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ACTION OF MINERALOCORTICOID AND SEX STEROIDS ON SODIUM TRANSPORT IN TOAD SKIN

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SUMMARY

Short circuit current (s.c.c.) and potential difference (PD) were measured in control toad skin and after the addition of aldosterone and deoxycorticosterone acetate. A significant effect was seen only when skins were preincubated for 12 h with the hormones. After this preincubation with mineralocorticoids or with sex steroids (estradiol, progesterone and testosterone), a kinetic study was performed measuring s.c.c. at different levels of Na^+ in the external medium. The results indicated that Na^+ transport followed Michaelis–Menten kinetics between 5 and 50 mequiv/l Na^+. V was significantly higher in all hormone-preincubated skins, the effect of mineralocorticoids being considerably greater than that of sex steroids. On the other hand, K_m was not significantly different from controls in most cases, and did not show a definite trend in the remainder, indicating no major alteration in the affinity between Na^+ and its transport system. Skin resistances fell in both chloride and sulfate Ringer solutions with mineralocorticoids, but only in the latter medium with estradiol and progesterone, and not at all with testosterone.

In conclusion, sex steroids stimulate Na^+ transport significantly, but to a lesser degree than mineralocorticoids; their effect on skin conductance is relatively smaller than that on Na^+ transport.

INTRODUCTION

The action of aldosterone and related steroids on biological membranes *in vitro* has contributed decisively to the understanding of their mechanism of action. The work of Edelman and co-workers[1,2] demonstrated that aldosterone stimulated protein synthesis in toad bladder, and its stimulating action on Na^+ transport could be prevented by the use of protein synthesis inhibitors such as actinomycin D and puromycin. Furthermore, these studies showed that the action of aldosterone was of slow onset, reaching its maximum only after several hours of incubation with the studied membrane. The work of Sharp and Leaf[3] as well as more recent investigations[4] have shown that aldosterone may also enhance the permeability of the apical (mucosal) cell membrane to Na^+. Studies concerning the action of this hormone on amphibian skin are

Abbreviations: PD, potential difference; s.s.c., short circuit current.

less numerous; Crabbé[5] showed a stimulatory action of aldosterone on toad skin which, however, was less marked than that on toad bladder. Nielsen[6], on the other hand, found that aldosterone stimulates moulting in frog skin after 2–4 h of incubation, and only after this period activation of Na^+ transport was prominent.

Several other steroid hormones were shown to have some action on ion transport in isolated biological membranes. Taubenhaus *et al.*[7] found stimulation of ion transport into frog skin sacs in the presence of desoxycorticosterone, fluorohydrocortisone and hydrocortisone, but the effect of estradiol and testosterone was minimal. On the other hand, Porter and Edelman[8] showed significant action of several steroids, including prednisolone, on Na^+ transport in the toad bladder. However, there was little, if any, effect of progesterone on this structure.

The purpose of the present paper is to obtain, first, an experimental procedure which is able to detect the effect of mineralocorticoids on toad skin Na^+ transport consistently, and subsequently, to compare this effect with that of the sex steroids estradiol, progesterone and testosterone.

METHODS

Ventral skins of toads of the species *Bufo ictericus* and *Bufo paracnemis* of both sexes and weighing more than 90 g were used. These toads were maintained in humid surroundings at temperatures between 20 and 25 °C for about 1 week prior to the experiments. These were performed in the months from September to April. Two to four segments were obtained from each toad for paired experiments. They were mounted in lucite chambers having a surface of 7 cm^2, according to the method of Ussing and Zerahn[9]. Potential differences (PD) between the two chambers were measured *via* Ringer–Agar bridges and calomel half-cells by means of a Keithley Model 610 C electrometer or of a Metrohm Model E 322 compensator. Short circuit current (s.c.c.) was measured by passing current through the preparation *via* Ringer–agar bridges and Ag/AgCl electrodes, using a current meter connected to a potentiometric circuit.

Segments of toad skin were preincubated for 12 h with chloride Ringer solution containing the hormone being studied in the internal compartment. At the time of the experiment, the kinetics of Na^+ transport were measured by replacing this solution in the external compartment by a series of solutions containing 115, 50, 20, 10, 5, 1, 0.5 and 0.1 mequiv/l Na^+, respectively, in the form of sulfate and in the indicated sequence, these solutions being brought to 115 mequiv/l SO_4^{2-} with $MgSO_4$. The external chamber was washed twice with every new solution, and the readings were performed 3 min after the addition of this solution, when the readings tended to stabilize.

