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STIMULATION AND INHIBITION OF THE SODIUM PUMP BY CARDIOACTIVE STEROIDS IN RELATION TO THEIR BINDING SITES AND THEIR INOTROPIC EFFECT ON GUINEA-PIG ISOLATED ATRIA

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1 The actions of ouabain, ouabagenin and dihydroouabain on the contractility and on the ionic content have been investigated in left guinea-pig atria stimulated at 3.3 Hz. The specific binding of ouabain and its displacement by the other cardenolides have been determined.

2 The action of either ouabain or ouabagenin on Na and K content was qualitatively different according to the concentration employed. Low doses evoked a reduction of Na_i whereas high doses produced an increase. Dihydroouabain evoked only a Na_i gain.

3 The increase of KCl concentration from 2.7 to 12 mM decreased Na_i in untreated atria and displaced ouabain dose-effect curves to the right.

4 ED_{50} values for the positive inotropic effect were lower than ED_{50} values for the inhibition of the pump and were not similarly affected by an increase in KCl concentration.

5 The specific binding of ouabain occurred at high and low affinity sites, related to Na pump stimulation and inhibition respectively.

6 The increase in KCl reduced the affinity of the two groups of sites for ouabain and increased the capacity of the high-affinity sites whereas the capacity of the other sites remained unchanged.

7 The results confirm the existence of two specific binding sites for ouabain in guinea-pig heart and suggest that the inhibition of the Na pump is not the only mechanism responsible for the positive inotropic effect of cardiac glycosides.

Introduction

Cardiac glycosides are known to increase the contractility of the heart and to inhibit the active transport of ions (Schatzmann, 1953; Glynn, 1957; 1964; Klaus, Kuschinsky & Lüllmann, 1961). The latter action is related to the inhibition of $(Na^+ + K^+)$-ATPase which has been proposed as the receptor both for the ionic and the inotropic action of the glycosides (Repke & Portius, 1963). This hypothesis has been supported by several lines of evidence (see Akera, Larson & Brody, 1970; Langer, 1972; Schwartz, 1976) but despite a great deal of work, it is still a matter of controversy (Lee & Klaus, 1971; Godfraind, 1975).

The concentration of free cardiac glycosides in the blood of patients treated for heart failure ranges between 10^{-9} and 5×10^{-9} mol/l.; higher concentrations are accompanied by symptoms of intoxication (Grahame-Smith & Everest, 1969; Lukas, 1971; Godfraind, 1973). Studies on human heart slices have shown that the sodium pump was inhibited by concentrations equivalent to those found in the blood of the intoxicated patients, whereas the therapeutic concentrations stimulated the activity of the sodium pump (Godfraind, 1972; 1973). Similar observations have been made with isolated, spontaneously beating atria of the guinea-pig: low concentrations of ouabain near 10^{-9} mol/l stimulated ^{42}K uptake whereas higher concentrations (from 10^{-8} mol/l) inhibited (Godfraind & Lesne, 1972). These findings are in agreement with electrophysiological studies on Purkinje fibres showing that low doses of ouabain produce changes in the K gradient that reflect stimulation of the sodium pump (Cohen, Daut & Noble, 1976). More recently, it has been shown in electrically driven left atria of guinea-pig, that the stimulation and the inhibition of the sodium pump were related to high- and low-affinity ouabain binding sites. Evidence has been given that these two bindings were to the sodium pump (Godfraind & Ghysel-Burton, 1977).

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In earlier experiments (Godfraind & Godfraind-De-Becker, 1965), it was observed that low doses of ouabain, which have now been found to stimulate the sodium pump, evoked a positive inotropic effect in spontaneously beating guinea-pig atria. More recently, it has been shown that such low doses increased the contractility of Purkinje fibres (Blood, 1975; Blood & Noble, 1977).

The inotropic effect of cardiac glycosides on the heart being dependent on the rate of beating (Koch-Weser & Blinks, 1962; Tuttle & Farah, 1962; Bentfeld, Lüllmann, Peters & Proppe, 1977), it seemed necessary to reinvestigate the inotropic action of such low doses under more controlled conditions in electrically driven left guinea-pig atria. Furthermore, in order to compare the properties of the high- and low-affinity ouabain binding sites, we have examined the influence exerted by extracellular KCl on the inotropic effect of the glycosides and on their action on the ionic composition of the tissue, in relation to their binding. In view of the determinant role played by the unsaturated lactone ring in $C_{17}\beta$ and by the sugar moiety in $C_3\beta$ (Portius & Repke, 1964; Godfraind, 1975; De Pover & Godfraind, 1976), the actions of dihydroouabain and of ouabagenin have been compared with those of ouabain.

