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The uptake of cardiac glycosides by intestinal smooth muscle of the guinea-pig in relation to digitalis receptors

T. GODFRAIND AND M. LESNE

Department of General Pharmacodynamics, University of Louvain, Louvain, Belgium

Summary

1. The accumulation and release of ^{3}H-digitoxin, ^{3}H-digoxin and ^{3}H-ouabain by isolated guinea-pig intestinal smooth muscle has been studied and compared with a pharmacological action due to inhibition of the sodium pump.

2. The uptake of labelled cardiac glycosides can be described by means of an exponential function. The t of uptake was similar for the three compounds and did not depend on the concentration.

3. Analysis of the curve relating the uptake of cardiac glycosides at equilibrium to the bath concentration enabled a non-saturable and a saturable binding site to be distinguished.

4. In contrast to the uptake observations, the onset of the pharmacological effect was dependent on the concentration, and furthermore the $t_{\frac{1}{2}}$ for this effect was shorter.

5. The release of cardiac glycosides proceeded more slowly than the uptake.

6. The uptake of a labelled glycoside was reduced in the presence of another glycoside. The amount of displaceable glycoside was nearly equivalent to the capacity of the saturable binding site.

7. The significance of these results is discussed.

Introduction

Cardiac glycosides exert a direct action on intestinal smooth muscle tone, and an indirect one on the response of this muscle to acetylcholine. These two actions are biphasic. As far as the indirect action is concerned, it was shown that the response to acetylcholine was at first potentiated, then reduced or abolished (Godfraind, 1964). Such an observation has also been made on synthetic steroids with cardiotonic properties (Godfraind & Burton, 1967), and has been extended to the heart (Godfraind & Godfraind-De Becker, 1965). The reduction of the contractile response to acetylcholine was attributed to the inhibition of the sodium pump (Godfraind & Godfraind-De Becker, 1962b, 1963 ; Godfraind, 1964).

We have now tried to see whether the amount of cardiac glycoside bound to smooth muscle cells was related to this inhibition of the sodium pump. The uptake of cardiac glycosides was monitored measuring the accumulation of either ^{3}H-digitoxin, ^{3}H-ouabain or ^{3}H-digoxin by isolated preparations in physiological solu-

tion. It was compared with the pharmacological action due to inhibition of the sodium pump. These experiments have enabled us to estimate the amount of specifically bound glycosides. A preliminary communication on some of this work was given at the Grenoble meeting of the Association des Physiologistes in June 1969.

Methods

Guinea-pig ileum preparations

Albino guinea-pigs weighing approximately 500 g were killed by a blow on the head and exsanguination. For uptake experiments, pieces of ileum 3 cm long were dissected and placed in a bath containing Tyrode solution, with known amounts of the cardiac glycoside to be studied. At the end of the appropriate incubation period, each piece was opened longitudinally, blotted on filter paper, and the mucosa and submucosa scraped off as already described (Godfraind & Godfraind-De Becker, 1962a). Smooth muscle only was used for measuring the uptake of tritiated drug.

For biological assay, pieces 4 cm long were suspended in a 50 ml bath.

Physiological solution

The composition of Tyrode solution was as follows (mmol): 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.

As digitoxin and digoxin have a low water solubility, they were dissolved in ethanol and this solution was added to Tyrode. In order to avoid any bias due to this solvent, all the incubating solutions containing either digitoxin, digoxin or ouabain contained 1% ethanol. The solutions were gassed with 95% oxygen and 5% carbon dioxide. All experiments were carried out at 37° C.

Biological assay

Recordings were made by an isometric lever with two strain gauges as part of a balanced bridge, the output of which was fed into a potentiometric recorder. In all experiments, muscles were initially loaded with 1 g.

The preparations were stimulated with acetylcholine 4×10^{-5}M, which evoked a maximum contraction.

Two control responses were determined, and after a resting period of 30 min the cardiac glycoside was added to the physiological solution for various times of incubation. Before adding the test dose of acetylcholine, the solution containing the glycoside was changed for fresh Tyrode, and by this procedure we avoided the influence of ethanol on the contractile response. Acetylcholine was kept in contact with the preparation for 15 s and the recorded response was therefore a phasic contraction. The response after treatment with cardiac glycoside was expressed as a percentage of the control response.

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 smooth muscle prepared as described above was found equal to 0·28 ml/g.