PD, s.c.c. and resistance were obtained under these conditions. The results were obtained according to Michaelis–Menten kinetics, plotting the reciprocal of s.c.c. against the reciprocal of the Na^+ concentration in the external compartment, thus obtaining a Lineweaver–Burk graph, from which the maximal transport velocity(V) and the Michaelis constant (K_m) were obtained[10]. Simultaneous measurements in control skins (preincubated only with Ringer solution) and hormone-incubated skins obtained from the same animal were made, the results being evaluated by the t test with paired data.

Solutions used: Chloride Ringer solution: NaCl, 115 mM; $KHCO_3$, 2.5 mM;

$CaCl_2$, 1.0 mM; glucose, 5.5. mM. Sulfate Ringer solution: Na_2SO_4, 57.5 mM; $KHCO_3$, 2.5 mM; $CaSO_4$; 1.0 mM. Solutions of 0.1–50 mM Na^+: same as sulfate Ringer solution, with the corresponding portions of Na_2SO_4 replaced by $MgSO_4$.

The final concentrations of the hormones used were: (+)-Aldosterone (Ciba): $1.38 \cdot 10^{-6}$ M; desoxycorticosterone acetate (Sigma): $1.35 \cdot 10^{-6}$ M; 17-β-estradiol (Sigma): $1.84 \cdot 10^{-6}$ M; testosterone (Sigma): $1.74 \cdot 10^{-6}$ M; progesterone (Sigma): $1.59 \cdot 10^{-6}$ M.

These concentrations are higher than those used in toad bladder ($7 \cdot 10^{-7}$ M, ref. 8), but lower than those referred to by Crabbé[5] for toad skin (10^{-5} M). Stock solutions containing 0.2 mg/ml in ethanol were used. The addition of ethanol alone in similar amounts did not interfere with Na^+ transport. Control skins received an equivalent amount of ethanol without hormone.

RESULTS

The addition of aldosterone or desoxycorticosterone at the indicated concentrations to the internal side of toad skins in the Ussing chamber did not produce significant alterations of PD or s.c.c. during observation periods of up to 6 h. For instance, 5 h after addition of desoxycorticosterone the ratio between the s.c.c. measured at this time to that at time zero (before addition of hormone) was 1.03 $\pm$ 0.40 (mean $\pm$ S.E. $n = 24$), compared to a value of 0.96 $\pm$ 0.27 ($n = 24$) for control skins. These values were not statistically different, as evaluated by the t test with paired data. The use of 10-fold higher hormone concentrations gave similar results. However, by means of the following procedure a significant effect of mineralocorticoid hormones on this structure could be observed. The skins were preincubated for 12 h with chloride

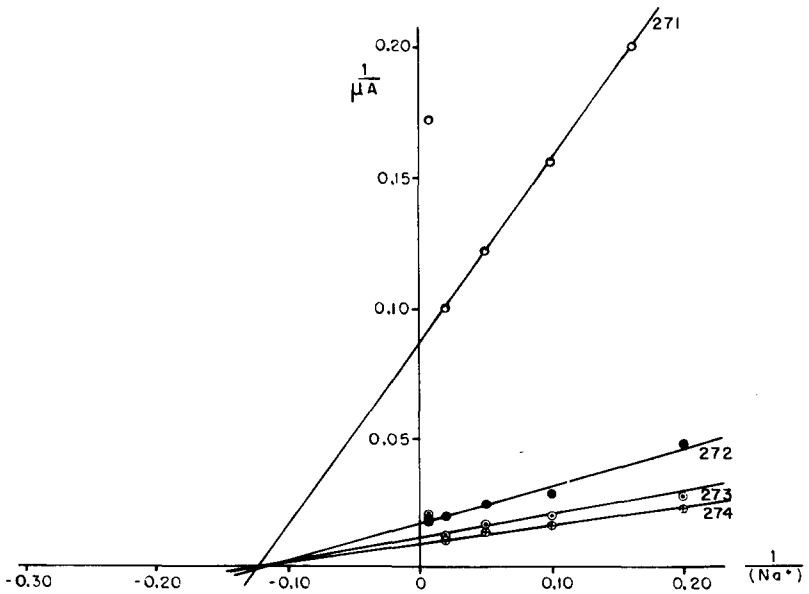

Fig. 1. Lineweaver–Burk plot of 1/s.c.c. (in μA) at different external Na^+ concentrations (1/Na^+), in skins preincubated for 12 h with desoxycorticosterone acetate. 271, control; 272–274, desoxycorticosterone acetate.

