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The uptake of cardiac glycosides in relation to their actions in isolated cardiac muscle

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Summary

1. The uptake of ^{3}H-digitoxin, ^{3}H-ouabain and ^{3}H-dihydro-ouabain by isolated guinea-pig atria has been studied and compared with the inhibition of the sodium pump and with the inotropic effect.

2. Analysis of the curve relating the uptake of digitoxin and ouabain at equilibrium to the bath concentration enabled a non-saturable and a saturable binding site to be distinguished.

3. The uptake of inactive doses of dihydro-ouabain was only by a non-saturable mechanism.

4. The uptake of labelled digitoxin and ouabain was reduced in the presence of another glycoside. The amount of bound glycoside was nearly equivalent to the estimated non-saturable uptake.

5. The uptake was reduced at 4° C to the clearance of the non-saturable site.

6. ED50 of digitoxin and of ouabain for inhibition of the sodium pump were measured and compared to the ED50 for inotropic effect and to the concentrations producing a half-saturation of the saturable binding site.

7. It is concluded that binding to the saturable site may be responsible for the cardiac actions of the glycosides.

Introduction

The mode of action of digitalis compounds on the cardiac contractility is still a great matter of controversy despite a great deal of work devoted to this problem (see, Godfraind, 1969 ; Lee & Klaus, 1971). One of the most intriguing questions is the role of the inhibition of the sodium pump in the inotropic action of cardiac glycosides. Since it was shown that cardiac glycosides were taken up from the perfusion medium by isolated heart muscle (Godfraind & Lesne, 1967 ; Kuschinsky, Lahrtz, Lüllmann & van Zwieten, 1967), several workers have analysed the factors controlling this process, as well as studying the subcellular localization of ^{3}H-glycoside (Dutta, Goswami, Datta, Lindower & Marks, 1968 ; Dutta, Goswami, Lindower & Marks, 1968).

These experiments and those analysing the interaction of ouabain with the Na-K-adenosine triphosphatase (Schwartz, Allen & Harigaya, 1969), suggest that some part of the sodium pump could act as a digitalis receptor, confirming the hypothesis of Repke & Portius (1963). It was therefore of interest to compare the apparent affinity constant determined by measuring either the inotropic effect or the inhibition of the sodium pump to the affinity constant estimated by analysing

^{3}H-cardiac glycoside uptake, as a similarity of these constants would be an argument in favour of the existence of only one receptor for digitalis action.

In a previous paper (Godfraind & Lesne, 1970a), we described the uptake of cardiac glycosides by the intestinal smooth muscle. Analysis of the curve relating this uptake at equilibrium to the bath concentration enabled a non-saturable and a saturable binding site to be distinguished. The same analysis has now been extended to isolated guinea-pig atria by measuring the uptake of ^{3}H-digitoxin, of ^{3}H-ouabain and ^{3}H-dihydro-ouabain. The inhibition of ^{42}K uptake in the presence of ouabain and digitoxin has also been measured.

The present experiments show that similar concentrations produce half saturation of the specific binding site, reduce ^{42}K uptake by 50% and produce 50% of the maximum inotropic effect.

Preliminary reports on some of this work have already been published (Godfraind & Lesne, 1970b and 1971).

Methods

Albino guinea-pigs weighing approximately 350 g were killed by a blow on the head, exsanguinated and the hearts rapidly removed. The atria were dissected out in Tyrode solution. Ventricular slices of 0·5 mm were prepared using a Stadie–Riggs tissue slicer.

Physiological solution

The composition of the Tyrode solution was (mM): NaCl 137, KCl 2·68, $CaCl_2$ 1·82, $MgCl_2$ 0·105, NaH_2PO_4 0·417, $NaHCO_3$ 11·9, glucose 5·55 ; it was equilibrated with a mixture of 95% O_2/5% CO_2.

Digitoxin was dissolved in ethanol since it is only slightly soluble in water. In order to avoid bias due to the solvent, 1% ethanol was added to all the incubating solutions containing digitoxin, ouabain or dihydro-ouabain in the uptake experiments.

