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BINDING OF DIHYDRODIGITOXIN TO BEEF AND HUMAN CARDIAC ($Na^+ + K^+$)-ATPase: EVIDENCE FOR TWO BINDING SITES IN CELL MEMBRANES

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Abstract—The specific binding of three cardiac glycosides, ^{3}H-ouabain, ^{3}H-digitoxin and ^{3}H-dihydrodigitoxin, to beef cardiac ($Na^+ + K^+$)-ATPase was compared. Non-specific binding was defined as that in the presence of 0.1 mM unlabelled compound, or in the absence of ligands. The dissociation constants (K_D-values) calculated from the inhibition of ^{3}H-ouabain binding were: ouabain, 2.9×10^{-9}M; digitoxin, 1.1×10^{-9}M; and dihydrodigitoxin 2.7×10^{-8}M.

The concentrations which inhibited beef cardiac ($Na^+ + K^+$)-ATPase by 50% were: ouabain, 5.9×10^{-9} M; digitoxin, 1.6×10^{-9} M; and dihydrodigitoxin, 2.5×10^{-8} M. Ouabain and digitoxin showed straight Scatchard plots for one site of high affinity (ouabain, $K_D = 2.6 \times 10^{-9}$ M; digitoxin, $K_D = 1.7 \times 10^{-9}$ M). However, dihydrodigitoxin gave a curved Scatchard plot. Analysis of this binding by the methods of M. J. Weidemann, H. Erdelt and M. Klingenberger (*Eur. J. Biochem.* **16**, 313 (1970)) for two binding sites gave the following results: for Mg^{2+},P_i-supported binding, the K_D of the high affinity site was 1.6×10^{-8} M with a capacity similar to that for ouabain of about 30 pmole/mg protein. For binding supported by Na^+,ATP,Mg^{2+}, the K_D-value of the high affinity site was 5.3×10^{-8}M of similar capacity. The low affinity binding site ($K_D = 4.0 \times 10^{-6}$ M for Mg^{2+},P_i; $K_D = 5.5 \times 10^{-6}$ M for Na^+,ATP,Mg^{2+}) bound about 350 pmole/mg protein. The low affinity site but not the high affinity site was also present in heat-denatured enzyme. Binding supported by Mg^{2+},P_i showed one low affinity site only for ouabain and dihydrodigitoxin in the presence of 200 mM Na^+. The high affinity sites for these three cardiac glycosides were further characterized by measurement of the association and dissociation rate constants.

The specific binding of ^{3}H-ouabain and ^{3}H-dihydrodigitoxin to human cardiac ($Na^+ + K^+$)-ATPase was measured. ^{3}H-Ouabain showed a straight Scatchard plot for one high affinity site only ($K_D = 4.5 \times 10^{-9}$ M, capacity about 15 pmole/mg protein). ^{3}H-Dihydrodigitoxin gave two binding sites: a high affinity site ($K_D = 1.8 \times 10^{-8}$ M) of similar capacity to ouabain, and a low affinity site ($K_D = 2.0 \times 10^{-6}$ M) of about 10-fold greater capacity.

We conclude that there are two binding sites for some cardiac glycosides in cardiac ($Na^+ + K^+$)-ATPase from the digitalis-sensitive beef and human: a dihydrodigitoxin high affinity site also occupied by ouabain and digitoxin, and a low affinity binding site, for dihydrodigitoxin only, whose biological significance has to be investigated further.

