Evidence for Concerted Effects of Aldosterone on a Target Sodium-transporting Epithelium

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ABSTRACT The sodium-transporting activity of toad skin is stimulated in vitro with aldosterone in the absence of energy-providing substrate; it can be stimulated further upon addition of glucose after prolonged (overnight) incubation. The magnifying effect exerted by glucose in these conditions could be blocked by inhibitors of ribonucleic acid and protein biosynthesis. In addition, exposure to cycloheximide prevented the increase in thermodynamic affinity resulting from aldosterone treatment.

A synthetic 19-nor steroid, (RU 24411), dimethyl-2,2-hydroxy-21-nor-19-pregnene-4-dione-3,20, also stimulated sodium transport by toad skin incubated in the absence of glucose, but there was no magnifying effect of this substrate. Furthermore, there was no change in thermodynamic affinity with RU 24411.

Therefore, the magnifying effect seen with glucose and the increase in thermodynamic affinity are not necessarily integral parts of the response of sodium-transporting epithelial to "mineralocorticoids."

INTRODUCTION

Soon after it proved possible to reproduce in vitro the stimulating effect of aldosterone on transepithelial sodium transport (1), the underlying mechanism started being a subject of controversy. Model preparations such as amphibian abdominal skin or urinary bladder carry out sodium transport from the epithelial (apical) to the corial (basal-lateral) side as the eventual consequence of increased activity of the sodium "pump." The limiting factor for the rate of sodium transport by these specialized cells is usually represented by the permeability for this ion at the apical border of the outermost cell layer (2). Thus, an increase in transepithelial sodium transport, as seen with aldosterone, could arise from increased apical permeability towards sodium; consequentially, there would be increased availability of this ion to the pump. Alternately, the hormone might somehow improve the interaction of sodium with the pump, leading to a decrease in the activity of the ion in the cytoplasm which, in turn, would accelerate transfer at the apical border as a consequence of a steeper electrochemical potential gradient across that border.

Some investigators are of the opinion that aldosterone acts at the apical border of target epithelia, while others favor the interpretation that the steroid hormone influences the sodium pump more directly (3). One significant argument for the latter view is that glucose or other energy-providing substrates magnify the aldosterone response of steroid-depleted amphibian epithelia (4). For this reason, long-term incubation is often performed in glucose-enriched media.

In contrast to the slowly developed aldosterone response (with or without glucose), addition of substrate to tissue incubated overnight with aldosterone alone leads rapidly to a significant further increase in sodium transport (5).

It will be shown that the additional response of aldosterone-treated toad skin to glucose could be blocked selectively with cycloheximide or actinomycin D when these drugs are added before glucose, yet after a stable aldosterone effect on transepithelial sodium transport was obtained. This is taken as evidence that more than one species of ribonucleic acids and proteins are under the regulatory influence of aldosterone, with appreciable differences in terms of biological half-life. These

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findings are in keeping with the observation that a synthetic steroid, dimethyl-2-2-hydroxy-21-nor-19-pregnene-4-dione-3,20 (RU 24411),[1] could bring about an increase in sodium-transporting activity after incubation overnight without subsequent substrate-elicited magnification of this activity.

METHODS

Toads, *Bufo marinus*, of both sexes from the Dominican Republic, were kept on peat with free access to water, and fed once weekly until death by pithing. Ventral skin was dissected and the appropriate number of fragments were incubated in Ringer's solution (composition: 115 mM NaCl, 2.5 mM $KHCO_3$, and 1 mM $CaCl_2$, pH 7.8 when aerated). One piece of skin per animal served as a reference for matched pieces treated with a steroid:aldosterone (from Sigma Chemical Co., St Louis, MO), RU 24411 from Roussel-UCLAF, Paris) and spirolactone (SC 14266; from Boehringer Mannheim, Mannheim, Federal Republic of Germany). Final concentration ranged from 2 nM to 50 μM; when solubility in Ringer's solution was low, the steroids were dissolved first in ethanol, with final concentration of this solvent in Ringer's solution never >0.1% (vol/vol).

