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EFFECT OF OUABAIN ON CORTICOSTERONE BIOSYNTHESIS AND ON POTASSIUM AND SODIUM CONCENTRATION IN CALF ADRENAL TISSUE *IN VITRO*

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SUMMARY

1. Corticosterone biosynthesis by slices of the outer cortex of calf adrenals is inhibited by low concentrations of ouabain added to the incubation medium.

2. Simultaneously ouabain inhibits the active cation transport system resulting in a loss of intracellular (non-inulin space) potassium and a gain of intracellular sodium.

3. The ouabain concentration causing a half-maximal inhibitory effect is about the same (10^{-7} M) for both corticosterone biosynthesis and cation transport.

4. Biosynthesis of corticosterone by a mitochondrial preparation was unaffected by ouabain.

5. The findings point to a connection between the two effects induced by ouabain.

INTRODUCTION

Systematic studies of the effects of cardiac glycosides on biosynthesis of corticosteroids *in vitro* have not been reported so far. The influence of cardiac glycosides administered *in vivo* on adrenocortical function[1–4] could be due to changes in cardiovascular[1] or hypophyseal[3,5] function.

It is well known that cardiac glycosides inhibit the active transport of potassium and sodium in many cells and tissues[6–13], although this has not yet been established for adrenocortical tissue. It is also known that potassium stimulates the biosynthesis of corticosteroids, *in vivo* as well as *in vitro*[14–17]. It therefore occurred to us that the effects of cardiac glycosides on corticosteroid biosynthesis might be mediated through their inhibitory effect on active cation transport in the adrenal cells.

The present study was therefore designed to examine whether ouabain added to adrenocortical tissue *in vitro* does indeed cause an efflux of intracellular potassium and an influx of intracellular sodium and whether such alterations in cation composition of the tissue are accompanied by a decrease in adrenocortical activity.

MATERIALS AND METHODS

Preparation of tissue slices and mitochondria and incubation procedure

Calf adrenals were obtained from the slaughterhouse as soon as possible after the death of the animals. The glands were transported to the laboratory in crushed ice. After the glands were freed of adhering fat, 0.5-mm thick slices of the outer cortex were cut by means of a Stadie–Riggs fresh-tissue microtome and placed in cooled incubation medium. After gentle blotting, 500-mg (± 1%) portions were prepared by weighing. These were incubated for 3 h in 4.0 ml Krebs–Ringer bicarbonate solution containing 200 mg/100 ml glucose at 37° in a Dubnoff metabolic shaker under O_2–CO_2 (95:5, v/v). Adequate amounts of a 10^{-3} M ouabain solution in Krebs–Ringer bicarbonate-glucose were added to the media to obtain the desired concentrations. Incubation was always started within 2 h of the death of the animals. After incubation the media were decanted from the tissue. The tissue was washed twice with 1 ml water, and the washings were added to the media. An aqueous solution containing a known amount of [1,2-$^3H_2$]corticosterone (specific activity, 25 C/mmole) was added to serve as an indicator of steroid loss during the isolation procedure.

For the preparation of mitochondria outer-cortex slices collected in ice-cold Krebs–Ringer bicarbonate–glucose were used. The cortical tissue was scraped from the capsule, and portions of about 300 mg of the scrapings were homogenized in a Ten Broeck all-glass homogenizer by three strokes of the pestle. The homogenates thus obtained were combined, and the mitochondrial pellet was prepared as described by RAMAN *et al.*[18] with minor modifications. The homogenate was first centrifuged for 15 min at 750 × *g* in an MSE refrigerated centrifuge, to remove cell debris and nuclei. The supernatant solution was centrifuged again for 30 min at 7200 × *g* and decanted. The sediment was resuspended in the same volume of 0.25 M sucrose and centrifuged for 30 min at the same speed. After this centrifugation the supernatant solution was decanted, and the pellet (designated "mitochondria") was resuspended in the incubation medium. Determination of the protein content of the mitochondrial preparation was performed by the method of LOWRY *et al.*[19]. The incubation medium consisted of Krebs–Ringer bicarbonate–glucose containing 4 mM ATP, 8 mM fumarate and 1 mM NADP. The pH was adjusted to 7.3. In this medium the mitochondrial preparation produced adequate amounts of corticosterone. Portions of 4 ml were incubated at 37° for 3 h in a Dubnoff metabolic shaker under O_2–CO_2 (95:5, v/v). Controls were compared with incubates containing 10^{-4} M ouabain. At the end of the incubation a known amount of [1,2-$^3H_2$]corticosterone was added to each vessel as an indicator of steroid loss during the purification procedure.