The present results confirm the existence of the high- and low-affinity binding sites for cardiac glycosides. They show that the stimulation of the sodium pump requires an unsaturated lactone ring. They indicate that the inhibition of the Na pump is not the only mechanism responsible for the positive inotropic effect of cardiac glycosides.

Some of this work has appeared in abstract form (Ghysel-Burton & Godfraind, 1975; 1977).

Methods

Preparation

Albino guinea-pigs weighing approximately 400 g were killed by a blow on the head, exsanguinated and the hearts rapidly removed. The atria were dissected out in physiological solution. The left atria were suspended in an organ bath between platinum electrodes under a resting tension of 500 mg. The atria were stimulated with rectangular pulses of 10 ms (strength at least twice threshold) at a rate of 3.3 Hz.

Physiological solution

The composition of the Tyrode solution was (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.82, $MgCl_2$ 0.105, NaH_2PO_4 0.417, $NaHCO_3$ 11.9, glucose 5.5. It was equilibrated at 30°C with a mixture of 95% O_2 and 5% CO_2. In some experiments, KCl was 6 or 12 mM.

Determination of [^{14}C]-inulin space

The procedure was similar to that followed with smooth muscle (Godfraind, 1976).

Uptake of [^3H]-ouabain

[^{3}H]-ouabain was added together with [^{14}C]-inulin to the incubation fluid. This procedure allowed tissue ouabain uptake to be corrected for extracellular ouabain content. At the end of the incubation period, the atria were blotted and weighed as described for Na determination. Each atrium was dissolved in 1 ml of a solution composed of equal parts of perchloric acid (37%, w/v) and H_2O_2 (30 vol). This solution was heated at 60°C for 30 min and after cooling was added to 10 ml of a scintillation solution (bis MSB 0.5 g, PPO 4 g, Triton X100 350 ml, toluene 650 ml).

When the radioactivity of the physiological solution was estimated, 0.1 ml was added to another scintillation solution (BBOT 0.3 g, PPO 5 g, Triton X100 360 ml, toluene 540 ml, water 100 ml).

The radioactivity of the samples was counted, taking into account the presence of both [^{3}H]-ouabain and [^{14}C]-inulin; the efficiency was determined with internal standards. Uptakes were expressed as:

$$\text{mmol/kg wet wt.} = \frac{\text{d/min in muscle}}{\text{wet wt. (kg)}} \times \frac{\text{mmol in medium}}{\text{d min}^{-1}\,\text{l}^{-1}\,\text{medium}}$$

The results of each determination have been converted to the apparent tissue content of [^{3}H]-ouabain after correction for the content in the inulin space.

As shown previously for ouabain concentrations higher than 3×10^{-8} mol/l (Godfraind & Lesne, 1972), the uptake of ouabain by guinea-pig atria followed the equation:

$$U = a\,Cm + \frac{b\,C_m}{C_m + K_b} \qquad (1)$$

where U is the tissue concentration corrected for cardiac glycoside content of inulin space, C_m is the cardiac glycoside concentration in the medium, a is the proportionality constant for the linear non-saturable uptake, b is the capacity and K_b is the dissociation constant for the saturable binding sites. Values of these constants have been computed as described previously (Godfraind, & Lesne, 1972). The value of parameter a has also been estimated by measuring the residual binding in the presence of a large excess of digitoxin used as a competitor.

Ionic content determinations

At the end of the incubation, the atria were removed from the organ bath and each preparation was blotted on filter paper and was pressed three times with a roller weighing 350 g. After weighing, each atrium was placed in a quartz crucible and left overnight at 100°C; it was then weighed. To remove organic material, it was left at 500°C for 18 h. The residue was dissolved in 1 ml HCl (1 N) and assayed by atomic absorption as described previously for another tissue (Godfraind, 1976).

Results are expressed in terms of intracellular water according to the formula

$$C_i = \frac{C_T - C_0 \times ECS}{(H_2O)_T - ECS} \text{ mmol/l fibre water}$$

where C_i is the cellular concentration, C_T the cation concentration of the preparation expressed as mmol/kg wet wt., C_0 the cation concentration in the perfusion fluid (mmol/l); ECS (inulin space) and $(H_2O)_T$ (water content of the atria) are expressed as l/kg wet wt. $(H_2O)_T$ and C_i were not affected by incubation for 3 to 4 h under the conditions described in this paper.

