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THE ROLE OF THE TRICARBOXYLIC ACID CYCLE IN THE ACTION OF ALDOSTERONE ON SODIUM TRANSPORT*

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SUMMARY

1. The effects of several substrates and inhibitors were studied in the isolated toad bladder system in an attempt to determine the metabolic reactions involved in mineralocorticoid regulation of Na^+ transport.

2. Of the substrates studied glucose, pyruvate, β-hydroxybutyrate, acetoacetate, and oxaloacetate were synergistic with aldosterone in regulating Na^+ transport, while acetate, α-ketoglutarate, succinate, and aspartate were relatively ineffective in both the presence and absence of exogenous steroid. Propionate, on the other hand, supported Na^+ transport in the absence of aldosterone but did not elicit a further effect in the presence of aldosterone.

3. Anoxia almost completely inhibited Na^+ transport both in steroid-free and in aldosterone-treated hemibladders. This inhibition was completely reversed by reaeration.

4. As oxythiamine or phenylpyruvate inhibition of steroid-dependent Na^+ transport was reversed by the addition of β-hydroxybutyrate or β-hydroxybutyrate and oxaloacetate, respectively, aldosterone does not regulate Na^+ transport by specific induction of pyruvate dehydrogenase or pyruvate carboxylase.

5. Aldosterone seems to act by stimulating a step or steps in the tricarboxylic acid cycle at a point between condensing enzyme and α-ketoglutarate dehydrogenase.

INTRODUCTION

A series of physiological, biochemical, and histological studies has been reported that supports the concept that aldosterone regulates Na^+ transport by a sequence

Abbreviation: scc, short-circuit current.

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of reactions initiated by steroidal stimulation of DNA-dependent RNA synthesis[1-4]. The present report explores the pathway from steroid-dependent synthesis of proteins to effects on the rate of Na^+ transport; in particular, the role of the tricarboxylic acid cycle in mineralocorticoid action.

From studies of the dependence of Na^+ transport on specific substrates in the presence and absence of metabolic inhibitors, MAFFLY AND EDELMAN[5] inferred that the supply of energy can regulate the rate of Na^+ transport in the isolated toad bladder. Subsequent studies showed that the response of the Na^+ transport apparatus to aldosterone is impaired in substrate-depleted toad bladders and that exogenous addition of glucose or pyruvate restores the response to aldosterone[1,6]. Accordingly, EDELMAN, BOGOROCH AND PORTER[1,7] proposed that oxidation of pyruvate and the generation of ATP or a similar high-energy compound were intermediate steps between steroid-induced protein synthesis and the subsequent rise in Na^+ transport.

Two enzymatic sites have been suggested for the coupling of metabolism and mineralocorticoid action. FELDMAN and co-workers[8,9] reported an increase in succinate dehydrogenase and cytochrome oxidase activity of rat kidney after injection of aldosterone *in vivo* and suggested that the mineralocorticoid effect could result from enhanced energy production by the action of these enzymes. SHARP AND LEAF[10] concluded from studies of substrate–steroid synergism, substrate utilization, and oxythiamine inhibition of the mineralocorticoid effect that aldosterone acts to couple the energy provided by oxidative decarboxylation of pyruvate to Na^+ transport (see NOTE ADDED IN PROOF).

We propose that aldosterone regulates Na^+ transport by enhancing the activity of the tricarboxylic acid cycle at some point or points between the formation of citrate from oxaloacetate and acetyl-CoA (condensing enzyme) and the formation of succinate (α-ketoglutarate dehydrogenase).

MATERIALS

The following compounds were obtained commercially and used without further purification: ATP (disodium salt), α-ketoglutaric acid, sodium propionate, reduced glutathione, acetyl-CoA, thiamine–HCl, and oxythiamine–HCl from Sigma Chemical Co.; sodium pyruvate, oxaloacetic acid, L-aspartic acid, sodium phenylpyruvate, and (+)-aldosterone from California Biochemical Co.; succinic acid and glucose from Baker and Adamson; and β-hydroxybutyric acid from Nutritional Biochemical Co.

Lithium acetoacetate was prepared by the method of HALL[11] and converted to sodium acetoacetate by treatment with Dowex-50 (Na^+, 200–400 mesh) before use.

METHODS AND RESULTS

In substrate-depleted bladders, mineralocorticoid activity is dependent on addition of substrate[6]. In substrate-enriched media the stimulatory effect of aldosterone on Na^+ transport is characterized by a latent period of 60–90 min. At the end of this latent period there is a linear increase in Na^+ transport over a period of 4.5–6.5 h.