Extraction of corticosteroids and purification of corticosterone

The media collected after incubation were extracted with 7 vol. of dichloromethane. After evaporation of the dichloromethane the crude residues were chromatographed on a silica gel layer by 3 successive runs in chloroform–ethanol (99:1, v/v), ethylacetate–ethylenedichloride–water (90:10:1, by vol.) and chloroform–methanol–water (94:6:0.5, by vol.), respectively[20]. The corticosterone zones (R_F 0.73) were detected under ultraviolet light and extracted according to the method described by MATTHEWS *et al.*[21]. Subsequently the corticosterone samples were rechromatographed on a second thin layer in the system chloroform–methanol–water (94:6:0.5, by vol., R_F 0.41) and on paper in a Busch-B_5 system (R_F 0.82) (ref. 22).

Incubated outer-cortex slices were removed from the medium, minced and homogenized. After addition of [1,2-$^3H_2$]corticosterone to the homogenate, extraction of corticosteroids was performed with 7 vol. of dichloromethane. The dichloromethane was evaporated, and the crude residues were dissolved in 5 ml 70% methanol and subsequently washed 2 times with 1 ml light petroleum (b.p. 80–100°)–toluene (1:1, v/v). The methanolic extracts were evaporated to dryness and the residues chromatographed as described above.

The mitochondrial incubates were extracted with dichloromethane, and the extracts were partitioned between 70% methanol and light petroleum–toluene as described for the tissue extracts. The chromatographic purification of the corticosterone formed during incubation was carried out as described above.

Fluorimetric assay of corticosterone

Corticosterone was determined by fluorimetry after the 3-stage chromatographic purification. The alcoholic eluate of the corticosterone area of the Bush-B_5 chromatogram was evaporated and the residue redissolved in dichloromethane. To an aliquot of this solution (10 ml) 2 ml fluorescence reagent (sulfuric acid–ethanol, 7:3, v/v) was added, and after 1 h at room temperature the fluorescence was read in an Aminco–Bowman fluorimeter (activating wavelength, 470 nm; fluorescence wavelength, 520 nm) (ref. 23). Filter paper blanks were run through the same procedure. In another aliquot of the dichloromethane solution the radioactivity was measured enabling correction for losses of corticosterone during extraction and purification.

Measurement of the sodium and potassium content of the tissue

In experiments in which the effect of ouabain on the sodium and potassium content of the incubated tissue was studied, about 250 mg of outer-cortex slices were incubated for 3 h in 2 ml Krebs–Ringer bicarbonate–glucose under the conditions described above. At the end of the incubation the vessels were successively removed from the incubator, and the tissue was separated from the medium by means of a Buchner funnel[24]. The tissue was weighed and dried overnight at 100° under reduced pressure. After measuring the dry weights the tissue samples were digested in 100 μl 70% HNO_3 at room temperature for at least 5 h (ref. 8). The HNO_3 digests were thoroughly mixed with 9.9 ml distilled, deionized water, and after centrifugation the clear supernatants were appropriately diluted and used for the estimation of the sodium and potassium contents in an Eppendorff flame photometer. The sodium and potassium concentrations of the incubation media were also measured.

