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THE EFFECT OF POLYENE ANTIBIOTICS ON THE ALDOSTERONE INDUCED CHANGES IN SODIUM TRANSPORT ACROSS ISOLATED FROG SKIN

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SUMMARY

It is shown that:

(1) The isolated *stratum corneum* has a very low electrical resistance.

(2) The passive sodium flux through the isolated *s. corneum* is 20–100 times higher than the active sodium transport.

(3) Polyene antibiotics (amphotericin B and filimarisin) abolish the aldosterone induced inhibition of the active sodium transport.

(4) Aldosterone induces a recovery in the electrical potential across skins where it had been reduced previously by the addition of a polyene antibiotic.

(5) An hypothesis is presented to explain these observations.

INTRODUCTION

IN PREVIOUS papers[1–3] we have shown that aldosterone in concentrations higher than $1{\cdot}5 \times 10^{-9}$ M induces a moult *in vitro* (i.e. a separation of the *stratum corneum* from the underlying cells). The moult is accompanied by characteristic changes in the electrical potential (P.D.) and the short-circuit current (s.c.c.). The sequence of changes can be divided into four parts: the first constant period, the inhibition period, the activation period and the second constant period[1]. At the end of the inhibition period it is rather easy to remove the *s. corneum* mechanically. If this is done one observes an immediate increase in the s.c.c. and the P.D.[1]. Thus, the inhibition of the Na^+-transport was probably not due to an effect on the Na^+, K^+-pump *per se*, but rather to an inhibition of the passive flux of Na^+ from the outside solution to the Na^+, K^+-pump. In order to get more information about the aldosterone induced inhibition of the Na^+ influx, the Na^+ flux across the isolated *s. corneum* and the effect of polyene antibiotics on the moulting cycle were investigated.

METHODS

The experiments were performed on male and female frogs (Rana temporaria). The frogs were kept partially immersed in tap water at about 4°C. The skins were dissected from pitched animals and divided into two or four pieces. The skins were mounted in perspex chambers (area 7 or 1 cm^2) and bathed in aerated Ringers solution ($Na^+ = 115{\cdot}0$, $K^+ = 2{\cdot}5$, $Ca^{++} = 1{\cdot}0$, $HCO_3^- = 2{\cdot}4$, $Cl^- = 117{\cdot}1$, mM, pH = 8·2).

The shortcircuit experiments were performed according to the method of Ussing and Zerahn[4], using an automatic voltage clamp apparatus which could be programmed to disconnect the short-circuit current every five minutes, thus allowing the potential to be measured for 15 sec. The fluxes are expressed in

terms of permeability coefficients as defined by Andersen and Ussing[5]:

$$P = (a_2V_2 - a_1V_1)/A \times a_s \times (t_2 - t_1).$$

Here a_1 and a_2 designate the radioactivity originating from side 2 in one ml of solution from side 1 at times t_1 and t_2 respectively; V_1 and V_2, the corresponding volumes of the solution on side 1; A, the area of the membrane; and a_s, the mean activity in one ml of solution from side 2 during the period from t_1 to t_2. The *stratum corneum* was isolated as described previously[1]. The Na^+-flux and the resistance across the isolated *s. corneum* were measured in a modified frog skin chamber (area 1·5 mm^2) with glass end pieces. The integrity of the membrane was inspected by means of a microscope (50× magnification, type MA 117 E, M. Aubert AG, Biel, Switzerland).

The polyene antibiotics used were amphotericin B and filimarisin. The amphotericin B (Squibb AB, Lindingö) was used as the water soluble preparation. Each vial contained 50 mg lyophilized amphotericin B powder, 41 mg sodium deoxycholate, 10 mg disodium phosphate, 0·89 mg monosodium phosphate, and 6·2 mg sodium chloride; the amphotericin B diluent had the same composition except that the amphotericin B was omitted. The filimarisin (Filipin) was a gift from Mr. Elleby (the Upjohn Company, Kalamazoo, Michigan, U.S.A.). The aldosterone used was "aldocorten" (Ciba Ltd.).

