-*O*-dibutyryl adenosine 3′,5′-phosphate.

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the effect on [6-^{14}C]glucose, was persistent even in the absence of Na^+ in the mucosal bathing medium and hence in the absence of Na^+ transport. As adenosine 3', 5'-phosphate (cyclic AMP) can stimulate sodium transport in the toad bladder[4] and has also been shown to decrease the evolution of $^{14}CO_2$ from [1-^{14}C]glucose in adipose tissue[5,6], the effect of cyclic AMP on glucose metabolism in toad bladder has been investigated. Further studies were performed on theophylline, because of its ability to inhibit phosphodiesterase, and on antidiuretic hormone, which is believed to act *via* the intermediacy of cyclic AMP [4,7,8].The results obtained gave rise to the possibility that cyclic AMP might mediate the stimulation of sodium transport by aldosterone. Consequently the concentration of cyclic AMP in the toad bladder and its transporting cells was measured in the presence and absence of aldosterone.

METHODS

Toads* used in these studies were kept partially immersed in 0.6 % saline solution for at least 2 days prior to use to decrease the endogenous secretion of aldosterone.

*To determine the effects of adenosine 3',5'-phosphate (cyclic AMP), N^6-2'-O-dibutyryl adenosine 3',5'-phosphate (dibutyryl cyclic AMP)[9], theophylline, and vasopressin** on $^{14}CO_2$ release from [1-^{14}C]- and [6-^{14}C]glucose****

Paired portions of bladder were incubated in Ringer's solution (NaCl, 113.5 mM; KCl, 2.5 mM; $NaHCO_3$, 2.4 mM; and $CaCl_2$, 0.89 mM; total solute concentration, 200 mosM/kg; pH in air 7.8) at room temperature for 6 h. Penicillin G and streptomycin (0.1 mg/ml each) were added to all incubation media. The measurements of glucose utilization were started by dropping the paired tissues into flasks containing fresh Ringer's solution, 5.5 mM glucose and either [1-^{14}C]glucose or [6-^{14}C]glucose. Where appropriate, 3 mM cyclic AMP, 3 mM dibutyryl cyclic AMP, 11 mM theophylline, or 100 munits/ml of vasopression was added. The flasks were sealed and incubated in a rotatory metabolic shaker at room temperature for 30 or 60 min. At the end of this time 0.1 ml of 1 M H_2SO_4 was injected into the medium of each flask and the flask shaken for an additional 30 min. The CO_2 liberated was trapped in a center well with 25 % KOH on a filter paper wick. The filter paper wick and the contents of the center well were quantitatively transferred, with 3.0 ml of methanol, to vials for liquid scintillation counting and 10 ml of a toluene scintillation mixture added. Samples of the medium were also taken for radioactive counting. The tissues were removed, blotted lightly, and transferred to tared weighing tubes, and dry weights obtained. Glucose utilization was calculated as μmoles/g dry weight per h. Similar incubations but without tissue were performed simultaneously in order to obtain blanks for the radioactivity.

In a further series of experiments, the effects of cyclic AMP and vasopressin in the presence of low concentrations of theophylline were determined. In these experiments theophylline was present in the Ringer's solution in all flasks, *i.e.* test and control flasks, and cyclic AMP or vasopressin was added to the test flasks only.

* Obtained from National Reagents, Bridgeport, Conn.

** Pitressin obtained from Parke-Davis and Company, Detroit, Mich.

*** [1-^{14}C]Glucose (4–10 mC/mmole) and [6-^{14}C]glucose (4–7 mC/mmole) were obtained from New England Nuclear Corp., Boston, Mass. Purity was greater than 99% and was checked by thin-layer silica-gel chromatography in an ethyl acetate–isopropanol–water solvent system (130:75:30, by vol.) followed by radioautography.

To determine the effect of aldosterone on the tissue content of cyclic AMP

By the method of PAUK AND REDDY[10]

Whole tissue. Paired bladders were obtained from 3–5 toads which were exsanguinated after rapid pithing. One half of the paired tissues was incubated with $5 \cdot 10^{-7}$ M aldosterone for 5 h. The other half was similarly incubated in Ringer's solution *plus* methanol diluent. Tissues were then transferred to flasks containing the same medium *plus* 5.5 mM glucose and incubated for an additional h. At 6 h, both aldosterone-treated and control tissues were dropped into beakers containing 4 ml of ice cold 10% trichloroacetic acid containing tracer amounts of cyclic [8-^{14}C] AMP. The contents of the beakers were quantitatively transferred to Potter–Elvehjem homogenizers and homogenized with teflon pestles. The homogenate was centrifuged at 3° at approx. 6000 × *g*. The precipitates were washed twice with distilled water, dried at 110°, and weighed.