Ringer solution in the absence or presence of the studied hormone in the internal compartment, whereupon the Na^+ concentrations of the external medium were progressively reduced in sulfate Ringer solution in order to study the kinetics of Na^+ transport under these conditions. A representative example of the Lineweaver–Burk type of graphs thus obtained is given in Fig. 1 in which $1/Na^+$ (external) on the abscissa is plotted against 1/s.c.c. in μA on the ordinate. A control skin (No. 271) is compared with three fragments of deoxycorticosterone acetate-preincubated skins from the same toad (272–274). It is clearly seen that there are considerable differences between the ordinate intercepts (V), but not between the abscissa intercepts (K_m). A similar graph concerning testosterone-preincubated skins is given in Fig. 2, in which the differences between control (393) and testosterone are also apparent. In all these experiments a good linearity of the Lineweaver–Burk graph was obtained only between 5 and 50 mequiv/l external Na^+ concentration; this was the range used for the evaluation of Michaelis–Menten kinetics. At higher Na^+ concentrations, transport rates were considerably lower than predicted from these graphs, as is shown on Figs 1 and 2 (see points next to the ordinate), suggesting the occurrence of self-depression of Na^+ transport at high external concentrations.

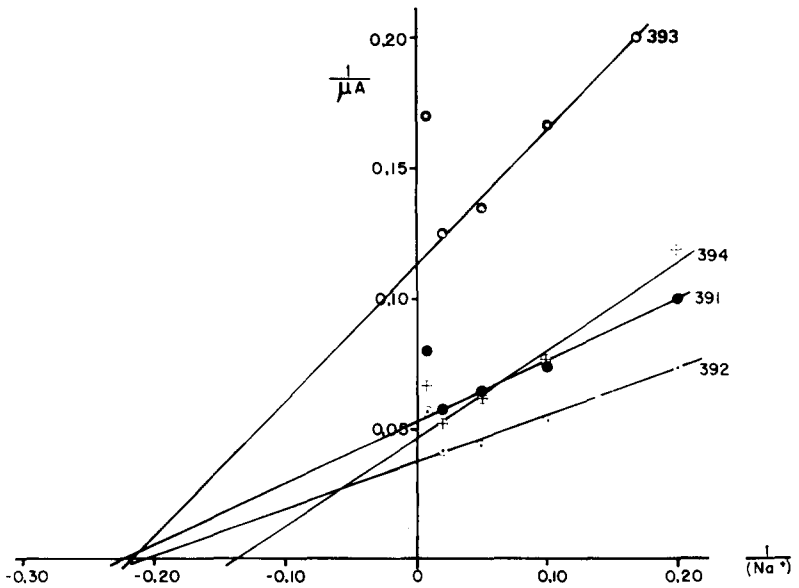

Fig. 2. Lineweaver–Burk plot of 1/s.c.c. at different external Na^+ concentrations, in skins preincubated for 12 h with testosterone. 393, control; 371, 392 and 394, testosterone.

Mean values of K_m and V obtained by this method are given on Table I. Standard errors and the number of skins used are also given. It is clear that preincubation with steroid hormones leads to a highly significant increase in V, but K_m values are only altered with deoxycorticosterone acetate and estradiol.

Although all V increases are significant, those obtained by aldosterone and deoxycorticosterone acetate preincubation are considerably greater than those obtained with the other hormones. On the other hand, the differences between the mean

TABLE I

EFFECT OF PREINCUBATION OF TOAD SKIN WITH STEROID HORMONES ON PARAMETERS OF MICHAELIS–MENTEN KINETICS

For hormone concentrations, see Methods. Means ± S.E. are given. Number of observations is given in parentheses.

Experiment	K_m (*mequiv/l*)	V (μA)
(1) Control	9.62 ± 1.09 (22)	140.5 ± 13.4 (22)
Aldosterone	9.28 ± 1.34 (18)	434.5 ± 41.4 (18) **
Desoxycorticosterone acetate	6.14 ± 0.57 (23) *	509.2 ± 47.2 (23) **
(2) Control	6.50 ± 1.12 (6)	167.2 ± 25.6 (6)
Estradiol	10.26 ± 1.02 (18) **	251.5 ± 20.2 (18) **
(3) Control	7.16 ± 0.99 (14)	221.1 ± 32.2 (14)
Testosterone	7.83 ± 1.49 (42)	315.3 ± 19.8 (42) **
(4) Control	9.44 ± 0.68 (4)	189.2 ± 20.4 (4)
	9.28 ± 0.91 (12)	391.5 ± 21.6 (12) **

* $0.05 > P > 0.01$.
** $P < 0.01$.