RESULTS

The first border the Na^+ was to cross before it reached the Na^+, K^+-pump was the *s. corneum*. The P.D. across the isolated *s. corneum* appeared to be 0 mV. The Na^+ permeability and the resistance of the isolated *s. corneum* were measured both when the *s. corneum* was isolated in the aldosterone-induced inhibition period and in the second constant period. The electrical resistance of the *s. corneum* was the same as the resistance of the Ringer's solution, both when the *s. corneum* was isolated in the inhibition period and in the second constant period. The Na^+ permeability of the *s. corneum*, isolated in the inhibition period, was $(3{\cdot}32 \pm 0{\cdot}26) \times 10^{-4}$ cm/sec and in the second constant period it was $(3{\cdot}12 \pm 0{\cdot}26) \times 10^{-4}$ cm/sec. These permeability coefficients correspond to a Na^+ flux which is 20–100 times higher than the Na^+ influx in the short-circuited frog skin. The data presented above, together with the recent data of Bracho *et al.*[6] which shows that the *s. corneum* also is permeable to La^{3+}, indicates that the *s. corneum* does not represent a major barrier for the diffusion of Na^+ into the underlying cells.

The next border through which the ions must pass is the outer cell membrane of the first living cell layer (the *s. granulosum*). In order to investigate whether or not the inhibition was due to changes in the *s. granulosum*, the effect of polyene antibiotics on the moulting cycle were investigated. The biological effects of the polyene antibiotics have been attributed to an increased permeability of the cell membranes[7–9]. We have shown previously[9] that the addition of 5×10^{-5} M amphotericin B to the outside bathing solution causes a highly significant reduction in the P.D. and resistance. Furthermore, we have presented evidence for an active outward transport of potassium during these conditions. The data in Table 1 show the Rb^+ influx and outflux (the Rb^+ was used as a substitute for K^+) after the addition of filimarisin. The flux measurements were first started after the

resistance of the skin had reached a steady-state following addition of the filimarisin. When the permeability of the membranes does not change during the flux measurements one can use the flux ratio equation[10] even during non steady-state conditions[11]. In experiments shown in Table 1, the skins were short-circuited and the solutions were identical on both sides of the skins. Under these circumstances, the flux ratio outflux/influx should be equal to 1 for passive fluxes[10, 11]. The fact that the flux ratio for Rb^+ was greater than unity during filimarisin treatment shows that an active outward Rb^+ transport took place during these conditions. Thus, filimarisin (polyene antibiotics) causes the formation of an unspecific pathway in the outer membrane of the *s. granulosum*.

Table 1. Effect of filimarisin on the non steady-state Rubidium influx and outflux

Rb^+ mM	K^+ mM	*P* out for rubidium cm × sec^{-1} × 10^{-7}		*P* in for rubidium cm × sec^{-1} × 10^{-7}		*P* out Rb^+/*P* in Rb^+	
min		0–30	30–60	0–30	30–60	0–30	30–60
0·5	2·0	92	175	5·1	11·8	18·0	14·7
2·0	0·5	234	249	19·9	20·4	11·8	12·2
1·25	1·25	32·2	100	5·4	10·4	6·0	9·6

The flux measurements were started and the isotope added (1–3 h after the addition of the filimarisin) when the resistance had reached a steady-state. The filimarisin was added to both sides of the skin to give a concentration of $1·2 \times 10^{-5}$ M.

