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INFLUENCE OF OUABAIN ON SODIUM TRANSPORT BY ALDOSTERONE-STIMULATED AMPHIBIAN EPITHELIA

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SUMMARY

Exposure to ouabain of isolated toad epithelia such as urinary bladder or abdominal skin, was regularly followed by decreases of short-circuit current and, to a lesser extent, of electrical potential difference; the inhibition was a function of ouabain concentration, and it proved reversible upon withdrawal of the glycoside from the incubation solution. Frog skin behaved similarly, with the notable exception that it was found to be much more sensitive to the glycoside.

When toad and frog preparations were treated with aldosterone, either *in vivo* or *in vitro*, the hormonal effect on transepithelial sodium transport was still detectable in the presence of ouabain. On the other hand, the increment in sodium transport occurring when glucose was added to aldosterone exposed substrate-depleted (toad) tissue, was quite sensitive to ouabain inhibition so that this increment could be eliminated at concentrations of the glycoside producing only moderate effects on baseline sodium-transporting activity.

Thus the influence exerted by aldosterone after prolonged treatment of the preparations *in vitro* appears complex: aside from hormone-induced changes at the sodium permeability barrier, the sodium "pump" might be involved. Whether these are manifestations of 2 distinct primary effects of aldosterone in the target cells is yet to be established.

INTRODUCTION

The action of cardiac glycosides on cells appears to be specifically concerned with inhibition of ion movement, mainly of active sodium transport across the plasma membrane[1, 2]. Furthermore, these compounds provide a means whereby the density in, and characteristics of, sodium "pumps" can be evaluated on intact cells [3, 4]. Ouabain also blocks transepithelial sodium transport as first shown by Koefoed-Johnsen with the isolated frog skin[5]. Advantage was therefore taken of this property in order to shed additional light on the mechanism(s) of aldosterone-induced stimulation of sodium transport across toad and frog bladder and skin[6]. Some investigators have concluded that this steroid hormone acts by facilitating sodium access to the "pump", through an effect at the apical cell border[7–9]; while others believe that sodium transport is stimulated through a hormonal effect at, or close to, the "pump" located at the other cell borders[10–12]. This of course means that the simplest formal approach was adopted to account for transcellular sodium movement: it is implied that the latter involves first carrier-mediated penetration from the outside into the cell proper at its apical pole, and that this is a passive step at least when the preparations are incubated in Ringer's fluid; then sodium somehow proceeds to the sodium "pump" located at the other cell borders; thus, provided fuel—ATP—is available, sodium is expelled from these specialized cells towards the *milieu intérieur*[13].

It was thought that, if aldosterone were to influence the operation of the "pump", this could be reflected in a change in the sensitivity of hormone-stimulated preparations to ouabain.

1. *Effects of ouabain on untreated amphibian epithelia*

As a preliminary, the effects of ouabain on active transepithelial sodium transport by untreated amphibian epithelia were studied using the incubation technique devised by Ussing and Zerahn[14].

In the case of toad (*Bufo marinus*) epithelia—bladder and skin—several characteristics deserve mention[15]:

(1) upon exposure to ouabain, a new steady state is attained (in terms of sodium transport) after a period of time of approximately 1 h for the urinary bladder

Table 1. Effect of ouabain on fresh toad bladder (Means ± S.E.)

	Short-circuit current ($\mu A/cm^2$)	Transmembrane electrical potential difference (mV)	Electrical resistance ($\Omega \cdot cm^2$)
Before ouabain	74·0 ± 7·0	62·1 ± 3·8	946 ± 112
	74·4 ± 7·6	62·1 ± 3·7	956 ± 112
	74·5 ± 8·1	61·6 ± 3·5	958 ± 111
Ouabain present	23·5 ± 3·8	27·4 ± 3·3	1281 ± 128
	22·1 ± 3·7	25·5 ± 3·1	1257 ± 123
	21·5 ± 3·7	25·1 ± 3·2	1275 ± 115
After removal of ouabain	58·6 ± 6·6	50·4 ± 5·4	934 ± 83
	59·6 ± 6·6	51·3 ± 5·4	906 ± 81
	59·9 ± 6·8	51·4 ± 5·0	902 ± 80

Ouabain, 5×10^{-5} M, was added to the solution on the inside after $3\frac{1}{2}$ h of incubation, and left in contact with the preparation for 2 h; thereafter solutions were replaced with fresh, glycoside-free Ringer's fluid and incubation continued for $1\frac{1}{2}$ h.