The enzyme system considered to be the sodium pump of intact cells, ($Na^+ + K^+$)-ATPase (EC 3.6.1.3), has been implicated in the pharmacological actions of digitalis [1–3]. Among recent investigations on cardiac ($Na^+ + K^+$)-ATPase, evidence has been presented for two receptors [4–7]. It is conceivable that binding of digitalis to the different receptor types may be responsible for the different pharmacological effects of digitalis such as the increase in force of contraction, arrhythmias and inhibition of ($Na^+ + K^+$)-ATPase. Cardiac ($Na^+ + K^+$)-ATPase from the relatively digitalis-insensitive rat showed both a high affinity receptor (K_D about 1×10^{-7} M) and a low affinity receptor (K_D about 3×10^{-5} M) [4]. In contracting rat heart ventricular muscle strips, occupation of the high affinity site corresponded to the positive inotropic effects of ouabain while occupation of the low affinity site corresponded to inhibition of $^{86}Rb^+$-uptake [4], an apparent measure of ($Na^+ + K^+$)-ATPase activity of intact cells [8]. The ouabain IC_{50} for partially purified rat cardiac ($Na^+ + K^+$)-ATPase was 4.0×10^{-5} M, a value similar to the low affinity K_D value.

Further studies on cardiac ($Na^+ + K^+$)-ATPase and contracting left atria from the moderately digitalis-sensitive guinea pig also indicated at least two different types of ouabain binding sites [5]. ^{3}H-Ouabain binding to the contracting left atria was closely related to the positive inotropic effects, while the $^{86}Rb^+$-uptake was decreased only at toxic ouabain concentrations. Binding to both contracting guinea pig left atria and to partially purified ($Na^+ + K^+$)-ATPase in the presence of Tyrode solution and 3 mM ATP gave curved Scatchard plots indicating at least two types of ouabain binding sites. Studies on guinea pig left atria by Ghysel-Burton and Godfraind [9] have shown two binding sites for ouabain. The high affinity site apparently stimulated the sodium pump while the lower affinity site produced pump inhibition. Two binding sites have also been demonstrated for Na^+, ATP, Mg^{2+}, K^+ and vanadate-supported ouabain binding to guinea pig cardiac ($Na^+ + K^+$)-

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ATPase [7]. The results of Fricke and Klaus [10], using guinea pig cardiac ($Na^+ + K^+$)-ATPase, indicated two binding sites activated by different Na^+ concentrations.

In contrast to these results with rat and guinea pig heart, most studies with digitalis-sensitive species such as human, beef, dog and cat have shown only one type of binding site of high affinity in cardiac ($Na^+ + K^+$)-ATPase [2, 11–14]. Two recent studies have shown two types of binding site in human cardiac ($Na^+ + K^+$)-ATPase [7] and canine ventricular sarcolemma [15].

These studies were performed with digitalis derivatives of high affinity such as ouabain, digitoxin and digoxin. Studies with these high affinity compounds on isolated organs have failed to show any differences in therapeutic index [16]. However, Vick *et al.* [17] reported that derivatives with a saturated lactone ring, although of much lower potency [18], showed an increased therapeutic index. Further, the unsaturated lactone ring has been shown to be a major determinant of digitalis binding to its receptor [19]. Thus, compounds such as dihydrodigitoxin may show a different attachment to the cardiac glycoside receptor or the cell membrane, that might be reflected in the appearance of two or more binding sites, in contrast to the high affinity compounds, ouabain and digitoxin. We have therefore measured the effects of these compounds on cardiac ($Na^+ + K^+$)-ATPase isolated from two digitalis-sensitive species, beef and human. Beef cardiac ($Na^+ + K^+$)-ATPase has been used because it has been relatively well-characterized [13, 14] and shows similar binding characteristics to human cardiac ($Na^+ + K^+$)-ATPase [14, 21].