The supernatant was extracted once with 2 vol. of ether, neutralized with 1 M KOH, and filtered with suction through Reeve Angel No. 984 H ultrafine glass fiber filters. The filtrate was analyzed for cyclic AMP according to the method of PAUK AND REDDY[10] with the following modification. After initial ion exchange chromatography of the neutralized trichloroacetic acid extract, the appropriate fraction was evaporated from a 50-ml round bottom flask at 45° or less. The residue was dissolved in 200 µl methanol and streaked onto Whatman 3 MM filter paper strips for further purification by paper electrophoresis in 0.1 M sodium borate. The flask was rinsed twice with 100 µl methanol. 700 V were applied for 1.5 h. The appropriate section of paper was eluted with water. The sample was then filtered with glass fiber filters and applied to a 1 cm × 5 cm Dowex 1 X8 formate form column. The column was eluted with 35 ml water and then with 1 M formic acid. 20 ml of the formic acid eluent were collected after discarding the first 2 ml. This fraction was evaporated and the residue applied to paper for final purification by descending paper chromatography.

Toad bladder mucosa. Bladders were incubated as described earlier. The mucosa was scraped off with the aid of glass slides. The mucosa was then dropped into 4 ml ice-cold 10% trichloroacetic acid containing tracer amounts of cyclic [8-^{14}C]AMP as already described.

By the method of BROOKER *et al.*[11]

Toad bladder mucosa. Toads were exsanguinated and paired hemibladders incubated for 6–7 h in Ringer's solution containing 5.5 mM glucose, and either $5 \cdot 10^{-7}$ M aldosterone in methanol or methanol alone. The mucosa was scraped off and dropped into ice-cold trichloroacetic acid containing a known amount of cyclic [^{3}H]AMP to monitor recovery. The cells were disrupted by sonication. Samples were centrifuged and the clear supernatant was extracted with ether to remove the trichloroacetic acid. The supernatant was then subjected to one $BaSO_4$ precipitation according to the method of KRISHNA *et al.*[12]. After centrifugation, the supernatant was applied to an AG50W X8 hydrogen form column and eluted with water. The appropriate fraction containing the cyclic AMP was collected in 2.5 ml and evaporated to dryness. Samples were then analyzed according to the method of BROOKER *et al.*[11]. A total of 16000 counts/min cyclic [^{3}H]AMP (Schwarz Bioresearch), specific activity 15.7 C/mmole, was added per test.

RESULTS

Effects of cyclic AMP, dibutyryl cyclic AMP and theophylline on glucose metabolism

In the interpretation of these experiments, $^{14}CO_2$ evolution from [6-^{14}C]glucose reflects the utilization of glucose *via* the Embden–Meyerhof pathway. $^{14}CO_2$ evolution from [1-^{14}]glucose reflects the utilization of glucose from the Embden–Meyerhof pathway *plus* alternate pathways, the principal one of which is the hexose monophosphate shunt pathway. As the C-1 and C-6 atoms of glucose are treated similarly *via* the Embden–Meyerhof pathway, the amount of $^{14}CO_2$ from [6-^{14}C]glucose can be subtracted from that of [1-^{14}C]glucose to represent $^{14}CO_2$ from the alternate pathways. This may be ascribed in large part to the hexose monophosphate shunt pathway. Documentation of inhibition by aldosterone by the methods of WOOD *et al.*[13] has been presented in the previous paper[16].

From the results in Table I, it can be seen that theophylline and dibutyryl cyclic AMP, but not vasopressin or cyclic AMP at the concentrations used, caused a significant reduction in the amount of $^{14}CO_2$ evolved from [1-^{14}C]glucose and in the [1-^{14}C]–[6-^{14}C] value. Theophylline also caused a decrease in $^{14}CO_2$ from [6-^{14}C]glucose, a finding in accord with the report of PARISI and BENTLEY[14] and ZOR *et al.*[15]. The inhibition of $^{14}CO_2$ from [1-^{14}C]glucose by dibutyryl cyclic AMP and theophylline

TABLE I

EFFECTS OF THEOPHYLLINE (11 mM), DIBUTYRYL CYCLIC AMP (3 mM), CYCLIC AMP (3 mM), AND VASOPRESSIN (100 munits/ml) ON THE METABOLISM OF [1-^{14}C]- AND [6-^{14}C]GLUCOSE IN TOAD BLADDER

Results are expressed as μmoles of glucose utilized per g dry weight of tissue per h.