Values given are for consecutive readings, taken at 15 min intervals, during the last half-hour of each of the 3 phases of the incubations; electrical resistance was calculated after Ohm's law. Preparations ($N = 14$) were kept short-circuited throughout. Incubation area was 2 cm^2.

and of 2–3 h for the abdominal skin; this has as a consequence that prolonged incubations could be carried out in the presence of ouabain, which offers a distinct advantage since the *in vitro* effect of aldosterone on sodium transport by these preparations is fully developed only after several hours[16];

(2) the relative insensitivity of toad tissues to the glycoside is such that concentrations approximating 5×10^{-5} M are required to obtain a 50% inhibition of the sodium-transporting activity to result;

(3) the ohmic resistance generally increases during exposure to ouabain;

(4) the effect of the glycoside is reversible—in less than one hour in the case of toad bladder, more slowly with skin.

Relevant data for toad bladder are summarized in Table 1.

When frog (*Rana esculenta*) skin is used instead, not only is the sensitivity to ouabain more pronounced—by 2 orders of magnitude at least—but obtaining a new

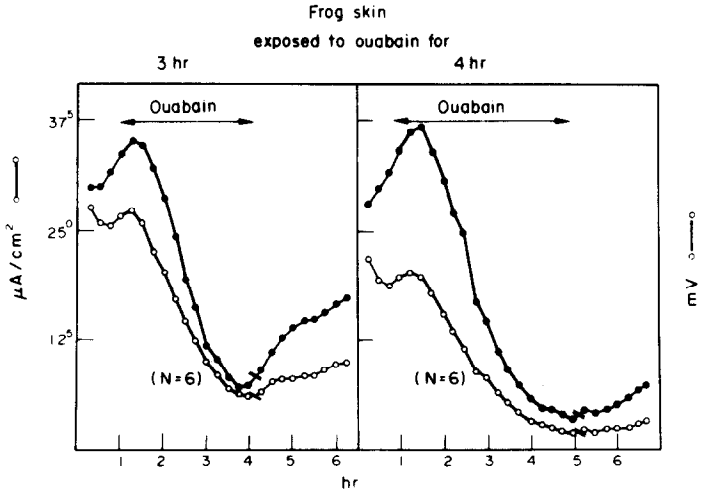

Fig. 1. Effects of ouabain on isolated frog skin.

When the abdominal skin of *Rana esculenta* was exposed to ouabain, 10^{-7} M, for 3 or 4 h, electrical potential difference and short-circuit current declined progressively; there was no indication for a sizeable concomitant rise in electrical resistance.

Upon removal of ouabain-containing Ringer's fluid, the preparations displayed signs of recovery. These experiments were carried out in early spring.

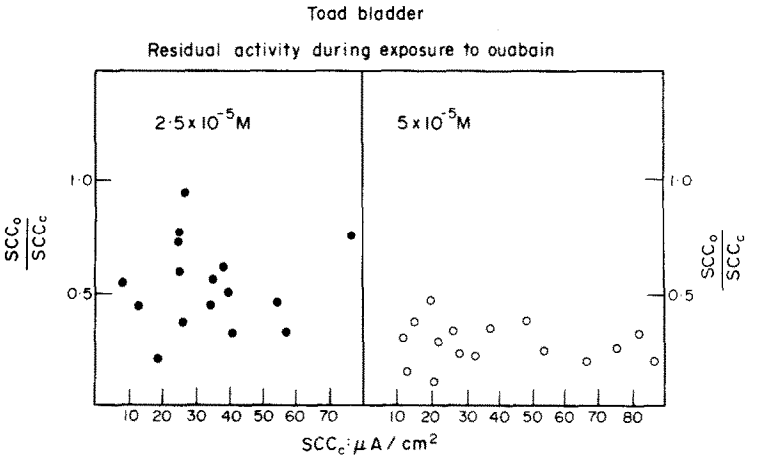

Fig. 2. Influence of ouabain as a function of spontaneous sodium-transporting activity of the isolated toad bladder.