METHODS

Beef hearts, obtained fresh from the slaughterhouse, were stored at −40° in portions of about 100 g. One portion was used for each partial purification procedure using deoxycholate and sodium iodide as described previously [4]. The final sediment, homogenized in 1 mM EDTA pH 7.25 (60 ml), was used for the experiments. The ($Na^+ + K^+$)-ATPase activity, determined by the coupled optical assay method [22], was between 0.2 and 0.3 μmole ATP hydrolysed/min per mg protein at 37°. About 90–95% of the total activity was inhibited by 1×10^{-3} M ouabain. For human heart, the deoxycholate-treated preparation [4], suspended in 1 mM EDTA pH 7.25 (60 ml), was used. The ($Na^+ + K^+$)-ATPase activity was about 0.07 μmole ATP hydrolysed/min per mg protein at 37°. About 80% of the total ATPase activity present was inhibited by 1×10^{-3} M ouabain. The procedures used for the binding studies with ^{3}H-ouabain, ^{3}H-digitoxin and ^{3}H-dihydrodigitoxin have been described previously [23, 24]. The membranes were incubated in 3 mM $MgCl_2$, 3 mM imidazole/PO_4, about 2×10^{-9} M ^{3}H-ouabain or ^{3}H-dihydrodigitoxin or about 5×10^{-10} M ^{3}H-digitoxin, in 50 mM imidazole/HCl buffer, pH 7.25. The total volume for all binding assays was 2.0 ml. Incubation time was 3 hr for beef cardiac membranes and 4 hr for human cardiac membranes. Bound drug was quantitated by a rapid filtration method (Whatman GF/C glass fibre membranes) to separate free drug from membrane-bound drug. The amount of drug bound in the presence of 1×10^{-4} M unlabelled ouabain or dihydrodigitoxin or 1×10^{-5} M unlabelled digitoxin was defined as the non-specific value. As a percentage of the total radioactivity added, this non-specific binding was about 1% for digitoxin, 0.5% for dihydrodigitoxin and less than 0.1% for ouabain. For all compounds, binding in the absence of ligands was approximately equal to this non-specific binding value. Experiments were performed in duplicate

Table 1. Binding of ouabain, digitoxin and dihydrodigitoxin to beef cardiac ($Na^+ + K^+$)-ATPase

		K_D value (maximal pmole bound/mg protein)		
	Binding medium	Ouabain	Digitoxin	Dihydrodigitoxin
Binding values (from IC_{50} value for ^{3}H-ouabain binding)*	Mg^{2+}, P_i	$2.9 \pm 0.15 \times 10^{-9}$ M (n = 4)	$1.1 \pm 0.2 \times 10^{-9}$ M (n = 4)	$2.7 \pm 0.4 10^{-8}$ (n = 4)
Binding values (from Scatchard plots)†	Mg^{2+}, P_i	$2.6 \pm 0.10 \times 10^{-9}$ M (25)	$1.7 \pm 0.2 \times 10^{-9}$ M (36)	1.6×10^{-8} M(29) 4.0×10^{-6} M(360)
	Na^+,ATP,Mg^{2+}	3.8×10^{-9} M (28)	2.5×10^{-9} M (38)	5.3×10^{-8} M(33) 5.5×10^{-6} M(360)
	Mg^{2+}, P_i + 200 mM Na^+	1.6×10^{-6} M (24)	‡	7.1×10^{-6} M(320)
	Mg^{2+}, P_i (with heat-denatured enzyme)	‡	‡	5.1×10^{-6} M(330)

* K_D values were calculated by the method of Erdmann and Schoner [24] from the concentration of unlabelled compound which inhibited by 50% the amount of ^{3}H-ouabain bound at equilibrium.

† The values for K_D and maximal amount of drug bound were calculated from Scatchard plots [26]. Curved Scatchard plots were analysed by the method of Weidemann *et al.* [27]. The values for the maximal number of picomoles of drug bound are given in brackets.

‡ No specific binding was measured in these experiments.

assays. For the experiments with heat-denatured membranes, the membranes were heated for 60 min at 60°. After heating, the membranes had no $(Na^+ + K^+)$-ATPase activity. The association and dissociation rate constants were calculated as described previously [23]. Protein was measured by the method of Lowry *et al.* [20], using bovine serum albumin as standard.

