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ALDOSTERONE EFFECT IN THE EPITHELIUM OF THE FROG SKIN—A NEW STORY ABOUT AN OLD ENZYME*

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SUMMARY

It is established that aldosterone stimulates active epithelial sodium transport. Evidence is accumulating that this effect on transport is mediated through an intermediate step of protein synthesis. So far, morphobioelectric results of our laboratory indicate that only one type of cell, namely the mitochondria-rich (MR) cell in all epithelia studied is involved. This cell responds in a characteristic manner to the hormone, with respect to induction and protein synthesis. Carbonic anhydrase activity is not only selectively located in the mitochondria-rich cells but its activity pattern parallels our earlier morphological observations. As there is evidence that sodium selection of the outer membrane in frog skin is strongly pH dependent[1], we tentatively propose that epithelial transport regulation by aldosterone is mediated through carbonic anhydrase which is induced, synthesized and secreted by the mitochondria-rich cells. Carbonic anhydrase may control the pH in the extracellular compartment next to the outer membrane and thus regulate the sodium permeability of the latter.

INTRODUCTION

It has been known for many years that aldosterone holds a key position in the regulation of epithelial sodium transport. That this is valid also for *in vitro* preparations has been shown to be the case for the epithelium of the toad bladder[2] and the frog skin[3]. It is presently accepted by most investigators in the field[*e.g.* 2, 4, 5] that the hormone effect on transport is an indirect one, mediated by an inductively synthesized protein. This view is supported mainly by the fact that protein synthesis inhibitors prevent hormone effect and also that between the addition of the hormone and the observed effect on transport there is a latent period of about 1 h.

With these facts in mind we first attacked the problem by morphophysiological techniques applied to the epithelia of the frog skin and the toad bladder[6, 7], and the following hypothesis was formulated:

During the latent period aldosterone promotes morphological changes in just one type of cell of amphibian epithelia, namely the mitochondria-rich cell (MR cell). Thus it is most likely that this cell population reacts as a hormone receptor for protein synthesis[7].

Recently Zeiske and Lindemann[1] observed that sodium transport in the epithelium of the frog skin is strongly dependent on the pH of the medium on the outside of the skin. This observation, together with our earlier one that the secretion product of the MR cells was always deposited next to the apical border of the outermost epithelial cell layer, *i.e.* to the supposedly sodium selective membrane, suggested carbonic anhydrase (CA) as a possible product of the aldosterone induced synthesis. As a working hypothesis, this idea prompted a histochemical search for CA in the MR cells, and an investigation on possible histo- and biochemical approaches to a correlation of CA content with various stages of the hormone effect.

While this work was in progress our attention was drawn to several reports[17–22], all of which supported our postulate.

MATERIALS AND METHODS

Skins of large animals (R. esculenta) were mounted in a special lucite chamber with a front surface of 10 cm^2. Ringer solutions containing no bivalent cations were used throughout. The epithelium was detached by treatment with collagenase, 45 U/ml (CLS grade 125 U/mg, Worthington, Freehold, New Jersey, U.S.A.), maintaining an inside positive hydrostatic pressure gradient of 15 cm H_2O, according to the method reported by Carasso *et al.*[10]. The fully detached epithelium was excised and gently shaken in Ringer solution containing collagenase (80 U/ml) until no further cells were liberated. The cells were rinsed off, centrifuged for 5 min (140 *g*), washed

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Table 1a. CO_2/H_2O equilibration in water and for cell suspensions not stimulated (control) or stimulated by aldosterone