Measurements of inulin space and calculation of intracellular sodium and potassium concentrations

In parallel experiments the extracellular space of the tissue was estimated in order to calculate sodium and potassium concentrations in the intracellular fluid (non-inulin space). In these experiments outer-cortex slices (250 mg wet wt. per vessel) were incubated in 2 ml Krebs–Ringer bicarbonate–glucose containing 0.2 μC [^{14}C]-carboxylinulin per ml (specific activity, 2.24 mC/g). At the end of the 3-h incubation the tissue was separated from the medium in the manner described above. After

weighing, the tissue was homogenized in 6.0 ml 10% trichloroacetic acid and after standing for several hours and subsequent centrifugation, 200-μl samples of the supernatant were taken for measurement of the ^{14}C radioactivity in BRAY's solution[25]. The radioactivity of the medium was also measured. The inulin space (extracellular compartment) was calculated from the relation:

$$\text{inulin space (in \% of wet wt.)} = \frac{\text{counts/min } ^{14}C \text{ per mg tissue}}{\text{counts/min } ^{14}C \text{ per } \mu l \text{ medium}} \times 100$$

The non-inulin space (intracellular compartment) can be calculated from the total tissue water and the inulin space. The sodium and potassium concentrations in the non-inulin space were calculated from the relation:

$$c_i = \left(c_t - \frac{E}{100} \cdot c_m\right) \cdot \frac{100}{I}$$

where c_i = intracellular cation concentration (mequiv/kg intracellular fluid); c_t = cation content of total tissue (mequiv/kg wet wt.); c_m = cation concentration of medium at termination of the incubation (mequiv/l); E and I = the extra- and intracellular fluid compartment, respectively (% of wet wt.).

Radioactivity measurements

Radioactive samples were counted in a Nuclear Chicago liquid scintillation spectrometer (system 725). Alcoholic solutions of the steroid (0.1 ml) were counted in 10 ml scintillation fluid (3 g 2,5-diphenyloxazole and 0.2 g 1,4-bis-(5-phenyloxazolyl-2)-benzene per l toluene). Aqueous solutions of the [^{14}C]carboxylinulin were counted in BRAY's solution[25].

Chemicals

[1,2-$^3H_2$]Corticosterone and [^{14}C]carboxylinulin were obtained from New England Nuclear Corp., ouabain from Nutritional Biochemicals Corp. and ATP as the disodium salt, fumarate as the disodium salt and NADP as the disodium salt were purchased from Boehringer, Mannheim.

RESULTS

Ouabain and corticosterone biosynthesis in vitro

Ouabain in a concentration of 10^{-8} M had no significant effect on corticosterone biosynthesis by the outer-cortex slices. At higher concentrations the cardiac glycoside caused a marked inhibition of corticosterone production (Table I). At concentrations ranging from 10^{-7} to 10^{-4} M a decrease of corticosterone production was observed from 36–75% of the control value. Although not quantitated, it was obvious from inspection of the first thin-layer chromatogram under ultraviolet light that apart from inhibition of corticosterone biosynthesis, ouabain caused a marked reduction of the amounts of 18-hydroxycorticosterone and aldosterone.

In the foregoing experiments only the corticosterone released into the medium was quantitated. In another experiment determination of corticosterone released into the medium was shown to be a valid parameter of corticosterone biosynthesis. In this experiment, in which 10^{-4} M ouabain was present, corticosterone was measured

TABLE I

EFFECT OF OUABAIN ON CORTICOSTERONE BIOSYNTHESIS BY OUTER SLICES OF CALF ADRENALS

Corticosterone was measured in the medium after incubation for 3 h in Krebs–Ringer bicarbonate–glucose medium containing various ouabain concentrations. Mean values ± S.E. are shown. Numbers in parentheses indicate number of observations.

Ouabain (*M*)	*Expt. No.*	*Corticosterone* (*μg/500 mg tissue per 3 h*)	*Change* (%*)	*P* (*Student t-test*)
0	1	8.67 ± 0.87 (5)		
	2	10.44 ± 0.80 (5)		
10^{-8}	1	7.19 ± 0.26 (5)	−17	>0.1
10^{-7}	1	5.53 ± 0.66 (5)	−36	<0.05
10^{-6}	1	3.08 (2)	−64	
	2	3.61 ± 0.21 (5)	−65	<0.001
10^{-5}	1	2.27 (2)	−74	
	2	2.63 ± 0.20 (5)	−75	<0.001
10^{-4}	1	2.61 (2)	−70	
	2	2.59 ± 0.21 (5)	−75	<0.001

* Percentage of appropriate control.