^{3}H-Ouabain (specific activity 16 Ci/mmol) and ^{3}H-digitoxin (specific activity, 13.8 Ci/mmol) were purchased from New England Nuclear, Dreieich, Germany. ^{3}H-Dihydrodigitoxin (specific activity, 9.2 Ci/mmol), prepared as described by Flasch and Heinz [25], was generously provided by Dr H. Flasch, Beiersdorf AG, Hamburg, Germany. A radiochromatogram of ^{3}H-dihydrodigitoxin on propanediol-impregnated cellulose plates using acetic acid (10%) in benzene as the mobile phase showed one peak only, with an R_f corresponding to unlabelled dihydrodigitoxin. According to u.v.-analysis, dihydrodigitoxin contained less than 0.5% digitoxin. All other chemicals were of analytical grade and were obtained from Boehringer-Mannheim, Mannheim, Germany or E. Merck, Darmstadt, Germany.

RESULTS

The binding of ^{3}H-ouabain, ^{3}H-digitoxin and ^{3}H-dihydrodigitoxin to beef heart $(Na^+ + K^+)$-ATPase has been plotted according to Scatchard [26]. Curved Scatchard plots were further analysed by the method of Weidemann *et al.* [27]. The values for the dissociation constant (K_D) and maximal amount of drug bound as calculated by these procedures are given in Table 1. The inhibition of ^{3}H-ouabain binding to partially purified $(Na^+ + K^+)$-ATPase by unlabelled digitalis derivatives can be used to calculate the dissociation constant (K_D) of these unlabelled derivatives [24]. The results from these indirect studies (Table 1) show that digitoxin is about three times more potent than ouabain, while dihydrodigitoxin is about ten times less potent than ouabain.

Direct binding studies using beef cardiac $(Na^+ + K^+)$-ATPase with the labelled compounds were performed in order to extend the indirect K_D data obtained by inhibition of ^{3}H-ouabain binding. For ouabain and digitoxin, with either Mg^{2+},P_i or Na^+,ATP, Mg^{2+}-supported binding, only one binding site of high affinity was observed (Fig. 1). The K_D-values are similar to those calculated by the indirect studies. Binding supported by Mg^{2+},P_i was of slightly greater affinity than binding supported by Na^+, ATP, Mg^{2+}, although the maximal amount of drug bound was similar (Table 1). However, dihydrodigitoxin binding, either supported by Mg^{2+},P_i (Fig. 2) or Na^+, ATP, Mg^{2+}, consistently gave a curved Scatchard plot. The results given in Table 1 have been calculated according to Weidemann *et al.* [27] with the assumption of only two classes of binding sites. These results are compatible with the presence in beef heart $(Na^+ + K^+)$-ATPase of a high affinity/low capacity site and a low affinity/high capacity site. The maximal amount of dihydrodigitoxin bound to the high affinity site was similar to the maximal amount of ouabain or digitoxin bound. The capacity of the low affinity site was about 10-fold greater.

For all three compounds, the number of counts bound in the presence of a high concentration of unlabelled compound (10^{-4} M ouabain or dihydrodigitoxin, 10^{-5} M digitoxin) or in the absence of Mg^{+2}, P_i or Na^+, ATP, Mg^{2+} was similar. This indicates that there was no specific binding in the absence