Aldosterone–substrate synergism

If aldosterone induces synthesis of proteins that regulate the supply of energy

to the Na^+ transport system, metabolic intermediates which yield substrates for the enzymes whose activities are altered will be synergistic with aldosterone in stimulating a rise in Na^+ transport. Metabolic intermediates which are products of these enzymes or subsequent enzymes in the metabolic pathway involved should give only an additive rise in Na^+ transport in the presence of aldosterone. Consequently, we tested the ability of several intermediates to potentiate the mineralocorticoid action of aldosterone in substrate-depleted hemibladders.

Pairs of hemibladders of the toad *Bufo marinus* were excised, mounted in glass chambers, and monitored electrically as described previously[3]. All hemibladders were incubated overnight in 40 ml of frog-Ringer solution containing penicillin G (0.5 mg/ml) and streptomycin sulfate (1.0 mg/ml), pH 7.8. The next morning fresh substrate-free Ringer solution was substituted. Approx. 1 h later, (+)-aldosterone was added to the serosal medium of one hemibladder (final concentration = 0.7 μM) and an equal volume of the diluent to that of the other hemibladder. 3 h after the addition of either aldosterone or diluent to the media, a substrate was added to the serosal and mucosal media of both hemibladders. Active Na^+ transport was measured by the short-circuit current (scc) method of USSING AND ZERAHN[12].

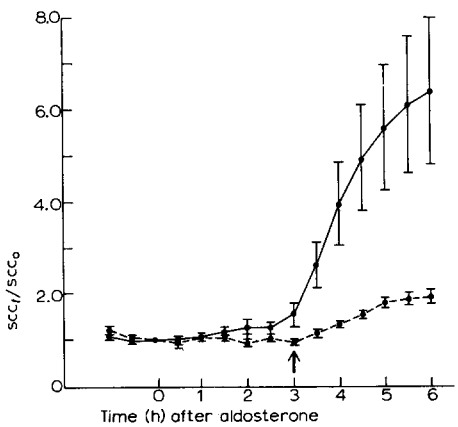

Fig. 1. Synergistic effects of oxaloacetate and (+)-aldosterone on the rate of active Na^+ transport across toad bladders. Paired hemibladders were incubated 16 h prior to time zero in frog-Ringer solution. At time zero 0.7 μM aldosterone was added to one hemibladder (●—●) and diluent to the other (●— —●). The arrow at $t = 3$ h indicates the addition of 4.8 mM sodium oxaloacetate to both hemibladders. The ratio scc_t/scc_0 denoted the short-circuit at time t divided by that observed in the same hemibladder at time zero. The points and vertical lines represent the mean ± 1 standard error of the mean. $n = 6$ pairs of hemibladders. The absolute scc's at time zero were 37 ± 10 and 38 ± 9 μA/2.54 cm^2 for the aldosterone-treated and control hemibladders, respectively.

The response to 4.8 mM oxaloacetate is shown in Fig. 1. The scc had been increasing minimally in the presence of aldosterone. Oxaloacetate produced a rapid curvilinear rise in Na^+ transport with almost no latent period; the rise in scc of the aldosterone-treated hemibladders was significantly greater than that of the controls within 30 min of the addition of substrate. Qualitatively, the response to oxaloacetate was the same as the response to glucose and pyruvate described by EDELMAN, BOGOROCH AND PORTER[1]. A comparison of the response to substrate of the control

TABLE I

SYNERGISTIC EFFECTS OF SPECIFIC SUBSTRATES AND (+)-ALDOSTERONE ON THE RATE OF ACTIVE Na^+ TRANSPORT ACROSS TOAD BLADDERS

For details of experimental procedure refer to legend of Fig. 1.