TABLE II

EFFECT OF 10^{-4} M OUABAIN ON CORTICOSTERONE RELEASED INTO THE MEDIUM AND ON CORTICOSTERONE WHICH COULD BE EXTRACTED FROM THE TISSUE

Slices were incubated for 3 h in Krebs–Ringer bicarbonate–glucose medium. After separation of medium and tissue, corticosterone was measured in both. Mean values ± S.E. are shown. Numbers in parentheses indicate number of observations.

	Corticosterone (*μg/500 mg tissue per 3 h*)	
	Medium	*Tissue*
Control	9.95 ± 0.64 (10)	4.02 ± 0.40 (9)
Ouabain	1.63 ± 0.54 (10)	0.77 ± 0.08 (9)
Change	−84% ($P<0.001$)	−81% ($P<0.001$)

TABLE III

EFFECT OF 10^{-4} M OUABAIN ON CORTICOSTERONE BIOSYNTHESIS BY A MITOCHONDRIAL PREPARATION AND BY OUTER-CORTEX SLICES OF CALF ADRENALS

Corticosterone was measured after 3 h incubation in Krebs–Ringer bicarbonate–glucose medium fortified with 4 mM ATP, 8 mM fumarate and 1 mM NADP. Mean values ± S.E. ($n = 5$) are shown.

	Costicosterone		*Change* (%)	*P* (*Student t-test*)
	Control	*Ouabain*		
Mitochondria, *μg*/9.2 mg protein per 3 h	26.96 ± 1.25	25.21 ± 0.33	−6	>0.2
Slices, *μg*/500 mg tissue per 3 h	14.32 ± 0.97	7.20 ± 0.32	−50	<0.001

in the medium as well as in the tissue homogenized after the incubation (Tabele II). The relative decrease of the corticosterone concentration in medium and tissue due to the presence of ouabain was 84 and 81% of the respective control values.

The effect of ouabain on corticosterone biosynthesis by an adrenal mitochondrial preparation was also studied (Table III). No effect of 10^{-4} M ouabain was observed. This result suggests that the effect of ouabain in the slices requires the presence of an intact plasma membrane. If tissue slices were incubated in this fortified medium, corticosterone biosynthesis was significantly inhibited (50% of control value), although considerably less than in the experiments with the normal Krebs-Ringer bicarbonate-glucose medium.

The effect of ouabain on sodium and potassium content of calf adrenal tissue

Addition of ouabain to the incubation medium resulted in a striking effect on the sodium and potassium content of the tissue (Table IV). At a concentration of 10^{-7} M the tissue sodium content was significantly increased (27% of control value).

TABLE IV

EFFECT OF OUABAIN ON POTASSIUM AND SODIUM CONTENT OF OUTER-CORTEX SLICES OF CALF ADRENALS

Outer slices were incubated for 3 h in Krebs-Ringer bicarbonate-glucose medium containing various ouabain concentrations. Mean values ± S.E. ($n = 5$) are shown.

Ouabain (M)	*Tissue sodium (mequiv/kg wet wt.)*	*Change (%)*	*P (Student t-test)*	*Tissue potassium (mequiv/kg wet wt.)*	*Change (%)*	*P (Student t-test)*
0	98.7 ± 2.16			38.1 ± 1.56		
10^{-8}	104.8 ± 3.78	+6	>0.1	39.6 ± 3.90	+4	>0.7
10^{-7}	125.2 ± 2.48	+27	<0.001	20.8 ± 0.84	−45	<0.001
10^{-6}	125.2 ± 2.53	+27	<0.001	11.6 ± 0.23	−70	<0.001
10^{-5}	122.8 ± 3.04	+24	<0.001	11.0 ± 0.48	−71	<0.001
10^{-4}	131.0 ± 6.98	+33	<0.01	11.3 ± 0.51	−70	<0.001
	Nonincubated slices					
	98.1 ± 1.19			26.4 ± 1.33		

Higher concentrations of ouabain did not further augment this sodium uptake by the tissue. Simultaneously the tissue potassium content was decreased by ouabain. At a concentration of 10^{-7} M the potassium loss of the tissue was 45% of the control value. At concentrations of 10^{-6} M and higher the potassium loss was increased to about 70%. In Table IV the sodium and potassium contents of non-incubated slices are also given. These slices were immersed in the cooled buffer while the slices were being prepared. Comparison of these values with those of the incubated slices shows that during incubation for 3 h the sodium content remained unchanged, while the potassium content was significantly increased ($P<0.001$).