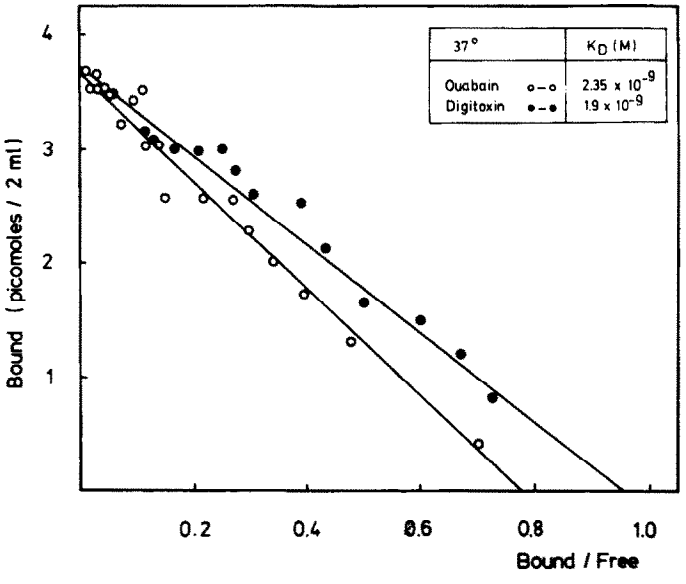

Fig. 1. Binding of ^{3}H-ouabain and ^{3}H-digitoxin to beef cardiac $(Na^+ + K^+)$-ATPase, plotted according to Scatchard [26]. Cardiac cell membranes (0.16 mg protein, $(Na^+ + K^+)$-ATPase activity 0.23 μmole ATP hydrolysed/mg protein per min at 37°) were incubated in Mg^{2+}, P_i-medium (3 mM $MgCl_2$, 3 mM imidazole/PO_4 in 50 mM imidazole/HCl buffer pH 7.25, final volume 2.0 ml) with either 2×10^{-9} M ^{3}H-ouabain or 5×10^{-10} M ^{3}H-digitoxin at 37° for 3 hr.

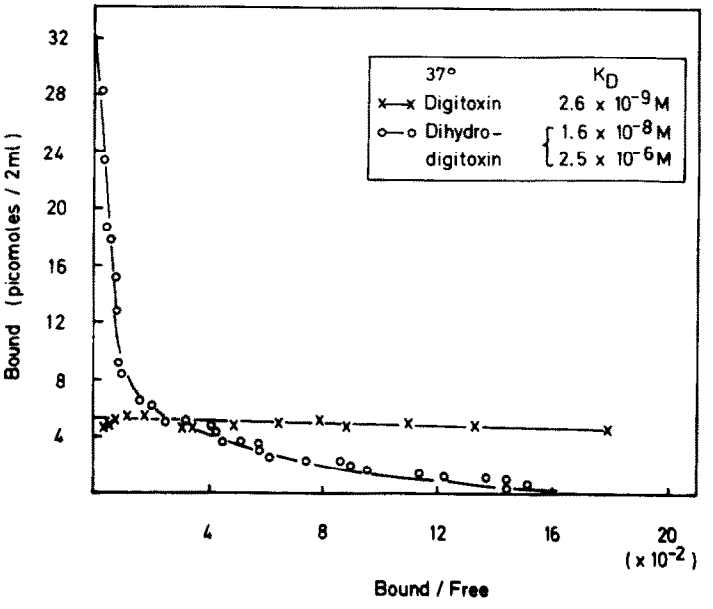

Fig. 2. Binding of ^{3}H-dihydrodigitoxin and ^{3}H-digitoxin to beef cardiac ($Na^+ + K^+$)-ATPase, plotted according to Scatchard [26]. Cardiac cell membranes (0.16 mg protein, ($Na^+ + K^+$)-ATPase activity 0.29 μmole ATP hydrolysed/mg protein per min at 37°) were incubated in Mg^{2+}, P_i-medium (3 mM $MgCl_2$, 3 mM imidazole/PO_4 in 50 mM imidazole/HCl buffer pH 7.25, final volume 2.0 ml) with either 2×10^{-9} M ^{3}H-dihydrodigitoxin or 5×10^{-10} M ^{3}H-digitoxin at 37° for 3 hr. The curvilinear Scatchard plot for ^{3}H-dihydrodigitoxin binding was calculated for two binding sites according to Weidemann *et al.* [27]; a high affinity site ($K_D = 1.6 \times 10^{-8}$ M, about 4.7 pmole bound/2 ml) and a low affinity site ($K_D = 2.5 \times 10^{-6}$ M, about 33 pmole bound/2 ml).

of ligands. No specific binding of ouabain or digitoxin to heat-denatured enzyme could be measured. However, dihydrodigitoxin did bind to heat-denatured enzyme, to one site of low affinity (Fig. 3). The affinity and capacity of this site were similar to the low affinity site observed under maximal binding conditions (Table 1). Dihydrodigitoxin binding to heat-denatured cell membranes was not observed in the absence of Mg^{2+} and P_i.

