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STUDIES ON THE KINETICS OF [^{3}H]-OUABAIN UPTAKE AND EXCHANGE IN THE ISOLATED PAPILLARY MUSCLE OF THE GUINEA-PIG

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1 The uptake, wash-out and exchange of [^{3}H]-ouabain was studied in isolated, resting, and electrically stimulated papillary muscles of the guinea-pig.

2 At the equilibrium level of uptake, a different tissue/medium ratio was obtained for each of the concentrations used, i.e. 3.4, 1.8 and 0.82 for 1×10^{-7} M, 7.7×10^{-7} M, and 5×10^{-6} M ouabain, respectively. Equilibrium was reached more rapidly at high concentrations of ouabain.

3 The maximum number of binding sites for ouabain was estimated to be 1×10^{15} binding sites/g wet weight.

4 No difference in [^{3}H]-ouabain uptake could be detected between resting and electrically stimulated papillary muscles.

5 The kinetics of the ouabain uptake, wash-out and exchange are discussed. The results suggest that there is a saturable compartment in papillary muscle which can best be demonstrated if low concentrations of ouabain are used. Because of its small size, the saturable compartment submerges in the process of the filling of the extracellular space at high concentrations (5×10^{-6} M).

Introduction

In spite of numerous investigations on the positive inotropic action of cardiac glycosides during recent years, their mode of action remains obscure (for review see Lee & Klaus, 1971). In particular, the site of action has been a matter of controversy.

Cardiac glycosides are taken up from the perfusion medium by isolated heart muscle (Kuschinsky, Lahrtz, Lüllmann & Van Zwieten, 1967a; Kuschinsky, Lüllmann, Schmitz & Van Zwieten, 1967b; Kuschinsky, Lüllmann & Van Zwieten, 1968; Dutta, Goswami, Datta, Lindower & Marks, 1968; Dutta & Marks, 1969; Godfraind & Lesne, 1972). However, according to their physico-chemical properties, cardiac glycosides show quite different distribution kinetics. This increases the difficulty of finding a site of binding, common to all digitalis compounds, which could mediate the effects on cardiac muscle.

Dutta *et al.* (1968) and Dutta & Marks (1969) suggested an intracellular site of action, because they found a good correlation between the uptake of digitalis glycosides into a microsomal fraction and their positive inotropic effect. Other authors (Kuschinsky *et al.*, 1967a; 1967b; 1968; Kuschinsky & Van Zwieten, 1969) came to the conclusion that only a modest amount of drug bound 'specifically' by the tissue is essential for its effect and that most of the digitalis glycoside taken up by the tissue is of no pharmacological importance.

Using autoradiographic techniques (Smith & Fozzard, 1963; Conrad & Baxter, 1964; Tubbs, Crevasse & Wheat, 1964; Fozzard & Smith, 1965; Waser, 1965; Löhr, Makoski, Göbbeler & Strötges, 1971) as well as differential centrifugation (Harvey & Pieper, 1955; Spratt & Okita, 1958; Gerber, Fricke, Klaus & Wollert, 1968; Fricke, Gerber, Klaus & Wollert, 1969), much effort was devoted to the intracellular localization of cardiac glycosides and the possible relation to some subcellular structure. However, the results obtained by these methods must be regarded with some caution, because disadvantages of the techniques such as loss of drug from the tissue and redistribution within the cell during the preparation procedures cannot be avoided.

The purpose of the present paper was to gain further evidence for the possible site of cardiac glycoside action. We therefore investigated the uptake of ouabain in order to avoid kinetics which are complicated by a large amount of 'unspecific' accumulation. Papillary muscles were preferred to

atria, because they are small ventricular muscle preparations of good integrity. It should also be kept in mind that the morphology of the ventricular muscle is rather different from atrial muscle: it possesses an abundant tubular system continuous with the extracellular space (Sperelakis & Rubio, 1971).

Methods

Right ventricular papillary muscles of less than 1 mm in diameter were dissected from the hearts of guinea-pigs weighing 300-400 grams.

For the determination of the uptake, wash-out and exchange of tritiated compounds, the papillary muscles were suspended vertically in a large organ bath containing oxygenated Tyrode solution at 32° C. A small weight of 340 mg tied to the free end of the muscles adjusted their resting tension. In some experiments the preparations were stimulated by 5 ms square wave impulses of suprathreshold voltage at a rate of 1 Hz. All other experiments were carried out with quiescent preparations.