Substrate	*Concentration (mM)*	*n*	*scc_0**		*% change in scc***	
			Control	*Aldosterone*	*Control*	*Aldosterone*
None		7	47 ± 24	31 ± 11	−42 ± 6	−5 ± 15
Glucose	6.0	13	48 ± 8	57 ± 10	42 ± 14	114 ± 20
Pyruvate	5.5	9	69 ± 13	70 ± 12	67 ± 19	128 ± 30
Oxaloacetate	4.8	6	38 ± 9	37 ± 10	105 ± 13	291 ± 42
Acetoacetate	5.4	6	47 ± 11	50 ± 12	53 ± 20	179 ± 26
β-Hydroxybutyrate	5.5	11	74 ± 9	67 ± 7	25 ± 3	46 ± 6
Acetate	5.7	9	53 ± 7	54 ± 8	6 ± 6	25 ± 9
α-Ketoglutarate	5.5	8	49 ± 7	50 ± 10	−20 ± 7	7 ± 8
Succinate	5.0	10	45 ± 17	31 ± 7	−33 ± 9	20 ± 14
L-Aspartate	5.5	5	67 ± 12	63 ± 8	−20 ± 4	−2 ± 5

* Absolute scc's ($\mu A/2.54\ cm^2$) at time zero.
** Per cent change in scc for 3 h from the time of addition of substrate to the media.

hemibladders and the aldosterone-treated hemibladders indicates that glucose, pyruvate, and oxaloacetate are synergistic with aldosterone. Identical experiments were performed with β-hydroxybutyrate, acetoacetate, acetate, aspartate, α-ketoglutarate, and succinate.

To provide a convenient method for expressing the results of the substrate–steroid experiments, the per cent change in scc 3 h after the addition of the substrate was computed for the control and steroid-treated hemibladders. The results are shown in Table I. In addition to glucose, pyruvate, and oxaloacetate, acetoacetate and to a lesser extent β-hydroxybutyrate supported the steroid-induced rise in Na^+ transport. The response to acetate, α-ketoglutarate and succinate, and aspartate in the presence of aldosterone did not differ significantly from the response of the substrate-free system to this steroid. In general, those substrates that induced a significant rise in scc in the steroid-free system were synergistic with aldosterone. These results agree with those of MAFFLY AND EDELMAN[5] who found that glucose, pyruvate, and β-hydroxybutyrate stimulated the scc of substrate-depleted bladders but that acetate and the tricarboxylic acid-cycle intermediates α-ketoglutarate, succinate, fumarate, and malate did not. Similarly, SHARP AND LEAF[10] found glucose, lactate, and oxaloacetate to be active in support of aldosterone-induced rises in scc, and acetate, β-hydroxybutyrate, fumarate, citrate, succinate, and α-ketoglutarate to be inactive. These results suggest, therefore, that an adequate supply of precursors of the tricarboxylic acid cycle (*i.e.*, oxaloacetate and acetyl-CoA) are required for mineralocorticoid action, but that the steps from α-ketoglutarate to malate may not be involved.

Additional evidence that the steps of the tricarboxylic acid cycle from succinate to malate are not directly involved in mineralocorticoid action has been obtained by FANESTIL*. Initial preparation of his hemibladders was identical to ours. 3 h after the

* Personal communication.

addition of aldosterone or diluent 4.25 mM sodium propionate was added to the media of both hemibladders. The scc's were monitored for another 3 h and then 10 mM glucose was added to the media of both hemibladders. As shown in Fig. 2, propionate increased Na^+ transport in the control and steroid-treated hemibladders to the same extent. The addition of glucose to the propionate-treated, steroid-free hemibladders produced only a small additional rise in scc, indicating that propionate had virtually eliminated the substrate deficiency. In contrast, the addition of glucose to the propionate-treated and steroid-treated hemibladders evoked a more than two-fold increase in scc, indicating that the metabolites of glucose but not of propionate were involved in the steroid-regulated process. As propionate enters the tricarboxylic acid cycle by the reaction sequence propionyl-CoA→methylmalonyl-CoA→succinyl-CoA→ succinate[13], these results are consistent with the inference that the tricarboxylic acid cycle from succinate to oxaloacetate (and the FAD-dependent electron-transport reactions) are not directly implicated in the response to aldosterone.

