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ON THE ROLE OF THE TRICARBOXYLIC ACID CYCLE IN THE STIMULATION OF SODIUM TRANSPORT BY ALDOSTERONE

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SUMMARY

One view of the mode of action of aldosterone is that the hormone stimulates sodium transport by enhancing the activity of the tricarboxylic acid cycle at some point between condensing enzyme and α-oxoglutarate dehydrogenase. The evidence supporting this view has been examined.

It is found that in the toad bladder: (1) acetoacetate stimulates sodium transport in the presence of aldosterone and oxythiamine. However, this does not imply an action of the hormone prior to α-oxoglutarate dehydrogenase in the cycle because it has been shown that oxythiamine does not inhibit α-oxoglutarate dehydrogenase in the intact tissue even though pyruvate dehydrogenase is blocked. (2) Malonate inhibits the hormonal stimulation of sodium transport when pyruvate is the exogenous substrate. (3) Propionate is synergistic with aldosterone.

The available evidence does not support the theory that aldosterone stimulates sodium transport by enhancing the activity of the tricarboxylic acid cycle at some point between condensing enzyme and α-oxoglutarate. Rather, the functioning of an intact tricarboxylic acid cycle is necessary for the full expression of the action of aldosterone.

INTRODUCTION

The sodium transport system of the toad bladder has been used to study the mode of action of aldosterone. Stimulation of sodium transport by the hormone has been shown to depend upon oxidative metabolism[1] and, when endogenous substrates are depleted, upon substrates such as pyruvate and acetoacetate which yield acetyl coenzyme A. These substrates can produce a marked rise in sodium transport in the presence of aldosterone while being ineffective in its absence[2–5]. The marked metabolic effects of aldosterone have led to the view that the hormone enhances the energy supply to the transport mechanism in such a manner that it increases the rate of sodium transport. On the other hand, an alternative hypothesis suggests that aldo-

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sterone acts to increase the rate of entry of sodium into a "transport pool" and that the increased availability of sodium drives the transport to a higher rate[6–11]. A consequence of the latter view is that the metabolic effects of aldosterone are secondary to the increased rate of sodium entry into the transport pool[10].

In accord with an action of the hormone to stimulate energy metabolism, it has been postulated that aldosterone enhances 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-oxoglutarate dehydrogenase)[5,12]. This proposal was suggested by the following evidence:

(1) Toad bladders depleted of endogenous substrate, treated with aldosterone for several hours, respond to the addition of either pyruvate or acetoacetate with a prompt increase of sodium transport. Bladders treated similarly but without the addition of aldosterone show no such increase. In the presence of oxythiamine, an inhibitor of pyruvate dehydrogenase and presumably of α-oxoglutarate dehydrogenase, the effect of pyruvate is blocked, but the effect of acetoacetate is not[3,4]. This suggests an action of aldosterone prior to α-oxoglutarate dehydrogenase in the tricarboxylic acid cycle[5,12].

(2) Malonate, a competitive inhibitor of succinate dehydrogenase activity, was reported not to block the hormonal stimulation of sodium transport when pyruvate was the exogenous substrate. This suggests an action prior to succinate dehydrogenase in the cycle[12].

(3) Propionate, a precursor of succinyl CoA, provides substrate for sodium transport in the steroid-free system but was reported not to be synergistic with aldosterone. This suggests an action of aldosterone prior to succinyl CoA in the cycle[5].

The following assumptions are crucial to the validity of the view that aldosterone stimulates sodium transport by enhancing the activity of the tricarboxylic acid cycle at some step between condensing enzyme and α-oxoglutarate dehydrogenase. In the experimental situation it is assumed that: (1) oxythiamine blocks the activity of α-oxoglutarate dehydrogenase; (2) malonate blocks the activity of succinate dehydrogenase; (3) the supply of energy from propionate is not limiting.

The following experiments were performed to test the validity of these assumptions and to examine the effects of propionate and malonate on sodium transport in the presence of aldosterone.