Fresh toad bladder preparations were incubated according to Ussing and Zerahn[14]; they were exposed to the glycoside after a suitable control period and the residual activity during the 2nd hour (SCC_o) was expressed relative to baseline short-circuit current (SCC_c).

Irrespective of the concentration of ouabain, there was no proportionality between these 2 parameters.

steady state in the presence of the inhibitor is quite problematic (Fig. 1). Furthermore, electrical resistance isn't increased by the glycoside as can be judged from Fig. 1. Finally, after removal of the drug, recovery is generally incomplete[17].

Since aldosterone stimulates sodium transport by these amphibian epithelia, the question of whether the inhibition exerted by ouabain was or was not a function of the rate of sodium transport was considered. For toad bladder, the effect of the glycoside appears independent of the baseline, as is illustrated by Fig. 2.

With amphibian skin, there is some interdependence: the higher the baseline sodium-transporting activity, the more pronounced the inhibitory influence exerted by ouabain. But the relationship is of borderline significance, both for fresh toad skin and fresh frog skin. Indeed, with toad preparations a regression equation reading as follows: $Y = 54{\cdot}2 - 0{\cdot}424X$ ($r = 0{\cdot}395$)—can be calculated when X stands for the baseline activity in $\mu A/cm^2$, and Y for short-circuit current after 2 h of exposure to 5×10^{-5} M ouabain expressed as per cent of X. Mean baseline current for this series ($N = 38$) was $36{\cdot}2\ \mu A/cm^2 \pm 2{\cdot}4$ (S.E.); 2 h

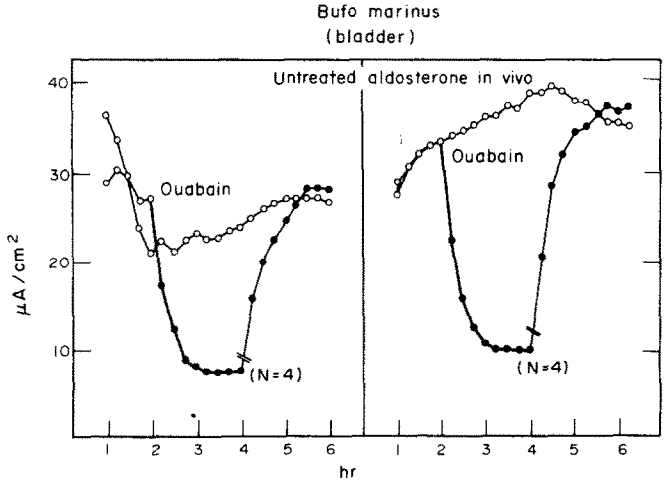

Fig. 3. Effect of ouabain on sodium transport by bladder from toads, *Bufo marinus*, treated or not with aldosterone.

Both lobes of the urinary bladder of each animal were incubated simultaneously according to Ussing and Zerahn[14]. After 2 h, one lobe was exposed to ouabain, 5×10^{-5} M, for another 2 h after which incubations continued in ouabain-free Ringer's fluid.

There were four toads used for each group.

after addition of ouabain, it had dropped to 38·9% ± 2·5 (S.E.).

When frog skin behaviour was analysed in a similar fashion, the following regression equation was obtained: $Y = 51{\cdot}0 - 0{\cdot}417X$ ($r = 0{\cdot}437$), with Y again expressed as a function of X, 2 h after addition of 5×10^{-7} M ouabain. Sodium-transporting activity of these frog preparations ($N = 20$) averaged 29·8 μA/cm^2 ± 4·1 (S.E.) prior to introduction of ouabain, and mean residual activity after 2 h of exposure to the glycoside was 38·6% ± 4·0 (S.E.) of baseline.