	Control	*Theophylline*	$\Delta \pm$ S.E.	*n*	*P*
C-1	3.39	2.64	-0.75 ± 0.27	17	<0.02
C-6	0.64	0.55	-0.09 ± 0.05	17	<0.2
C-1 – C-6	2.75	2.09	-0.66 ± 0.24	17	<0.02
	Control	*Dibutyryl cyclic AMP*	$\Delta \pm$ S.E.	*n*	*P*
C-1	4.67	3.91	-0.76 ± 0.22	14	<0.01
C-6	1.20	1.19	-0.01 ± 0.10	14	0.7
C-1 – C-6	3.47	2.72	-0.75 ± 0.23	14	<0.01
	Control	*Cyclic AMP*	$\Delta \pm$ S.E.	*n*	*P*
C-1	1.67	1.78	$+0.11 \pm 0.15$	8	0.5
C-6	0.29	0.41	$+0.12 \pm 0.02$	8	<0.001
C-1 – C-6	1.40	1.38	-0.02 ± 0.14	8	0.9
	Control	*Vasopressin*	$\Delta \pm$ S.E.	*n*	*P*
C-1	2.44	2.66	$+0.22 \pm 0.25$	7	0.5
C-6	0.50	0.66	$+0.16 \pm 0.09$	7	<0.2
C-1 – C-6	1.94	2.00	$+0.06 \pm 0.30$	7	0.8

but not by cyclic AMP or vasopressin raised the following considerations. If the action of theophylline is to raise the intracellular concentrations of cyclic AMP by inhibition of phosphodiesterase activity, then it is possible that the failure of exogenous cyclic AMP and vasopressin, which also increases the tissue level of cyclic AMP[7], to decrease the $^{14}CO_2$ from [1-^{14}C]glucose might be due to rapid breakdown of the cyclic AMP by phosphodiesterase. Because of this possibility, the effects of cyclic AMP and vasopressin in the presence of low concentrations of theophylline were tested. The results of these experiments are shown in Figs. 1 and 2.

It is clear that under these conditions both antidiuretic hormone and cyclic AMP cause a significant reduction in the amount of $^{14}CO_2$ evolved from [1-^{14}C]glucose by pathways other than the Embden–Meyerhof pathway. As cyclic AMP also stimulates sodium transport in the toad bladder[11] the possibility exists that cyclic AMP could be the common mediator of the stimulation of Na^+ transport by aldosterone and the effect of the hormone on glucose metabolism. For this reason the effect of aldosterone upon tissue cyclic AMP concentrations was determined.

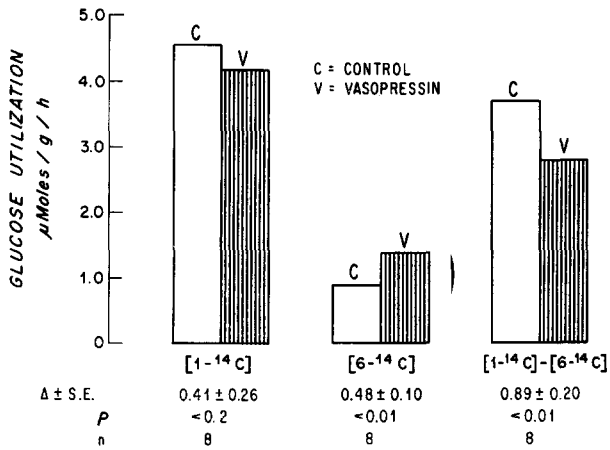

Fig. 1. Effect of vasopressin (100 munits/ml) on the utilization of [1-^{14}C]- and [6-^{14}C]glucose by toad bladder in the presence of 5.5 mM theophylline.