EXPERIMENTAL PROCEDURE

Measurement of sodium transport

Sodium transport across the toad bladder was measured as short-circuit current in double chambers, as has been described previously[6]. Briefly, mounting one half of the bladder across a double chamber allows one quarter to be used as a test tissue and the other quarter as a control. After the spontaneous membrane potential is reduced to zero in the short-circuited preparation, the electrical current required to maintain the potential at zero is equal to the sodium transport across the bladder.

Toads used for these studies were kept partially immersed in 0.6 % saline solution for 2 days prior to use, so as to decrease the endogenous secretion of aldosterone. Experiments were carried out using a Ringer's solution containing Na^+, 115; K^+, 3.5; Cl^-, 116.5; HCO_3^-, 2.4 mequiv per l; Ca^{2+}, 0.89 mM and phosphate, 3 mM

at pH 7.6, except for the studies with malonate which were carried out at pH 7.0. Total solute concentration was 230 mosM per kg of water.

The effect of propionate on sodium transport

The half bladders were mounted on double chambers for the measurement of sodium transport. (+)-Aldosterone was added to the serosal medium bathing one quarter bladder to a final concentration of $2 \cdot 10^{-7}$ M. After incubation for 12 h, sodium propionate (10 mM) was added to both tissues. Sodium transport was monitored for a further period of 2 h.

Effects of oxythiamine on α-oxoglutarate dehydrogenase and pyruvate dehydrogenase activity

For each experiment, 4 toads were pithed and their bladders quartered. Four quarter bladders, one from each toad, were incubated in each of 4 erlenmeyer flasks containing 5 ml of frog Ringer's solution with 0.1 mg/ml of penicillin and of streptomycin and $2 \cdot 10^{-7}$ M (+)-aldosterone. 3 mM phosphate buffer maintained the pH at 7.6. Sodium pyruvate (0.5 mM) was added to two of the flasks and ethyl acetoacetate (0.5 mM) to the remaining two. To one flask containing pyruvate and to one flask containing acetoacetate, oxythiamine to a final concentration of 2 mM was added. All the tissues were then incubated for 6 h on a rotary shaker. At the end of this period the tissues were transferred to fresh solutions and 1 μC of [3-^{14}C]-pyruvate and 1 μC of [3-^{14}C]acetoacetate were added, respectively, for an additional 60 min. The tissues were then removed, rinsed twice, and rapidly frozen on solid CO_2.

Tricarboxylic acid cycle intermediates were isolated by the method of Busch, Hurlbert and Potter[13], as follows. The frozen tissues were homogenized and extracted at 0–2°, first with 0.66 M and then with 0.33 M perchloric acid. The perchloric acid-precipitable material was dried at 100° for 48 h and weighed. The extracts were then neutralized with 25% potassium hydroxide solution using phenol red as an indicator, and succinic, malic and fumaric acids separated by elution from a 15-cm Dowex 1-X8, 200–400 mesh, ion–exchange column in formate form, using a 0 to 6 M formic acid gradient[13]. 120 fractions of 1.7 ml were collected automatically. 0.5-ml aliquots in 15 ml scintillation fluid (5 g naphthalene, 0.7 g 2,5-diphenyloxazole, and 0.005 g *p*-bis-(2-(5-phenyloxazolyl))-benzene per 100 ml dioxane were counted in a scintillation spectrometer (Nuclear Chicago Corp., Ill.). ^{14}C-labeled intermediates in the effluent were identified by comparison with known, isotopically labeled intermediates separated under the same conditions. The labeling of the intermediates was expressed as counts/min per 100 mg of perchloric acid-insoluble material.

The effect of malonate on sodium transport

Two series of experiments were performed, one on fresh bladders stimulated with aldosterone, in the presence of pyruvate (1 mM), and one on bladders which had been incubated for 12–15 h prior to the addition of pyruvate (1 mM) and aldosterone ($2 \cdot 10^{-7}$ M). Ringer's solution at pH 7.0 was used as the bathing medium. In both series, when the hormonal stimulation of sodium transport was maximal, the effect of 5, 10 and 20 mM sodium malonate on transport was studied on paired tissues. Sodium malonate was added to the medium in isotonic solution.