Ouabain-sensitive K content is defined as the cellular K content of guinea-pig atria which is lost during incubation of atria in the presence of ouabain concentrations producing the maximum K loss; this is the same concentration as the one which evoked a maximal inhibition of ^{42}K uptake (Godfraind & Lesne, 1972). Once the equilibrium effect of the glycoside has been reached the changes in cellular K and Na content, expressed as a percentage of the maximal change with the most effective drug, probably reflect the percentage change in the rate of the Na pump. The estimate of the ED_{50} for cardenolide inhibition of the Na pump, obtained by measuring changes in Na_i, might be biased if the incubation time is not sufficient to reach equilibrium effect. Therefore, prolonged incubation periods were used.

Recording of the contractions and experimental protocol

Recordings of the contractile activity were made by an isometric lever using two strain gauges as part of a balanced bridge, the output of which was fed into a potentiometric recorder.

When the experiments were carried out with KCl 6 mM or 12 mM, the dissection was performed in KCl 2.7 mM. The atria were stimulated for 30 min in this solution which was then changed for the required one.

The systolic tension was sensitive to the change in KCl. A time of 30 min was sufficient to reach the new level of contractility which with KCl 6 mM was constant for a further 90-min observation period. Expressed in g/100 mg, the systolic tension was equal to 1.13 ± 0.02 ($n = 193$) for KCl 2.7 mM; to 0.75 ± 0.05 ($n = 109$) for KCl 6 mM; to 0.54 ± 0.08 ($n = 30$) for KCl 12 mM. Changes in systolic tension evoked by ouabain and its derivatives were expressed as a percentage of the tension developed before the addition of the drug. This appeared to be an adequate procedure for KCl 6 and 12 mM for which the contractility of the controls was not altered during the observation period. This was not the case with KCl 2.7 mM; therefore, a correction was made for the decline in contractility of the untreated atria.

Statistical methods

Whenever possible values are presented as means $\pm$ s.e. mean. Significance of differences between means was checked by Student's *t*-test. For uptake experiments, standard errors of parameters are indicative of the goodness of fit. The method of calculation used did not allow their estimation. The goodness of fit was checked by the agreement between expected and observed values.

Drugs

Ouabain, ouabagenin and dihydroouabain were a gift from Nativelle (France).

[^{3}H]-ouabain (12 Ci/mmol) was obtained from New England Nuclear Corp. The stock solution was kept in ethanol:benzene (9:1). Before use, this solvent was evaporated with nitrogen. The purity of [^{3}H]-ouabain was checked by thin-layer chromatography (Godfraind & Lesne, 1970).

Results

Ionic content of atria incubated in the presence of cardioactive steroids

The Na, K, Mg, and Ca contents were determined in guinea-pig atria incubated for 3 h in physiological solutions containing KCl 2.7, 6 or 12 mM and various concentrations of ouabain. This 3-h incubation time was found to be required in order to observe the ouabain equilibrium effect (Godfraind & Ghysel-Burton, 1977). The action of ouabain on Na and K contents was qualitatively different according to the ouabain concentration employed. For KCl 2.7 mM, ouabain 10^{-9} mol/l evoked a reduction of Na content and a gain of K ($P < 0.01$); ouabain 3×10^{-9} mol/l or higher concentrations evoked a gain of Na and a loss of K. Maximum changes in monovalent cation contents were evoked by ouabain 10^{-4} mol/l. The dose of ouabain producing an increase in intracellular Na content equal to 50% of the maximum increase was

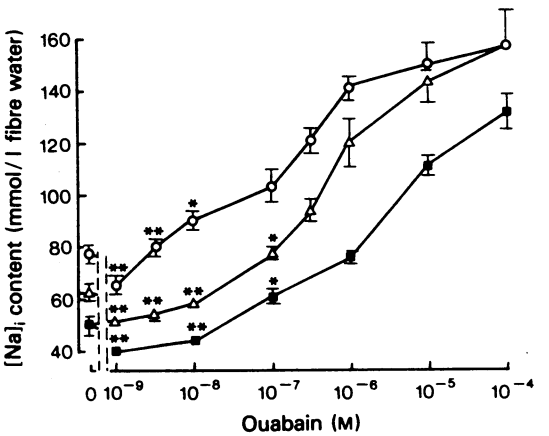

Figure 1 Cellular sodium content (mmol/l fibre water) of stimulated left guinea-pig atria incubated for 3 h in physiological solution at 30°C containing KCl 2.7, 6 and 12 mM. Abscissa scale: ouabain concentration; ordinate scale: Na_i content of atria for K_o 2.7 (○), 6 (△) and 12 (■) mM. Each point is the mean of at least 4 experiments. The limits of s.e. are shown where they exceed the diameter of the symbol. Difference between treated and untreated: * $P < 0.05$; ** $P < 0.01$. For ouabain concentrations higher than 10^{-7} M, $P < 0.01$.

