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EVIDENCE FOR AN APICAL MEMBRANE EFFECT IN THE REGULATION OF THE HEXOSE MONOPHOSPHATE SHUNT PATHWAY IN TOAD BLADDER STUDIES WITH AMILORIDE

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SUMMARY

Aldosterone stimulates Na^+ transport in toad bladder and, simultaneously with a coincident dose-response relationship, inhibits the hexose monophosphate shunt pathway. Amiloride, an acylguanidine diuretic, inhibits sodium transport when applied to the apical surface of the bladder. In this study, amiloride was found to partially reverse the inhibitory effect of aldosterone on the hexose monophosphate shunt pathway. The amiloride effect upon glucose metabolism was detected when it was applied to both surfaces of the bladder simultaneously, in flask experiments, and when it was applied to the apical surface. No effect of amiloride on the shunt pathway was detected when it was applied to the serosal surface only, even at very high concentrations. It may be, but has not been proven, that the effects of aldosterone and amiloride on the hexose monophosphate shunt pathway are mediated by a common site at the apical membrane.

INTRODUCTION

The stimulation of Na^+ transport by aldosterone in toad bladder is characterized by a latent period of approximately 1 h and a progressive increase in transport to peak rates at 3–5 h [1–3]. Oxygen consumption and substrate utilization increase as the increased Na^+ transport demands more energy [4–8]. Thus $^{14}CO_2$ evolution from [6-^{14}C]glucose increases as Na^+ transport increases, whereas in the absence of Na^+ for transport, as for example when choline Ringer's solution is used instead of Na^+ Ringer's on the apical surface, aldosterone has no effect upon $^{14}CO_2$ evolution from [6-^{14}C]glucose [8]. Aldosterone also has an effect upon the hexose monophosphate shunt pathway [7,9]. This is an inhibition of the shunt pathway that occurs with the same time course as the stimulation of Na^+ transport and exhibits the same steroid specificity and concentration dependence; it is inhibited by actinomycin D

and occurs even in the absence of Na^+ [7]. The inhibition of the hexose monophosphate shunt pathway has been followed by a study of $^{14}CO_2$ evolution from [1-^{14}C]-glucose and [6-^{14}C]glucose and validated by ^{14}C-labeling of lactate from [1-^{14}C]-glucose and [6-^{14}C]glucose [10]. In the interpretation of $^{14}CO_2$ evolution studies, we have assumed that $^{14}CO_2$, derived from [6-^{14}C]glucose, indicates the activity of the Embden–Meyerhof pathway while that from [1-^{14}C]glucose is derived from the Embden–Meyerhof pathway and alternate pathways, the principal one being the hexose monophosphate shunt pathway. As the C-1 and C-6 atoms of glucose are metabolized similarly by the Embden–Meyerhof pathway the $^{14}CO_2$ from [6-^{14}C]glucose can be subtracted from that derived from [1-^{14}C]glucose to obtain a representation of the hexose monophosphate shunt pathway. The assumptions underlying this technique have been discussed in a previous paper [11].

In an attempt to explore further the relationship between aldosterone, the stimulation of Na^+ transport and activity of the hexose monophosphate shunt pathway we have studied the effect of amiloride, an acylguanidine derivative, which affects Na^+ transport by an effect at the apical membrane. Amiloride is inhibitory when applied at low concentrations to the apical membrane and acts by preventing Na^+ entry into the transporting epithelial cells [12, 13]. It has little effect when applied to the basal (serosal) surface of the cells.

With the possibility of an important connection between the apical membrane, the hexose monophosphate shunt pathway and the action of aldosterone we have examined the effect of amiloride on both Na^+ transport and glucose metabolism. Also, by a modification of the technique used previously we have investigated the effects of amiloride on glucose metabolism when the amiloride was applied to either the apical (mucosal) or basal (serosal) sides of the epithelial cells of toad bladder.

METHODS

All experiments were performed on bladders of the toad, *Bufo marinus*, obtained from National Reagents, Bridgeport, Conn.

Measurement of Na^+ transport

Na^+ transport across the toad bladder was measured as the short circuit current in double chambers, a technique that has been described in detail previously [14].