The effect of malonate on succinate dehydrogenase activity

After overnight incubation, the bladders of 4 toads were quartered. Four quarter bladders, one from each toad, were placed in each of 4 flasks containing (+)-aldosterone, $5 \cdot 10^{-7}$ M, and 1 mM sodium pyruvate in 5 ml of Ringer's solution at pH 7.0. After 4 h of incubation at room temperature on a rotary shaker, the tissues were transferred to fresh solutions and two different experiments performed. In one, 1 μC of [3-^{14}C]pyruvate was added to each flask. After an additional 45 min, sodium malonate was added to final concentrations of 0, 5, 10 and 20 mM. 15 min later, the tissues were rapidly frozen and the ^{14}C-labeled intermediates separated as previously described. In the other, sodium malonate was added 30 min prior to the 1 μC of [3-^{14}C]pyruvate and 1 mM sodium pyruvate. Labeling was permitted for 30 min before the tissues were rapidly frozen and the ^{14}C-labeled intermediates extracted.

The effect of aldosterone on the activity of the tricarboxylic acid cycle as reflected by the ^{14}C-labeling of succinate and malate with [3-^{14}C]pyruvate

For each experiment, 4 toads were pithed and paired half bladders incubated in erlenmeyer flasks containing 5 ml of frog Ringer's solution with penicillin and streptomycin and $2 \cdot 10^{-7}$ M (+)-aldosterone where appropriate. 3 mM phosphate buffer maintained the pH at 7.6. After 12 h the tissues were transferred to fresh solutions containing 2 mM sodium pyruvate. At the end of a further 60 min the tissues were again transferred to fresh solutions containing in addition 1 μC of [3-^{14}C]-pyruvate. Incubation was continued for 45 min before isolation of the ^{14}C-labeled intermediates as described previously.

RESULTS

The effect of propionate on sodium transport

The response to 10 mM propionate is shown in Fig. 1. Propionate produced a significant increase in short-circuit current in the presence of aldosterone. No such

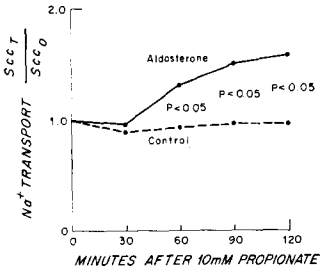

Fig. 1. Sodium transport, after the addition of 10 mM sodium propionate in the presence and absence of (+)-aldosterone, expressed as the short-circuit current at any particular time (Scc_T) divided by the short-circuit current at time zero (Scc_0). Probability of the difference in short-circuit current occurring by chance is less than 5 % at 60, 90 and 120 min.

increase occurred with propionate in the absence of aldosterone. Thus, propionate supports the energy requirements for the stimulation of sodium transport by aldosterone.

Effects of oxythiamine on α-oxoglutarate dehydrogenase and pyruvate dehydrogenase activity

The results of typical experiments are shown in Figs. 2 and 3. Oxythiamine markedly reduced ^{14}C-labeling of succinate, malate, and fumarate when [3-^{14}C]-pyruvate was used as the precursor (Fig. 2), while there was no inhibition of labeling of these intermediates when [3-^{14}C]acetoacetate was used (Fig. 3).

Fig. 4 summarizes the results of 4 experiments, contrasting a 70 % inhibition of [3-^{14}C]pyruvate-labeling of succinate, malate and fumarate with no inhibition of