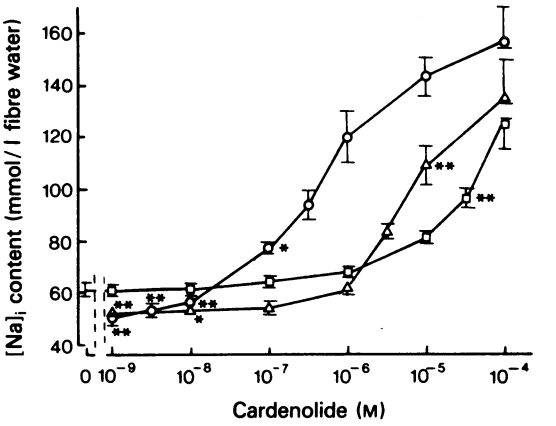

Figure 2 Cellular sodium content (mmol/l fibre water) of stimulated left guinea-pig atria incubated for 3 h in physiological solution at 30°C containing KCl 6 mM and various concentrations of ouabain (○), ouabagenin (△) and dihydroouabain (□). Abscissa scale: cardenolide concentration; ordinate scale: Na_i content of atria. Each point is the mean of at least 4 experiments. The limits of s.e. are shown where they exceed the diameter of the symbol. The sodium content of untreated atria is shown on the ordinate axis. Difference between treated and untreated: * $P < 0.05$; ** $P < 0.01$. For concentrations higher than ouabain 10^{-7} M and ouabagenin and dihydroouabain 10^{-5} M, $P < 0.01$.

2×10^{-7} mol/l (Figure 1). This dose also evoked a 50% decrease in ouabain-sensitive K content. As the two opposite actions of ouabain here described cannot be accounted for by changes in extracellular space, they may be attributed, as already proposed, to stimulation or inhibition of the Na pump activity (Godfraind & Ghysel-Burton, 1977).

Changes in divalent cations were also noticed: a dose-dependent increase in Ca content occurred down to ouabain 10^{-7} mol/l. A decrease in the Mg content of atria was evoked by the highest ouabain concentrations (10^{-5} and 10^{-4} mol/l, $P < 0.01$). The increase in K_o from 2.7 to 12 mM decreased Na_i in untreated atria and displaced ouabain dose-effect curves to the right (Figure 1).

The actions of ouabagenin and dihydroouabain on the ionic content of atria have been examined for 6 mM KCl and compared with ouabain (Figure 2). The concentrations producing a 50% inhibition of the sodium pump were 5.4×10^{-7} mol/l for ouabain, 1.4×10^{-5} mol/l for ouabagenin and 4.2×10^{-5} mol/l for dihydroouabain. Within the range of concentrations between 10^{-9} and 10^{-8} mol/l, both ouabain and ouabagenin evoked a stimulation of the Na pump as shown by the decrease in Na content compensated for by an increase in K content ($P < 0.05$). The action of dihydroouabain was only inhibitory; the threshold concentration producing an increase of Na_i content was approx 10^{-6} mol/l.

The action of cardioactive steroids on the contractility of guinea-pig isolated atria

The contractility of electrically driven guinea-pig left atria has been monitored in physiological solutions containing KCl 2.7, 6 or 12 mM and various concentrations of ouabain ranging from 10^{-9} to 10^{-4} mol/l.

For KCl 6 mM, the threshold effect was found with ouabain 10^{-9} mol/l which evoked a 10% increase in systolic tension (Figures 3 and 5). The onset of this action was observed 30 min after the addition of ouabain, the peak effect being reached after 1 h. When the ouabain concentration was increased, the time to onset and the time to peak decreased in a dose-dependent manner. The magnitude of the inotropic effect was related to the concentration of the glycoside. However, for ouabain concentrations higher than 3×10^{-7} mol/l the increase in systolic tension was not sustained as it was for the lower concentrations. As shown for ouabain 10^{-6} mol/l (Figure 4), the systolic tension decreased once the diastolic tension increased. The increase in the diastolic tension and its time to onset were dependent on ouabain concentration. The ouabain concentration producing an