K_m values observed during these experiments are of relatively small magnitude, without showing a definite trend in one or the other direction.

From the relation between PD and s.c.c., an evaluation of skin resistances was obtained in the studied experimental conditions, assuming linearity between current and voltage over the used range of values. Such data, expressed as $\Omega \cdot cm^2$, were obtained in chloride and in sulfate Ringer solution. They are given in Table II for hormone-preincubated skins and for their respective controls. Statistical evaluation of the difference between these values by the t test is also included. It is seen that aldos-

TABLE II

EFFECT OF PREINCUBATION WITH STEROID HORMONES ON ELECTRICAL RESISTANCE OF TOAD SKIN

Means ($\Omega \cdot cm^2$) ± S.E. are given. Number of observations is given in parentheses.

Experiment	*Chloride Ringer's*	*Sulfate Ringer's*
(1) Control	2242 ± 350 (14)	3140 ± 217 (14)
Aldosterone	1814 ± 203 (18) *	2136 ± 175 (18) *
(2) Control	1435 ± 161 (8)	3886 ± 189 (8)
Desoxycorticosterone acetate	827 ± 56 (23) *	1884 ± 119 (23) *
(3) Control	1324 ± 217 (6)	3886 ± 329 (6)
Estradiol	1178 ± 84 (18)	2838 ± 182 (18) *
(4) Control	1626 ± 168 (14)	2908 ± 350 (14)
Testosterone	1450 ± 105 (41)	2600 ± 126 (42)
(5) Control	1668 ± 182 (4)	3799 ± 371 (4)
Progesterone	1345 ± 168 (10)	2652 ± 189 (12) *

* $P < 0.01$.

terone-desoxycorticosterone acetate-preincubated skins show highly significant reductions in resistances in both bathing fluids. Preincubation with estradiol and progesterone leads to significant reductions only in sulfate Ringer solution, whereas testosterone preincubation does not significantly change this parameter.

DISCUSSION

The results presented in this paper indicate, in the first place, that a kinetic analysis according to Michaelis and Menten of Na^+ transport in toad skin after long-term (12 h) preincubation with steroid hormones is a valuable means to study the action of hormones on this preparation. The differences obtained between hormone-incubated and control skins are highly significant, which was not the case in experiments on the "acute" preparation, in which the effect of hormones is observed within several hours of its addition to the bathing medium. The absence of a satisfactory response during short-term experiments may be due to the moulting phenomenon observed by Nielsen[6], involving a phase of s.c.c. reduction 2–4 h after addition of aldosterone.

With regard to the measurements of Na^+ transport kinetics, some considerations concerning the experimental conditions could be made. It is known that a small part of the s.c.c. in toad skin can be due to transport of other ions, such as Cl^-(ref. 11). This could affect the values of Na^+ transport, especially at low external Na^+ concentrations, where net Na^+ transport is low. It is possible that this was the reason for deviations from linearity of the Lineweaver–Burk plot observed at external Na^+ concentrations below 5 mequiv/l; therefore, we have chosen not to use this lower range for our calculations. Another source of error could be the passive Na^+ back-flux from the internal to external side of the skin due to the concentration gradient created under these experimental conditions. It has been shown, however, that at external concentrations of 1 mequiv/l the passive efflux is still low in comparison with the measured net flux, and therefore should not affect our results significantly [12].

The transport mechanism studied under the present experimental conditions is the limiting step in the Na^+ transfer process, and it need not be the Na^+ pump, believed to be located at the internal cell membrane. Isotope flux studies by Frazier *at al.*[13] have given some indication that this limiting step might be located at the external side (mucosal cell membrane in the toad bladder), and that it consisted of a saturable process. On the other hand, Cereijido *et al.*[14] have shown that the Na^+ permeability of the external membrane decreased with increasing Na^+ concentrations in the external compartment between 7 and 115 mequiv/l, an observation that could correspond to the existence of a saturable process, such as facilitated diffusion, at this site. Furthermore, the observation of these authors could explain our finding of "self-depression" of Na^+ transport at high external Na^+ concentrations. It is, therefore, very possible that the saturable process we are dealing with might not be the Na^+ pump, but a transfer mechanism at the external cell membrane. This cannot be differentiated using the present means, for the measured K_m is a parameter referred to the epithelium as a whole, and could be dependent on any process within this structure; it should, however, be related to the limiting step of the Na^+ transport mechanism.