Urinary bladders were excised from doubly pithed female toads. The excised hemibladders were placed in continuously aerated amphibian Ringer's solution containing $5 \cdot 10^{-7}$ M aldosterone, 5.5 mM glucose, 0.1 mg/ml penicillin G and 0.05 mg/ml streptomycin for 4 to 5 h. The Ringer's solution had the composition NaCl, 113.5 mM; KCl, 2.5 mM; $NaHCO_3$, 2.4 mM; and $CaCl_2$, 0.89 mM; total solute concentration, 220 mosM/kg; pH in air, 7.8.

Half bladders were then mounted across a double chamber, one quarter serving as a test tissue and one as a control in the same bathing solution as mentioned above. When the spontaneous membrane potential is reduced to zero in the short circuited preparation, the electrical current required to maintain the potential at zero is equivalent to the Na^+ transport across the bladder. Amiloride was added

in concentrations ranging from 10^{-4} to 10^{-6} M either to the mucosal or serosal side. The effect of amiloride on Na^+ transport was determined by comparing the amount of Na^+ transported over a 1-h period with and without amiloride.

Measurement of $^{14}CO_2$ from [1-^{14}C]glucose and [6-^{14}C]glucose

(*a*) *Flask experiments.* Toads used in these studies were kept partially immersed in 0.6% saline solution for at least 48 h prior to use to decrease the endogenous secretion of aldosterone, then rapidly pithed.

Toad bladders were divided into eight and incubated in Ringer's solution at room temperature for 6 h. Penicillin G (0.1 mg/ml) and streptomycin (0.05 mg/ml) were added to all incubation media. Four pieces of bladder were incubated with $5 \cdot 10^{-7}$ M aldosterone. The remaining 4 pieces served as control tissue.

The measurement of glucose utilization was started by dropping the tissue into flasks of fresh Ringer's solution containing 5.5 mM glucose and either [1-^{14}C]-glucose or [6-^{14}C]glucose. Where appropriate, amiloride ($1 \cdot 10^{-6}$ M) was added. The flasks were sealed and incubated in a rotatory metabolic shaker at room temperature for 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 scintillation mixture [2,5-diphenyloxazole (PPO), 29 mM; 1,4-bis-2-(5-phenyloxazole 1)-benzene (POPOP) 0.35 mM; and naphthalene, 0.81 M; toluene–dioxane, 1:1, v/v] were added. Samples of the medium were also taken for measurement of radioactivity. The tissues were blotted lightly, transferred to tared weighing tubes, and dry weights obtained. Glucose utilization was expressed as μmoles per g dry weight per h. Similar incubations were performed simultaneously without tissue in order to obtain blanks for the radioactivity.

(*b*) *Experiments with serosal or mucosal addition of amiloride.* Paired half bladders were tied as bags, serosa out, on the ends of glass tubes fitted with rubber sleeves. Each glass tube was suspended by means of a wire loop attached to it. Bags were filled with Ringer's solution containing streptomycin sulfate (0.05 mg ml) and penicillin G (0.1 mg/ml). Phenol red (5 mg/40 ml) was also added so that if leaks occurred they could readily be detected. Each bag was then suspended in a beaker containing Ringer's solution, antibiotics, and $5 \cdot 10^{-7}$ M aldosterone for 5–6 h at room temperature. Bladders were kept oxygenated during the pre-incubation period. At the end of this time small balloons were tied off with silk suture thread from each half bladder. This was achieved by passing a loop of thread over a portion of the bladder so that as the loop was tightened and tied, a portion of the bladder was 'pinched off'. After other balloons were tied, all were cut from the bladder bag. Measurements of glucose utilization were begun by dropping each balloon into a flask containing 10 ml of Ringer's solution with penicillin and streptomycin and 5.5 mM glucose to which [1-^{14}C]glucose or [6-^{14}C]glucose had been added. Amiloride was present either in the serosal medium or inside the bag (mucosal surface), depending on the experiment. The flasks were sealed with rubber stoppers and the CO_2 liberated from the tissue was collected in polyethylene cups containing fluted filter paper wicks soaked in 25% KOH. These were hung inside the flasks above the

media. Incubations were performed at room temperature for 60 min on a rotatory metabolic shaker. At the end of this time 0.2 ml of 1.0 M H_2SO_4 was injected into each flask, and they were shaken for an additional 30 min. The filter paper wicks and the contents of the cups were quantitatively transferred with 3 ml methanol to scintillation vials for counting. Scintillation fluid, 10 ml, was then added to each vial. Flasks containing all ingredients except tissue were run as blanks simultaneously in all experiments. Medium samples were also taken for counting. Tissues were blotted and dried for 24 h at 100° C to obtain dry weights.