Extraction and estimation of ^{3}H-glycosides

The tissue samples (weighing approximately 150 mg) were shaken overnight in 1·5 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 litre).

The radioactivity of the samples was counted as usual, with appropriate controls, and the efficiency was determined with internal standards.

Recovery

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 $95{\cdot}1 \pm 1{\cdot}3\%$ ($n = 60$), and experimental values here reported were corrected for recovery.

Identification of cardiac glycosides in incubation solutions and in extracts

The purity of the solutions containing either ^{3}H-digitoxin, ^{3}H-digoxin or ^{3}H-ouabain was checked by thin-layer chromatography. The following solvents were used for the development of the chromatograms: for ^{3}H-digitoxin and ^{3}H-digoxin: cyclohexane/acetone/glacial acetic acid (49/49/2); for ^{3}H-ouabain chloroform/methanol/water (65/30/5). The dried chromatograms were scanned by means of a thin layer scanner LB 2721 (Berthold) system. Thereafter, the chromatograms were revealed by a saturated chloroform solution of $SbCl_3$ and examined under ultraviolet light.

The three drugs gave rise to a single peak on radiochromatograms, and they correspond to a single spot. The following R_F values were obtained: ^{3}H-digitoxin, 0·45; ^{3}H-digoxin, 0·36; ^{3}H-ouabain, 0·40.

These 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.

Drugs

^{3}H-digoxin (126 μCi/mg) was kindly supplied by Burroughs Wellcome and Co., U.S.A. ^{3}H-digitoxin (945 mCi/mmol) and ^{3}H-ouabain (620 mCi/mmol) were obtained from New England Nuclear Corp. Digitoxin, digoxin and ouabain were gifts of Nativelle, France.

Results

Uptake of cardiac glycosides

The uptake of digitoxin, digoxin and ouabain was investigated using different concentrations of each drug in the bathing fluid. The onset of the inhibition produced by cardiac glycosides on the contractile response to acetylcholine was measured in other experiments.

For concentrations from 10^{-8} g/ml up to 10^{-5} g/ml, the uptake of the three glycosides reached equilibrium after 3 to 4 hr (Fig. 1) and was fitted by the equation

$$y=A(1-e^{-\alpha t}) \qquad (1)$$

where y is the tissue concentrations of the glycoside at time t, A the concentration at equilibrium and α the rate constant (min^{-1}). As shown in Table 1 the half time did not differ significantly either for the different concentrations or for the nature of the three cardiac glycosides. The total binding (A), however, varied according to the dose and the nature of the glycoside.

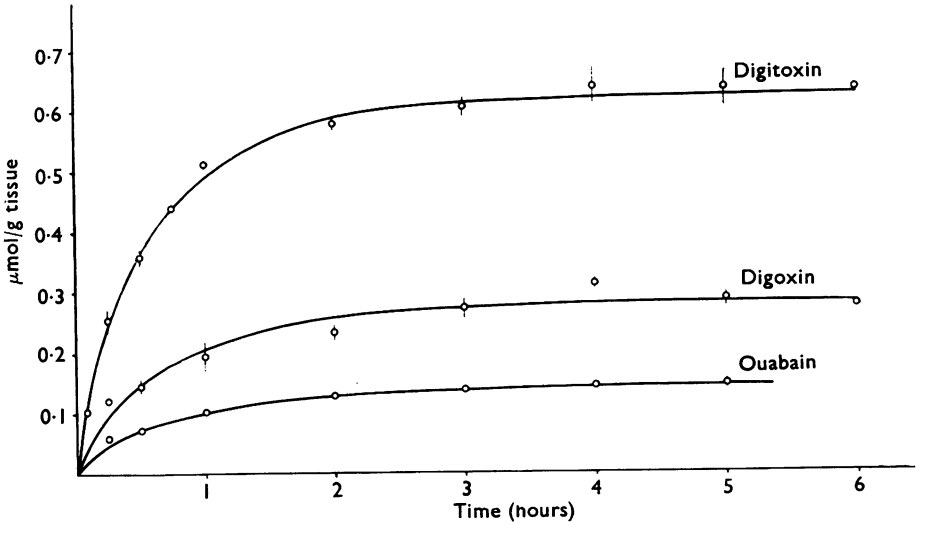

FIG. 1. Uptake of cardiac glycosides by isolated smooth muscle. Ordinate: tissue content, abscissa: duration of incubation, at 37° C, in Tyrode solution plus $1{\cdot}3\times10^{-7}$M glycoside. Each value (±S.E.) is the mean of at least five determinations.