^{42}KCl Tyrode was prepared by replacing KCl by the same amount of ^{42}KCl, in order to obtain a specific activity of about 10 μCi/litre.

Uptake of ^{3}H-cardiac glycosides

Unless otherwise stated, the experiments were performed at 37° C on spontaneously beating atria ; at the end of the incubation, the atria were quiescent. Left and right atria were kept together.

Extraction and estimation of ^{3}H-glycosides

At the end of the appropriate incubation period, each atrium was blotted on filter paper and weighed. The tissue samples were shaken overnight in 2 ml ethanol. The extracted tissue was discarded and the ethanol extract was added to 8 ml of a scintillation solution (dimethyl POPOP 0·5 g, PPO 10 g, naphthalene 100 g, dioxane 1 l.). The radioactivity of the samples was counted and the efficiency determined with internal standards.

The recovery of the extraction was estimated either by addition of known amounts of ^{3}H-glycoside before extraction or by dissolution of extracted samples

with hyamine and counting the residual radioactivity. Recovery was 96±1% (n=10) and experimental values were corrected for recovery.

The purity of the solutions containing ^{3}H-digitoxin was checked by thin-layer chromatography, as reported by Godfraind & Lesne (1970a). The purity of the solution containing ^{3}H-ouabain and ^{3}H-dihydro-ouabain was checked by the continuous paper chromatography method developed by Dutta, Marks & Smith (1963). The three drugs gave rise to a single peak on radiochromatograms and they corresponded to a single spot. Rf values were identical with those of the non-radioactive glycosides. Tissue extracts were submitted to the same manipulations. In all experiments, the radioactivity was found to be due to the ^{3}H-glycoside dissolved in the incubation medium, and there was no evidence of metabolism.

Determination of inulin space

The inulin space was estimated after equilibration of tissues for 90 min in an incubation fluid containing 1% inulin. The inulin concentration was determined according to Gillis (1964). The inulin space of the atria blotted on filter paper as above was equal to 0·340±0·005 ml/g wet weight (n=4).

Computation of parameters

As discussed in the **Results**, the observed uptake could be described by the sum of a linear and a saturable component:

$$U = aC_m + \frac{bC_m}{C_m + K_b}$$

where U=total uptake, C_m=glycoside concentration in medium, a=proportionality constant for linear uptake, b=capacity of saturable site, K_b=equilibrium constant for saturable site. This equation can be rearranged to give:

$$U = aC_m - K_b \cdot \frac{U}{C_m} + aK_b + b$$

Multiple regression analysis was carried out to obtain least squares estimates of the regression coefficient of U against C_m and against U/C_m respectively. Even though the dependent variable, U, appears on both axes, this method has been found to give a reasonably unbiased estimate of K_b (see Colquhoun, 1971). From the two regression coefficients, a and K_b, and the intercept, aK_b+b, the estimate of b was determined.

Counting of ^{42}K

At the end of the appropriate incubation period, each atrium was blotted on filter paper, weighed and dissolved in 10% NaOH. The activity of samples containing ^{42}K was measured by a gamma detecting scintillation counter.

Statistical method

Whenever possible, values are presented as means±S.E. of mean. Significance of differences between means was checked by Student's t test.

Drugs

^{3}H-Digitoxin (0·945 Ci/mmol), ^{3}H-ouabain (11·7 Ci/mmol) and ^{3}H-dihydro-ouabain (1·0 Ci/mmol), were obtained from New England Nuclear Corp. ^{42}KCl was obtained from Mol Atomic Center (Belgium).