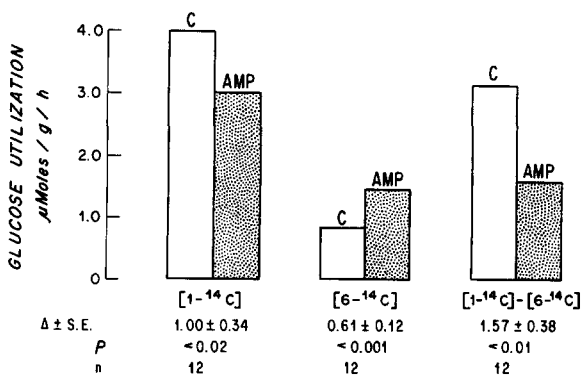

Fig. 2. Effects of cyclic AMP (3 mM) on the utilization of [1-^{14}C]- and [6-^{14}C]glucose by toad bladder in the presence of 2 mM theophylline.

The concentration of cyclic AMP in toad bladder

In order to check the methods used and for comparison with data previously reported for this tissue, bladders were paired and one group treated with 100 munits/ml vasopressin. Cyclic AMP in the control tissue was estimated at $1.4 \cdot 10^{-9}$ g/mg dry weight of trichloroacetic acid precipitate. This value is similar to that reported by HANDLER *et al.*[7]. Vasopressin significantly increased this value by 40% at 6 min after administration to $1.9.10^{-9}$ g/mg dry weight of trichloroacetic acid precipitate.

In contrast to vasopressin, aldosterone failed to affect the tissue concentration of cyclic AMP. After incubation for 6 h with $5 \cdot 10^{-7}$M (+)-aldosterone no change in cyclic AMP content was found by either the method of PAUK and REDDY[10] (Table II) or by the method of BROOKER *et al.*[11] (Table III).

TABLE II

LACK OF EFFECT OF ALDOSTERONE ($5 \cdot 10^{-7}$ M) ON THE CONCENTRATION OF CYCLIC AMP IN TOAD BLADDER AND IN MUCOSAL CELLS OF THE TOAD BLADDER

	Cyclic AMP (10^{-9} g/mg dry wt. trichloroacetic acid ppt.)				
	Control	*Aldosterone*	*Δ ± S.E.*	*n*	*P*
Whole tissue	1.5	1.4	-0.12 ± 0.15	6	0.5
Scraped mucosal cells	11.8	11.1	-0.63 ± 1.64	6	0.8

TABLE III

LACK OF EFFORT OF ALDOSTERONE ($5 \cdot 10^{-7}$ M) ON THE CONCENTRATION OF CYCLIC AMP IN MUCOSAL CELLS OF THE TOAD BLADDER

Protein was determined according to the method of LOWRY *et al.*[17].

Cyclic AMP (10^{-12} moles/mg protein)				
Control	*Aldosterone*	*Δ ± S.E.*	*n*	*P*
102.4	100.7	-1.7 ± 13.2	8	>0.9

DISCUSSION

It has been shown previously[3] that aldosterone has a marked effect upon the metabolism of glucose in the toad bladder. The stimulation of Na^+ transport by aldosterone in this tissue is accompanied by an increase in the evolution of $^{14}CO_2$ from [6-^{14}C]glucose and by a decrease in the evolution of $^{14}CO_2$ from [1-^{14}C]glucose. The increased $^{14}CO_2$ from [6-^{14}C]glucose is secondary to the increased rate of Na^+ transport, whereas the reduction of $^{14}CO_2$ from [1-^{14}C]glucose is not secondary to the Na^+ transport. The reduction of $^{14}CO_2$ from [1-^{14}C]glucose is most simply expressed as the $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]glucose in order to account for the amount of $^{14}CO_2$ evolution from [1-^{14}C]glucose *via* the Embden–Meyerhof pathway. The inhibition of $^{14}CO_2$ evolution, therefore, does not take place in the Embden–Meyerhof pathway but in some other pathway of glucose metabolism, the most likely of which is the hexose monophosphate shunt pathway[3].

The primary nature of the action of aldosterone on glucose metabolism led us to perform several series of experiments in order to assess the relationship between this metabolic effect of the hormone and the stimulation of Na^+ transport[3]. It was found that the two effects had a similar time course and concentration dependence, and were inhibited by actinomycin D. Furthermore, dexamethasone and deoxycorticosterone which, like aldosterone, stimulate sodium transport in toad bladder, both decreased $^{14}CO_2$ from [1-^{14}C]glucose whereas progesterone and cortisone which do not affect Na^+ transport did not affect glucose metabolism[3,16]. Spirolactones have been shown to inhibit the actions of aldosterone on both Na^+ transport and on glucose metabolism[16].