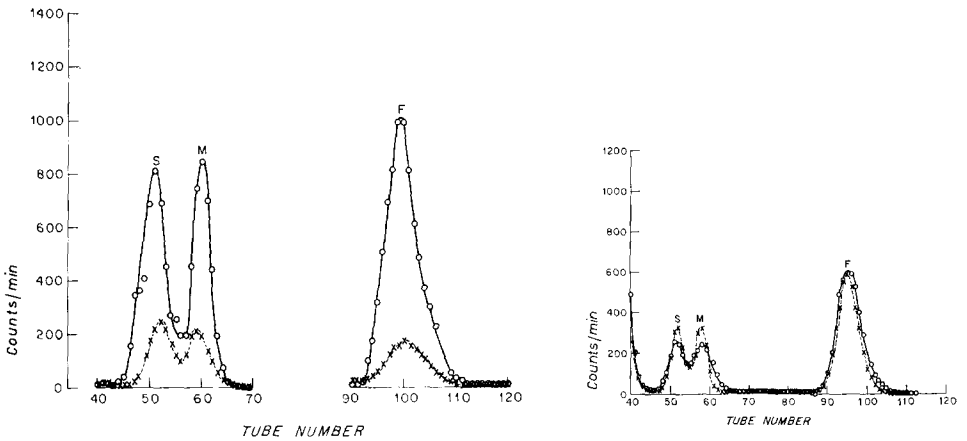

Fig. 2. Inhibition of [3-^{14}C]pyruvate-labeling of succinic (S), malic (M) and fumaric (F) acids in presence of oxythiamine (2 mM), expressed as counts/min per 100 mg perchloric acid-precipitable material. O—O, [3-^{14}C]pyruvate; ×--×, [3-^{14}C]pyruvate *plus* oxythiamine.

Fig. 3. Absence of inhibition of [3-^{14}C]acetoacetate-labeling of succinic (S), malic (M), and fumaric (F) acids, expressed as counts/min per 100 mg perchloric acid-precipitable material. O—O, [3-^{14}C]acetoacetate; ×--×, [3-^{14}C]acetoacetate *plus* oxythiamine.

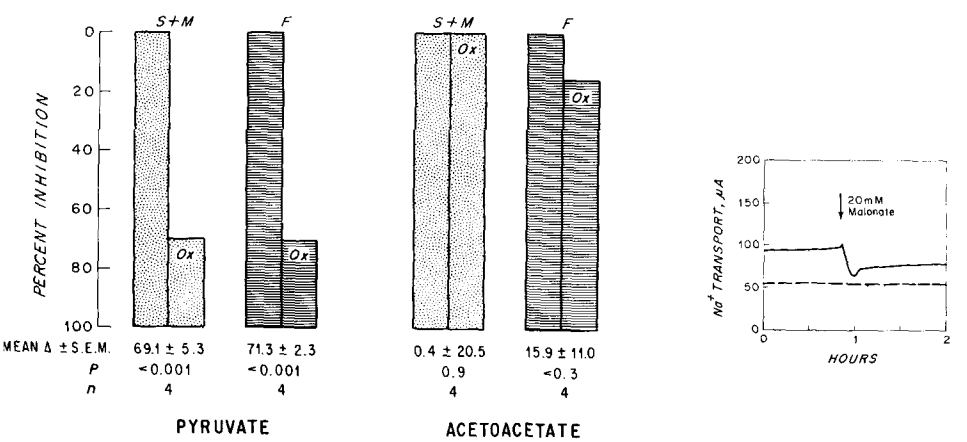

Fig. 4. Summary of 4 experiments demonstrating inhibition of [3-^{14}C]pyruvate-labeling of succinic (S), malic (M), and fumaric (F) acids in presence of oxythiamine (Ox) (2 mM) and absence of inhibition of [3-^{14}C]acetoacetate-labeling of the same acids.

Fig. 5. The effect of malonate, 20 mM, on sodium transport in the presence of (+)-aldosterone ($2 \cdot 10^{-7}$ M) and pyruvate (1 mM).

the [3-^{14}C]acetoacetate-labeling of the same intermediates. Thus, under the conditions of these experiments, oxythiamine produced no detectable inhibition of the α-oxoglutarate dehydrogenase.

Effect of malonate on sodium transport

5 mM malonate had no effect upon sodium transport. Both 10 and 20 mM sodium malonate inhibited sodium transport. Maximum inhibition occurred after approx. 15 min followed by a recovery phase. In the case of 10 mM malonate the recovery was essentially complete whereas with 20 mM malonate the recovery was slight (see Fig. 5).