The effect of anaerobiosis on Na^+ transport

Since an adequate supply of tricarboxylic acid-cycle precursors is required for

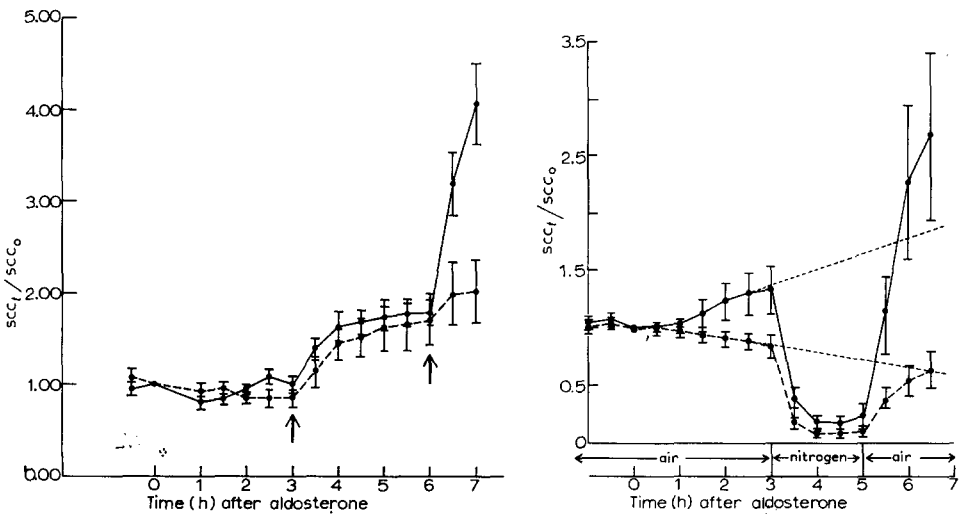

Fig. 2. A comparison of the synergistic effects of propionate *versus* glucose and (+)-aldosterone on the rate of active Na^+ transport across toad bladders. Paired hemibladders were incubated 16 h prior to time zero in frog-Ringer solution. At time zero 0.7 μM aldosterone was added to one hemibladder (●—●) and diluent to the other (●— —●). The first arrow at $t = 3$ h indicates addition of 4.25 mM sodium propionate and the second arrow at $t = 6$ h addition of 10 mM glucose to both hemibladders. The conventions used in the construction of this figure are described in the legend of Fig. 1. $n = 11$ pairs of hemibladders. The absolute scc's at time zero were 32 ± 5 and 35 ± 8 μA/2.54 cm^2 for the aldosterone-treated and control hemibladders, respectively.

Fig. 3. The effect of anaerobiosis and re-aeration on the action of aldosterone on active Na^+ transport across toad bladders. Paired hemibladders were incubated 16 h prior to time zero in frog-Ringer solution with 10 mM glucose. At time zero 0.07 μM aldosterone was added to one hemibladder (●—●) and diluent to the other (●— —●). The conventions used in the construction of this figure are described in the legend of Fig. 1. $n = 7$ pairs of hemibladders. The dotted lines represent extrapolations of the curves from the values recorded in the first 3 h in air. The absolute scc's at time zero were 89 ± 27 and 79 ± 14 μA/2.54 cm^2 for the aldosterone-treated and control hemibladders, respectively.

steroidal activity on Na^+ transport, it is reasonable to assume that oxidative pathways mediate the response. SHARP AND LEAF[10] observed that the toad bladder does not respond to aldosterone in O_2-deficient media. We studied the requirement for molecular O_2 in the action of aldosterone on Na^+ transport and the pattern of recovery from anaerobiosis.

In these experiments, all hemibladders were incubated overnight in frog-Ringer solution containing 10 mM glucose. The following morning all solutions were exchanged for fresh frog-Ringer solution containing 5.5 mM glucose. 1 h later aldosterone was added to both the serosal and mucosal media of one hemibladder (final concentration 0.07 μM) and diluent to the media of the control hemibladder. 3 h after the addition of aldosterone or diluent all chambers were fitted with stoppers with constricted openings and water-pumped N_2 in place of air was bubbled through the solutions. Anaerobic conditions were reversed after 2 h.

As shown in Fig. 3, the hemibladders responded to aldosterone as usual until the N_2 replaced the air. Anaerobiosis promptly decreased the scc of both the control and steroid-treated hemibladders. Moreover, the rates of decline as well as the absolute magnitudes of the declines were comparable in both populations. When air was restored, the rate of Na^+ transport rose promptly in both sets of hemibladders. The rate of the response was similar to that of the aldosterone–substrate synergism studies (Fig. 1). In the control population, the re-introduction of air returned the scc to the value predicted by extrapolation of the curve from its pre-anaerobic values. Thus, anaerobic inhibition was fully reversible. In the steroid-treated group, however, the scc was distinctly greater than the values predicted by linear extrapolation of the pre-anaerobic curve. Both the effects of anaerobiosis and the pattern of response to re-aeration support the inference that oxidative metabolism mediates the action of aldosterone on Na^+ transport.