Incubation usually proceeded overnight at room temperature in beakers before transfer (the following morning) to incubation chambers designed for measurement of transepithelial electrical potential and of short-circuit current (6). Exposed area was usually 3.14 cm^2. Occasionally, the preparations were set up in the chambers from the outset in which case they were left in the open-circuit state during the night. Glucose, introduced on the corial side as the energy-providing substrate at the final concentration of 10 mM, was usually added in the morning. For those experiments with glucose-containing Ringer's solution from the beginning, antibiotics (1,000 U/ml penicillin and 1 mg/ml streptomycin) were added to the incubation solution.

When used, cycloheximide and actinomycin D were introduced in the solution on the corial side several hours after starting incubation with aldosterone, as noted in Results. Final concentrations were 20 and 5 μM, respectively.

Oxygen consumption was measured by polarography under short-circuit conditions, as well as during short periods of voltage clamping, for calculation of the thermodynamic affinity of the system (7, 8).

For statistical analysis, whenever possible, the difference in short-circuit current (SCC) across matched preparations was considered before glucose:$SCC_{steroid} - SCC_{control}$. The effect of the substrate was evaluated by subtracting the resulting change noted occasionally in controls from that occurring concomitantly in matched steroid-treated preparations: $\Delta SCC_{steroid}$(after − before glucose) − $\Delta SCC_{control}$(after − before glucose).

RESULTS

Aldosterone and sodium-transporting activity of toad skin: effect of glucose. As is the case for the toad bladder (4) the response to aldosterone of toad skin incubated overnight is magnified in the presence of glucose (5). The final sodium-transporting activity of skin treated overnight with aldosterone is the same whether glucose was present from the outset or was added in the morning (Fig. 1). Incidentally, after overnight exposure to glucose, the transepithelial electrical potential difference was lower than shortly after addition of this substrate (Fig. 1) so that ohmic conductance was significantly higher in the former case (1.66 mS/cm^2 vs. 0.99 mS/cm^2; $\Delta\pm$SE: 0.67±0.21; $P < 0.02$). On the other hand, aldosterone alone led to an increase in conductance by the end of the incubation (0.71 mS/cm^2 for treated preparations vs. 0.52 mS/cm^2 for controls, $\Delta\pm$SE: 0.19±0.04; $P < 0.01$).

Since the addition of glucose in the morning makes it possible to assess separately the influence on sodium transport of aldosterone and the magnifying effect of glucose, this two-step approach was usually adopted.

The following experiments were designed to evaluate the effect of aldosterone concentration on the subsequent glucose response. Four pieces of the abdominal skin of *B. marinus* were incubated overnight simultaneously, with one serving as a reference for three pieces exposed to 1, 10, and 100 nM aldosterone, respectively; 10 mM glucose was added in the morning. As seen from Table I, there was no significant stimulation of sodium transport at the low steroid concentration selected, either before or after glucose. On the other hand, while aldosterone was not significantly more potent at 100 nM than at 10 nM before glucose