TABLE 1. *Uptake of cardiac glycosides by smooth muscle*

Cardiac glycoside	Concentration (M)	A μg/g wet weight	$t_{\frac{1}{2}}$ min uptake	$t_{\frac{1}{2}}$ min effect
^{3}H-Digitoxin	$1{\cdot}3\times10^{-8}$	0·085	41	—
	$1{\cdot}3\times10^{-7}$	0·487	33	27
	$1{\cdot}3\times10^{-6}$	3·99	41	25
	$1{\cdot}3\times10^{-5}$	31·90	39	9
^{3}H-Digoxin	$1{\cdot}3\times10^{-8}$	0 046	41	—
	$1{\cdot}3\times10^{-7}$	0·223	32	—
	$1{\cdot}3\times10^{-6}$	1·30	36	—
	$1{\cdot}3\times10^{-5}$	10·20	39	9
^{3}H-Ouabain	$1{\cdot}3\times10^{-8}$	0·017	—	—
	$1{\cdot}3\times10^{-7}$	0·115	34	21
	$1{\cdot}3\times10^{-6}$	0·98	—	—
	$1{\cdot}3\times10^{-5}$	7·03	—	—

Values of A and of $t_{\frac{1}{2}}$ for uptake obtained from equation (1) and of $t_{\frac{1}{2}}$ for effect obtained from equation (3).

The relation between the tissue content in ^{3}H-glycoside at equilibrium and the concentration in the medium was found to fit the equation

$$U=aC_m+\frac{b\,C_m}{C_m+K_b} \qquad (2)$$

where U is the tissue concentration at equilibrium corrected for cardiac glycoside content of extracellular space, C_m is the cardiac glycoside concentration in the medium, a is the capacity constant for a non-saturable binding site, b and K_b are capacity and equilibrium constants for a saturable binding site. Values of these constants are given in Table 2. In Table 2 are also reported the uptake capacity of the saturable binding site in molecules per cell and the corresponding covered surface expressed in per cent of cell surface according to Paton and Rang (1965). The surface covered by one molecule of cardiac glycoside was estimated as the projection of the genine, it equals approximately 10^{-14} cm^2.

As shown by comparison of the data reported in Table 3, calculated values of U are in good agreement with experimental data.

The rate of onset of the reduction of the maximum phasic contraction induced by acetylcholine was fitted using the equation

$$E=E_{eq}(1-e^{-\alpha' t}) \qquad (3)$$

where E is the reduction at time t, E_{eq} the reduction at equilibrium and α' the rate constant (min^{-1}). Equilibrium effect was attained after 45 minutes of incubation for the three cardiac glycosides, tested at concentrations down 10^{-7} to 10^{-5} g/ml;

TABLE 2. *Computed estimates of parameters describing cardiac glycosides uptake and of capacity of the saturable binding site*

		Digitoxin	Digoxin	Ouabain
a	(ml/g wet weight)	2·902	0·773	0·459
b	(nmol/g wet weight)	0·92	0·358	0·357
K_b	(nmol)	172	93	357
Capacity of saturable binding site (molec./cell)		$7·67\times10^5$	$2·98\times10^5$	$2·98\times10^5$
Covered surface of saturable binding site (% of cell surface)		0·093	0·036	0·036

TABLE 3. *Comparison of experimental data with computed estimates of tissue concentration and of saturable and non-saturable components*

Cardiac glycoside	C_m nmol/ml	$U_{exp.}$ nmol/g wet weight	U_{calc} nmol/g wet weight	aC_m nmol/g wet weight	$\frac{bC_m}{C_m+K_b}$ nmol/g wet weight	Maximum displaceable amount of ^{3}H glycoside nmol/g wet weight
Digitoxin	0·013	0·107±0·004 (18)	0·106	0·038	0·069	
	0·13	0·61 ±0·03 (14)	0·77	0·38	0·39	0·24
	1·3	4·9 ±0·10 (16)	4·6	3·8	0·80	1·04
	13·0	38·6 ±1·8 (9)	38·6	37·7	0·90	
Digoxin	0·013	0·057±0·007 (16)	0·059	0·010	0·049	
	0·13	0·26 ±0·02 (12)	0·31	0·10	0·21	0·15
	1·3	1·4 ±0·14 (14)	1·33	1·0	0·33	0·36
	13·0	10·4 ±0·39 (15)	10·4	10·0	0·40	
Ouabain	0·013	0·019±0·001 (5)	0·019	0·006	0·013	
	0·13	0·12 ±0·005 (5)	0·16	0·06	0·10	0·10
	1·3	0·99 ±0·03 (5)	0·88	0·60	0·28	0·84
	13·0	6·3 ±0·68 (5)	6·32	5·97	0·35	