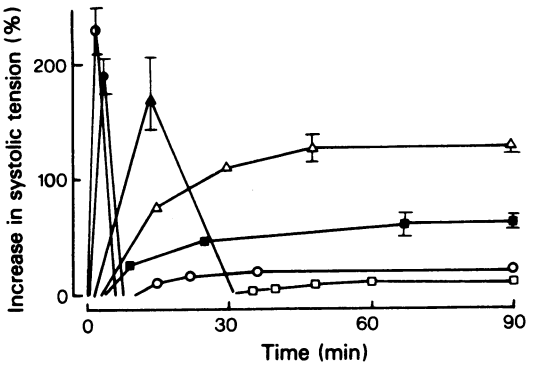

Figure 3 The time course of the increase in systolic tension of stimulated left guinea-pig atria evoked by various doses of ouabain at 30°C in the presence of 6 mM KCl. Abscissa scale: time after the addition of ouabain to the bathing fluid; ordinate scale: changes in sytolic tension as % of the tension recorded before the addition of ouabain. Each point is the mean of different experiments for ouabain 10^{-9} M ($n = 7$) (□); 10^{-8} M ($n = 8$) (○); 10^{-7} M ($n = 6$) (■); 3×10^{-7} M ($n = 4$) (△); 10^{-6} M ($n = 5$) (▲); 10^{-5} M ($n = 4$) (●) and 10^{-4} M ($n = 4$) (◑). The limits of the s.e. are shown when they exceed the diameter of the symbol.

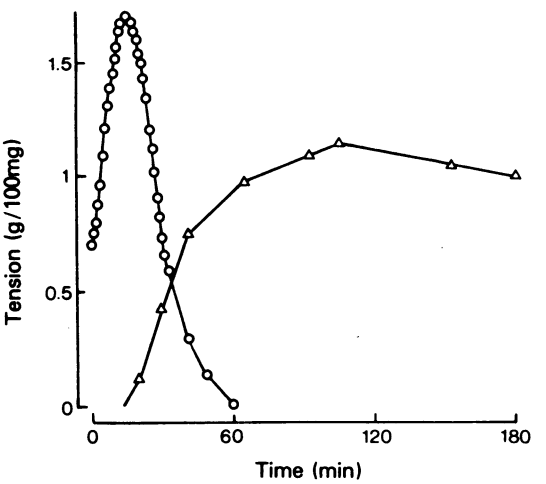

Figure 4 The time course of the systolic and diastolic tensions of stimulated left guinea-pig atria evoked by ouabain 10^{-6} mol/l at 30°C in the presence of 6 mM KCl. Abscissa scale: time after the addition of ouabain 10^{-6} mol/l; ordinate scale: systolic tension (○) and diastolic tension (△) from which was subtracted the initial resting tension of 0.5 g. Each point is the mean of 5 experiments. The limits of the s.e. did not exceed the diameter of the symbols.

Table 1 Parameters of ouabain binding to guinea-pig atria bathed at 30°C in physiological solutions containing KCl 2.7, 6 and 12 mmol/l

KCl concentration (*mmol/l*)	(*a*) (*l/kg wet wt.*)	(*b*) (*nmol/kg wet wt.*)	*U* (*nmol/kg wet wt.*)	K_b (*nmol/l*)
(a) Left atria				
2.7	0.394	424	4.350	256
6	0.319	372	3.550	389
12	0.309	237	3.320	750
(b) Right atria	Isotopic dilution:			
2.7	0.266 ± 0.009 ($n = 15$)		3.373 ± 90 ($n = 15$)	
6	0.260 ± 0.008 ($n = 42$)		3.450 ± 80 ($n = 42$)	
12	0.209 ± 0.012 ($n = 14$)		3.111 ± 117 ($n = 14$)	
	Competition:			
2.7	0.298 ± 0.006 ($n = 7$)			
6	0.313 ± 0.010 ($n = 35$)			
12	0.285 ± 0.010 ($n = 12$)			

For electrically stimulated left atria, the parameters were calculated from equation 1. Estimations of U were obtained after 4 h incubation in the presence of the following [^{3}H]-ouabain concentrations expressed in mol/l: for KCl 2.7 mmol/l: 3×10^{-8} ($n = 4$), 10^{-7} ($n = 4$), 10^{-6} ($n = 4$), 10^{-5} ($n = 4$); for KCl 6 mmol/l: 10^{-8} ($n = 8$), 3×10^{-8} ($n = 4$), 10^{-7} ($n = 4$), 10^{-5} ($n = 4$); for KCl 12 mmol/l: 3×10^{-8} ($n = 4$), 10^{-7} ($n = 4$), 10^{-6} ($n = 4$), 10^{-5} ($n = 4$). For spontaneously beating right atria, the non-saturable clearance (a) was calculated by isotopic dilution and by competition. For the latter case, (a) corresponded to the non-displaceable amount of [^{3}H]-ouabain measured after 4 h of incubation in the presence of 10^{-9} mol/l [^{3}H]-ouabain and 10^{-5} mol/l digitoxin. U was the amount bound in the presence of [^{3}H]-ouabain 10^{-5} mol/l.