After an equilibration period of 30 min in Tyrode solution, the muscles were incubated for various periods up to 3 h in a medium which contained the tritiated compound to be investigated. At the end of the incubation period the muscles were assayed for their content of radioactivity. When high concentrations of glycoside were necessary, additional inactive drug was used from a separate stock solution.

In the wash-out experiments, the papillary muscles were preloaded in an incubation medium containing radioactive ouabain and were then washed for various periods in glycoside-free Tyrode solution. In two other experimental groups, the preparations were preloaded for 3 h with either active or inactive ouabain and were then allowed to exchange with identical concentrations of inactive or active ouabain.

The tritium content of the papillary muscles was assayed in the following manner. After well-defined periods of incubation the muscles were removed from the bath, pressed carefully for 90 s between blotting paper and weighed immediately. They were dissolved in 1 ml of Soluene or Hyamine [*p*-(diisobutylcresoxyethyl)-dimethylbenzylammonium hydroxide, 1 M in methanol] at 50° C for 2-4 hours. After addition of 2 ml ethanol and 10 ml scintillation phosphor (4 g 2,5-diphenyl-oxazole (PPO) and 0.5 g 1,4-di-(2(5-phenyloxazole))-benzene (POPOP) dissolved in 1 litre toluene) the samples were counted in a Packard Tricarb Liquid Scintillation Spectrometer. The individual quenching of each sample was corrected by means of an internal tritium standard. The radioactivity of the papillary muscles was expressed as nCi/g tissue (wet weight). The tissue/medium ratio was determined by dividing the radioactivity of the tissue by the radioactivity in the incubation medium measured in nCi/ml. The uptake, wash-out and exchange were plotted as a function of time.

[^{3}H]-ouabain, [^{3}H]-digitoxin and [^{3}H]-sucrose were labelled according to the Wilzbach exchange procedure by New England Nuclear Corporation, Chicago. Purity of the radioactive material was more than 99.9% (NEN, Chicago). Previous experiments (Kuschinsky *et al.*, 1967a; Lüllmann, Peters & Van Zwieten, 1969) have shown that the glycosides are not metabolized and all radioactive material detected represents the original tritiated compound.

Unlabelled ouabain was dissolved in distilled water, unlabelled digitoxin in ethanol: water (70 : 30). The final alcohol concentration in the incubation medium did not exceed 0.1%, which in this system is biologically ineffective.

The composition of the Tyrode solution (mmol/litre) was as follows: NaCl 137.0, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 12.0, NaH_2PO_4 0.21, glucose 5.5. The pH of the solution was adjusted to 7.35 by gassing with a mixture of 95% O_2 and 5% CO_2.

Results

Measurement of the size of the extracellular space

From previous experimental results (Lüllmann & Van Zwieten, 1967) the size of the extracellular space (ECS) of guinea-pig atria was estimated to be 0.35 ml/g wet weight. Atrial tissue, however, does not possess the well-developed transverse tubular system which is found in mammalian ventricular muscle (Sperelakis & Rubio, 1971). Therefore, the ECS in papillary muscle was determined since we expected to find the size of this compartment different from atrial tissue.

The uptake of [^{3}H]-sucrose (0.5×10^{-7} M) was measured after various periods of incubation and plotted as the tissue/medium radioactivity ratio (T/M ratio) against incubation time (Figure 1). A maximum T/M ratio of 0.54 was attained after 120 minutes. Uptake experiments with [^{3}H]-inulin yielded essentially the same results, i.e. the time course and the final T/M ratio were identical to those determined with [^{3}H]-sucrose. From these data, the size of 0.54 ml/g wet weight for the ECS of papillary muscle can be calculated (Kleinzeller & Knotkova, 1966).

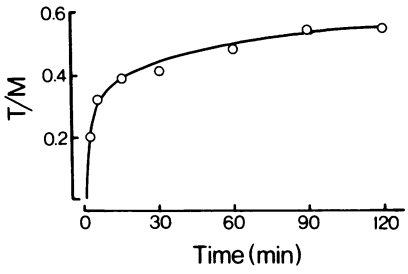

Figure 1 Time course of [^{3}H]-sucrose uptake by guinea-pig isolated papillary muscle. T/M = tissue/medium radioactivity ratio. The experimental points are mean values of at least 8 single determinations; standard error <5%. After 120 min, the maximum T/M value attained was 0.54.