Aldosterone and amiloride were kindly provided by Dr M. M. Pechet (Research Institute for Medicine and Chemistry, Cambridge, Mass.) and Dr J. Baer, (Merck, Sharp and Dohme, West Point, Pa.) respectively. Isotopically labeled glucose was obtained from the New England Nuclear Corporation, Boston, Mass.

RESULTS

(*1*) *Effect of amiloride on sodium transport*

In Table I are shown the effects of three concentrations of amiloride on Na^+

TABLE I

EFFECT OF AMILORIDE ON SODIUM TRANSPORT IN TOAD BLADDERS WHEN APPLIED TO EITHER THE MUCOSAL OR SEROSAL SURFACE

Concentration (M)	Na^+ transported (μEquiv. per h per 2 cm^2)		$\bar{x} \pm$ S.E.	P	Inhibition (%)	n
	Adjusted* control	Test				
Amiloride mucosal						
10^{-6}	3.78	1.17	-2.61 ± 0.69	<0.01	69	11
10^{-5}	3.60	0.36	-3.24 ± 0.73	<0.001	85	14
10^{-4}	2.26	0.20	-2.06 ± 0.85	<0.05	89	8
Amiloride serosal						
10^{-6}	4.18	4.28	$+0.10 \pm 0.09$	N.S.**	0	11
10^{-5}	4.34	4.36	$+0.02 \pm 0.14$	N.S.	0	17
10^{-4}	5.39	4.59	-0.80 ± 0.25	<0.02	15	7

* Normalized to the starting current of the test tissue.

** N.S., not significant.

transport. When applied to the mucosal surface of the bladders, Na^+ transport was rapidly and markedly inhibited. The inhibition was 69% at 10^{-6} M, 85% at 10^{-5} M, and 89% at 10^{-4} M. In contrast, the effects from the serosal side were slight and gradual. No inhibition of Na^+ transport was observed at 10^{-6} or 10^{-5} M, while at 10^{-4} M an inhibition of only 15% was observed. This inhibition of transport was different in character to that observed when amiloride is added to the mucosal surface. In the latter situation the inhibition of transport is extremely rapid and most of the inhibition occurs within the first minute after application. In contrast, serosal addition of the amiloride results in a slow and progressive decrease in transport.

(2) Effects of amiloride on glucose metabolism

In these experiments paired pieces of toad bladders were incubated for 5 h in the presence or absence of aldosterone. Amiloride ($1 \cdot 10^{-6}$ M) was then added where appropriate so that $^{14}CO_2$ evolution from [1-^{14}C]glucose and [6-^{14}C]glucose could be measured under four sets of conditions; namely under control conditions, in the presence of amiloride, and after aldosterone treatment in the presence and absence of amiloride. The incubations were performed in flasks in the conventional manner so that amiloride bathed both the serosal and mucosal surfaces of the tissue.

TABLE II

EFFECTS OF ALDOSTERONE ($5 \cdot 10^{-7}$ M) AND AMILORIDE ($1 \cdot 10^{-6}$ M) ON THE METABOLISM OF [1-^{14}C]GLUCOSE AND [6-^{14}C]GLUCOSE IN TOAD BLADDER