Block	exp.	Time required for acid production, sec: Water $\bar{w} \pm s$	N	Control $\bar{u} \pm s$	N	Aldosterone $\bar{v} \pm s$	N	Significance of the difference (Δ_t) between the averages. ($\bar{u}, \bar{v}$). p double-sided
A	1	53·60 ± 1·51	5	53·50 ± 1·00	4	51·50 ± 0·92	8	$p < 0·02$
	2	57·58 ± 0·49	6	54·91 ± 1·11	6	53·83 ± 1·47	6	$p < 0·2$
	3	61·00 ± 1·26	6	59·40 ± 1·51	5	57·50 ± 2·51	4	$p < 0·3$
	4	56·60 ± 1·14	5	56·33 ± 1·63	6	54·62 ± 1·92	8	$p < 0·2$
	5	68·60 ± 0·89	5	65·60 ± 1·51	5	66·00 ± 1·89	6	$p < 0·7$
	6	64·60 ± 1·14	5	62·00 ± 1·87	5	59·60 ± 1·89	5	$p < 0·1$
	7	64·00 ± 2·07	8	60·66 ± 1·96	6	59·83 ± 1·60	6	$p < 0·4$
	8	59·88 ± 1·61	9	59·60 ± 1·51	5	58·40 ± 1·81	5	$p < 0·3$
	9	57·37 ± 1·40	8	61·66 ± 1·86	6	58·40 ± 1·34	5	$p < 0·01$
	10	64·57 ± 0·97	7	60·50 ± 1·73	4	60·25 ± 0·50	4	$p < 0·7$
B	11	109·00 ± 3·16	5	76·80 ± 2·16	5	68·00 ± 2·91	5	$p < 0·001$
	12	102·50 ± 1·76	6	72·40 ± 3·84	5	67·00 ± 3·39	5	$p < 0·05$
	13	101·85 ± 1·57	7	67·80 ± 1·64	5	64·20 ± 1·09	5	$p < 0·01$
	14	93·42 ± 2·14	7	87·50 ± 2·08	4	87·33 ± 2·08	3	$p < 0·9$
	15	95·00 ± 1·58	5	73·40 ± 2·79	5	66·75 ± 1·25	4	$p < 0·01$
C	16	131·28 ± 0·56	7	116·16 ± 2·56	6	103·83 ± 1·32	6	$p < 0·001$
	17	129·60 ± 0·89	5	84·40 ± 1·14	5	76·20 ± 2·58	5	$p < 0·001$
	18	133·60 ± 1·96	6	103·00 ± 1·73	5	93·80 ± 3·03	5	$p < 0·001$
	19	134·00 ± 3·22	6	110·00 ± 4·85	5	100·00 ± 4·63	5	$p < 0·02$
	20	130·40 ± 1·24	5	92·60 ± 2·07	5	87·00 ± 3·74	5	$p < 0·02$

Table 1b. The significance of the mean difference ($\bar{\Delta}_t$) between the time averages ($\bar{u}$, $\bar{v}$) required for acid production by control and by aldosterone stimulated cells

Block	$\bar{\Delta}_t$	$s_{\bar{\Delta}_t}$	Significance of $\bar{\Delta}_t$
A	1·42	0·33	$p < 0·01$
B	4·92	1·46	$p < 0·05$
C	9·06	1·10	$p < 0·01$

several times with Ringer solution, and used immediately. In case of the combined frog experiments (4 and 6 frogs), the epithelia were stored overnight in an oxygenated calcium-free Ringer solution at 4°C., the collagenase treatment and further processing described above being postponed to the following day. Before use, the cells were carefully re-suspended. Two equal samples were pipetted; to one sample aldosterone was added (10^{-7} M) and to the other the same amount of Ringer solution. The incubation time was 45 min at room temperature. Biochemical CA determinations were then carried out after repeated freezing and thawing by the method of Wilbur and Anderson[11], and in experiments using one frog by the micromethod of Maren[12]. In both procedures the time required for a predetermined pH change in a bicarbonate/CO_2 solution is measured, with and without previous addition of cell suspension to be tested for CA (cf. Table 1a, b).

Samples processed identically, were treated by the histochemical method of Hansson[13] and the modified version of Rosen[14]. The same methods were applied to demonstrate CA activity in cryostat sections of the intact tissue (Fig. 1).

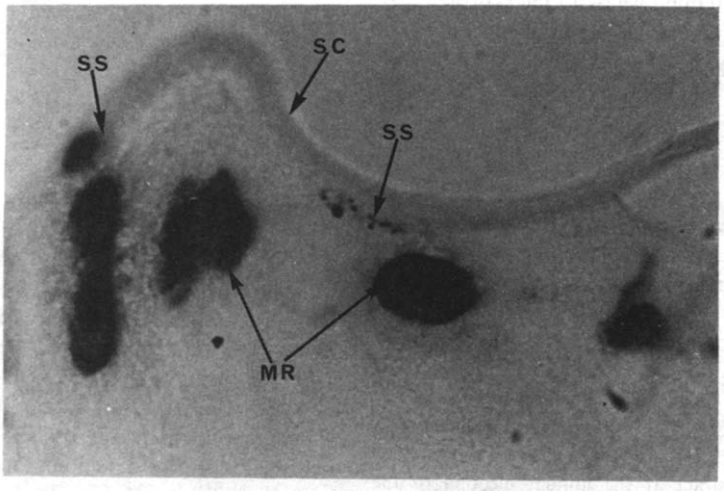

Fig. 1. Histochemical location of CA activity in the epithelium of the frog skin. SC = stratum corneum SS = subcorneal space (note CA positive spots). MR = CA positive MR cells.