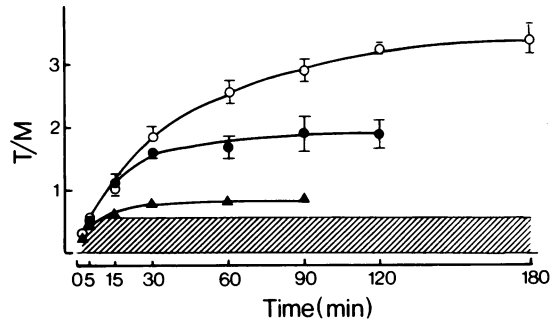

Figure 2 Time courses of the uptake by guinea-pig papillary muscle with a different concentration of [^{3}H]-ouabain in the incubation medium: 1×10^{-7} M (○), 7.7×10^{-7} M (●), and 5×10^{-6} M (▲). T/M = tissue/medium radioactivity ratio. The individual points represent mean values of at least 6 separate measurements. Vertical bars show s.e. mean. With increasing concentrations of ouabain, the T/M ratio approached the value of the extracellular space (hatched area). The time required to reach an equilibrium level decreased.

Accumulation of [^{3}H]-ouabain at different concentrations of the drug in the incubation medium

The uptake of [^{3}H]-ouabain by ventricular muscle was investigated at different drug concentrations in the medium: 5×10^{-8} M, 1×10^{-7} M, 7.7×10^{-7} M, and 5×10^{-6} M. The time courses of the T/M ratios for these concentrations were compared in Figure 2. The uptake of the lowest concentration of ouabain (5×10^{-8} M) was not included in Figure 2 because its time course cannot be distinguished from the uptake of 1×10^{-7} M ouabain. The T/M ratios reached an equilibrium level which depended on the concentration studied. In the presence of ouabain, 1×10^{-7} M, the T/M ratio at equilibrium was 3.4. This value decreased to 1.8 and 0.82 respectively, when ouabain concentrations of 7.7×10^{-7} M and 5×10^{-6} M were used. In other words, at high concentrations of ouabain the T/M ratio at equilibrium level approached that of the ECS as established with [^{3}H]-sucrose. Furthermore, the time required to reach a steady tissue level of ouabain became shorter with increasing concentrations. With ouabain 1×10^{-7} M, the equilibrium level was attained after 120 min; in the presence of 7.7×10^{-7} M and 5×10^{-6} M, the times required decreased to 90 and 60 min respectively.

For an estimation of the amount of drug taken up by or bound to the cells only, it had to be assumed that ouabain was distributed in the ECS similarly to sucrose or inulin which seemed justified since the latter two compounds of different molecular size occupied a tissue space of equal size. Thus the uptake of ouabain in equilibrium was corrected for the amount of drug present in the ECS (Godfraind & Lesne, 1972) and plotted versus its concentration in the incubation medium (Figure 3a). This curve was found to fit a single Langmuir adsorption term:

$$U = ax/x + K_a$$

where U = measured uptake, a = a constant describing the capacity of the adsorption site and K_a = equilibrium constant (Paton & Rang, 1965). On rearranging,

$$\frac{1}{U} = \frac{1}{a} + \frac{K_a}{a}\frac{1}{x}.$$

Plotting 1/uptake versus 1/concentration in the medium (Figure 3b) a straight line with a regression coefficient of $r = 0.997$ was obtained in the concentration range which is limited either by the specific activity of obtainable [^{3}H]-ouabain or by intoxication of the muscles. Within this biologically relevant range the uptake of ouabain by papillary muscles was a single saturable process. Furthermore, from the intercept of the straight line with the abscissa scale the equilibrium constant can be calculated. Thus the concentration of half maximal binding is 6×10^{-7} M. Similarly from the intercept with the ordinate scale the maximum number of binding sites could be estimated: it amounted to 1×10^{15}/g wet weight. This corresponds to a binding capacity of 1.9×10^{-6} mol ouabain/kg tissue. The number of binding sites occupied at various concentrations of ouabain during steady-state conditions (Table 1) can be calculated by correcting the total tissue content of ouabain for the amount present in the ECS as described above.