One conclusion of these studies was that a common precursor, produced by aldosterone in the tissue, could be responsible for both the stimulation of Na^+ transport and the inhibition of $^{14}CO_2$ evolution from [1-^{14}C]glucose. As cyclic AMP is known to inhibit the hexose monophosphate shunt pathway in adipose tissue[5,6] and is capable of stimulating Na^+ transport in the toad bladder[7], this compound could be an intermediate in the stimulation of Na^+ transport by aldosterone. In the first series of experiments performed here, the effects of theophylline, cyclic AMP, and vasopressin were examined. It was found that theophylline and dibutyryl cyclic AMP, like aldosterone, decreased the amount of $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]-glucose. On the other hand, cyclic AMP and vasopressin had no such effect. The failure of cyclic AMP and vasopressin to decrease the $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]-glucose when theophylline and dibutyryl cyclic AMP both caused a decrease, required an explanation. Theophylline has been shown to increase the concentration of cyclic AMP in the toad bladder[7], presumably by inhibition of phosphodiesterase. Dibutyryl cyclic AMP is relatively resistant to phosphodiesterase and crosses cell membranes more readily than cyclic AMP[9]. Because of its slow rate penetration and rapid breakdown by phosphodiesterase, cyclic AMP may not achieve an intracellular concentration sufficiently high to affect glucose metabolism. Similarly, vasopressin may not raise the intracellular cyclic AMP levels sufficiently, or perhaps, the cyclic AMP released might be compartmentalized or, again, rapidly broken down. The second series of experiments, shown in Figs. 1 and 2, was performed to examine this point. Experiments were carried out in the presence of low concentrations of theophylline. These concentrations were gauged such that phosphodiesterase activity would be partially inhibited, and although $^{14}CO_2$ from [1-^{14}C]- and [6-^{14}C]glucose would be decreased, this decrease would be slight. The ability of endogenous cyclic AMP, as released by vasopressin, and exogenous cyclic AMP to further reduce the $^{14}CO_2$ from [1-^{14}C]- and [6-^{14}C]glucose was now tested. It is clear that under these conditions both cyclic AMP and vasopressin can reduce the $^{14}CO_2$ evolution and in this respect mimic the action of aldosterone. Therefore, cyclic AMP, whether endogenous, as a consequence of the actions of theophylline and vasopressin, or exogenous, as seen with the dibutyryl derivative or with theophylline and cyclic AMP, can mimic the action of aldosterone to stimulate Na^+ transport and to decrease the $^{14}CO_2$ from [1-^{14}C] — [6-^{14}C]glucose.

It is possible that cyclic AMP is the common mediator of these two effects. However, when this possibility was tested by measuring the concentration of cyclic AMP in the tissue, we were unable to demonstrate any effect of aldosterone on cyclic AMP levels either in the whole tissue or the scraped mucosal cells. Consequently, we cannot confirm the idea that aldosterone acts to stimulate Na^+ transport by

raising the intracellular cyclic AMP concentration. Furthermore, in view of the differences in Na^+ transport when stimulated by aldosterone rather than by cyclic AMP or vasopressin, the greater magnitude of inhibition of the hexose monophosphate shunt pathway by aldosterone than by cyclic AMP and the lack of direct effect of aldosterone on water permeability in contrast to cyclic AMP, it seems unlikely that cyclic AMP mediates the effects of aldosterone.

ACKNOWLEDGMENTS

The authors are grateful to Dr. W. J. Reddy and Dr. G. Brooker for their valuable advice concerning the determination of cyclic AMP, and to Dr. M. M. Pechet for generous supplies of (+)-aldosterone.

This work was supported in part by grants from the John A. Hartford Foundation, Inc., by the Public Health Service research grants HE-06664 from the National Heart Institute and AM-04501 from the National Institute of Arthritis and Metabolic Diseases, and by the Massachusetts Kidney Foundation. Performed by M.A.K. partly during the tenure of a postdoctoral fellowship of the National Institute of Arthritis and Metabolic Diseases AM-33916.

G.W.G.S. was supported by a Public Health Service Research Career Development Award of the National Institute of Arthritis and Metabolic Diseases, 1 K04 AM-42376.

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