Table 2a. Relative number of CA-secreting cells in cell suspensions not stimulated (control), and stimulated by aldosterone

Block	exp.	Control x_1 ‰	x_2 ‰	x_3 ‰	$\bar{x} \pm s$ ‰	Aldosterone y_1 ‰	y_2 ‰	y_3 ‰	$\bar{y} \pm s$ ‰	Significance of the difference (Δ_c) between the averages, ($\bar{y}$, $\bar{x}$). p double-sided
A	1	9	5	12	8·66 ± 3·51	10	14	16	13·33 ± 3·05	$p < 0.2$
	2	12	13	15	13·33 ± 1·52	17	18	16	17·00 ± 1·00	$p < 0.05$
	3	21	15	12	16·00 ± 4·58	31	18	20	23·00 ± 7·00	$p < 0.3$
	4	4	7	3	4·66 ± 2·08	51	52	45	49·33 ± 3·78	$p < 0.001$
	5	18	17	15	16·66 ± 1·52	40	45	43	42·66 ± 2·51	$p < 0.001$
	6	2	1	0	1·00 ± 1·00	14	11	12	12·33 ± 1·52	$p < 0.001$
	7	29	31	26	28·66 ± 2·51	32	34	28	31·33 ± 3·05	$p < 0.4$
	8	4	1	6	3·66 ± 2·51	25	18	20	21·00 ± 3·60	$p < 0.01$
	9	10	15	18	14·33 ± 4·04	38	37	40	38·33 ± 1·52	$p < 0.001$
	10	33	35	26	31·33 ± 4·72	41	49	42	44·00 ± 4·35	$p < 0.05$
B	11	3	3	2	2·66 ± 0·57	22	24	15	20·33 ± 4·72	$p < 0.01$
	12	4	1	6	3·66 ± 2·51	21	17	15	17·66 ± 3·05	$p < 0.01$
	13	6	11	6	7·66 ± 2·88	12	15	16	12·33 ± 2·51	$p < 0.2$
	14	4	7	3	4·66 ± 2·08	17	16	14	15·66 ± 1·52	$p < 0.01$
	15	21	24	19	21·33 ± 2·51	26	30	25	27·00 + 2·64	$p < 0.1$
C	16	7	4	6	5·66 ± 1·52	25	23	27	25·00 ± 2·00	$p < 0.001$
	17	3	9	7	6·33 ± 3·05	43	49	47	46·33 ± 3·05	$p < 0.001$
	18	5	1	3	3·00 ± 2·00	8	5	6	6·33 ± 1·52	$p < 0.1$
	19	21	26	24	23·66 ± 2·51	27	38	35	33·33 ± 5·68	$p < 0.1$
	20	10	9	8	9·00 ± 1·00	28	20	26	24·66 ± 4·16	$p < 0.01$

Table 2b. The significance of the mean difference ($\bar{\Delta}_c$) between the averages (y, x) of CA-active cells per 1000 cells

Block	$\bar{\Delta}_c$	$s_{\bar{\Delta}_c}$	Significance of $\bar{\Delta}_c$
A	15·40	4·15	$p < 0.01$
B	10.60	2·46	$p < 0.02$
C	17·60	6·22	$p < 0.05$
A–C	14·75	2·60	$p < 0.001$

Differential cell counts were done with a Leitz microscope at a total magnification of 400. At each of three different loci, both in the control and in the aldosterone treated preparation the number of cells in which CA activity could be detected in a total of 1000 individually observed cells was counted. The results were statistically evaluated (Table 2a, b). Absolute cell counts were performed in a Neubauer counting chamber.

RESULTS

Figure 1 illustrates the histochemical location of CA in a section of an intact epithelium. The reaction product (black areas) was limited to the MR cells and the tip of their neck in the subcorneal space.

Figure 2 shows a suspension of separated cells after fixation and exposure to the Hansson–Rosen histochemical reagent for CA activity[13, 14].* Only about

* The reagent is a solution of cobalt sulfate in a carbonic acid/bicarbonate buffer which deposits cobalt carbonate at CA active sites; the deposit becomes visible after treatment with ammonium sulphide.