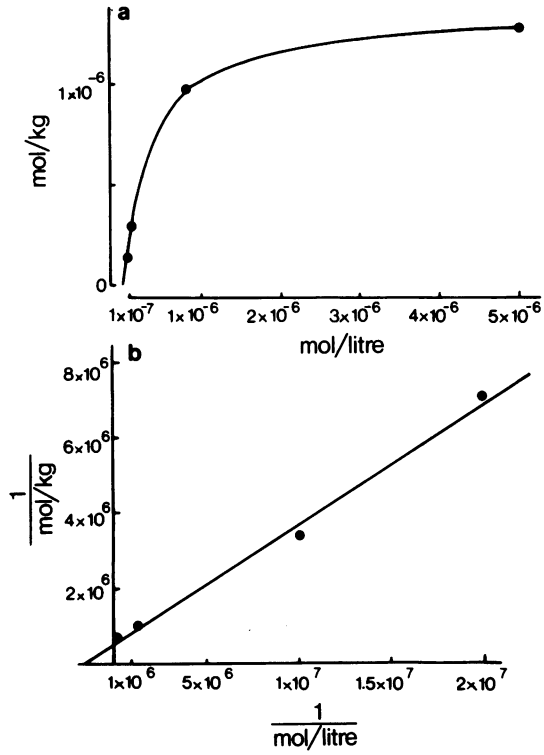

Figure 3 Uptake of [^{3}H]-ouabain by isolated papillary muscles of guinea-pig. (a) Ordinates: tissue content in mol/kg wet weight of tissue at equilibrium. Abscissae: ouabain concentration (in mol/l) in the perfusion medium. The uptake of ouabain was not directly proportional to its concentration in the perfusion fluid and could be described by a Langmuir adsorption term (b). (b) Reciprocal plot of the uptake. Ordinates: ouabain bound by the tissue (corrected for ouabain located in the ECS) as 1/mol/kg. Abscissae: concentration of ouabain in the incubation medium as 1/mol/litre. The linear regression coefficient of the straight line ($y = 0.32\,x + 5.3 \cdot 10^5$) equals $r = 0.997$.

Comparison of [^{3}H]-digitoxin and [^{3}H]-ouabain uptake

In ventricular tissue, digitoxin was accumulated to a much higher degree than ouabain which confirms the findings in atria. Figure 4 shows a comparison of the uptake of [^{3}H]-digitoxin and [^{3}H]-ouabain at 5×10^{-7} M and 7.7×10^{-7} M respectively. Digitoxin did not reach an equilibrium level during the 180 min period of observation. The T/M ratio for digitoxin uptake was 10 times higher than that for ouabain, which can only be explained by an intracellular accumulation of the hydrophobic digitoxin.

Influence of stimulation on the [^{3}H]-ouabain uptake

All experiments described so far were done with resting papillary muscles. However, it seemed important to investigate the influence of stimulation on the uptake of ouabain since mechanical and electrical activity might well be able to modify this process. Rhythmic contraction, for example, could facilitate the exchange of the ECS with the incubation medium and depolarization of the membrane might render it permeable to ouabain by some unknown mechanism. We therefore studied the ouabain uptake at 1×10^{-7} M in electrically driven papillary muscles. The results are presented in Figure 5. There was no difference between the uptake curves for stimulated and quiescent muscles, which indicates that the distribution of ouabain within the tissue must be essentially the same.

Exchange of tissue-bound ouabain with free ouabain in the medium

Previous studies in atria revealed a striking difference between the mere uptake of ouabain on

Table 1 Binding capacities for ouabain in ventricular and atrial muscle of guinea-pig

Tissue	Ouabain concentration in the incubation medium (M)	T/M at equilibrium	Maximal* membrane-bound ouabain (mol/g wet wt.)	Occupied† binding sites per g wet wt.
Papillary muscle	1×10^{-7}	3.4	3×10^{-10}	1.8×10^{14}
	7.7×10^{-7}	1.8	1×10^{-9}	6.0×10^{14}
	5×10^{-6}	0.82	1.4×10^{-9}	8.4×10^{14}
Atria‡	1.7×10^{-7}	0.58	4×10^{-11}	2.4×10^{13}

* Calculated from the total tissue content at equilibrium and corrected for the amount present in the ECS (see results section).
† Calculations based on Avogadro's number (6×10^{23}).
‡ Data taken from Kuschinsky *et al.*, 1968.