Results

Uptake of cardiac glycosides at 37° C

Previous studies (Godfraind & Lesne, 1967 and 1968; Kuschinsky *et al.*, 1967; Kuschinsky, Lüllmann & van Zwieten, 1968) have shown that equilibrium uptake of cardiac glycosides requires at least three hours to be achieved. The uptake of ^{3}H-digitoxin, ^{3}H-ouabain and ^{3}H-dihydro-ouabain after 4 h incubation was investigated at different drug concentrations (Table 1). Uptake of the inactive derivative, dihydro-ouabain, was studied over a range of concentrations (Reiter, 1967), and was found to be linearly proportional to the drug concentration. After subtracting the amount of glycoside dissolved in the inulin-space, the mean tissue clearance was about 0·08 ml/g wet weight. With ouabain, the tissue content after subtraction of the glycoside in the inulin-space was not a linear function of the medium concentration (Fig. 1), but was found to fit the equation already proposed for smooth muscle (Godfraind & Lesne, 1970a):

$$U = aC_m + \frac{bC_m}{C_m + K_b} \qquad (1)$$

where U is the tissue concentration at equilibrium corrected for cardiac glycoside content of inulin space, C_m is the cardiac glycoside concentration in the medium,

TABLE 1. *Uptake of cardiac glycosides by isolated guinea-pig atria in Tyrode solution at* 37° *C*

Glycoside concentration (μM)	Tissue content of glycoside (nmol/g wet weight)		
	Digitoxin	Ouabain	Dihydro-ouabain
0·003	0·027±0·002 (3)	—	—
0·01	0·076±0·002 (3)	0·009±0·0004 (7)	0·004±0·0004 (6)
0·1	0·683±0·044 (6)	0·083±0·002 (12)	0·042±0·003 (6)
0·3	1·77 ±0·01 (3)	—	—
1·0	5·66 ±0·04 (3)	0·560±0·018 (9)	0·43 ±0·02 (6)
10·0	51·6 ±1·4 (6)	4·55 ±0·14 (6)	—

TABLE 2. *Computed estimates of parameters describing uptake of cardiac glycosides by guinea-pig atria*

	Digitoxin	Ouabain
a (ml/g wet weight)	4·764±0·046	0·100±0·002
b (nmol/g wet weight)	0·652	0·158
K_b (nmol/ml)	208±43	312±44

TABLE 3. *Uptake of cardiac glycosides by isolated guinea-pig atria at* 37° *C in Tyrode solution containing a large excess of another non-radioactive glycoside, and the estimated non-saturable uptake*

Medium concentration in ^{3}H-glycoside	Tissue content of radioactive glycosides (nmol/g wet weight)				
	^{3}H-digitoxin + 10^{-4} M ouabain	Estimated non-saturable ^{3}H-digitoxin uptake	^{3}H-ouabain + 10^{-5} M digitoxin	Estimated non-saturable ^{3}H-ouabain uptake	^{3}H-dihydro-ouabain + 10^{-5} M ouabain
0·01	0·055±0·001 (3)	0·051	0·005±0·0001 (3)	0·004	0·004±0·0002 (6)
0·1	0·483±0·023 (3)	0·51	0·049±0·0009 (6)	0·044	0·047±0·003 (3)
1·0	5·10 (2)	5·10	0·473±0·013 (3)	0·440	0·460±0·016 (3)

a is the proportionality constant for the linear non-saturable uptake, b and K_b are capacity and equilibrium constants for the saturable binding site.

