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ALDOSTERONE STIMULATES Na^+ TRANSPORT WITHOUT AFFECTING CITRATE SYNTHASE ACTIVITY IN CULTURED CELLS

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Aldosterone increases citrate synthase activity in toad urinary bladder and mammalian kidney. It has been suggested that this action is important to aldosterone stimulation of Na^+ transport, and it has been used as a marker of those epithelia which are stimulated by aldosterone. We describe three continuous lines of cultured cells derived from toad urinary bladder and toad kidney in which aldosterone increases active Na^+ transport but does not increase the activity of citrate synthase. Therefore, in cultured cells at least, citrate synthase is not a critical enzyme for, or a suitable marker of, aldosterone stimulation of Na^+ transport.

Considerable evidence has accumulated which indicates that induction of citrate synthase is important in the stimulation of Na^+ transport by aldosterone [1]. Aldosterone increases the activity of citrate synthase in toad urinary bladder [2], rat kidney [3,4] and isolated nephron segments from rabbit kidney [5]. In rat and rabbit kidney, these responses appear to be specific for aldosterone, as they are not produced by comparable doses of dexamethasone and may be blocked by spironolactone, an inhibitor of binding to the mineralocorticoid receptor [3–5]. The increase in citrate synthase activity correlates with both the dose-response and the time course of aldosterone-induced changes in Na^+ transport and has been demonstrated to be a consequence of induction of the synthesis of this enzyme [2,3]. Based on these observations it has been proposed that aldosterone regulates mitochondrial metabolism which in turn modulates active Na^+ transport [1]. Moreover, it has been suggested that the induction of citrate synthase is sufficiently specific to represent an enzymatic marker for sites sensitive to the mineralocorticoid actions of aldosterone [5]. We have examined the effect of aldosterone on citrate synthase activity in intact toad urinary bladder and in three continuous lines of cultured cells derived from toad urinary bladder and toad kidney.

Cell lines TB-6C and TB-M derived from the epithelial cells of the toad urinary bladder [6] and line A-6 derived from toad kidney [7] form oriented epithelia in culture and actively transport Na^+ from apical to basal cell surface [6,8]. The time course and the concentration dependence of the response to aldosterone are similar to that of the intact toad urinary bladder. An effect on I_{sc} can be seen following 1.5 h incubation with $1 \cdot 10^{-7}$ M aldosterone, which reaches a plateau within 6 h [9]. Concentration for half-maximal aldosterone effect on Na^+ transport is $8 \cdot 10^{-9}$ M in TB-6C, $4 \cdot 10^{-8}$ M in TB-M and $1 \cdot 10^{-8}$ M in A-6 [9,10].

Cells on 10 cm petri dishes which had been con-

Abbreviations: EGTA, ethyleneglycolbis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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fluent for 2–7 days were incubated for 6 or 18 h in growth medium [6] with or without $1 \cdot 10^{-8}$ M or $1 \cdot 10^{-7}$ M aldosterone. At the end of the incubation, dishes were rinsed three times with ice-cold homogenizing solution containing 5 mM Tris-HCl/1 mM EGTA, pH 7.4. The cells were scraped from the dish and allowed to stand at 4°C for 10 min in homogenizing solution before being collected and disrupted with three strokes of a loose-fitting pestle in a Dounce homogenizer. The solution was centrifuged at $500 \times g$ for 20 min to remove nuclei and cell debris. The supernatant solution was centrifuged at $20\,000 \times g$ for 20 min, resuspended in homogenizing solution and centrifuged again at $20\,000 \times g$ for 20 min. The final pellet was resuspended in 1 ml homogenizing solution and sonicated for 15 s four times in a Bransom sonicator at a setting of 7. The sonicate was quickly frozen to −70°C and assayed within 3 days. Storage for this period of time had no effect on enzyme activities. Ratio of activity of citrate synthase between mitochondrial pellet and crude homogenate was 1.5 for intact toad bladder and 2.3 for cultured cells. There was minimal citrate synthase activity in the supernatant of the mitochondrial pellet, ratios of activity between pellet and supernatant being 5.3 and 7.9 for intact road bladder and cultured cells, respectively. Urinary bladders from the toad (*Bufo marinus*) were excised and incubated for 18 h in flasks with or without $1 \cdot 10^{-7}$ M aldosterone as described by Kirsten et al. [2]. Epithelial cells were then scraped off the bladders with a glass slide and mitochondrial enriched pellets were prepared as described above. Citrate synthase activity was assayed by the method of Srere [11]. Assays were conducted in a final volume of 1 ml containing 100 μM acetyl-coenzyme A, 230 μM oxaloacetate, 100 μM DTNB and 200 mM Tris-HCl (pH 8.1) in a cuvette in a Gilford Spectrophotometer at room temperature. Cultured cells were also assayed with 47 μM or 1 mM acetyl-CoA and showed no difference in enzyme activity between control and aldosterone-treated cells. Initial velocity was estimated by recording change in absorbance at 412 nM every 30 s for 2 min. The reaction rate was linear over this time. There was no significant activity when oxaloacetate was omitted. Succinic dehydrogenase was measured by the method of Pennington [12].