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Figure 1 shows the effect of aldosterone ($1·4 \times 10^{-6}$ M) and filimarisin ($1·2 \times 10^{-5}$ M) on the P.D. across 3 pieces of the same frog skin. At the arrow A filimarisin was added to the outside bathing medium of skin pieces 1 and 2. 15 min after the addition of the filimarisin the P.D. started to decrease, reaching a new stable level 1–2 h after the addition. At the arrow B aldosterone was added to both the outside and the inside bathing media of skin pieces 1 and 3. Skin piece 3, to which aldosterone had been added but no filimarisin, showed the normal aldosterone induced changes in the P.D.: the inhibition of the P.D. started 200 min after the addition of the aldosterone and the activation 250 min after the addition of aldosterone. Skin piece 1, to which there had been added both aldosterone and filimarisin, did not show the aldosterone induced inhibition of the P.D. 200 min after the addition of aldosterone, but rather an activation. This aldosterone induced activation of the P.D. was also observed when the P.D. across the skin was reduced by addition of amphotericin B. In 16 out of 20 experiments where the P.D. had been reduced either by addition of filimarisin or amphotericin B, the aldosterone induced activation of the P.D. started before the first 50% of the aldosterone induced inhibition period had elapsed. In one experiment the aldosterone induced activation occurred at the same time in both skin pieces, and in 3 experiments aldosterone did not activate the P.D. in the skin piece to which the polyene antibiotic had been added.

Figure 2A shows the effect of aldosterone on one skin half (skin A), and Fig. 2B shows the combined effect of aldosterone and amphotericin B. At the arrow

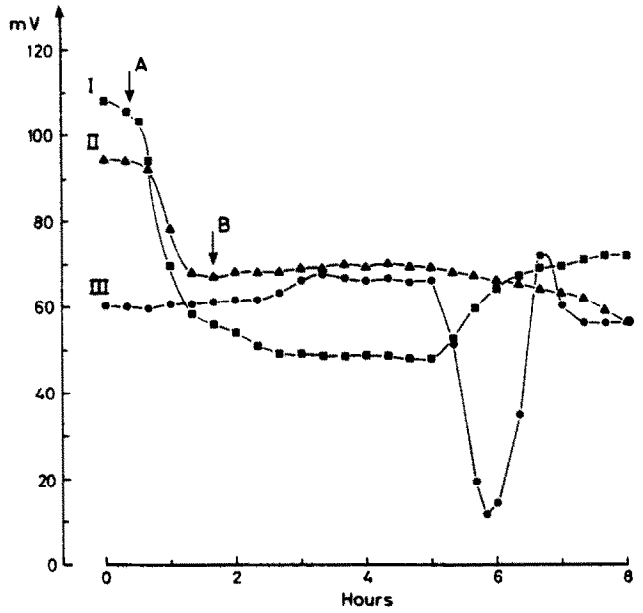

Fig. 1. Effect of filimarisin ($1{\cdot}2 \times 10^{-5}$ M) and aldosterone ($1{\cdot}4 \times 10^{-6}$ M) on the P.D. across 3 pieces of the same frog skin. At arrow A filimarisin was added to skin pieces 1 and 2. At arrow B aldosterone was added to skin pieces 1 and 3.

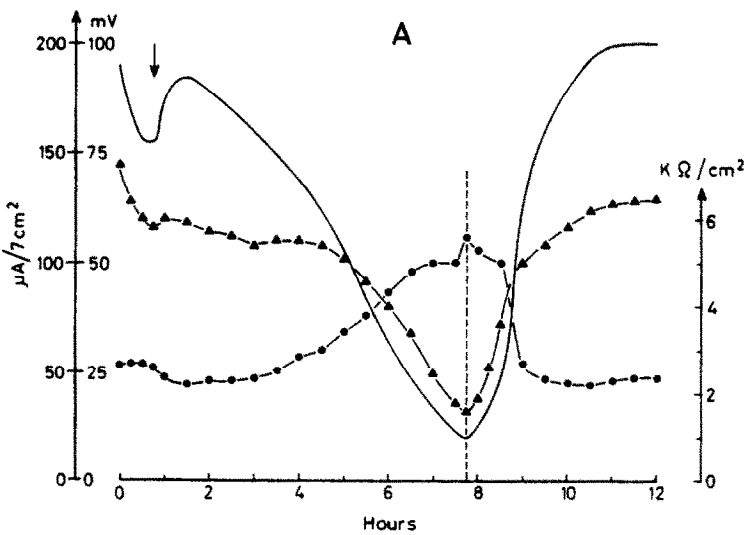

Fig. 2A. Effect of aldosterone on the s.c.c. and the P.D. across the frog skin. At the arrow amphotericin B diluent and aldosterone were added.
——— s.c.c. μA/7 cm². ▲——▲ P.D. mV. ●——● Resistance kΩ/cm².