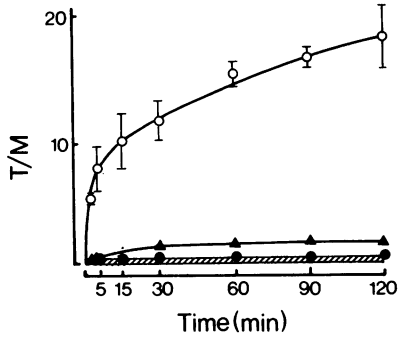

Figure 4 Comparison of the uptake of [^{3}H]-digitoxin (5×10^{-7} M) and [^{3}H]-ouabain (7.7×10^{-7} M) by isolated papillary muscles. Layout as in Figure 2. Digitoxin (○) was accumulated to a much greater extent in the tissue than ouabain (▲), and it did not reach an equilibrium level during the period of observation.

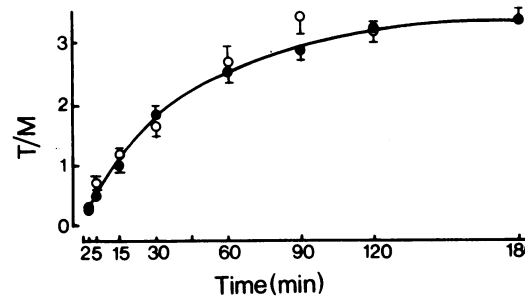

Figure 5 Uptake of [^{3}H]-ouabain (1×10^{-7} M) by quiescent (●) and stimulated (○) papillary muscles. Layout as in Figure 2. No difference in the rate of uptake could be found between quiescent and stimulated papillary muscles.

the one hand and the exchange of ouabain with previously bound, non-radioactive ouabain on the other hand. After preincubation with non-radioactive ouabain (0.5 μg/ml), the uptake of labelled drug proceeded more slowly (Kuschinsky *et al.*, 1968). In a similar experiment this 'exchange' of ouabain was studied in papillary muscles at a concentration of 1×10^{-7} M (Figure 6a). In order to ensure similar experimental conditions for all preparations in which either direct uptake or exchange were studied, the equilibration period for the control muscles was prolonged for an additional period of 180 min in normal Tyrode solution before determination of ouabain uptake. This was the same period of time required for equilibration with unlabelled ouabain in the exchange group. As illustrated in Figure 6a the differences between direct uptake and exchange were small but particularly obvious after 15 min of uptake or exchange.

The exchange of previously bound labelled ouabain with unlabelled ouabain was also studied at 1×10^{-7} M and compared with the corresponding wash-out curve (Figure 6b). The disappearance of radioactivity from the tissue occurred faster if unlabelled ouabain was present in the washing medium. No attempt was made to study the exchange of ouabain at higher concentrations, because contracture would develop during the long course of these experiments.

Wash-out curves for [^{3}H]-ouabain

In order to study the time courses of ouabain wash-out, the papillary muscles were preincubated with tritium-labelled ouabain until an equilibrium level was attained and then transferred into glycoside-free Tyrode solution for various periods of time. Again, the preparations were assayed for their content of radioactivity. The resulting wash-out curves for papillary muscles which had been preincubated at the concentrations 1×10^{-7} M, and 5×10^{-6} M are depicted in Figure 7a, where the ouabain content of the tissue is expressed as percent of the preload equilibrium level and plotted versus time in a semilogarithmic coordinate system. Analysis of the resulting curves revealed that initially ouabain was removed from the tissue at a higher rate than during the later phase of wash-out. In each case the experimental values could be fitted by a complex exponential curve: separating the two components by subtraction yielded two straight lines of different slopes. The initial rate of the wash-out was faster than the corresponding uptake; the late phase of the wash-out, however, occurred very slowly with a half life of 135 and 117 min for 1×10^{-7} M and 5×10^{-6} M ouabain respectively. Furthermore, this presentation of the data provides evidence that about 40%-60% of the tissue content of ouabain was released at a very slow rate and may therefore be bound rather tightly.

Figure 7b presents a similar plot of the time courses of the wash-out and of the exchange of labelled ouabain with unlabelled drug at 1×10^{-7} M. The graphical analysis of the data of the exchange experiments likewise revealed two phases of disappearance of radioactivity from the tissue. However, each phase was much faster than the corresponding wash-out phase. The half lives amounted to 3 and 65 min for the exchange as compared to 16 and 135 min respectively for the wash-out.