The effect of aldosterone on mitochondrial citrate synthase activities in cultured cell lines is shown in Table I. None of the cultured cell lines showed any change in citrate synthase activity following 18 h incubation with $1 \cdot 10^{-7}$ M aldosterone, though an effect similar to that previously reported could be demonstrated in intact toad urinary bladder. Because of the possibility that the response could have become refractory to prolonged stimulation with this concentration of aldosterone, enzyme activity was also measured following incubation with $1 \cdot 10^{-7}$ M aldosterone for 6 h or following 18 h incubation with $1 \cdot 10^{-8}$ M aldosterone. In no case was there any increase in citrate synthase activity. Similarly, no change in enzyme activity could be demonstrated with 2 h incubation with $1 \cdot 10^{-7}$ M aldosterone (data not shown). The K_m for oxaloacetate and acetyl-CoA were determined for lines TB-M and A-6 and epithelial cells from intact toad urinary bladder (Table II). K_m values for each substrate in the cultured cell lines were about an order of magnitude higher than values reported for rat and rabbit kidney [3,5], but similar to values obtained from intact toad urinary bladder. We also measured the activity of a second enzyme, succinic dehydrogenase, in the presence and absence of aldosterone. Activities of succinic dehydrogenase were similar in cells incubated in control medium or medium supplemented with $1 \cdot 10^{-7}$ M aldosterone (Table I).

There are several possible explanations for this apparent dissociation between the Na^+ transport and citrate synthase responses to aldosterone. One possibility is that the increase in Na^+ transport induced by aldosterone in cultured cells might be mediated through a fundamentally different hormone-receptor complex than the mineralocorticoid receptors described for toad urinary bladder or mammalian kidney. Compounds which are relatively 'pure' glucocorticoids in mammals are known to have significant effects on sodium transport in toad bladder [13], frog skin [14] and the three cultured cell lines employed in this study [9,10]. In frog skin, dexamethasone is a more potent stimulator of Na^+ transport than aldosterone, though the effects of both may be inhibited by spironolactone [14]. In intact toad urinary bladder, dexamethasone stimulates both Na^+ transport and citrate synthase activity [15]. It has been observed that the classic distinctions of corticosteroids as either mineralocorticoid or glucocorticoid are somewhat blurred in amphibians [14,16]. Whether

TABLE I

EFFECT OF ALDOSTERONE ON CITRATE SYNTHASE ACTIVITY AND SUCCINATE DEHYDROGENASE ACTIVITY IN CULTURED EPITHELIAL CELLS

$n = 4$ for each observation, except intact toad urinary bladder where $n = 5$. Values are ± S.E.

	Toad bladder	TB-6C	TB-M	A-6
Citrate synthase activity (nmol/min per mg protein)				
Control	304 ± 24	187 ± 4.7	144 ± 5.6	205 ± 2.1
10^{-7} M aldosterone, 18 h	422 ± 46 [a]	178 ± 1.3	145 ± 3.2	193 ± 3.2
Control	–	–	221 ± 3.6	185 ± 4.8
10^{-8} M aldosterone, 18 h	–	–	226 ± 6.8	184 ± 12.1
Control	–	–	267 ± 4.2	234 ± 1.7
10^{-7} M aldosterone, 6 h	–	–	282 ± 11.8	234 ± 1.6
Succinate dehydrogenase activity (nmol/min per mg protein)				
Control	16.6 ± 2.8	5.3 ± 0.20	6.4 ± 0.42	4.5 ± 0.03
10^{-7} M aldosterone, 18 h	20.2 ± 3.6	5.2 ± 0.01	6.2 ± 0.40	5.0 ± 0.60

[a] $P < 0.025$ compared to control.

the physiologically mineralocorticoid action of aldosterone on cultured cells is mediated via a classical mineralocorticoid or glucocorticoid receptor remains to be determined. Such a sodium transport response, if it occurs through a glucocorticoid receptor and hence via a different pathway (in this case not involving citrate synthase), has not been previously described.

A second possibility is that the previously described effects of aldosterone on Na^+ transport and citrate synthase may be independent effects which are not causally related. We have observed stimulation of Na^+ transport without an effect on citrate synthase by aldosterone. Marver and colleagues have observed effects of aldosterone on citrate synthase not only on nephron segments which have identifiable mineralocorticoid receptors and a transport response to aldosterone [5], but also in medullary collecting tubule and thick ascending limb of Henle [17], sites which demonstrate either no apparent transport response to mineralocorticoids [18] or no apparent mineralocorticoid receptors [19], respectively. We conclude that, in cultured cells at least, citrate synthase is not a critical enzyme for aldosterone-stimulated Na^+ transport and would not represent an adequate marker for sites responsive to aldosterone.

TABLE II

K_m OF SUBSTRATES FOR CITRATE SYNTHASE

K_m (X10^{-5}) for each substrate was determined by varying the substrate concentration over a range of 25–200 μM at two fixed concentrations of the cosubstrate, either 100 μM (A) or 200 μM (B). K_m was computed from Lineweaver-Burk plots. $n = 4$ for each point tested. Data are presented as means ± S.E.

	Acetyl Co-A		Oxaloacetate	
	A	B	A	B
Toad bladdder	5.8 ± 0.2	5.6 ± 0.8	3.2 ± 0.6	3.1 ± 1.1
TB-M	8.8 ± 0.5	7.6 ± 1.8	3.2 ± 0.7	2.1 ± 2.4
A-6	9.3 ± 0.4	11.5 ± 0.3	4.8 ± 0.4	4.3 ± 0.3

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