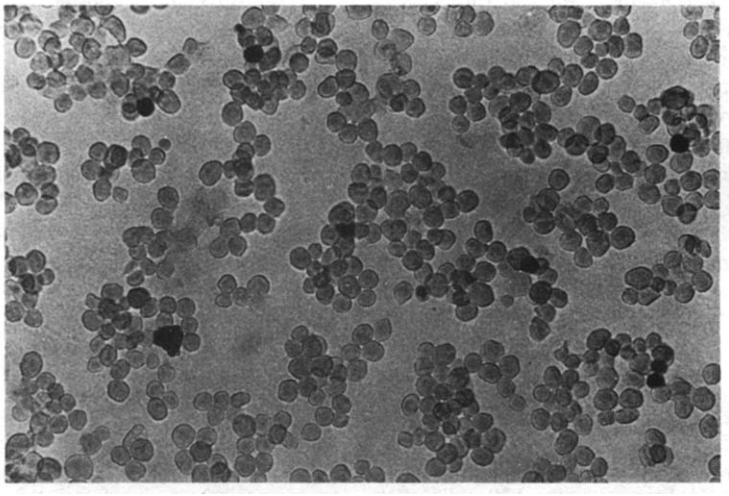

Fig. 2. Suspension of separated epithelial cells reacted histochemically for CA activity. Preparation used for counts.

1–3% of all cells were coated by the typical brown-black precipitate. Evidently all other cells were completely devoid of any CA activity. Preparations such as these were used for counts (cf. Table 2a, b).

In series A (experiments 1–10), the skin of one single frog was used for biochemical and histochemical evaluation. In series B and C (experiments 11–15, 16–20), 4 and 6 skins respectively were combined for every experiment. In the individual experiments of series A the difference in the amount of enzyme present in aldosterone activated and non-activated cell suspensions was evidently too small to produce individually significant differences as estimated by the analytical methods used[12]. The mean time difference ($\bar{\Delta t}$ = 1·42 s) however, in experiments 1–10 was well beyond the S.D. $s_{\bar{\Delta t}}$ = 0·33 and significant. It was now felt that at least 4 combined frog skins were needed to obtain enough enzyme to produce large enough individual time differences. Indeed in B and C there was a considerable improvement, the time differences now being significant in most cases where four and significant in all cases where six combined skins were used. Note the approximate proportionality of the averaged time difference and the number of skins involved.

Histochemically, the significance of observed CA increase after aldosterone activation is generally of the same order. However, no quantitative correlation could be found with respect to results of single biochemical and histochemical experiments. One or several of the following factors might be responsible:

1. Methodological differences (including counting and measuring errors).

2. Heterogenous distribution of CA among MR cells. Yet low and high activity cells shows up identically with histochemical techniques and thus could all be counted. Heterogeneity could only be detectable biochemically.

3. Incomplete CA recovery and/or aspecific background CA activity. But when parallel biochemical tests for CA activity were performed on identical samples, after freezing and thawing them (usual procedure) or after sonication, there was no difference in CA recovery. Another check for CA recovery and possible aspecific background was done by inhibiting CA with acetazolamide at 4°C. After 10 min 90% of the CA was inhibited, after 24 h 100%.

In order to estimate the CA activity of one cell, we counted in experiments 16–20 the total number of cells in each ($2–3 \times 10^7$). Taking into account this number, the percentage of CA active cells (cf. Table 2a) and the enzymatic activity found (Table 1a) we calculated that there is per CA active MR cell an amount of 1–10 μU of CA. The unit U is defined as $(t_{water} - t_{sample})/t_{sample}$ [15].

DISCUSSION

After presentation of our results depicting the effect of aldosterone on CA activity in the MR cells of the frog skin epithelium, it seems convenient to subdivide relevant data into four major groups: 1. Aldosterone induced acidification; 2. Epithelial cell aldosterone receptors; 3. Distribution of carbonic anhydrase activity; 4. The sodium selective outside membrane and its response to pH changes.

In 1972 Fanestil and collaborators[17, 18] showed that aldosterone promotes acidification of the mucosal bath in the toad bladder. They offered evidence that in the absence of sodium transport the reversed short circuit current (RSCC) paralleled significantly this acidification process and that RSCC is (at least partially) acetazolamide sensitive. No direct relationship could be found between acidification and CA content of the tissue. They concluded that aldosterone stimulated acidification and aldosterone effect on sodium transport represented two independent mechanisms.