(Fig. 2A) both amphotericin B diluent and aldosterone were added. At the arrow in Fig. 2B amphotericin B (5×10^{-5} M) was added to the outside bathing media and aldosterone was added both to the inside and the outside bathing media. After the addition of amphotericin B diluent (Fig. 2A) and the amphotericin B (Fig. 2B)

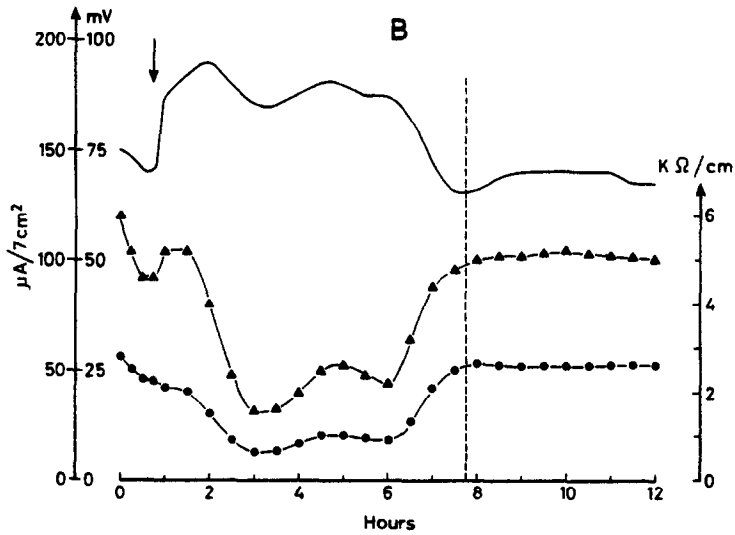

Fig. 2B. Effect of aldosterone and amphotericin B on the s.c.c. and P.D. across the frog skin. At the arrow amphotericin B (5×10^{-5} M) was added to the outside bathing media and aldosterone was added both to the inside and the outside bathing media. Symbols as in Fig. 2A.

one observes the onset of a small increase in the s.c.c. The increase in the s.c.c. is, however, somewhat bigger in the skin half to which the amphotericin B was added. If we compare the s.c.c. in Fig. 2A with the s.c.c. in Fig. 2B, it appears that the addition of amphotericin B nearly abolishes the aldosterone induced inhibition of the s.c.c. As in Fig. 1 we observe an activation of the P.D., across the skin where it had been reduced previously, by the addition of the polyene antibiotic (Fig. 2B). Furthermore, the activation of the P.D. started 70 min earlier in skin B than in skin A. In both skin halves the resistance was about 2·5 kΩ/cm² before the addition of amphotericin B and aldosterone. In skin A the resistance remained relatively constant during the first 3 h of incubation; thereafter, it started to increase, reaching a maximum of 5·5 kΩ/cm² when the aldosterone induced inhibition was maximal. In the activation period the resistance decreased to 2·3 kΩ/cm² and then remained practically constant the following 2 h. After the addition of amphotericin B, the resistance in skin B decreased to about 1 kΩ/cm² and stayed at that level for about 3 h. Later (after 6 h of incubation) when the aldosterone treated skin half (skin A) was in the inhibition period, both the resistance and the potential started to increase in skin B, reaching a new stable level after 8 h of incubation. At that time the aldosterone induced inhibition had its maximum in skin A.