The role of pyruvic dehydrogenase in the action of aldosterone on Na^+ transport

To infer that a specific reaction mediates the action of aldosterone, two criteria should be met: (1) an inhibitor of the reaction should inhibit the aldosterone-induced rise in Na^+ transport, and (2) the product of the reaction should not act as a synergistic substrate for the mineralocorticoid effect. SHARP AND LEAF[10] observed inhibition of the action of aldosterone with oxythiamine, a potent inhibitor of pyruvate dehydrogenase, which meets the first criterion. We have tried to meet the second criterion by testing the response to substrates capable of forming acetyl-CoA by a pathway not dependent on thiamine.

Pairs of hemibladders were preincubated overnight in frog-Ringer solution fortified with 10 mM glucose. The following morning all solutions were replaced with fresh 5.5 mM glucose–frog-Ringer solution. 1 h later 0.07 μM aldosterone and/or 0.3 mM oxythiamine were added to the serosal and mucosal media as indicated in Fig. 4. Oxythiamine significantly inhibited the scc in the control (steroid-free hemibladders) and in the aldosterone-treated hemibladders. The addition of 0.14 mM thiamine to the media at the time zero completely reversed this inhibition. Thus, both basal Na^+ transport and steroid-dependent Na^+ transport require intact thiamine-dependent pathways.

The conversion of β-hydroxybutyrate and acetoacetate to acetyl-CoA do not depend on thiamine. Therefore, the possibility that aldosterone acts specifically at the

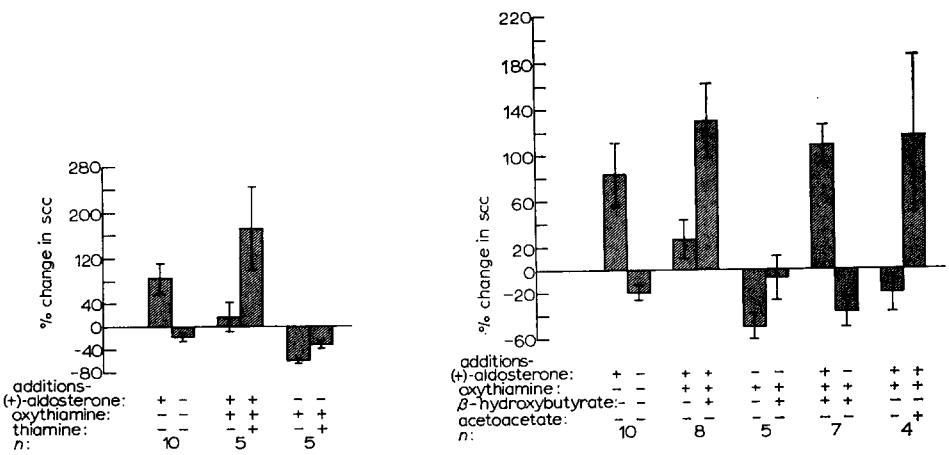

Fig. 4. The inhibitory effect of oxythiamine and its reversibility by thiamine on the action of aldosterone on active Na^+ transport across toad bladders. The per cent change in scc was computed for a period of 6 h after the addition of aldosterone or the intermediates to the media. The final concentrations of the added components were 0.07 μM (+)-aldosterone, 0.30 mM oxythiamine, and 0.14 mM thiamine. All additions were made to both the serosal and mucosal media. The height of the bar and the vertical lines represent the mean $\pm$ 1 standard error of the mean. n denotes the number of pairs of hemibladders in each group. The absolute scc's (in μA/2.54 cm^2) at time zero were 108 $\pm$ 17 and 104 $\pm$ 15; 68 $\pm$ 11 and 82 $\pm$ 22; and 141 $\pm$ 27 and 135 $\pm$ 28, respectively, for the pairs of hemibladders treated as indicated from left to right.