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

			± S.E.	n	P
	Control	Aldosterone			
C-1	0.98	1.12	+0.14 ± 0.12	12	0.3
C-6	0.26	0.80	+0.54 ± 0.11	12	< 0.001
C-1 − C-6	0.72	0.32	−0.40 ± 0.10	12	< 0.01
	Control	Amiloride			
C-1	0.98	1.02	+0.04 ± 0.07	12	0.6
C-6	0.26	0.14	−0.12 ± 0.03	12	< 0.01
C-1 − C-6	0.72	0.88	+0.16 ± 0.08	12	< 0.1
	Aldosterone	Aldosterone + amiloride			
C-1	1.12	0.86	−0.26 ± 0.10	12	< 0.02
C-6	0.80	0.27	−0.53 ± 0.10	12	< 0.001
C-1 − C-6	0.32	0.59	+0.26 ± 0.10	12	< 0.02
	Amiloride	Aldosterone + amiloride			
C-1	1.02	0.86	−0.16 ± 0.09	12	< 0.2
C-6	0.14	0.27	+0.14 ± 0.05	12	< 0.05
C-1 − C-6	0.88	0.59	−0.29 ± 0.10	12	< 0.02

It can be seen from Table II that amiloride significantly inhibited $^{14}CO_2$ evolution from [6-^{14}C]glucose, in the presence or absence of aldosterone. Aldosterone significantly increased $^{14}CO_2$ from [6-^{14}C]glucose, while exerting its usual inhibitory effect upon the hexose monophosphate shunt pathway ([1-^{14}C] − [6-^{14}C]) as seen by a change from 0.72 μmoles/g dry weight per h under control conditions to 0.32 after treatment with aldosterone. Amiloride was found to partially reverse the inhibition induced by aldosterone, $^{14}CO_2$ evolution increasing from 0.32 to 0.59 μmoles/g dry weight per h. No significant increase was detected in control tissue treated with amiloride.

With the demonstration of an inverse relationship between sodium transport and the hexose monophosphate shunt pathway, as evidenced by stimulation of the shunt pathway with amiloride in the presence of aldosterone, it became important to define the amiloride effect. Thus the effect of either mucosal or serosal application was studied.

(3) *Effects of amiloride on glucose metabolism*

(a) *When applied to the mucosal surface.* From Table III it can be seen that

TABLE III

EFFECTS OF AMILORIDE WHEN APPLIED TO THE MUCOSAL SURFACE OF TOAD BLADDER ON THE METABOLISM OF [1-^{14}C]GLUCOSE AND [6-^{14}C]GLUCOSE

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

Aldosterone + amiloride concentration		Aldosterone effect	Aldosterone + amiloride effect	$\Delta \pm$S.E.	n	P
$1 \cdot 10^{-6}$ M	C-1	0.86	0.65	-0.21 ± 0.12	7	< 0.2
	C-6	0.48	0.20	-0.28 ± 0.11	7	< 0.05
	C-1 − C-6	0.38	0.44	$+0.06 \pm 0.17$	7	< 0.8
$1 \cdot 10^{-5}$ M	C-1	0.54	0.49	-0.04 ± 0.09	9	< 0.7
	C-6	0.27	0.18	-0.09 ± 0.01	9	< 0.001
	C-1 − C-6	0.26	0.31	$+0.05 \pm 0.09$	9	< 0.7
$5 \cdot 10^{-5}$ M	C-1	0.93	1.47	$+0.53 \pm 0.20$	6	< 0.1
	C-6	0.44	0.18	-0.26 ± 0.10	6	< 0.05
	C-1 − C-6	0.49	1.28	$+0.80 \pm 0.22$	6	< 0.02
$1 \cdot 10^{-4}$ M	C-1	0.86	1.04	$+0.18 \pm 0.10$	10	0.1
	C-6	0.26	0.12	-0.14 ± 0.05	10	< 0.05
	C-1 − C-6	0.60	0.92	$+0.32 \pm 0.13$	10	< 0.05

amiloride on the apical surface consistently inhibited the evolution of $^{14}CO_2$ from [6-^{14}C]glucose at all four concentrations tested. This presumably reflects the inhibition of Na^+ transport and reduced requirement for energy. Effects on the $^{14}CO_2$ evolution from the hexose monophosphate shunt pathway (C-1 minus C-6) were only observed at the two highest concentrations tested, $5 \cdot 10^{-5}$ M and $1 \cdot 10^{-4}$ M. At these concentrations amiloride increased the activity of the shunt pathway, thus reversing the inhibitory effect of aldosterone. No effect was observed with 10^{-5} and 10^{-6} M amiloride.