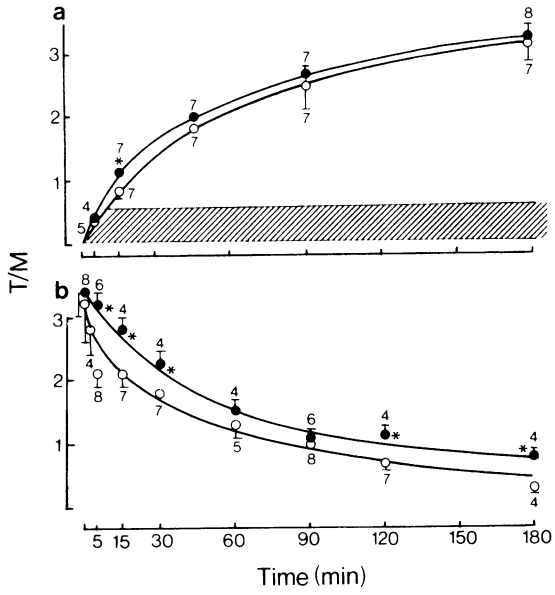

Figure 6 Comparison of (a) the uptake and 'exchange' and (b) the wash-out and 'exchange' of [^{3}H]-ouabain (1×10^{-7} M) in isolated quiescent papillary muscles. Lay-out as in Figure 2. The digits indicate the number of experiments for each point. (a) The exchange (○) of active ouabain with inactive drug with which the muscles had been preloaded proceeded more slowly than the uptake (●) of radioactive ouabain. (b) The disappearance of radioactivity from the tissue was faster, if unlabelled drug was present in the washing medium.
* differences of experimental values statistically significant on a 5% level ($P < 0.05$).

Discussion

Recent investigations on the localization of tritiated cardiac glycosides in the heart muscle by means of autoradiography (Löhr *et al.*, 1971) revealed that ouabain is mainly accumulated in the ECS and partly also at the plasma-membranes. Even after an 18 h incubation period during which marked intracellular oedema cannot be avoided, an intracellular location of glycoside close to the plasma-membrane is seen only occasionally and is very likely to be an artefact. The relatively small amount of ouabain found near the plasma-membrane, which would correspond to a 'membrane-bound' fraction, can readily be explained by the very high concentrations of ouabain (200 μg/l) used for these experiments. Due to its relatively small size, at high concentrations of ouabain the saturable compartment can