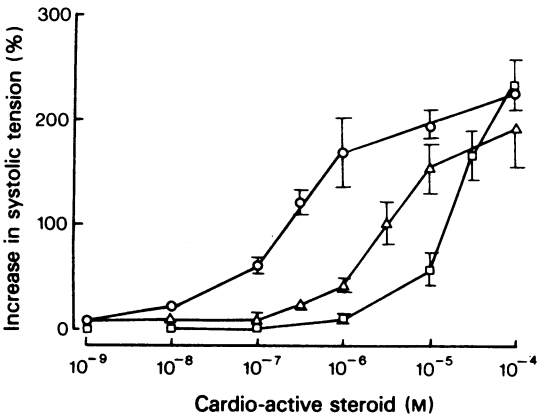

Figure 5 Dose-response relation of the effect of cardioactive steroids on the increase in systolic tension of stimulated left guinea-pig atria at 30 C in the presence of KCl 6 mM. Abscissa scale: drug concentration; ordinate scale: increase in systolic tension as °₀ of initial tension for ouabain (○), ouabagenin (△) and dihydroouabain (□). Each point is the mean of at least 4 experiments. The limits of the s.e. are shown where they exceed the diameter of the symbol.

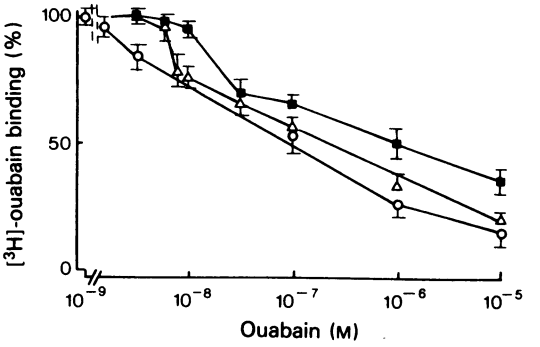

Figure 6 The effect of non-radioactive ouabain on the binding of [^{3}H]-ouabain to stimulated left guinea-pig atria in the presence of KCl 2.7, 6 and 12 mM. The preparations were immersed for 4 h in a medium containing [^{3}H]-ouabain 10^{-9} M and increasing amounts of non-radioactive ouabain. Abscissa scale: concentration of non-radioactive ouabain; ordinate scale: [^{3}H]-ouabain tissue content as °₀ of controls after correction for [^{14}C]-inulin space; for KCl 2.7 (○), 6 (△) and 12 (■) mM. Each value is the mean of at least 5 determinations. Vertical lines show s.e. mean.

increase in systolic tension equal to 50% of the maximum observed was 3.4×10^{-7} mol/l for KCl 6 mM whereas it was 10^{-7} mol/l for KCl 2.7 and 12 mM. The action of ouabain on the diastolic tension was dependent on K_o, increase of which displaced the dose-effect curves to the right and enhanced the magnitude of the contracture (Table 1).

The positive inotropic effect of cardioactive steroids was dependent on their chemical structure; their order of potency was ouabain > ouabagenin > dihydroouabain (Figure 4). However, ouabagenin, which was 10 times less potent than ouabain in producing an increase in systolic tension equal to 50% of the maximum, evoked a rather similar inotropic effect at 10^{-9} mol/l (Figure 5). The lowest concentration at which dihydroouabain evoked a positive inotropic effect was approx. 10^{-6} mol/l. The increase in diastolic tension evoked by ouabagenin and by dihydroouabain required higher doses than ouabain, although for the three compounds here studied, the maximum effect on tension was of the same magnitude.

Table 2 Comparison of cardioactive steroids concentrations producing a 50°₀ inhibition of the sodium pump, 50°₀ of the maximum increase in systolic tension and in diastolic tension, 50°₀ of the saturation of the low-affinity binding sites and 50°₀ displacement of [^{3}H]-ouabain binding

		Concentration producing 50% of the maximum (ED_{50}) (mmol/l)				
Drug	*KCl concentration in medium (mM)*	*Inhibition of Na pump*	*Increase in systolic tension*	*Increase in diastolic tension*	*Saturation of low-affinity sites*	*Displacement of [^{3}H]-ouabain*
Ouabain	2.7	2×10^{-7}	10^{-7}	2×10^{-7}	2×10^{-7}	10^{-7}
	6	5.4×10^{-7}	3.4×10^{-7}	7×10^{-7}	3.9×10^{-7}	4×10^{-7}
	12	10^{-6}	10^{-7}	5×10^{-6}	7.5×10^{-7}	10^{-6}
Ouabagenin	6	1.4×10^{-5}	3×10^{-6}	1.4×10^{-5}	—	10^{-5}
Dihydroouabain	6	4.2×10^{-5}	2.2×10^{-5}	8.5×10^{-5}	—	$>10^{-5}$