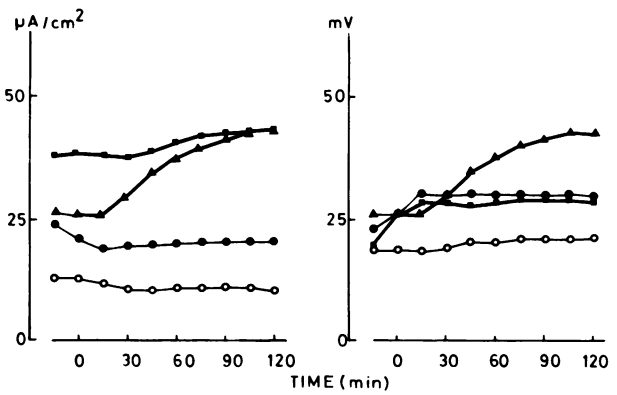

FIGURE 1 The effect of glucose on the aldosterone-induced sodium transport increase in isolated toad skin. In nine instances, four fragments of the abdominal skin of *B. marinus* were incubated simultaneously according to Ussing and Zerahn (6). One piece served as control (○), while the three other pieces (■, ▲, ●) were exposed to 10 nM aldosterone. Glucose was present on the corial side throughout in one case (■), or it was added in the morning (at "zero" time: ▲). The third piece exposed to aldosterone did not receive glucose (●). Short-circuiting was briefly interrupted at 15-min intervals for transepithelial electrical potential readings. Thick lines indicate addition of glucose.

[1] *Abbreviations used in this paper:* RU 24411, dimethyl-2,2-hydroxy-21-nor-19-pregnene-4-dione-3,20.

TABLE I

Stimulation by Aldosterone of Sodium Transport by the Isolated Toad Skin: Role of Concentration of the Hormone

Concentration of aldosterone	Steroid effect on short-circuit current	
	Base-line conditions*	Response to glucose‡
nM	*μA/cm²±SE*	
1	1.5±0.9	1.4±1.7
10	12.4±2.6	14.9±3.1
100	17.2±3.8	28.9±4.4

In 10 instances, four fragments of the abdominal skin of *B. marinus* were incubated overnight in Ringer's fluid, one piece serving as a reference for the other ones exposed to aldosterone at the concentrations stated. The following morning, short-circuit current was recorded first for half an hour before glucose, and during the 4th half-hour that followed addition of the substrate on the corial side (10 mM). The hormonal effect in base-line conditions (*) was evaluated by subtracting short-circuit current values of matched untreated skin; for the response to glucose (‡), the occasional change in short-circuit current observed with the untreated preparations was subtracted from the change in the matched aldosterone-treated preparation: see Methods.

(Δ±SE: 4.8±3.1 μA/cm²), the magnifying effect of this substrate was more apparent at 100 nM than at 10 nM (Δ±SE: 14.0±4.3 μA/cm²).

Complementary experiments consisted of incubating toad skin overnight with 5, 50, or 500 nM aldosterone, vs. control, 10 mM glucose being present throughout (n = 6). In these conditions, at the low hormone concentration, the effect was two thirds of what it was at 50 nM (16.7 vs. 23.9 μA/cm²; Δ±SE: 7.2±2.8; P = 0.05). When aldosterone concentration was raised from 50 to 500 nM, short-circuit current failed to increase further.

Specificity of the glucose effect. The magnifying effect of glucose on sodium-transporting activity of toad skin incubated with aldosterone overnight could still be demonstrated when the metabolic expenditure of this tissue had been reduced by absence of sodium on the epithelial side overnight (9). On the other hand, there is no magnifying effect of glucose with indanone, a drug that increases apical conductance for sodium when added to the epithelial side (10, 11). Indeed, for indanone-treated toad skin incubated overnight, short-circuit current increased to the same extent whether or not Ringer's solution contained glucose (Δ±SE: 4.1±5.4 μA/cm²; n = 5).

Selective block of the glucose effect on aldosterone-treated toad skin with inhibitors of ribonucleic acid and of protein biosynthesis. (*a*) Cycloheximide: moderately short treatment (3–6 h) with cycloheximide after aldosterone had exerted its effect on sodium transport by toad skin made it possible to selectively block the increase in sodium-transporting activity that results from addition of glucose to toad skin incubated overnight with aldosterone.

As seen in Fig. 2, when cycloheximide was left in contact with toad skin for 6 h after incubation overnight with aldosterone, the hormonal effect was unaffected (14.0±2.7 μA/cm² vs. 11.5±2.9 μA/cm²). Treatment with cycloheximide for 15 h did decrease the hormonal effect obtained in the absence of glucose (from 9.5±4.2 μA/cm² to 2.8±3.0 μA/cm²). Incidentally, some recovery could still be observed upon removal of cycloheximide after this period of time, since the hormonal effect had again reached statistical significant 4 h after withdrawal of cycloheximide (Δ±SE: 5.9±2.2 μA/cm²).