Figures in column 3 are means ±S.E. The number of experiments are given in brackets.
Figures in column 7 were obtained from experiments described in Table 5.

as tested for digitoxin, the half-lives ($t_{\frac{1}{2}}$) varied according to the dose. With the highest concentrations there was a clear cut dissociation between the rate of uptake and the rate of onset of the inhibitory effect (Table 1).

Washout of tissue-bound glycosides in the absence or in the presence of glycoside in the medium

After incubation until equilibrium (5 hr) in the presence of radioactive glycoside, the preparations were transferred either to a bath with a continuous flow of Tyrode solution or to a similar solution which also contained the same amount of non-radioactive glycoside as that used for the uptake. The residual radioactivity of the preparations was determined at various time intervals. The washout curves were fitted by two exponentials (Fig. 2), half-lives of which are presented in Table 4. There was no difference for washout in the presence or in the absence of non-radioactive glycoside.

Binding of cardiac glycosides in the presence of other glycosides

Incubation of a ^{3}H-glycoside was performed in the presence of 1, 10, and 100 times the concentration of another non-radioactive glycoside.

^{3}H-glycoside tissue content measured at equilibrium (after an incubation of 5 hr) was reduced in the presence of another glycoside (Table 5). With the exception of ouabain 1·3 nmol/ml, the maximum amount of displaced ^{3}H-glycoside was found to

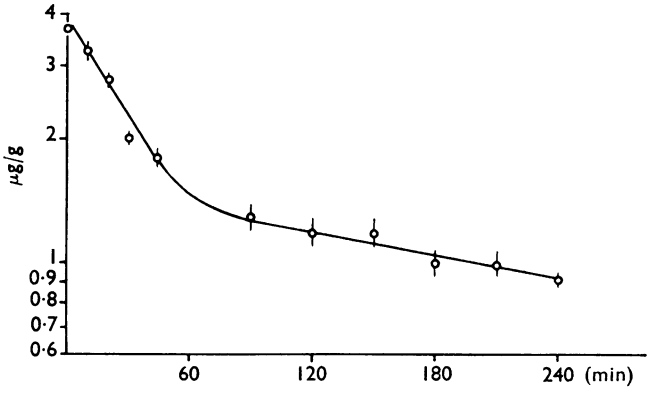

FIG. 2. Release of previously bound ^{3}H-digitoxin. After 5-hr incubation in a medium containing 10^{-6} g/ml ^{3}H-digitoxin, the preparations were transferred to glycoside-free solution and incubated for various periods. Each point on the curve represents the mean value ($\pm 2 \times$ S.E.) of three preparations.

TABLE 4. *Half-lives ($t_{\frac{1}{2}}$) of the washout processes*

		Washout in glycoside free Tyrode		Washout in Tyrode+non-radioactive glycoside	
Glycoside	Concentration in the medium	First period $t_{\frac{1}{2}}$ min	Second $t_{\frac{1}{2}}$ min	First $t_{\frac{1}{2}}$ min	Second $t_{\frac{1}{2}}$ min
Digitoxin	$1{\cdot}3 \times 10^{-7}$M	21	230	20	250
	$1{\cdot}3 \times 10^{-6}$M	22	275		
Digoxin	$1{\cdot}3 \times 10^{-5}$M	18	250		
Ouabain	$1{\cdot}3 \times 10^{-7}$M	18	275	19	280

be nearly equivalent to the capacity of the saturable binding site (Table 3). Furthermore, the amount of displaced ^{3}H-glycoside depended on the nature of the non-radioactive glycoside and on its concentration in the medium. The order of potency as binding antagonist followed the order digitoxin>ouabain>digoxin.