Intracellular sodium and potassium concentrations were calculated from the figures presented in Table IV, the medium cation concentrations at termination of incubation and measurement of the non-inulin space of the tissue in parallel experiments. The total tissue water content and the non-inulin space were not influenced

by ouabain (average values 83 and 37%, respectively, of tissue wet weight at termination of incubation). The calculated intracellular cation concentrations are depicted in Fig. 1. This figure shows that ouabain has a considerable effect on the intracellular sodium and potassium content. Ouabain added in concentrations of 10^{-7} M and higher caused an influx of sodium and an efflux of potassium. The sodium and potassium concentrations of the media measured at termination of the incubation are given in the same figure.

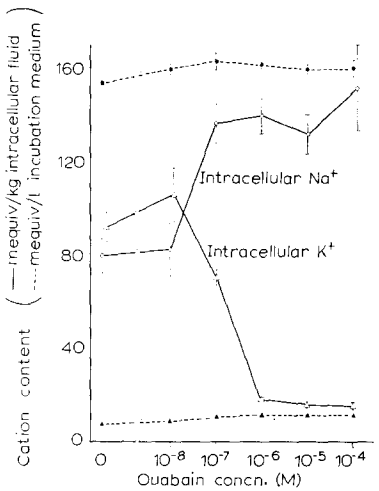

Fig. 1. Effect of ouabain on intracellular potassium and sodium concentration in outer slices of calf adrenals incubated for 3 h in Krebs–Ringer bicarbonate–glucose medium. The mean values of intracellular potassium and sodium measured at various ouabain concentrations are connected by uninterrupted lines. Dotted lines connect the potassium (K^+_m) and sodium (Na^+_m) concentrations in the medium at the end of incubation. Each point represents the mean of 5 observations. Length of vertical lines represents $2 \times$ S.E. The standard errors of K^+_m were too small to be shown. The half-maximal inhibition concentrations for potassium and sodium are about $2 \cdot 10^{-7}$ and $3 \cdot 10^{-8}$ M, respectively. ▲---▲, K^+_m concentration; ●---●, Na^+_m concentration.

DISCUSSION

The results of the present experiments demonstrate that ouabain markedly inhibits the biosynthesis of corticosterone by beef adrenal outer-cortex slices. The observation that the biosynthesis of corticosterone by a mitochondrial preparation was unaffected by ouabain suggests that the glycoside has no direct influence on the biosynthetic steps occurring in these subcellular particles. An intact plasma membrane thus appears to be an essential prerequisite for ouabain action on corticosteroidogenesis.

Furthermore the findings reported demonstrate that the active cation transport system of the adrenal tissue is inhibited by ouabain. The observed loss of intracellular potassium and gain of intracellular sodium is consistent with the presence of an ouabain-sensitive ($Na^+ + K^+$)-activated ATPase system as found by BONTING *et al.*[26] in cat adrenals and by PISAREVA[27] in adrenal cortex of guinea pigs.

The observation that the ouabain concentration causing a half-maximal inhibitory effect is about the same (approx. 10^{-7} M, compare Table I with Fig. 1) for

both corticosterone biosynthesis and cation transport, strongly suggests that these effects are in some way connected with each other. In this regard it is of importance to note that KAPLAN[16] showed that outer slices of beef adrenals produced markedly less corticosterone and aldosterone, when the potassium concentration of the incubation medium was lowered from 5.9 (*i.e.*, potassium concentration of Krebs–Ringer bicarbonate–glucose solution) to 1.5 mequiv/l.

Further studies are indicated in order to clarify whether the observed inhibition of biosynthetic function of the cortex slices by ouabain is indeed caused by changes in intracellular cation concentration.

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