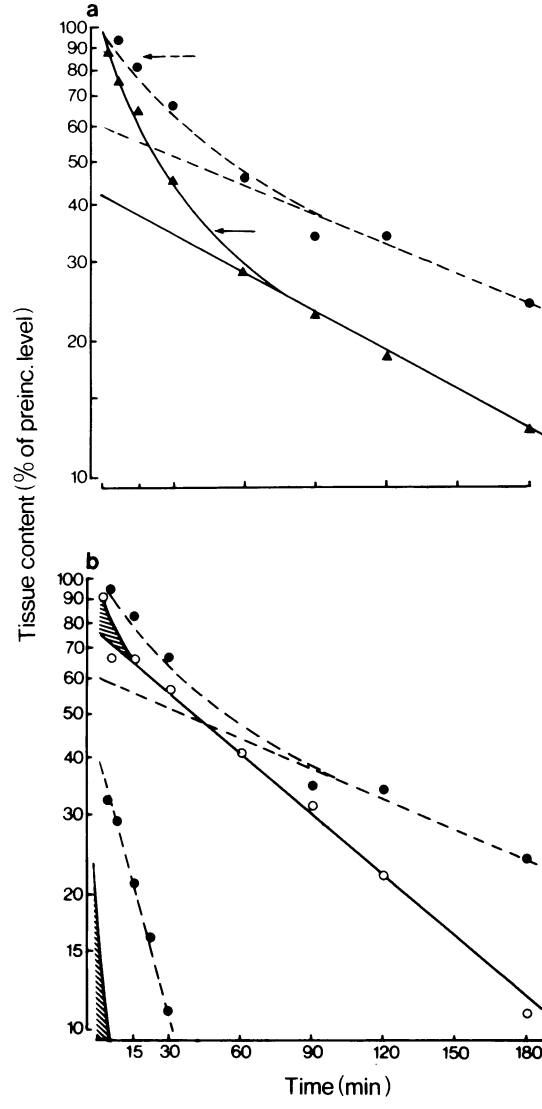

Figure 7 Semilogarithmic plots of the time courses of the wash-out and exchange of ouabain in isolated papillary muscle. (a) Time course of the wash-out [^{3}H]-ouabain after preincubation with 5×10^{-6} M (▲) and 1×10^{-7} M (●). Ordinates: ouabain tissue content expressed as percentage of the preincubation equilibrium level. The arrow indicates the relative size of the ECS at the preincubation equilibrium level: 1×10^{-7} M (dotted arrow) and 5×10^{-6} M (continuous arrow). For each wash-out curve, 2 separate phases could be distinguished. Neither wash-out curve yielded a straight line in the semi-logarithmic plot, i.e. they do not follow a simple exponential function. (b) Time courses of the wash-out of ouabain 1×10^{-7} M (●) and the 'exchange' from active to inactive ouabain (○). Lay-out as in (a). See text for further explanation.

hardly be separated from the drug present in the extracellular space.

In uptake experiments with different glycosides including ouabain, Dutta *et al.* (1968) found the major portion of their labelled material in a subcellular fraction which they assumed to be sarcoplasmic reticulum. Using similar homogenization procedures we could also find ouabain within a microsomal fraction. However, sucrose and inulin were traced in the same fraction (Lüllmann & Peters, 1974), which indicates that the lumen of the structure giving rise to the formation of microsomes during homogenization must have been continuous with the ECS. Therefore, it is more reasonable to assume that the prevailing structures of the microsomal fraction are plasma-membranes and sarcolemmal tubular systems rather than sarcoplasmic reticular membranes. The uptake experiments presented in this paper strongly suggest that in heart muscle there is a saturable compartment for ouabain (see also Figure 3). There are several possibilities for the localization of a saturable compartment. It could be located for example within the cell or it could represent either a saturable transport mechanism or an adsorption to the cell surface. We think our experimental data provide evidence for the latter possibility.

If it is assumed, for the sake of argument, that ouabain was accumulated at some membranous structure accessible only via the intracellular space, it follows that ouabain should have been present in the cell water before binding to this hypothetical site could occur. Furthermore, the bulk of cell water would be expected to be in equilibrium with the ECS and thus contain the same concentration of ouabain. This, however would be apparent as a further non-saturable compartment in the uptake kinetics. Our data provide no evidence for a compartment linearly dependent on the concentration in the medium. Due to its strong hydrophilic character ouabain does not penetrate the plasma-membrane unless transported by a specific mechanism. Such a transport mechanism, however, would be expected to transfer the drug from the ECS to some intracellular compartment until the latter is saturated. In such a model some drug would always be present intracellularly. Therefore, even at very high drug concentrations the T/M ratio could not attain a value below 1. With ouabain 5×10^{-6} M we found a considerably lower T/M ratio of 0.82; in other words, the T/M ratio was approaching the value of the ECS.

Digitoxin was accumulated to a much higher degree (almost 10 fold) than ouabain in papillary muscle. Furthermore, the uptake did not attain an equilibrium level after 3 h whereas with a similar concentration of ouabain the uptake had reached equilibrium after 90 min of incubation. We concluded that digitoxin must be taken up by the cells which is supported not only by the results of previous uptake experiments (Godfraind & Lesne, 1967; Kuschinsky *et al.*, 1968) but also by autoradiography studies (Waser, 1965; Löhr *et al.*, 1971). Although ouabain and digitoxin exhibit such different distribution patterns, their pharmacological action is essentially the same. In fact, the plasma-membrane may be the site of action common to all cardiac glycosides; however, the uptake kinetics of hydrophobic glycosides are complicated by intracellular accumulation. This large 'unspecific' compartment makes it very difficult to estimate their membrane-bound fraction which should correspond to the membrane-bound fraction of ouabain and is probably responsible for its pharmacological effect (Kuschinsky *et al.*, 1968).

Kuschinsky *et al.*, (1967b) demonstrated that the uptake of [^{3}H]-digoxin by resting left auricles proceeds more slowly than by beating auricles. Thus the uptake process of digoxin seems to be related in some way to the excitation process of the membrane. It may well be possible that only the intracellular uptake of digoxin is facilitated by excitation, since the uptake of ouabain which cannot pass through the plasma-membrane into the intracellular space remains unaffected by the excitation process.