It should be remembered that unlike what is seen with toad skin, the inhibitory action of ouabain on sodium transport by frog skin is far from fully expressed after 2 h of exposure to the glycoside (Fig. 1).

2. *Effects of ouabain on aldosterone-stimulated amphibian epithelia*

With this information as a background, the case of aldosterone-stimulated amphibian epithelia was investigated. The hormonal effect was brought about either by treating the animal (toad) prior to sacrifice, or by exposure of the epithelia to the hormone *in vitro* for several hours—overnight usually.

(a) First, an examination was conducted of the inhibition exerted by ouabain on sodium transport by bladder from *Bufo marinus* treated with aldosterone; 5 μg of the steroid hormone in aqueous solution had been injected subcutaneously the evening before the animal was killed. As can be seen on Fig. 3 this had as a result an increase in the short-circuit current, from 23·3 μA/cm^2 to 37·2 μA/cm^2, during the fourth hour of incubation; ouabain-inhibited matched preparations had at that time reached a new steady state which seemed slightly higher, by one third, for preparations from aldosterone-treated toads: mean values were 7·6 vs 10·1 μA/cm^2.

(b) This kind of observation led to an evaluation of the influence exerted by ouabain on the aldosterone effect developed *in vitro*. As reported in detail elsewhere [15], ouabain failed to eliminate the hormone-induced stimulation of sodium transport on bladder and skin; this applies to frog skin as well[17].

Even when preparations were exposed to ouabain through the entire period of treatment with aldosterone, i.e. overnight, a residual effect of the hormone could still be demonstrated.

(1) In eight instances, four fragments of the ventral skin of a given toad were incubated simultaneously overnight: two pieces underwent prolonged (overnight) exposure to ouabain, 5×10^{-5} M; one of them as well as one of the two other pieces were treated in addition with aldosterone, 5×10^{-7} M. In relative terms, the effect of aldosterone was as evident in the presence of ouabain (65% ± 24) as in its absence (53% ± 17)

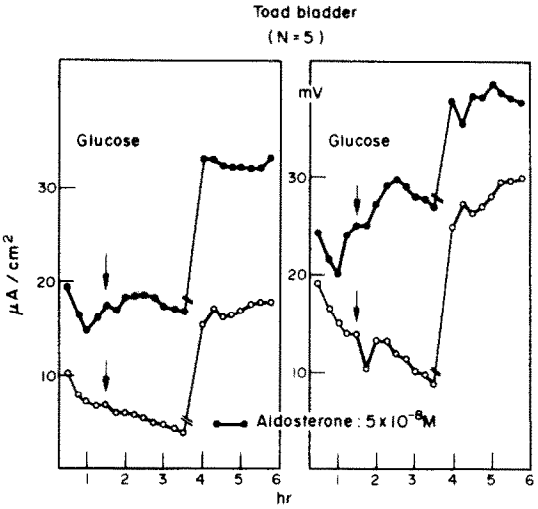

Fig. 4. Effect of aldosterone on toad bladder exposed overnight to ouabain.

In five instances, incubation was carried out overnight with toad bladder exposed to ouabain, 10^{-7} M, throughout; one of the lobes of each pair was treated in addition with aldosterone, 5×10^{-8} M. The following morning, a clearcut difference in electrical activity could be observed immediately. Glucose, 10 mM, failed to enhance the effect of aldosterone on short-circuit current in these circumstances; note, however, the rise in electrical potential difference.

Removal of ouabain-containing solutions after 3·5 h (breaks in the tracings) and replacement with fresh frog Ringer's led to a brisk rise in activity; yet the effect of aldosterone was not thereby made more apparent.

despite the fact that ouabain treatment had produced a drop in baseline activity, from 12·7 ± 2·0 μA/cm^2 to 8·2 ± 1·5 μA/cm^2.

(2) The same was observed with toad bladder incubated overnight in the presence of ouabain, 10^{-5} M. As seen on Fig. 4, the following morning the hormonal effect was evident even before removal of ouabain which resulted in a prompt increase of the sodium-transporting activity of both hemibladders.