Fig. 5. The reversal of oxythiamine inhibition of the action of aldosterone on active Na^+ transport across toad bladders by β-hydroxybutyrate and acetoacetate. The final concentrations of the added components were 0.07 μM (+)-aldosterone, 0.30 mM oxythiamine, 5.5 mM β-hydroxybutyrate and 5.5 mM acetoacetate. The conventions used in the construction of this figure are described in the legend of Fig. 4. The absolute scc's (in μA/2.54 cm^2) at time zero were 108 $\pm$ 17 and 104 $\pm$ 16; 83 $\pm$ 13 and 82 $\pm$ 16; 89 $\pm$ 9 and 73 $\pm$ 5; 77 $\pm$ 8 and 88 $\pm$ 13; and 117 $\pm$ 30 and 113 $\pm$ 32, respectively, for the pairs of hemibladders treated as indicated from left to right.

pyruvate dehydrogenase locus was tested by adding either 5.5 mM β-hydroxybutyrate or 5.5 mM acetoacetate to the media of hemibladders treated with oxythiamine and/or aldosterone at time zero as indicated in Fig. 5. β-Hydroxybutyrate completely reversed the inhibitory effect of oxythiamine both in the control and in the aldosterone-treated hemibladders; acetoacetate completely reversed oxythiamine inhibition in the aldosterone-treated group. Thus, pyruvate dehydrogenase does not mediate the action of aldosterone on Na^+ transport. These results indicate, however, that an adequate supply of acetyl-CoA is required for the mineralocorticoid effect. Similar results with acetoacetate and β-hydroxybutyrate in the presence of oxythiamine and aldosterone were recently reported by SHARP AND LEAF[14].

The role of pyruvate carboxylase in the action of aldosterone on Na^+ transport

The findings of steroid–substrate synergism with oxaloacetate and acetoacetate (see Fig. 2) suggest that pyruvate carboxylase (pyruvate + ATP + $CO_2 \xrightarrow{\text{acetyl-CoA}}$ oxaloacetate + ADP + P_i) is not directly involved in the mechanism of action of aldosterone. To provide a more stringent test of this inference, we studied the effect of phenylpyruvate on the response to aldosterone. KREBS AND DE GASQUET[15] introduced phenylpyruvate as a competitive analogue of gluconeogenesis from pyruvate, and SEUBERT AND HUTH[16] have suggested that phenylpyruvate is a potent inhibitor

TABLE II

PHENYLPYRUVATE INHIBITION OF RAT-KIDNEY PYRUVATE CARBOXYLASE

Complete system: Tris buffer (pH = 7.4), 50 μmoles of $MgCl_2$, 5 μmoles of ATP, 1.25 μmoles of acetyl-CoA, 0.35 μmole of sodium pyruvate, 15 μmoles (specific activity = $1.54 \cdot 10^5$ counts/min per μmole) of $NaH^{14}CO_3$ and 0.5 ml of water. Incubated at 30° for 10 min.

System	*Phenylpyruvate (μmoles)*	*$^{14}CO_2$ incorporated (mμmoles/min per mg protein)*
Complete	0	10.1
Complete	5.7	5.3

of pyruvate carboxylase activity. To ensure that this analogue did inhibit pyruvate carboxylase, we tested the effect of phenylpyruvate in the rat kidney. A pair of kidneys taken from a fasted Sprague–Dawley male rat were homogenized in 3 vol. of 0.1 M Tris buffer (pH 7.4) containing 1 mM glutathione for 2 min, sonicated at 20 000 cycles/sec for 1 min and centrifuged at 105 000 × *g* for 1 h at 0°. Pyruvate carboxylase activity of the supernatant fraction was measured by the method of UTTER AND KEECH[17] with and without 11.4 mM phenylpyruvate in the medium. The results are shown in Table II and confirm those of SEUBERT AND HUTH[16] of inhibition of the biotin-dependent, acetyl-CoA-dependent pyruvate carboxylase.