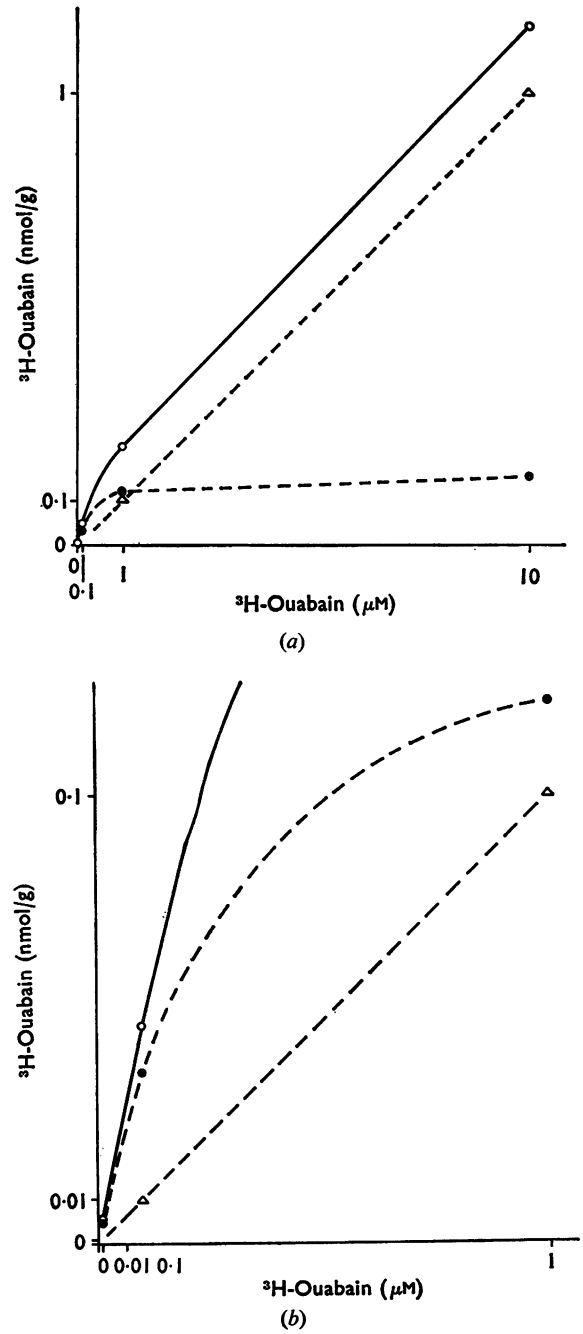

FIG. 1. Uptake of ^{3}H-ouabain by isolated atria. Ordinate: tissue content at equilibrium corrected for cardiac glycoside in solution in the inulin space. Abscissa: concentration of ^{3}H-ouabain in the perfusion fluid. Experiments were performed at 37° C. ○, Experimental data; ●, estimated content of the saturable binding site; △, estimated content of the non-saturable binding site. The lower concentrations in the range covered in Fig. 1(a) are shown on an expanded scale in Fig. 1(b).

Values of the constants computed as described under **Methods**, are reported in Table 2, together with those of digitoxin, whose uptake was also found to fit equation (1).

The main difference between the two glycosides is in the clearance of the non-saturable site, which could represent a non-specific uptake. This view is favoured by the similarity of the uptake of the inactive dihydro-ouabain and of the estimated non-saturable uptake of ouabain (Tables 1 and 3). The saturable binding site could therefore be the specific digitalis binding site. To test these conclusions, the uptake was studied in the presence of large amounts of another glycoside.

Uptake of cardiac glycosides in the presence of another glycoside

The uptake of ^{3}H-glycosides was analysed in the presence of a large excess of another non-radioactive glycoside (Table 3). A large excess of ouabain reduced ^{3}H-digitoxin uptake; digitoxin also reduced ^{3}H-ouabain uptake. However, the uptake of dihydro-ouabain was not changed by ouabain (Table 3) or by digitoxin. In the presence of a competing glycoside, the ^{3}H-glycoside uptake was nearly equivalent to the estimated non-saturable uptake (Table 3).

These results suggest that the non-saturable uptake is non-specific as it is not changed by large amounts of another glycoside. They show that there is a common binding site for cardiac glycosides which appears to be the saturable binding site considered above.

The uptake of ouabain at low temperature or in the presence of metabolic inhibitors

The binding of cardiac glycosides to microsomal preparations requires the activation of Na-K-ATPase (Schwartz, Matsui & Laughter, 1968). The uptake of ouabain was studied at 4° C, since low temperatures inactivate the sodium pump (Glynn, 1964).

^{3}H-Ouabain content of atria was measured after incubation for 4 h in Tyrode solution at 4° C containing ^{3}H-ouabain with or without a large excess of non-radioactive digitoxin. The results (Table 4) show that the uptake was linearly related to the medium concentration. At 4° C, the inulin-space of atria was reduced to 132 ± 5 ($n=4$) ml/kg wet weight instead of 340 ml at 37° C. After subtracting the content of the glycoside in the inulin-space from the total tissue content, the mean tissue clearance was about 0·15 ml/g wet weight. As also shown in Table 4, there was no decrease of ^{3}H-ouabain bound at 4° C in the presence of a large excess of digitoxin.