Comparing the result of our present investigation in papillary muscle with those found previously in atria (Kuschinsky *et al.*, 1967a, b, 1968; Kuschinsky & Van Zwieten, 1969) no qualitative though considerable quantitative differences could be demonstrated.

The ECS of atria was 0.35 ml/g wet weight (Lüllmann & Van Zwieten, 1967) whereas in papillary muscle an ECS of 0.54 ml/g wet weight was determined. The large size of the ECS in ventricular muscle was confirmed by estimations in the Langendorff preparation of the guinea-pig heart, where about 50% of the total tissue volume was found to represent this compartment (Peters, unpublished observation). The well-developed transverse tubular system in ventricular muscle (Sperelakis & Rubio, 1971) may account for this difference in the size of the ECS.

In order to compare the binding capacities for ouabain in ventricular and atrial muscle, we calculated the number of binding sites occupied at equilibrium level for different concentrations of ouabain and listed these values together with the estimate for atria based upon the data of Kuschinsky *et al.* (1968) in Table 1. Although the T/M ratios established for the uptake of different concentrations of ouabain in atria varied within such a small range (0.5-0.7) that no significant

concentration-dependent differences could be measured, the maximum number of binding sites could be roughly estimated. It amounted to approximately 2×10^{13} binding sites/g wet weight. This value is only 1/40 of the number calculated for ventricular tissue, i.e. 8×10^{14} binding sites/g wet weight. These estimates are supported by the results of Rieger & Kuschinsky (1972) who also found a greater accumulation of glycoside in the ventricle than in atria. Some of the results reported in this paper are not in agreement with the findings of Godfraind & Lesne (1972) in atria, which may be explained by the different concentration ranges investigated.

The differences in the time courses of the exchange and wash-out experiments (Figure 7b) require some further considerations. Since our uptake kinetics revealed a single saturable compartment the two phases of ouabain wash-out cannot be explained by an unsaturable intracellular compartment (Marks, 1972). We have rather to assume two sets of binding sites with similar association constants but different dissociation constants, i.e. 'fast' and 'slow' binding sites.

If a labelled molecule of ouabain dissociates from a 'slow' binding site, it has a reasonable chance to be reassociated to either a 'fast' or a 'slow' binding site when no other glycoside is present in the medium. This rebinding will delay the disappearance of radioactivity from the tissue during wash-out. However, if unlabelled ouabain is present in the medium and therefore also in the ECS, the concentration of dissociating labelled molecules will be diluted in the vast reservoir of inactive molecules. This in turn diminishes their chances of reassociation considerably and thus radioactivity will disappear faster from the tissue. For these reasons, the second slow phase of exchange of active to inactive drug will give a better estimate of the rate of dissociation from the binding sites than the wash-out curve. Thus the half life of dissociation from the 'slow' binding sites is more likely to be 65 min than 135 minutes.

The following numerical example is an attempt to explain the differences in the time courses of uptake and exchange of ouabain (Figure 6a) assuming a half life of 65 min for the slowly dissociating binding sites. During the wash-out of ouabain 1×10^{-7} M (Figure 7) almost 60% of all binding sites are slowly dissociating. Thus, in the beginning only fast dissociating bindings sites (i.e. 40% of all binding sites) can take part in the exchange. With time, an increasing fraction of slowly dissociating binding sites is also involved: after 1 half life (65 min) half of them resulting in a total of 70% of available binding sites, after 3 half lives (195 min) 9/10 of them, rendering a total of 94% of the binding sites available for exchange. This rough estimate may explain why the delay in the uptake of radioactivity after preincubation with inactive ouabain is most prominent during the early phases of exchange (Figure 6a) when a relatively large number of the binding sites available under these conditions is still occupied by inactive drug which dissociates slowly.

From our data presented in this paper we can conclude that in papillary muscle there must be a saturable compartment which clearly can be distinguished from the ECS. Its filling is associated with the binding of ouabain to some structure located probably at the plasma-membrane and including the tubular system. The time course of dissociation of ouabain from the binding sites suggests that they can exhibit two different kinetic properties. The significance of these membrane located binding sites for the site of action for the positive inotropic effect is still under investigation.

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