Pairs of hemibladders were preincubated in 10 mM glucose–frog-Ringer solution

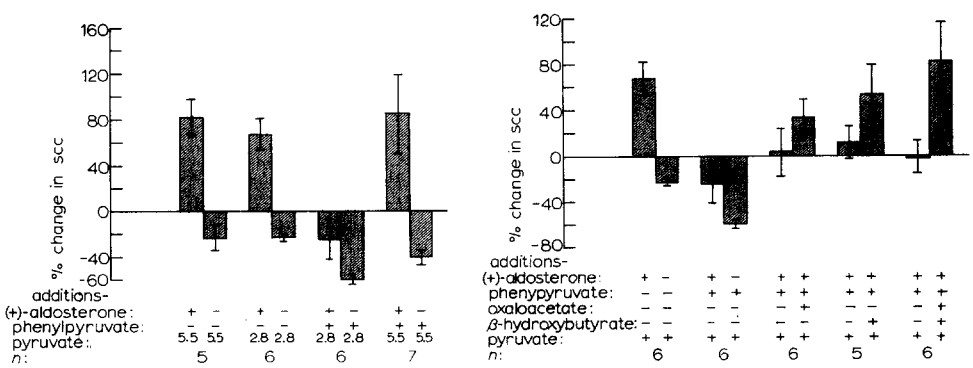

Fig. 6. The inhibitory effect of phenylpyruvate and its reversibility by pyruvate on the action of aldosterone on active Na^+ transport across toad bladders. The final concentrations of added components were 0.07 μM (+)-aldosterone and 11.5 mM phenylpyruvate. The conventions used in the construction of this figure are described in the legend of Fig. 4. The absolute scc's (in μA/2.54 cm^2) at time zero were 97 ± 20 and 95 ± 28; 127 ± 24 and 145 ± 27; 136 ± 19 and 125 ± 14; and 60 ± 14 and 68 ± 11, respectively, for the pairs of hemibladders treated as indicated from left to right.

Fig. 7. The reversal of phenylpyruvate inhibition of the action of aldosterone on active Na^+ transport across toad bladders by oxaloacetate and β-hydroxybutyrate. The final concentrations of added components were 0.07 μM (+)-aldosterone, 11.5 mM phenylpyruvate, 5.5 mM oxaloacetate, 5.5 mM β-hydroxybutyrate and 2.8 mM pyruvate. The conventions used in the construction of this figure are described in the legend of Fig. 4. The absolute scc's (in μA/2.54 cm^2) at time zero were 124 ± 24 and 145 ± 27; 136 ± 19 and 125 ± 14; 124 ± 22 and 118 ± 18; 94 ± 20 and 104 ± 25; and 101 ± 28 and 80 ± 18, respectively, for the pairs of hemibladders treated as indicated from left to right.

overnight. The following morning all of the chambers were drained, rinsed with substrate-free frog-Ringer solution, and then refilled with pyruvate-enriched (either 2.8 or 5.5 mM) frog-Ringer solution. 1 h later 0.07 μM aldosterone was added to the serosal and mucosal media as shown in Fig. 6. 2 h after aldosterone addition, 11.5 mM phenylpyruvate was added. At a pyruvate concentration of 2.8 mM, phenylpyruvate inhibited Na^+ transport in both the presence and absence of aldosterone. The scc fell 60% in the control and 24% in the aldosterone-treated hemibladders. When the concentration of pyruvate was increased to 5.5 mM, the scc of the control hemibladders fell 40% in 6 h whereas that of the steroid-treated group rose 85%. Thus phenylpyruvate acts as a competitive analogue of pyruvate in this system. The relevance of the pyruvate carboxylase step to the action of aldosterone was then tested by adding either 5.5 mM oxaloacetate, 5.5 mM β-hydroxybutyrate or both to the media at time zero (Fig. 7). The result was complete reversal of phenylpyruvate inhibition of the mineralocorticoid effect (scc increased 82% in 6 h). The following conclusions, therefore, may be inferred: (1) the precursors of the tricarboxylic acid cycle (oxaloacetate and acetyl-CoA) are needed for the mineralocorticoid effect to occur, and (2) the action of aldosterone on Na^+ transport is not mediated by activation or induction of pyruvate carboxylase.

DISCUSSION

We propose that aldosterone acts by inducing the synthesis of a protein involved in the tricarboxylic acid cycle at a point or points between the condensing enzyme and α-ketoglutarate dehydrogenase steps. This postulate is supported by the following findings: (1) Substrate–aldosterone synergism is limited to oxaloacetate, precursors of oxaloacetate, and precursors of acetyl-CoA (*i.e.*, glucose, pyruvate, acetoacetate, and β-hydroxybutyrate). (2) Inhibition of the action of aldosterone by oxythiamine is completely reversed by acetoacetate or β-hydroxybutyrate, indicating that neither pyruvate dehydrogenase nor α-ketoglutarate dehydrogenase is necessary to the action of aldosterone. (3) Inhibition of the action of aldosterone by phenylpyruvate is completely reversed by oxaloacetate and β-hydroxybutyrate in combination indicating the need for C_4 as well as C_2 precursors of the tricarboxylic acid cycle. (4) The findings of FANESTIL* (Fig. 2) probably eliminate the steps from succinate→malate as mediators of the action of aldosterone because propionate, a precursor of succinyl-CoA, provides adequate substrate for Na^+ transport in the steroid-free system but is not synergistic with aldosterone. (5) The supply of O_2 must be adequate for the mineralocorticoid effect and the kinetics of the response to O_2 and to substrates are impressively similar in the hemibladders pretreated with aldosterone, suggesting that oxidative metabolism mediates the response to the steroid.