TABLE 4. *Uptake of ^{3}H-ouabain by isolated guinea-pig atria in Tyrode solution at 4° C*

Medium concentration of ^{3}H-ouabain (μM)	Tissue content of ^{3}H-ouabain (nmol/g wet weight)
0·1	0·027 ± 0·0007 (3)
0·1 + digitoxin 10^{-5} M	0·028 ± 0·0009 (3)
1·0	0·288 ± 0·008 (3)
10·0	2·85 ± 0·06 (3)

Monoiodoacetic acid (1 mM) or dinitrophenol (1 mM) also reduced the uptake of ^{3}H-ouabain at 37° C. Monoiodoacetic acid had a greater effect than dinitrophenol (Table 5).

TABLE 5. *Uptake of ^{3}H-ouabain by isolated guinea-pig atria in Tyrode solution at 37° C in the presence of metabolic inhibitors*

Concentration of ^{3}H-ouabain (μM)	Metabolic inhibitor	Tissue content of ^{3}H-ouabain (nmol/g wet weight)
0·01	Iodoacetic acid 1 mM	0·006±0·0002 (3)
0·01	Dinitrophenol 1 mM	0·009±0·0004 (3)
0·10	Iodoacetic acid 1 mM	0·060±0·005 (3)
0·10	Dinitrophenol 1 mM	0·072±0·002 (3)

The action of ouabain and digitoxin on ^{42}K uptake

Isolated atria were preincubated for 4 h in Tyrode solution containing different concentrations of ouabain or digitoxin. Thereafter, they were transferred to the ^{42}KCl solution. After 30 min, they were blotted on filter paper, weighed, dissolved in 10% NaOH and counted for radioactivity. The effect was expressed as the percentage inhibition of K^+ uptake by the glycoside; the maximal inhibition was the same for both ouabain and digitoxin, and the fraction of K^+ uptake sensitive to the glycosides corresponded to 60% of total K^+ uptake. As shown in Fig. 2, digitoxin was a more potent inhibitor than ouabain, the concentrations producing a 50% inhibition being respectively 0·24 μM and 0·60 μM.

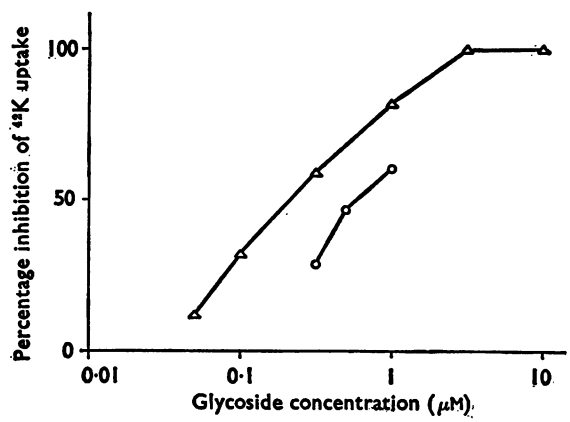

FIG. 2. Comparison of the effect of digitoxin (△) and of ouabain (○) on the ^{42}K uptake of isolated guinea-pig atria in Tyrode at 37° C. Ordinate: percentage inhibition of the K^+ uptake sensitive to cardiac glycosides. Abscissa: cardiac glycoside concentration in the perfusion fluid. Each point is the mean inhibition of at least 5 atria.

TABLE 6. *Guinea-pig heart muscle. Comparison of the concentrations producing a half-saturation of the saturable binding site, 50% diminution of ^{42}K uptake and 50% of the maximum inotropic response of papillary muscle. Concentrations are expressed in μM*

Cardiac glycosides	Half-saturation (atria)	50% inhibition		50% inotropic effect (papillary muscle)
Digitoxin	0·208	Atria	0·24	0·28*
		Ventricles	0·21	
Ouabain	0·312	Atria	0·60	0·34*
		Ventricles	0·35	

Data of Reiter (1967).