Na^+ has been shown to reduce the affinity of

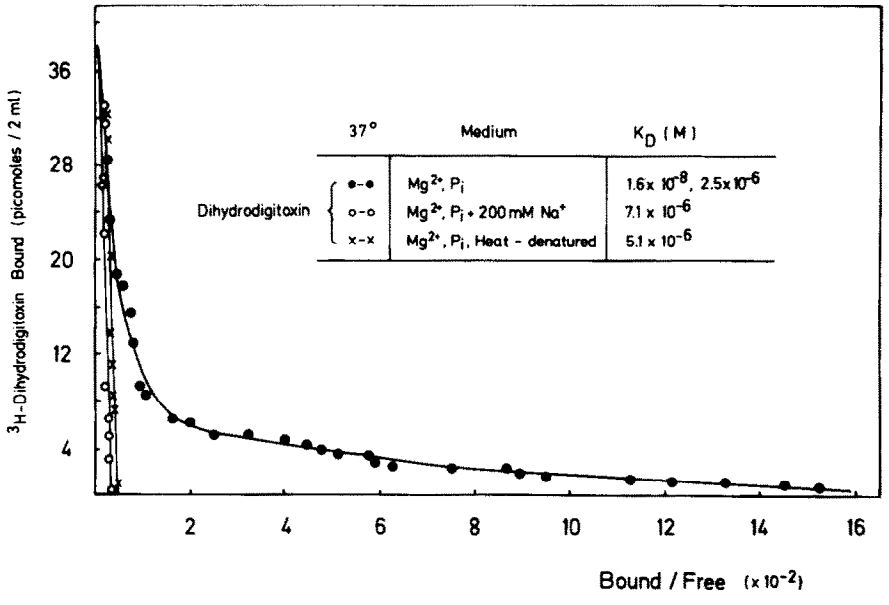

Fig. 3. Binding of ^{3}H-dihydrodigitoxin to beef cardiac ($Na^+ + K^+$)-ATPase, plotted according to Scatchard [26]. Cardiac cell membranes were incubated as described in the legend to Fig. 1 with either Mg^{2+},P_i (●—●) or Mg^{2+},P_i + 200 mM Na^+ (○—○). Heat-denatured membranes were incubated in Mg^{2+},P_i-medium (×—×). The curvilinear Scatchard plot (Mg^{2+},P_i-supported binding) was calculated for two binding sites according to Weidemann *et al.* [27]; the results are given in Fig. 2.