DISCUSSION

The reduction in the P.D. by the polyene antibiotics occurs together with a decrease in the transepithelial resistance. The reduction in P.D. induced by aldosterone occurs together with an increase in the transephithelial resistance. This indicates that these two ways of reducing the P.D. occur by two different mechanisms. However, since the polyene antibiotics are able to abolish the aldosterone-induced inhibition of the s.c.c., one might suggest they act at the same "level". The sodium flux and the resistance measurements on the isolated *stratum*

corneum indicate that the inhibition probably was not due to changes in that layer. The data in Table 1 and previous experiments[9] indicate that the polyene antibiotics cause the formation of an unspecific pathway in the outer membrane of the first living cell layer (the *stratum granulosum*). A hypothesis to account for these observations has to provide answers to the following questions:

(1) Why does the transepithelial resistance first increase and then later decrease during the aldosterone-induced moult?

(2) Why does the resistance increase together with the aldosterone-induced P.D. recovery in skins where the P.D. had previously been reduced by the addition of a polyene antibiotic (Figs. 2A and 2B)?

The following working hypothesis would explain the above observations:

Fig. 3A shows the frog skin during the normal transport conditions, the first constant period. The top layer (the hatched layer with the broken lines) is the *s. corneum*. The broken lines indicate that the cells are very permeable and non-selective. According to "the two membrane hypothesis"[12, 13] the "outward-facing membrane" of the next layer (the *s. granulosum*) is selectively, but passively, permeable to Na^+ (indicated by the dots). The outward facing membrane is separated from the "inward-facing membrane" by "tight seals"[13, 14]. The inward-facing membrane (the continuous line) is permeable to K^+, but imperme-

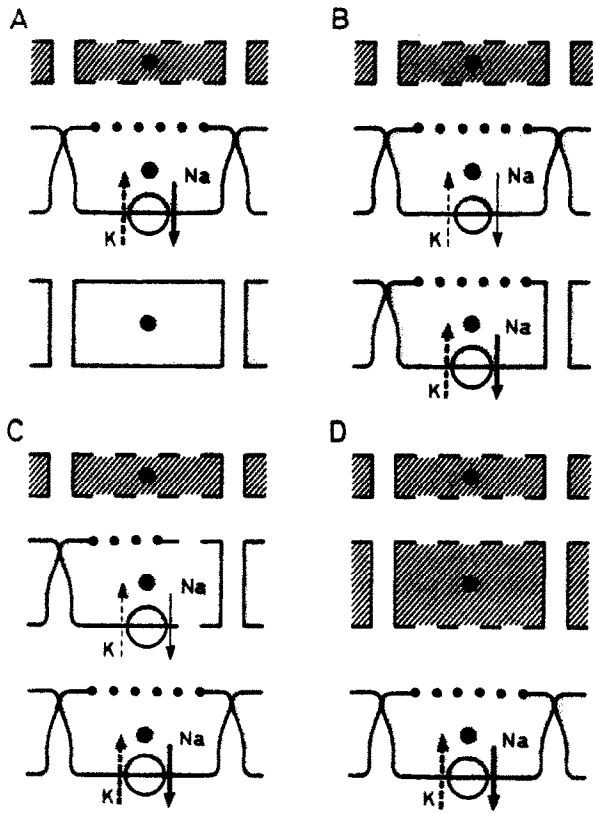

Fig. 3. Diagram of the 3 outermost layers of the isolated frog skin during aldosterone treatment. A. The first constant period. B. The inhibition period. C. The activation period. D. The second constant period. For further information see text.

able to free Na^+. Thus, the major Na^+ flux across the inward-facing membrane takes place via the Na^+, K^+-pump.

During the first constant period and the inhibition period an enzyme which attacks the material between the *s. granulosum* and the *s. corneum* is released. Moreover in the inhibition period (Fig. 3B) the outermost layer of the *s. granulosum* cells starts to turn into a new cornified layer. Furthermore, the next layer of the *s. granulosum* starts to form tight seals. During these processes the activity of the Na^+, K^+-pump decreases (the cornified layer consists of dead cells). Accordingly, the Na^+ flux across the inward facing membrane (the s.c.c.) decreases, and since the inward facing membrane is impermeable to free Na^+, the transepithelial resistance therefore increases. The formation of new "tight seals" in the second layer of the *s. granulosum* gives two cell layers in series, namely, the new transporting cell layer and the newly formed *s. corneum*. This would also cause an increase in the resistance. In the activation period (Fig. 3C) the former, first layer of the *s. granulosum* becomes very leaky (indicated by the hole in the inward and outward facing membrane). Thus, the Na^+ begins to diffuse to the new transporting cell layer causing an increase in the Na^+ transport and a decrease in the resistance (cf. Fig. 2A, vertical broken line). In the second constant period (Fig. 3D) the skin is back in its normal transport situation but with two cornified layers. Thus, one of the reasons why aldosterone activates the Na^+ transport in the isolated frog skin might be that the transport is performed by a new layer of cells.