After overnight incubation with aldosterone and subsequent exposure to cycloheximide for 1–2 h, the effect of glucose was already appreciably blunted with the current barely rising, from 22.2 to 24.0 μA/cm², while it increased from 24.2 to 35.8 μA/cm² for matched aldosterone-treated preparations in the absence of cycloheximide (difference in glucose effect: 10.5 μA/cm²±1.7; n = 4).

This block might be selective for glucose as aldosterone-treated preparations exposed to cycloheximide for 3–4 h responded to indanone in that short-circuit current increased 28±10% (SE) within the ensuing half hour (n = 8). Such results point at the short half-life of the protein species involved in this glucose effect.

(*b*) Actinomycin D: since cycloheximide made it possible to interfere with the glucose effect without significant attenuation of the base-line hormonal stimulation of sodium transport, additional experiments were carried out with actinomycin D, which inhibits ribonucleic acid (RNA) synthesis.

As appears from Fig. 3, when this antibiotic was added 5 or 10 h before glucose (yet several hours after aldosterone) the hormone was still fully active in the absence of substrate; on the other hand, the response to glucose was quite reduced, possibly more so when exposure to the inhibitor lasted for 10 rather than 5 h.

Lack of glucose effect on toad skin exposed to other steroids. A synthetic steroid first considered as interfering with the action of aldosterone in vivo (12), proved capable of bringing about a stimulation of sodium transport by toad skin, without a demonstrable glucose effect.

As illustrated on Fig. 4, RU 24411 appeared as capable of stimulation of sodium transport as aldosterone in the absence of glucose; only with the hormone was there a response to glucose.

RU 24411 seems to interact with toad skin in a standard manner for steroids, as the stimulation of sodium

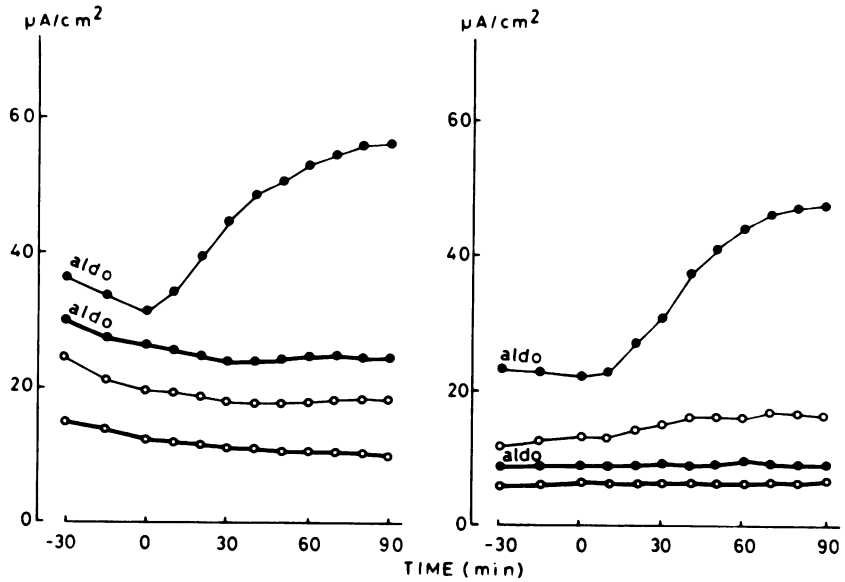

FIGURE 2 Effects of cycloheximide on the sodium-transporting activity of toad skin incubated overnight with aldosterone. Four pieces of the ventral skin of *B. marinus* were incubated simultaneously with (two pieces: ●) or without (two pieces: ○) 50 nM aldosterone for ~24 h. After 14 (*left*, $n = 9$) or 7 h (*right*, $n = 6$), one of the untreated pieces as well as one of the pieces treated with the hormone, were exposed to cycloheximide, 20 μM (heavy tracing). 6 (*left*) or 15 h later (*right*) at 0 min on the time scale, glucose was added on the corial side in all cases; final concentration was 10 mM.