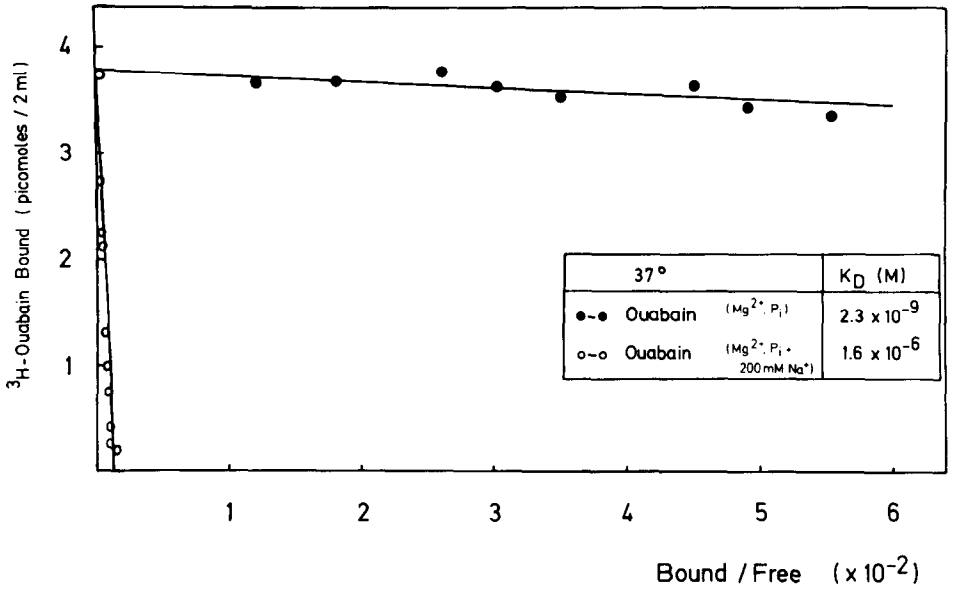

Fig. 4. Binding of ^{3}H-ouabain to beef cardiac ($Na^+ + K^+$)-ATPase, plotted according to Scatchard [26]. Cardiac cell membranes were incubated as described in the legend to Fig. 1. Binding supported by Mg^{2+}, P_i was measured in the presence or absence of 200 mM Na^+.

Mg^{2+},P_i-supported ouabain binding [11]. At a concentration of 200 mM Na^+, the affinity of ouabain was reduced about 700-fold, although the maximal amount of ouabain bound was not significantly altered (Table 1, Fig. 4). No specific digitoxin binding was observed in the presence of Mg^{2+},P_i and 200 mM Na^+. Mg^{2+},P_i-supported dihydrodigitoxin binding in the presence of 200 mM Na^+ showed only one site of low affinity (Fig. 3), similar to the binding site observed with heat-denatured cell membranes for dihydrodigitoxin.

The concentrations of the three compounds which inhibited beef heart ($Na^+ + K^+$)-ATPase activity by 50% are given in Table 2. Digitoxin was almost four times more potent than ouabain while dihydrodigitoxin was more than four times less potent than ouabain.

The association and dissociation rate constants for ouabain, digitoxin and dihydrodigitoxin binding to the high affinity binding sites on beef heart ($Na^+ + K^+$)-ATPase are given in Table 2. As shown in Fig. 5, dihydrodigitoxin association was a second-order process while dihydrodigitoxin dissociation was a first-order process, as previously shown for ouabain [23]. The ratio of the dissociation rate constant to the association rate constant gives a value for the dissociation constant (K_0). For ouabain and digitoxin, this value is slightly lower than the value determined from the Scatchard analysis. Since a low concentration of ^{3}H-dihydrodigitoxin (about 2×10^{-9}M) was used for these experiments, only binding to the high affinity site will be measured. The K_D-values calculated from the kinetic measurements and from the Scatchard plots are similar. The

Table 2. Kinetic constants and inhibitory concentrations of ouabain, digitoxin and dihydrodigitoxin with beef cardiac ($Na^+ + K^+$)-ATPase*

	Ouabain	Digitoxin	Dihydrodigitoxin
Association rate constant (k_{+1})	1.0×10^5/M per sec	2.7×10^5/M per sec	1.8×10^5/M per sec
Dissociation rate constant (k_{-1})†	1.6×10^{-4}/sec	2.6×10^{-4}/sec	3.5×10^{-3}/sec
K_D (from k_{-1}/k_{+1})	1.6×10^{-9} M	9.4×10^{-10} M	2.0×10^{-8} M
IC_{50}‡	5.9×10^{-9} M	1.6×10^{-9} M	2.5×10^{-8} M

* All values are taken from experiments with Mg^{2+}, P_i-supported binding.

† Association and dissociation rate constants were calculated by the methods of Erdmann and Schoner [23].