Having dealt with the effect of aldosterone we can now go on to consider the effect of the polyene antibiotics. Suppose that the polyene antibiotics, besides making a non-specific pathway in the outer membrane of the outermost cell layer of the *s. granulosum* also make an unspecific pathway in the inward-facing membrane, or "open" the tight seals. Then the first cell layer of the *s. granulosum* would present no barrier to the movement of Na^+ (this cell layer becoming like that shown in Fig. 3C). The aldosterone-induced inhibition of the s.c.c. would therefore not occur. Furthermore, if the tight seals are formed in the underlying cell layer at this time a rise in P.D. would be expected, as observed in Figs. 1 and 2B.

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DISCUSSION

Leaf: I just wonder where you get the experimental evidence which would support your reporting all these things?

Nielsen: Why shouldn't it be so? The hormone acts as an inducer. Specially the amphotericin data strongly suggest that we are in presence of the formation of new tight seals: the resistance and the potential difference recover after the addition of aldosterone.

Leaf: Do you think that the whole induction system for new cells is turned on by hormones?

Nielsen: In a way. But I would at the present stage prefer to say that one of the effects of aldosterone in the isolated frog skin is to increase the turnover of the already existing epithelial cell layers.

Wiederholt: In your last paper you suggested a Na/K exchange pump. Would you speculate whether it is a coupled Na/K pump, an active sodium pump or an active sodium-active potassium pump?

Nielsen: It is a coupled pump, I think. Whether it is a one to one, a three to two or whatever degree of coupling, I don't know. We have an active outward transport of potassium when we open up the outside membrane with the addition of amphotericin B and this active outward transport of potassium can be inhibited by the addition of ouabain. This strongly indicates that it is pumped in by the sodium pump, or there might be a potassium pump which might be inhibited by this drug.

Nutbourne: We have examined the effect of aldosterone on skins obtained from frogs which had been kept under different conditions. We found 3 different effects of aldosterone. If we kept the frogs at 4°C in the dark in either dilute saline or water, then aldosterone caused the typical moulting effect just shown by Nielsen. This moult was not prevented by the presence of the spirolactone SC 14266. However, if the frogs were kept at room temperature in the light, then in the great majority of skins aldosterone had no effect and did not cause a moult. In two skins from "cold" frogs which had just completed a *spontaneous* moult, aldosterone caused a prolonged delayed rise is s.c.c. similar to that observed in toad bladder. This rise in s.c.c. was inhibited by Spirolactone S.C. 14266 (2×10^{-5} M).

Edelman: The frog skin contains calcium deposits that may change in amount during slough reaction. Have you considered the possible role of these deposits in the permeability changes during moulting?

Nielsen: I must admit that we have done only one experiment in calcium free Ringer's so far but have seen no difference either in P.D., resistance of s.c.c. as compared to normal Ringer's.

Edelman: The amount of calcium in frog skin is so high that changes in the calcium content of the medium may have no influence on a calcium dependent process. Have you measured the amount of calcium in frog skin at different stages during moulting?

Nielsen: No.

Ussing: Different species of frogs differ very much with respect to their contents of calcium. Thus *Rana temporaria* has very little calcium in contrast to e.g. *Rana pipiens*. As shown by Zadunaiski and others practically all calcium in frog skins is located in a layer of calcium phosphate crystals in the connective tissue. This calcium thus cannot be involved in the active sodium transport. On the other hand the layer of crystals may have disasterous effects on the study of calcium fluxes through the skin.