The Lineweaver-Burk plots of 1/uptake in the saturable binding site against 1/concentration in the medium gave a straight line for each glycoside. In the presence of another glycoside, the line became steeper, whereas its ordinal intercept was unchanged. However, the increase in slope at different concentrations of non-radioactive glycoside did not follow the relation of a competitive antagonism.

Discussion

Experimental results reported here have revealed some aspects of the uptake of digitoxin, digoxin and ouabain by intestinal smooth muscle. The main difference between the three compounds was the relative accumulation achieved in the equilibrium phase of the uptake process. A similar observation has previously been made with cardiac muscle (Dutta, Goswami, Datta, Lindower & Marks, 1968; Godfraind & Lesne, 1968; Kuschinsky, Lahrtz, Lüllmann & Van Zwieten, 1967; Kuschinsky, Lüllmann & Van Zwieten, 1968; Lüllmann & Van Zwieten, 1969).

The rate of the uptake process was similar for the three glycosides. In this respect, there was a difference between cardiac and smooth muscle, as in cardiac muscle, the $t_{\frac{1}{2}}$ of uptake varied according to the nature of the glycoside (Kuschinsky, *et al.*, 1967; Kuschinsky *et al.*, 1968; Lüllmann & Van Zwieten, 1969). However, the $t_{\frac{1}{2}}$ of the inhibitory effect increased with the concentration in smooth muscle. The difference between the $t_{\frac{1}{2}}$ of the uptake and the $t_{\frac{1}{2}}$ of the effect, as well as the polyphasic nature of the washout curves, are indicative of the existence of a distribution of the glycosides in different phases of the cell. This is confirmed by the fact

TABLE 5. *Tissue content of ^{3}H-cardiac glycosides of smooth muscle incubated for 5 hr at 37° C in a medium containing the radioactive glycoside plus a non-radioactive glycoside*

Cardiac glycosides		Tissue content: $1{\cdot}3\times10^{-7}$M ^{3}H-glycosides	Tissue content: $1{\cdot}3\times10^{-6}$M ^{3}H-glycoside
^{3}H-Ouabain		0·112±0·004	0·955±0·05
+Digitoxin	$1{\cdot}3\times10^{-7}$M	0·064	
	$1{\cdot}3\times10^{-6}$M	0·023	0·270
	$1{\cdot}3\times10^{-5}$M	0·009	0·112
+Digoxin	$1{\cdot}3\times10^{-7}$M	0·116	
	1·3 10^{-6}M	0·047	0·328
	$1{\cdot}3\times10^{-5}$M	0·030	0·133
^{3}H-Digitoxin		0·649±0·012	4·97±0·16
+Ouabain	$1{\cdot}3\times10^{-7}$M	0·563	
	$1{\cdot}3\times10^{-6}$M	0·464	4·17
	$1{\cdot}3\times10^{-5}$M	0·407	3·93
+Digoxin	$1{\cdot}3\times10^{-7}$M	0·630	
	$1{\cdot}3\times10^{-6}$M	0·540	4·76
	$1{\cdot}3\times10^{-5}$M	0·461	4·48
^{3}H-Digoxin		0·226±0·007	1·27±0·05
+Ouabain	$1{\cdot}3\times10^{-7}$M	0·235	
	$1{\cdot}3\times10^{-6}$M	0·159	1·13
	$1{\cdot}3\times10^{-5}$M	0·113	0·99
+Digitoxin	$1{\cdot}3\times10^{-7}$M	0·131	
	$1{\cdot}3\times10^{-6}$M	0·104	1·01
	$1{\cdot}3\times10^{-5}$M	0·077	0·91

Values reported (±S.E.) are mean of five determinations. These values, corrected for cardiac glycosides content of extracellular space, are expressed in nmol/g wet weight.

that the relation between the tissue concentration at equilibrium and the concentration in the medium fit well equation (2). The non-saturable component of the binding is related to the polarity of the molecules. Its saturable component appears to be related to their pharmacological activity, for which the order is digitoxin> ouabain>digoxin (Bieltvedt, 1967). It is therefore likely that the saturable uptake is on to a specific site. This conclusion is reinforced by the observation that the amount of displaceable glycoside is nearly equivalent to the capacity of the saturable binding site.

The major structural difference between the cardiac glycosides here studied is provided by $-CH_3$ and $-OH$ groups on the steroid nucleus. The non-competitive nature of the depression of their binding in the presence of another glycoside could be due to the formation of hydrophilic and hydrophobic bonds with the saturable binding site.

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