(3) Frog skin was assayed the same way. Here difficulties arose due to its sensitivity to ouabain: for one thing, as already commented on, steady state residual sodium-transporting activity is reached quite late after addition of the glycoside. With low concentrations of this inhibitor of sodium transport it was possible to reproduce with this preparation the observation made on toad bladder and skin, thus demonstrating an effect of aldosterone after prolonged incubation carried out in the presence of ouabain, as appears from Table 2; an attempt at "protecting" frog skin by increasing the concentration of potassium in the incubation fluid—while not detrimental in terms of the effect of aldosterone—proved fruitless. This is in keeping with observations of Marro *et al.*[18] who empha-

Table 2. Residual influence of aldosterone on frog skin in the presence of ouabain
The results are expressed in $\mu A/cm^2 \pm$ S.E.

Experimental conditions		Untreated	+ Aldosterone	Hormonal effect
Ouabain				
absent	[K] = 2·5 mEq/L*	10·6 ± 1·7	16·5 ± 4·8	5·9 ± 2·4
	[K] = 25 mEq/L†	13·1 ± 2·9	18·1 ± 3·5	5·0 ± 1·5
present (4×10^{-8} M)	[K] = 2·5 mEq/L*	3·9 ± 0·6	5·9 ± 0·7	2·0 ± 0·6
	[K] = 25 mEq/L†	2·6 ± 1·0	7·1 ± 1·8	4·5 ± 2·0

The ventral skin of *Rana esculenta* was divided in 2 pieces incubated simultaneously overnight according to Ussing and Zerahn[14]. One preparation of each pair was exposed from the outset to aldosterone, 5×10^{-8} M. Experiments were conducted in standard and in potassium-enriched Ringer's; ouabain when used, was added at the start of the incubation.

Six animals were used per experiment.

These studies were carried out in late winter.

* Composition of incubation solution was: NaCl, 115 mM; $KHCO_3$, 2·5 mM; $CaCl_2$, 1 mM.

† Composition of incubation solution was: NaCl, 92·5 mM; KCl, 22·5 mM; $KHCO_3$, 2·5 mM; $CaCl_2$, 1 mM.

sized that changes in the concentration of potassium influenced the rate of ouabain-induced decrease in electrical activity, rather than the extent of inhibition eventually achieved.

Thus the effect of aldosterone is not eliminated by ouabain even at concentrations of the glycoside which influence significantly baseline sodium-transporting activity.

(c) It should be pointed out that the preparations were exposed *in vitro* for several hours to aldosterone and/or ouabain, in the absence of energy-providing substrate. Actually, in these circumstances the effect of glucose, which usually magnifies the influence of aldosterone on sodium transport by toad bladder[19] and skin[20, 21], proved remarkably sensitive to ouabain.* This is quite apparent from Fig. 5 in the case of bladder: glucose was almost totally ineffective on aldosterone-stimulated preparations as long as ouabain was present, while the glycoside concentration was such that barely any inhibition of baseline sodium transport had ensued.

With toad skin, the situation appears to be similar. Experiments were conducted on four pieces of the ventral piece of a given animal; all were exposed to aldosterone, 5×10^{-8} M, overnight. The following morning, one was used as a reference for the three other matched preparations treated with ouabain, at three different concentrations. As summarized in Table 3, the enhancing effect exerted by glucose on short-circuit current was quite blunted at concentrations of ouabain devoid of influence on the sodium-transporting activity prior to introduction of glucose.

This striking change brought about by glucose led to an examination of the possibility that the substrate