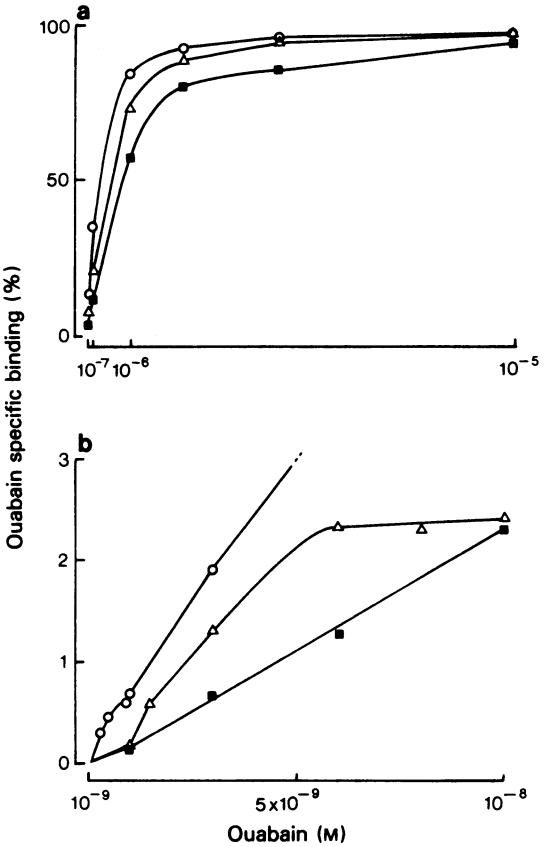

Figure 7 Specific uptake of ouabain by stimulated left guinea-pig atria. Abscissa scale: concentration of ouabain in the incubation fluid. The lower concentrations in the range covered in (a) are shown on an expanded scale in (b). Ordinate scale: tissue content of ouabain corrected for cardiac glycoside concentration in the inulin space and for the non-saturable uptake; the content is expressed as % of the maximum binding capacity for KCl 2.7 (○), 6 (△) and 12 (■) mM. Each value is the mean of at least 5 determinations. The limits of s.e. are shown where they exceed the diameter of the symbol. The atria were incubated for 4 h, a time sufficient to reach equilibrium.

The binding of ouabain

As shown previously, the specific binding of ouabain to guinea-pig atria occurs at high and low affinity sites, responsible respectively for the stimulation and the inhibition of the sodium pump. Both bindings were sensitive to changes in K_o which affected the apparent affinity of both groups of sites for ouabain (Godfraind & Ghysel-Burton, 1977). As Figure 6 illustrates, when a constant amount of [^{3}H]-ouabain was added to the incubation medium in the presence of increasing concentrations of non-radioactive ouabain, the amount of [^{3}H]-ouabain bound to the tissue after 4 h decreased. For KCl 6 mM, the curve relating the binding of [^{3}H]-ouabain to the concentration of cold ouabain showed two slopes. The steepest one was observed at ouabain concentrations lower than 10^{-8} mol/l and it corresponded to a displacement of 20% of [^{3}H]-ouabain. A similar pattern was observed for KCl 12 mM, the steepest slope being seen with ouabain concentrations lower than 3×10^{-8} mol/l which displaced 30% of the radioactivity. For KCl 2.7 mM, the steepest slope was apparent with ouabain 3×10^{-9} mol/l which displaced 15% of the radioactivity. The less steep parts of the displacement curves were nearly parallel. The concentrations of ouabain at which [^{3}H]-ouabain binding was reduced to 50% were increased when the KCl concentration was changed from 2.7 to 12 mM (Table 2).

The action of KCl on ouabain binding parameters is shown in Table 1. As the computed data seemed to indicate an action of KCl on the linear non specific uptake (parameter a) and on the maximum receptor capacity (parameter b), some experiments have been performed at random with right atria. Observations showed that the increase in medium KCl from 2.7 to 12 mM was without significant influence on parameter a. Furthermore, the uptake for ouabain 10^{-5} mol/l was not significantly different at the three KCl concentrations. This suggests that the maximum receptor capacity was not altered by KCl. These results therefore indicate that changes in KCl affected only the affinity of the receptors sites for ouabain (Figure 7).

Figure 7b illustrates the high-affinity binding of ouabain in the range of ouabain concentrations lower than 10^{-8} mol/l. It shows that the affinity of these binding sites for ouabain was also decreased by KCl which displaced the uptake curve to the right. The capacity of these sites for ouabain was apparently increased by increasing KCl concentration.