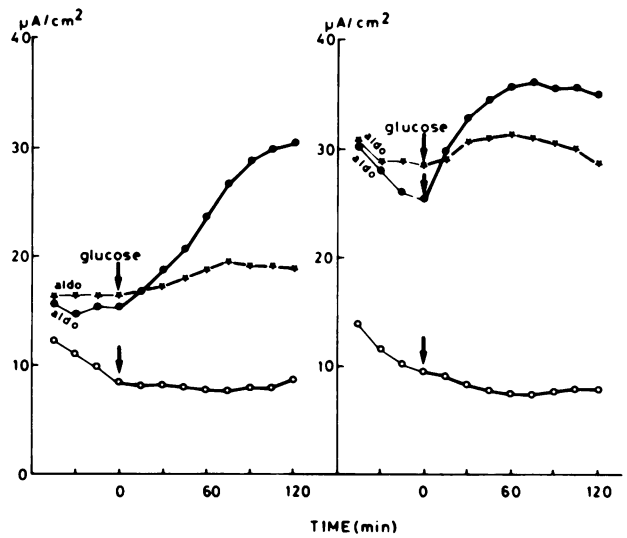

FIGURE 3 Effects of actinomycin D on the sodium-transporting activity of toad skin incubated overnight with aldosterone. Three pieces of the ventral skin of *B. marinus* were incubated simultaneously with (two pieces ●, ★) or without (one piece: ○) 50 nM aldosterone for ~20 h. After 8 h (*left*; $n = 5$) or 14 h (*right*; $n = 5$), one of the hormone-treated pieces (★) was exposed to 5 μM actinomycin D. 10 (*left*) or 5 h later (*right*), glucose was added on the corial side everywhere, at 0 min on the time scale; final concentration was 10 mM.

transport is demonstrable only after a latency period, and is inhibited by a spirolactone. Indeed, overnight incubation of toad skin in the presence of 50 μM spirolactone led to total inhibition of the effect of 1 μM RU 24411 ($\Delta \pm SE$: 3.0 ± 4.4 μA/cm²; $n = 6$).

To shed additional light on the mechanism of action of RU 24411, experiments were conducted with a large excess of this compound with respect to aldosterone so as to check whether it would interfere with the full effect of aldosterone. As indicated by Table II, it did not. In other words, the glucose effect, strikingly absent upon prolonged incubation of toad skin with RU 24411 alone, while present with aldosterone alone, was as apparent as in the latter case when the preparation was exposed to both steroids. This suggests that the mechanism underlying the substrate effect might depend on a distinct chain of events with respect to the stimulation demonstrable in glucose-free media.

Thermodynamic affinity. Thermodynamic affinity has been proposed as a measure of the metabolic force driving sodium transport (7). We have confirmed that aldosterone increases thermodynamic affinity in toad skin (8, 13). Even though the precise meaning of this parameter is debated, as the increase observed with aldosterone appears rather specific, it was thought of interest to measure thermodynamic affinity in hormone-treated, cycloheximide-exposed toad skin prep-