TABLE I

THE EFFECTS OF SODIUM MALONATE ON SODIUM TRANSPORT IN TOAD BLADDER IN THE PRESENCE OF ALDOSTERONE ($2 \cdot 10^{-7}$ M) AND PYRUVATE (1 mM)

Tissue preparation	*Concentration of malonate (mM)*	*No. of expts.*	*Maximum % inhibition*	*% Inhibition after recovery*
Depleted tissue	10	9	12.1 ± 2.8	5.3 ± 1.8
Depleted tissue	20	7	26.6 ± 4.0	17.8 ± 3.9
Fresh tissue	10	5	9.5 ± 3.0	3.1 ± 1.9
Fresh tissue	20	5	20.2 ± 4.2	16.7 ± 2.3

A summary of the results obtained with malonate is shown in Table I. In depleted tissue stimulated with aldosterone in the presence of pyruvate 10 and 20 mM malonate caused an early maximal inhibition of 12 % and 27 % of sodium transport, respectively. Subsequent recovery to 5 % and 18 % inhibition, respectively, occurred. Similar results were obtained with fresh tissue.

A possible reason for the recovery of sodium transport in the presence of malonate is that succinate concentrations in the tissues rise sufficiently to compete successfully with malonate for sites on the enzyme. Consequently, the labeling of succinate in the presence of malonate was investigated. The results illustrated in

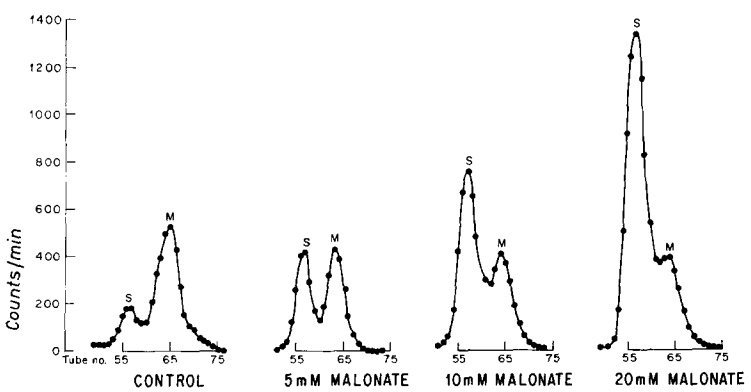

Fig. 6. Labeling of succinic (S) and malic (M) acids by [3-^{14}C]pyruvate in presence of different concentrations of sodium malonate, expressed as counts/min per 100 mg perchloric acid-precipitable material.

Fig. 6 show that succinate-labeling increases almost linearly with increasing malonate concentrations from 5 to 20 mM. Furthermore, experiments in which malonate was added 30 min prior to the ^{14}C-labeled pyruvate showed that fumarate, the product of succinate dehydrogenase activity, was still labeled (see Table II). Therefore, succinate dehydrogenase activity is not markedly blocked even in the presence of 20 mM malonate.

TABLE II

THE INCORPORATION OF ^{14}C, FROM [3-^{14}C]PYRUVATE, INTO FUMARATE AFTER 30 min OF INCUBATION OF THE TOAD BLADDER WITH SODIUM MALONATE IN THE PRESENCE OF ALDOSTERONE

Expt.	*Counts/min in fumarate per 100 mg perchloric acid-insoluble material*		
	Control	*Malonate*	
		10 mM	*20 mM*
1	5910	3471	2286
2	4346	3524	3411

Effect of aldosterone on ^{14}C-labeling of succinate and malate

Table III shows that aldosterone significantly increases the labeling of succinate and malate in the presence of [3-^{14}C]pyruvate. Therefore the increased activity of the tricarboxylic acid cycle which is caused by aldosterone in this tissue[1] is faithfully reflected by increased labeling.

TABLE III

THE INCORPORATION OF ^{14}C, FROM [3-^{14}C]PYRUVATE, INTO SUCCINATE AND MALATE IN THE TOAD BLADDER IN THE PRESENCE AND ABSENCE OF ALDOSTERONE

	Counts/min in succinate and malate per 100 mg perchloric acid-insoluble material				
	Control	*Aldosterone*	*$\Delta \pm$ S.E.*	*P*	*Number of experiments*
Succinate	217	341	124 ± 44	<0.02	11
Malate	442	598	156 ± 50	<0.02	11

DISCUSSION

The stimulation of sodium transport by aldosterone has been shown to depend upon oxidative metabolism. Enhanced activity of the tricarboxylic acid cycle accompanies the hormonal stimulation of transport[1,10,11]. One hypothesis of the mode of action of this hormone is that it stimulates a step or steps in the tricarboxylic acid cycle at a point between condensing enzyme and α-oxoglutarate dehydrogenase[5,12]. This hypothesis rests upon three arguments.