In 1969 and 1972 our group[6, 7] published data favouring the view that in the epithelia of the frog skin and toad bladder there was, both *in vitro* and *in vivo*, just one type of cell responding to aldosterone, *i.e.* the mitochondria-rich cell (MR cell). The response to the hormone was observed to occur within the latent phase and to be morphologically characteristic.

The MR cells change from their round shape in the resting state to become flask-like after induction. They extend their neck toward the subcorneal space (frog skin) or toward the lumen (bladder). After stimulation, there is an increase not only of their number of mitochondria but, most drastically, of their content in ribosomes. The secretory phase in frog skin is typical: at the tip of the neck a subcorneal "lake" forms containing amorphous secretion material.

Scott and co-worker[19, 20, 21] recently provided impressive evidence (on MR cells of the toad bladder separated by density gradient centrifugation) that the MR cells are specifically induced by aldosterone to synthesize CA and that specific aldosterone binding occurs in these cells only. Their conclusion is that both proton secretion and increment in sodium transport induced by aldosterone might represent a function of these MR cells only.

The histochemical and biochemical results reported in this paper show that the enzyme CA is solely found in the MR cells and that its activity distribution after aldosterone stimulation changes in a way similar to that of the morphological pattern reported earlier[6, 7].

CA has been located histochemically in a large variety of epithelia by Rosen and co-workers[14, 22] with the help of an improved method of Hansson[13]. They concluded that proton secretion must be mediated by MR cells but they did not mention aldosterone induction.

In epithelia of the frog skin Zeiske and Lindemann[1] observed the effect of rapid pH changes in the outside bath on the sodium selective membrane. Decreasing the pH from 9 to 5 increases, from 5 to 3 reversibly decreases the sodium current, with a concomitant increase of electrical resistance in the latter case. Upon further decrease to pH 1, there is a drop

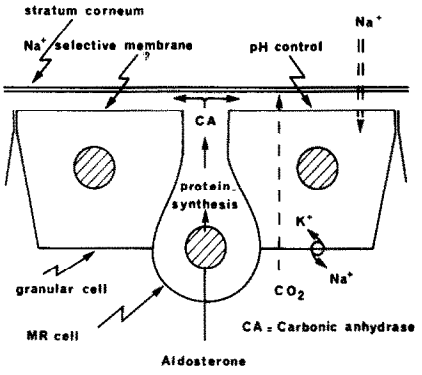

Fig. 3. Illustration of our proposed model of aldosterone mediated stimulation of sodium transport in the epithelium of the frog skin.

in resistance, and the increasing current is no longer sodium specific.

Some years ago Leaf and co-workers[23] reported similar findings on toad bladder. Contradictory results of other groups[24–26] are not easily interpreted because of widely varying experimental approaches.

SUMMARY

Aldosterone, in all epithelia studied by us so far, appears to influence just one type of cell, *i.e.* the MR cell; this is strongly supported by the additional results presented in this paper and the reports of Scott and collaborator. Material secreted by these cells has been shown to contain the enzyme carbonic anhydrase. Morphological observations and histochemical findings indicate that the enzyme is always secreted and deposited next to the outside of the sodium selective outer (or luminal) membrane. This leads to the assumption that its *locus agendi* must be there. As the pNa^+ of this membrane appears increased by a pH drop at its outer surface (according to Lindemann), the hypothesis is formulated that a correlation exists between a primary aldosterone effect, *i.e.* CA induction, leading to acidification next to the sodium selective membrane, and final sodium transport stimulation (see Fig. 3).

To some extent our interpretation is divergent from that of Scott:

We have good reason to believe[8, 9, 16, 27, 28] that in the epithelium of the frog skin asymmetric active sodium transport is performed by *all* granular cells of the 1st living cell layer next to the corneum with the exception of the MR cells. Consequently the aldosterone induced sodium transport increment is also carried by the granular cells and not the MR cells. The latter are secreting cells (thus transporting in the opposed direction) first for carbonic anhydrase and then for cations (possibly the carriers of the RSCC) as reported by Fanestil and co-workers[17, 18].

Hydration of freely diffusible CO_2 catalyzed by CA outside the sodium selective membrane would produce much larger quantities of acid than if hydration were limited to the MR cells themselves. Thus the term "proton secretion" (at least in frog skin and may be also in the toad bladder) may have to be exchanged for the term "luminal CO_2 hydration". The fate of the few bicarbonate ions generated by dissociation of some of the formed H_2CO_3 need not concern us since their function might merely consist in maintaining electroneutrality in the acidified subcorneal space. In any event, bicarbonate does not seem to permeate through the epithelium of the frog skin[26].

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