Recent studies of FANESTIL, PORTER AND EDELMAN[18] are also consistent with the concept that specific steps of the tricarboxylic acid cycle mediate the action of aldosterone on Na^+ transport. FANESTIL, PORTER AND EDELMAN[18] obtained physiological evidence that aldosterone provokes a rise in the electromotive force of the Na^+ pump. This effect is consistent with an increase in the rate of the transport reaction

* Personal communication.

(*i.e.*, ATP–Na^+–K^+–Mg^{2+}–ATPase reaction) because of a rise in ATP concentration at the pump site.

Some of the observations, however, do not fit the proposal of steroidal action *via* the tricarboxylic acid cycle. Acetate should have been synergistic with aldosterone, as it is readily oxidized by the toad bladder[5]. Moreover, SHARP AND LEAF[10] found an increase in oxidation of [^{14}C]acetate with the addition of aldosterone to toad bladders. It is possible, however, that the anomalous results obtained with acetate reflect both the inability of the epithelial cells involved in active Na^+ transport to form acetyl-CoA directly from acetate, and the ready oxidation of acetate by the non-transporting cells, *e.g.*, mucus-secreting cells, connective tissue cells, smooth muscle cells.

The results obtained with oxythiamine and phenylpyruvate preclude the possibility that either pyruvate dehydrogenase or pyruvate carboxylase is the key site of action of aldosterone. However, the possibility of simultaneous induction of either of these enzymes together with another enzyme that is rate limiting for Na^+ transport still exists. It is apparent that steroidal action by an effect at the level of the tricarboxylic acid cycle can be achieved by induction of a cytoplasmic protein as well as the induction of one of the enzymes of the tricarboxylic acid cycle or of the electron-transport system. Steroid induction could lead to the synthesis of a cytoplasmic activator or cofactor of one of the reaction steps of the tricarboxylic acid cycle or of the electron-transport chain. All of these hypotheses are consistent with the available evidence.

FELDMAN and co-workers[8,9] reported an increase in the activities of succinate dehydrogenase and cytochrome oxidase in homogenates of rat kidney after injection of aldosterone *in vivo* and proposed that the hormone acts by affecting these oxidative enzymes. In the toad bladder system, the results obtained by FANESTIL* with propionate seem to contradict this possibility. Also, the physiological significance of the observations on the oxidative enzymes must be considered doubtful as huge doses of aldosterone (*i.e.*, from 100 to 200 μg per rat) were used to elicit measurable increases in enzyme content. Furthermore, DOMJÁN AND FAZEKAS[19] found no rise in rat kidney succinate dehydrogenase after 4 days of administration of physiological levels of aldosterone (1 μg/kg) to adrenalectomized rats.

CONCLUSIONS

In the isolated toad bladder system, glucose, pyruvate, β-hydroxybutyrate, acetoacetate, and oxaloacetate were synergistic with aldosterone in regulating Na^+ transport. In contrast, acetate, α-ketoglutarate, succinate, and aspartate were almost inactive in support of Na^+ transport both in the steroid-free and in the aldosterone-treated hemibladders. Propionate, on the other hand, supported Na^+ transport in the absence of aldosterone but did not elicit a mineralocorticoid effect in the presence of aldosterone. Thus, it is proposed that aldosterone acts *via* stimulating a step or steps in the tricarboxylic acid cycle at a point between condensing enzyme and α-ketoglutarate dehydrogenase. Consistent with this proposal are the observations on the absolute need for O_2 for the expression of the mineralocorticoid effect on Na^+ transport.

* Personal communication.

and the evidence that neither pyruvate dehydrogenase nor pyruvate carboxylase are primarily involved in the mechanism of action.

NOTE ADDED IN PROOF

(Received January 13th, 1967). Recently, SHARP *et al*, proposed that aldosterone increases the entry of Na^+ across the apical surfaces of the mucosal epithelial cells and that the metabolic effects of aldosterone are secondary to the entry of Na^+ into the tissue. Their proposal rests on similarities in the metabolic effects of aldosterone and amphotericin B and on the supposition that amphotericin B increases Na^+ transport by reducing the permeability barrier to Na^+ at the apical surface. However, no direct evidence was obtained to support the "permease" theory of the action of aldosterone on Na^+ transport.

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