At concentrations higher than 10^{-6} mol/l, ouabagenin and dihydroouabain competed with [^{3}H]-ouabain binding. Their potency ratio as competitors was similar to the one found for inhibition of the Na pump (Table 2).

Discussion

The inhibition of the Na pump by cardiac glycosides is well documented. The study of this action has improved our knowledge of cellular ionic regulation (see Glynn, 1964; Glynn & Karlish, 1976). In guinea-pig atria, our observations summarized in Table 2 show

that the concentrations of ouabain producing an inhibition of 50% of Na pump activity lie close to the ones which produced a half-saturation of the low-affinity saturable binding sites or a displacement of 50% of the binding of [^{3}H]-ouabain. This suggests that the low-affinity saturable binding sites so far identified are the inhibitory sites.

The observation that potassium does affect the apparent affinity of these sites for ouabain is in agreement with others (Baker & Willis, 1970; Brading & Widdicombe, 1974). It has been previously reported (De Pover & Godfraind, 1976) that the cardioactive steroids studied here inhibited guinea-pig heart ($Na^+ + K^+$)-ATPase according to the sequence ouabain > ouabagenin > dihydroouabain. The present results are in agreement with these observations.

It has been proposed that the positive inotropic effect of cardiac glycosides is related to the inhibition of the sodium pump (Langer, 1968; Akera *et al.*, 1970; Besch, Allen, Glick & Schwartz, 1970). According to this hypothesis, such an inhibition might increase the transmembrane Na–Ca exchange described by several authors (Baker & Blaustein, 1968; Reuter & Seitz, 1968; Glitsch, Reuter & Scholz, 1970; Baker, 1972; Brading, 1973; Langer, 1973; Reuter, Blaustein & Haeusler, 1973; Reuter, 1974). This hypothesis implies that the concentration parameters (ED_{50}) describing the inhibition of the Na pump and the inotropic effect should be quantitatively similar and that any change in the affinity of the receptors should produce an identical shift of these parameters. Affinity changes were obtained by modifying K_o concentration and by using cardenolides with different structures. It is noteworthy that the ED_{50} for inhibition of the Na pump and for the inotropic effect were dissimilar, the latter being lower than the former. This indicates that the inhibition of the Na pump is not the only mechanism responsible for the positive inotropic effect.

The present experimental results confirm and extend the previous observations of a stimulation of the heart Na pump by low concentrations of cardiac glycosides (Godfraind & Lesne, 1972; Godfraind, 1973; Cohen, Daut & Noble, 1976; Godfraind & Ghysel-Burton, 1977). Previous observations with ^{42}K (Godfraind & Lesne, 1972; Godfraind, 1973) or with electrophysiological methods (Cohen *et al.*, 1976) did not allow an analysis of the transient effects of ouabain. The present data show that in the range of stimulatory doses, ouabain was bound to high affinity binding sites sensitive to K_o which reduced their affinity but increased their binding capacity. In this respect high-affinity and low-affinity saturable sites behaved differently.

It is likely that the low-affinity sites are a part of ($Na^+ + K^+$)-ATPase as a great many experimental results have demonstrated that inhibition of the Na pump is due to inhibition of this enzyme (Glynn, 1964; Portius & Repke, 1964; Repke, 1964; Schatzmann, 1965). As far as the high-affinity sites are concerned, there is at present no direct biochemical indication that they could constitute one of the reactive sites of this enzyme. A few biochemical studies have reported stimulation of ($Na^+ + K^+$)-ATPase by cardiac glycosides, but they have not been confirmed (Repke, 1964; Schwartz, 1976). It might be that the receptor properties *in situ* are different from those studied on an enzyme prepared by detergent and salt treatment. Recent reports have shown that several sites are involved in the action of cations on the Na pump (Cavieres & Ellory, 1975; Glynn & Karlish, 1976). Using an isolated enzyme preparation, Hansen, (1976) and Charnock, Simonson & Almeida, (1977) have reported that some ouabain binding sites were sensitive to phospholipase and others were not. However, it is difficult to speculate from the actual biochemical data, as under the present conditions, the stimulatory sites are only 1 to 4% of the total ouabain binding sites and they could be lost during purification procedures.

The absence of a Na pump stimulation by dihydroouabain, as described in this paper, shows that cardiac glycosides may have not only quantitative but also qualitative differences, according to the unsaturation of the lactone ring. The presence of a sugar moiety in $C_3\beta$ increases the affinity for the inhibitory sites but is not required for an interaction with the stimulatory sites as shown by the action of ouabagenin.

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