TABLE IV

EFFECTS OF AMILORIDE ($1 \cdot 10^{-4}$ M) WHEN APPLIED TO THE SEROSAL SURFACE OF TOAD BLADDER ON THE METABOLISM OF [1-^{14}C]GLUCOSE AND [6-^{14}C]-GLUCOSE

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

	Aldosterone	Aldosterone + amiloride	$\Delta \pm$S.E.	n	P
C-1	0.61	0.57	-0.04 ± 0.09	9	N.S.*
C-6	0.21	0.18	-0.03 ± 0.01	9	< 0.02
C-1 − C-6	0.40	0.40	0.00 ± 0.10	9	

* N.S., not significant.

(*b*) *When applied to the serosal surface.* 10^{-4} M amiloride at the serosal surface caused a small (15%) but significant decrease in $^{14}CO_2$ evolution from [6-^{14}C]glucose (Table IV). This is a greater percentage fall in glucose metabolism than would be anticipated from the 15% reduction in Na^+ transport that was also observed at this concentration of amiloride. Strikingly, no effect was observed on the C-1 minus C-6 parameter reflecting hexose monophosphate shunt activity.

DISCUSSION

Using $^{14}CO_2$ evolution from [6-^{14}C]glucose and [1-^{14}C]glucose it has been demonstrated that aldosterone inhibits the hexose monophosphate shunt pathway [7] and that an inverse relationship exists between this and the steroid induced stimulation of Na^+ transport [10]. As a preliminary study [15] had indicated that amiloride increased hexose monophosphate shunt activity while simultaneously decreasing Na^+ transport, the possibility existed that the mechanism by which the rate of Na^+ transport was regulated at the apical membranes was affecting shunt pathway activity and that aldosterone and amiloride were acting on a common site on the apical membranes. This study proves that such an inverse relationship exists between the rate of Na^+ transport and the activity of the hexose monophosphate shunt pathway after treatment with aldosterone and amiloride. However it has not been possible to associate definitively the effects of amiloride at the apical surface, by a study of the dose–response characteristics. Evidence that amiloride could affect the hexose monophosphate shunt pathway at a concentration which causes sub-maximal inhibition of Na^+ transport was obtained in the flask experiments, i.e., with both surfaces exposed to the diuretic. For instance in Table II, a partial reversal of the aldosterone-induced inhibition of the shunt pathway was obtained at an amiloride concentration of $1 \cdot 10^{-6}$ M. At this concentration Na^+ transport is inhibited by 69%. It should be noted that when amiloride was added only to the serosal surface, even at 10^{-4} M, no effect upon the shunt pathway was observed. Thus, it is most probable that the effect seen at $1 \cdot 10^{-6}$ M was exerted at the apical surface.

Experiments were performed in which different concentrations of amiloride were added only to the apical surface. Under these conditions, only slight, non-significant increases in hexose monophosphate shunt activity were detected at concentrations of 10^{-6} M and 10^{-5} M. At $5 \cdot 10^{-5}$ M and 10^{-4} M, however, a significant reversal of the aldosterone effects were seen. Thus with the 'balloon' technique we were unable to demonstrate apical effects of amiloride over the same dose range which causes minimal to maximal inhibition of Na^+ transport. While this discrepancy between the flask experiments and the balloon experiments might be ascribed to differences in sensitivity between the two techniques, it does mean that we were unable to conclusively demonstrate a direct association between the effects of aldosterone and amiloride on the hexose monophosphate shunt pathway. Despite this, the results show a remarkable association between the apical cell membrane and the shunt pathway. From the mucosal bathing medium amiloride fails to enter the cells. Its effects on Na^+ transport are rapid, reversible and exerted from the outer surface of the cell membranes, yet it has a large effect upon $^{14}CO_2$ evolution from C-1 minus C-6 glucose. At the same concentration from the serosal

medium, it enters and accumulates in the cells yet has no effect upon $^{14}CO_2$ from C-1 minus C-6 glucose. Thus this work demonstrates an effect of amiloride on the activity of the hexose monophosphate shunt pathway which is exerted only from the outer surface of the apical cell membranes.

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