It is quite clear from our results that both mineralocorticoid and sex steroid

hormones significantly enhance the maximal velocity of Na^+ transport in the toad skin, the former leading to a greater effect than the latter. This might be due to an increase of active sites available for transport, to an increased access of Na^+ to these active sites, or to an increased amount of energy-rich substrates available for transport. On the other hand, the affinity between these transport sites and the transported cation, commonly considered to be evaluated by the Michaelis constant, does not appear to be consistently altered.

The data given on Table II might give an indication concerning possible differences in the mechanism of action of the studied hormones. The electrical resistance of the toad skin is commonly considered to be related to overall ionic permeability, when similar electrolytic solutions are in contact with the membrane. It should be remembered, however, that the skin conductance could also be dependent on a carrier-mediated transfer of some of the involved ions across one or more of the membrane barriers within this epithelium. Aldosterone and desoxycorticosterone acetate lead to considerably decreased resistances both in chloride and in sulfate Ringer solution, indicating the occurrence of increased ionic conductivities. These findings agree with those of Civan and Hoffman[15], which showed decreased resistances of toad bladder due to action of aldosterone, and also with the results of Eigler[16], showing that aldosterone increases transepithelial water flux along an osmotic gradient in the skin of *Rana temporaria*. On the other hand, sex steriods did not change resistances in chloride Ringer solution, and only estradiol and progesterone reduced resistances in sulfate Ringer solution; the latter medium, due to the presence of a less permeant anion, permits greater sensitivity in the assessment of resistance changes. These results indicate that the changes in ion permeability produced by sex steroids are considerably less than those produced by mineralocorticoids. The observed differences might be related simply to the intensity of the action of these hormones on the same site; however, it is possible that the observed differences in the effect on skin resistances might reflect an action of the mineralocorticoids on an additional site within the epithelium, which would lead specifically to the observed decrease in skin resistance. The observed alteration of the skin resistance could, furthermore, also be due to changes in the cellular interspaces of the epithelium, which are the cause of an important electrical shunt across this membrane[17].

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REFERENCES

1 I. S. Edelman, R. Bogoroch and G. A. Porter, *Proc. Natl Acad. Sci. U.S.*, 50 (1963) 1169.
2 G. A. Porter, R. Bogoroch and I. S. Edelman, *Proc. Natl Acad. Sci. U.S.*, 52 (1964) 1326.
3 G. W. G. Sharp and A. Leaf, *Physiol. Rev.*, 46 (1966) 593.
4 L. Lipton and I. S. Edelman, *Am. J. Physiol.*, 221 (1971) 733.
5 J. Crabbé, *Hormones and the Kidney*, Academic Press, London, 1963, p. 75.
6 R. Nielsen, *Acta Physiol. Scand.*, 77 (1969) 85.
7 M. Taubenhaus, I. B. Fritz and J. V. Morton, *Endocrinol.*, 59 (1956) 458.
8 G. A. Porter and I. S. Edelman, *J. Clin. Invest.*, 43 (1964) 611.
9 H. H. Ussing and K. Zerahn, *Acta Physiol. Scand.*, 23 (1951) 110.

10 H. R. Mahler and E. H. Cordes, *Biological Chemistry*, Harper and Row, New York, 1966, p. 250.
11 C. R. House, *Biochim. Biophys. Acta*, 173 (1969) 344.
12 T. U. L. Biber, R. A. Chez and P. F. Curran, *J. Gen. Physiol.*, 49 (1966) 1161.
13 H. S. Frazier, E. F. Dempsey and A. Leaf, *J. Gen. Physiol.*, 45 (1962) 529.
14 M. Cereijido, F. C. Herrera, W. J. Flanigan and P. F. Curran, *J. Gen. Physiol.*, 47 (1964) 879.
15 M. M. Civan and R. E. Hoffman, *Am. J. Physiol.*, 220 (1971) 324.
16 J. Eigler, *Pfluegers Arch.*, 317 (1970) 236.
17 H. H. Ussing and E. E. Windhager, *Acta Physiol. Scand.*, 61 (1964) 484.