(1) Since the inhibition of the action of aldosterone by oxythiamine is completely reversed by acetoacetate, it is inferred that neither pyruvate dehydrogenase nor α-oxoglutarate dehydrogenase is necessary to the action of aldosterone.

(2) Since malonate was reported not to block the effect of aldosterone in the presence of pyruvate, it was assumed that energy from the tricarboxylic acid cycle beyond succinate was not used to support the stimulation of sodium transport by the hormone.

(3) Since propionate, a precursor of succinyl CoA, was not found to be synergistic with aldosterone a hormonal action prior to succinyl CoA in the tricarboxylic acid cycle was assumed.

Each of these arguments has been examined and found to be inadequate.

Although oxythiamine clearly blocks pyruvate entry into the tricarboxylic acid cycle, the lack of depression of the incorporation of ^{14}C from [3-^{14}C]acetoacetate into succinate and malate in the presence of oxythiamine indicates that α-oxoglutarate dehydrogenase was unaffected. The findings here are in accord with those of GUBLER[14] who found that, in studies *in vivo*, the ability of oxythiamine to inhibit pyruvate dehydrogenase is considerably greater than its inhibitory effect on α-oxoglutarate dehydrogenase. Thus, it cannot be argued that energy for the effect of aldosterone on sodium transport is derived from only that portion of the tricarboxylic acid cycle between condensing enzyme and the α-oxoglutarate dehydrogenase.

In contrast to the results reported by EDELMAN AND FIMOGNARI[12] malonate did inhibit the effect of aldosterone on sodium transport but recovery from initial inhibition was almost complete except with 20 mM malonate. The rise in tissue succinate levels was sufficient to overcome the malonate blockade of succinate dehydrogenase at concentrations of malonate of 10 mM, and even with 20 mM malonate the inhibition was incomplete. This is shown by recovery of sodium transport and incorporation of ^{14}C into fumarate and malate.

Finally, it has been reported that 4.25 mM propionate is not synergistic with aldosterone in the stimulation of sodium transport[5] unlike the precursors of acetyl coenzyme A. This, it is claimed, eliminates the steps from succinate to malate as a requirement for the action of aldosterone because propionate enters the tricarboxylic acid cycle by the route propionyl coenzyme A, methylmalonyl coenzyme A, succinyl coenzyme A, succinate. However, when maximally stimulated by propionate, the tissue in the absence of hormone, could be further stimulated by glucose (see ref. 5, Fig. 2) showing that even the control tissue was substrate limited. This limitation by substrate in both the control tissue and the tissue treated with aldosterone would naturally result in an equal rise in sodium transport. In the experiments reported here using a concentration of propionate of 10 mM, a significantly greater stimulation of sodium transport occurred in the presence of aldosterone than in the control.

The results show that the assumptions upon which was built the hypothesis implicating the first portion of the tricarboxylic acid cycle, as the site of action of aldosterone, are not valid. Furthermore, since malonate does inhibit aldosterone-stimulated transport, and since propionate in sufficient concentration is synergistic with aldosterone, it might be argued that the portion of the cycle beyond succinate is necessary for aldosterone to exert its full effect. It seems likely, therefore, that the presence of a complete and intact tricarboxylic acid cycle is necessary to the action of aldosterone. This conclusion is compatible with an alternative view of the mode of action of aldosterone, namely that the hormone stimulates the entry of sodium ions into the so-called transport pool, probably by the synthesis of a protein permease[10,11]. The consequence of this theory is that the enhanced activity of the

tricarboxylic acid cycle seen in the presence of aldosterone is secondary to the sodium entry step[11].

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