Similar experiments were performed using ventricular slices instead of atria, the concentrations producing a 50% inhibition being 0·21 μM for digitoxin and 0·35 μM for ouabain (Table 6). The ED50 for inhibition of K^+ uptake and the concentration for half-saturation of the binding site were close together. A similar observation was made by Baker & Willis (1970) on HeLa cells. We also observed a stimulation of pumping with the lowest glycoside concentrations; in the presence of either ouabain 0·003 μM or digitoxin 0·003 μM, K^+ influx in ventricle slices was 120% of controls, and this increase was significant ($P<0·01$).

Discussion

The present experiments were designed to compare uptake and action of cardiac glycosides. The three compounds studied show certain dissimilarities. Thus, digitoxin is a non-polar compound and shows high affinity for proteins, whereas ouabain is a polar compound with low affinity for proteins (Lüllmann & van Zwieten, 1969). Saturation of the ouabain lactone ring yields dihydro-ouabain and drastically reduces the positive inotropic action, dihydro-ouabain being 50 times less potent than ouabain (Reiter, 1967); Dunham & Glynn (1961) have shown that the non-saturated lactone ring of Scillaren A was required for Na-K-ATPase inhibitory activity, hexa-hydro-Scillaren A being at least ten times less potent. Similar results have been obtained by comparing ouabain and dihydro-ouabain (Godfraind, unpublished).

The uptake of ^{3}H-ouabain and of ^{3}H-digitoxin by isolated atria is a complex process involving a saturable and a non-saturable binding site; in this respect, cardiac cells behave like smooth muscle cells. There is strong evidence suggesting that binding to the saturable site is responsible for the characteristic effects of the glycosides. It seems reasonable to assume that ouabain and digitoxin act on the same receptor since their effects are governed by the same factors (Godfraind, 1969; Glynn, 1964; Lee & Klaus, 1971); it may therefore be inferred that they compete for a single binding site so that a large excess of one compound may displace a small amount of the other. The amount of radioactive glycoside bound in the presence of a large excess of a competing drug therefore represents a non-specific uptake. On the other hand, dihydro-ouabain, which is inactive up to 10^{-6} mol/l. (Reiter, 1967), shows a linear relationship between tissue uptake and medium concentration; its tissue clearance is not affected by other glycosides and is very close to the computed estimate of the non-saturable ouabain clearance.

At 4° C, the tissue clearance of ouabain is similar to that of the non-saturable binding site and is not influenced by a large excess of digitoxin. It has been shown on microsomal preparations that the presence of ATP is required for the activation of Na-K-ATPase and for the binding of cardiac glycoside to this enzyme (Schwartz, Matsui & Laughter, 1968); at low temperature, the sodium pump is inactive (Glynn, 1964), because of the small amount of available ATP, so that the Na-K-ATPase might be in a conformation which does not allow the binding of ouabain. The reduction of the binding in the presence of metabolic inhibitors is consistent with this view.

Comparison of the concentrations of cardiac glycosides producing a half-saturation of the saturable binding site with the ED50 for the inotropic effect and for inhibition of K^+ uptake (Table 8) shows that all these values lie close together,

suggesting that a single receptor may be responsible for the actions of cardiac glycosides.

Considering atrial cells as cylinders 10 μ in diameter and 85 μ in length between intercalated discs, comprising 70% of the wet tissue (Potter, 1967), the receptor capacity for ouabain appears to be $9{\cdot}5 \times 10^5$ molecules/cell. From measurement of molecular models, the area covered by the genin is approximately 10^{-14} cm^2, so $9{\cdot}5 \times 10^5$ molecules could cover about 0·034% of the plasma membrane of atrial cells. As far as digitoxin is concerned, the receptor capacity appears to be 4 times higher.

Lukas (1971) has reported that the plasma concentration of digitoxin attained during treatment is about 20 ng/ml (0·026 μM). Since 80–90% is bound to protein, the free concentration is 0·0025–0·005 μM. This is sufficient to occupy only about 1% to 2% of the binding sites, but causes a measurable stimulation of K^+ uptake by isolated atria, a small increase of the contractile tension and a large potentiation of the inotropic response to adrenaline (Godfraind & Godfraind-De Becker, 1965).

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