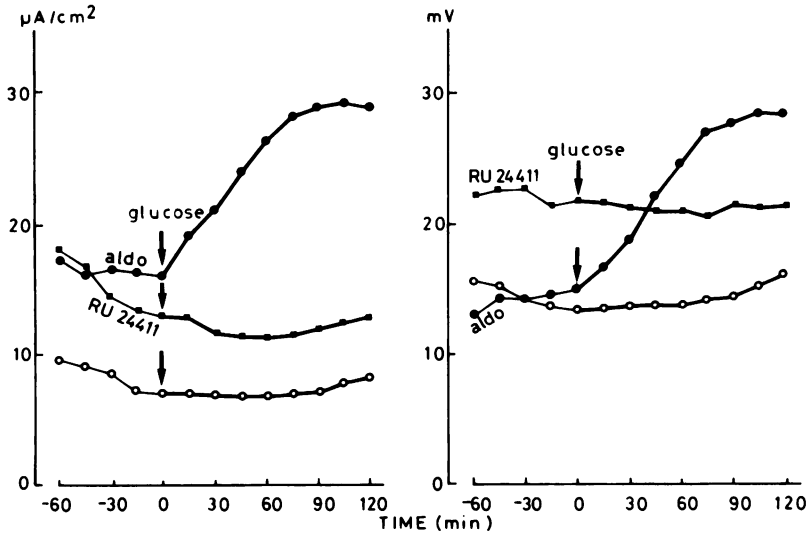

FIGURE 4 Lack of effect of glucose on toad skin incubated overnight with RU 24411. Three pieces of the abdominal skin of *B. marinus* were incubated simultaneously overnight in seven instances; one served as a reference (○) for the fragments exposed from the outset to 50 nM aldosterone (●) vs. 1 μM RU 24411 (■). The following morning, all preparations were short-circuited, save for brief interruption at 15-min intervals so as to measure the transepithelial electrical potential difference. 1 h later, glucose was added to the compartment corresponding to the corial side, final concentration being 10 mM. Just before addition of glucose, the effect of aldosterone averaged 8.8 $\mu A/cm^2 \pm 3.1$ and that of RU 24411, 6.1 $\mu A/cm^2 \pm 1.3$, with respect to matched reference preparations.

arations as well as in preparations treated with RU 24411. In neither case was there an increase in this parameter (Table III).

TABLE II
Effects of Aldosterone and RU 24411, Isolated or Combined, on Sodium Transport by the Isolated Toad Skin

Steroid assayed	Steroid effect on short-circuit current	
	Base-line conditions*	Response to glucose‡
	$\mu A/cm^2$	
Aldosterone	15.0±3.9	12.2±5.2
RU 24411	6.7±3.2	−1.7±7.6
RU 24411 + aldosterone	21.3±4.3	10.0±5.0

In eight instances, four fragments of the abdominal skin of *B. marinus* were incubated overnight in Ringer's fluid, one piece serving as a reference for the other pieces exposed to aldosterone (20 nM), RU 24411 (20 μM) or both. The following morning, short-circuit current was recorded for half an hour before adding glucose, at the final concentration of 10 mM on the corial side, and during the 4th half-hour that followed. The hormonal effect in base-line conditions (*) was evaluated by subtracting short-circuit current values of matched untreated skin; for the response to glucose (‡), the occasional change in short-circuit current observed with the untreated preparation was subtracted from the change obtained in the matched aldosterone-treated one (see Methods).

DISCUSSION

It appears justified to distinguish two components in the response of toad skin to aldosterone in vitro: (*a*) a stimulation of transepithelial sodium transport after exposure to the hormone overnight in base-line conditions (i.e., in the absence of energy-providing substrate such as glucose), and (*b*) a prompt magnification of this aldosterone effect subsequent to the addition of glucose. Only occasionally do untreated toad skin preparations react to glucose by an increase in sodium transporting activity, while this is the rule for hormone-exposed preparations (5). Similarly, the aldosterone-induced stimulation of hydrogen ion secretion by turtle bladder is magnified by glucose (14).