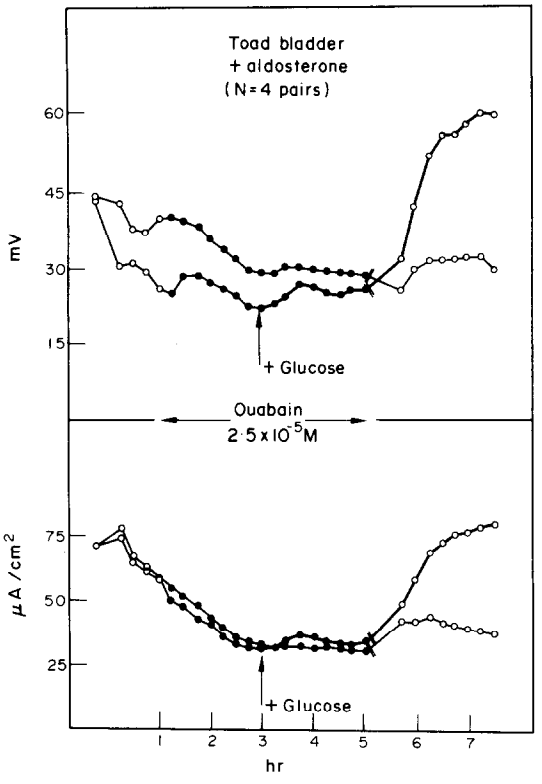

Fig. 5. Inhibition by ouabain of the effect exerted by glucose on electrical activity of aldosterone-stimulated toad bladder.

In four instances, both hemibladders of *Bufo marinus* were incubated overnight according to Ussing and Zerahn [14] in the presence of aldosterone, 7×10^{-7} M. The following morning, after a suitable control period, ouabain was added and left in contact with the preparations for 4 h. Halfway through this period of time, one hemibladder of each pair was treated with glucose, 10 mM.

Only after removal of ouabain did glucose produce its effect on short-circuit current and electrical potential difference.

* It is noted that aldosterone-exposed frog skin reacts much less to glucose after prolonged incubation.

Table 3. Inhibition by ouabain of the effect exerted by glucose on sodium transport by toad skin stimulated with aldosterone.

Concentration of ouabain*	Before glucose ($\mu A/cm^2$)	After addition of glucose ($\mu A/cm^2$)	Glucose response (% ± S.E.)
None	25·7	42·7	+ 46·3 ± 19·3
10^{-6}M	25·8	32·8	+ 14·7 ± 19·9
10^{-5}M	25·9	29·3	+ 7·2 ± 11·6
10^{-4}M	10·0	7·4	− 32·5 ± 16·4

* Ouabain was added to the solution coming into contact with the inner surface of toad skin (N = 8) 2½ h prior to introduction of glucose (final concentration, 10 mM). Short-circuit current readings dealt with here correspond to the last half-hour preceding glucose treatment and to the 4th half-hour of the latter.

could modify the sodium-transporting properties of the toad epithelia *in toto*: if this were the case, residual activity of glucose-treated preparations exposed to ouabain would be expected eventually to drop below that of appropriate controls. This was not observed, however[15]. Thus, although one cannot help but postulate an influence of glucose on the "pump" (especially in the presence of aldosterone) after protracted incubation, it is unlikely that the sodium-transporting activity of the tissue is modified in a homogeneous way.

From data such as discussed above, the conclusion can be formulated that two populations of cells equipped with sodium "pumps" whose sensitivity to ouabain differs by two orders of magnitude approximately, are involved in the full physiological expression of aldosterone action on transepithelial sodium transport. It appears difficult to ascribe such a difference in sensitivity merely to changes, which would occur in certain cells of the micro-environment of the ATPase system implicated in active sodium movement: neither cell ATP[8, 22], nor cell potassium concentrations [6, 9, 23] vary to a large, detectable extent, in the experimental conditions selected; as to changes in cell sodium they are unlikely to account for the observation, as Hoffman has shown this parameter to influence the rate rather than the extent of ouabain-dependent inhibition of sodium transport by erythrocytes[24].

It is tempting to reflect in this context on the two-fold increase in the number of mitochondria-rich cells observed by Voûte in amphibian epithelia exposed to aldosterone[25].

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DISCUSSION

Sjövall:

Has anyone ever looked at sulfatides and sulfatide turnover in relation to aldosterone action. I am thinking of the striking correlation between salt excreting function and sulfatide concentrations e.g. in salt glands of herring gull and spiny dogfish demonstrated by K. A. Karlsson's group in Gothenburg.

Crabbé:

No, not to my knowledge at least.