Electrophysiological data led investigators to suggest that this glucose effect on toad skin corresponds to an enhancement of the activity of the sodium "pump" in these circumstances (15), while prolonged aldosterone treatment alone results mainly in increased apical conductance for sodium (16). Thus, the two schools of thought alluded to in the Introduction, concerning the site of action of aldosterone on target sodium-transporting epithelia, need not be incompatible (17). Rossier et al. (18) have recently raised the possibility of dual effects of aldosterone, since transepithelial conductance increased only during the early phase of the

TABLE III

Effect of Aldosterone and Cycloheximide, and of RU 24411 on Sodium Transport and Thermodynamic Affinity: Studies on the Isolated Toad Skin

	Number of paired preparations	Short-circuit current	Thermodynamic affinity*
		$\mu A/cm^2$	*kcal/mol* O_2
Untreated		15	21
Aldosterone		41	49
Δ±SE	10	16±6‡	28±7‡
Cycloheximide		31	35
Aldosterone + cycloheximide		48	37
Δ±SE	7	17±7‡	2±5
Untreated		19	32
RU 24411		36	35
Δ±SE	6	17±6‡	3±10

Toad skin was incubated for ~18 h with or without 50 nM aldosterone, or 1 μM RU 24411, on the corial side. The aldosterone response was tested in the presence of 20 μM cycloheximide added to the serosal solution after 9 h incubation with or without aldosterone, and 9 h before these measurements.

* Calculated according to Essig and Caplan (7).

‡ Statistically significant difference between matched preparations at the 0.05 level or less.

response of toad bladder to aldosterone. In this respect, it is noteworthy that conductance across toad skin, increased after prolonged exposure to aldosterone, changed no further during the 2-h period that followed addition of glucose in the morning despite a further rise in sodium-transporting activity (Fig. 1).

The glucose-dependent component could be blocked almost selectively by short treatment with cycloheximide and actinomycin D, while preparations treated that way could still respond to indanone, thus implying the integrity of the metabolic machinery of sodium-transporting cells. It is therefore suggested that a protein material of relatively short half-life is involved in this glucose effect, whereas that of the protein(s) held responsible for the hormonal effect, demonstrable in the absence of glucose, would be much longer.

Interestingly, the influence of glucose is clear-cut only after a long incubation (4); we have confirmed that this substrate failed to alter the responsiveness to aldosterone of fresh toad skin and bladder. It may be that the effect of glucose becomes significant, in the conditions defined, only after the supply of some endogenous substrate is exhausted; yet, even so, aldosterone remains essential for the glucose effect to develop. For instance, indanone, a drug stimulating transepithelial sodium transport sustainedly when applied on the epithelial surface (8), failed to bring it about. Furthermore, there is a glucose effect when overnight incubation was conducted with aldosterone but without sodium available for transport (9), which again rules out increased metabolic fuel consumption as the factor underlying this effect. It is tempting to relate the responsiveness to glucose of aldosterone-treated amphibian epithelia to "improved" energetics of the sodium pump, located at the basal-lateral border of target cells. In this respect, the increase in thermodynamic affinity occurring after prolonged treatment (8, 13) possibly reflects an increase in ATP availability (19) itself being the consequence of hormone-dependent induction of mitochondrial enzymes (20, 21).

In view of such results, the hypothesis could be raised that the glucose effect and the increase in thermodynamic affinity, both of which appear to be selectively blocked by cycloheximide, are reflections of increased availability of ATP to the sodium pump.

It was therefore of particular interest that RU 24411, a synthetic 19-nor steroid (12), reproduced only the base-line effect of aldosterone on sodium transport by toad skin and failed to increase thermodynamic affinity.

Since RU 24411 failed to bring about the glucose effect seen with aldosterone without interfering with the full hormonal response, the possibility must be considered that RU 24411 reacts with only part of the mineralocorticoid receptor complex, rather than fitting the definition of a "partial agonist." This hypothesis deserves specific examination.

There was but a small difference in the dose-response relationship when the two components of the full aldosterone effect on sodium transport by toad skin

are examined separately (Table I). Thus, it is unlikely that the glucose effect results from interaction with the low affinity binding sites reported for toad bladder, since in terms of binding characteristics these sites differ from the high affinity sites by almost three orders of magnitude (22).

Since the induction hypothesis has been proposed (23), biochemical studies have indicated that more than one protein species is under the regulatory influence of aldosterone (24, 25); the data presented and discussed here suggest that these proteins assume different physiological roles in the sodium-transporting process.

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