‡ The IC_{50} value is the concentration necessary to inhibit beef cardiac ($Na^+ + K^+$)-ATPase activity by 50%. For these experiments, the suspension containing compound and enzyme was heated at 37° for 2 hr to obtain equilibrium binding conditions before activity was measured by the coupled optical assay method [22].

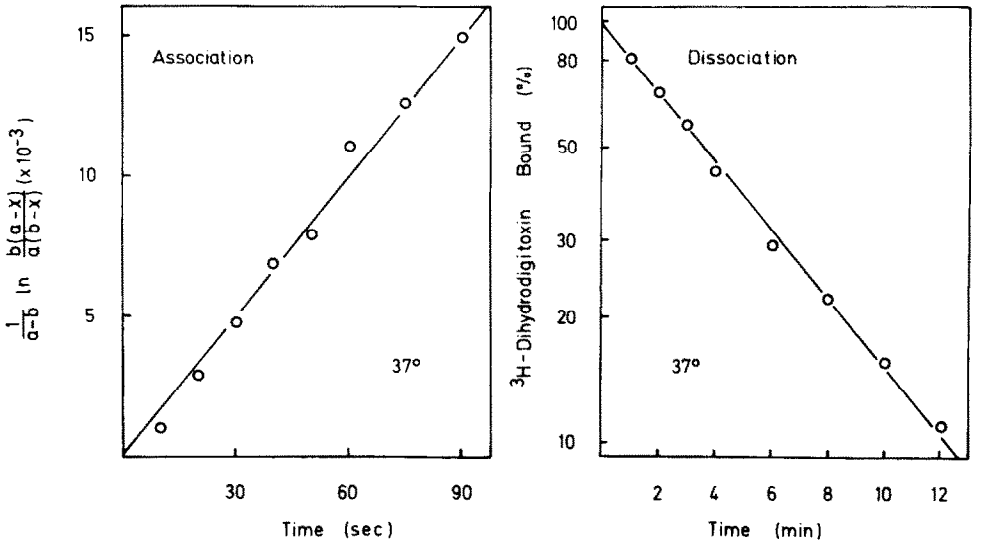

Fig. 5. Association and dissociation of ^{3}H-dihydrodigitoxin from beef cardiac (Na^- + K^+)-ATPase. Association rate constant: cardiac cell membranes (0.22 mg protein, (Na^+ + K^-)-ATPase activity 0.32 μmole ATP hydrolysed/mg protein per min at 37°, initial concentration of high affinity receptors = a = 5.7 nM) were incubated in 3 mM $MgCl_2$, 3 mM imidazole/PO_4, 2.05 nM ($=b$) ^{3}H-dihydrodigitoxin in 50 mM imidazole/HCl buffer pH 7.25 at 37°, final volume 2.0 ml for the stated times. The membrane bound drug was measured after rapid filtration. The association rate constant (1.77×10^5/M per sec) is the slope of the graph in Fig. 5. Dissociation rate constant: cardiac cell membranes (7.6 mg protein, (Na^- + K^-)-ATPase activity 0.32 μmole ATP hydrolysed/mg protein per min at 37°) were incubated with 3 mM $MgCl_2$, 3 mM imidazole/PO_4, 6.8×10^{-9} M ^{3}H-dihydrodigitoxin in 50 mM imidazole/HCl buffer pH 7.25 at 37° for 3 hr. After 30 min centrifugation at 100,000 g, the sediment was homogenized in 6 ml water at 0°. A portion of this suspension (0.2 ml, 0.26 mg protein) of drug-receptor complex was incubated for the times shown, in 3 mM $MgCl_2$, 3 mM imidazole/PO_4, 10^{-4} M unlabelled dihydrodigitoxin in 50 mM imidazole/HCl buffer pH 7.25 at 37°, final volume 2.0 ml. The membrane bound drug was measured after rapid filtration. The dissociation rate constant (3.46×10^{-3}/sec) is the slope of the graph in Fig. 5.

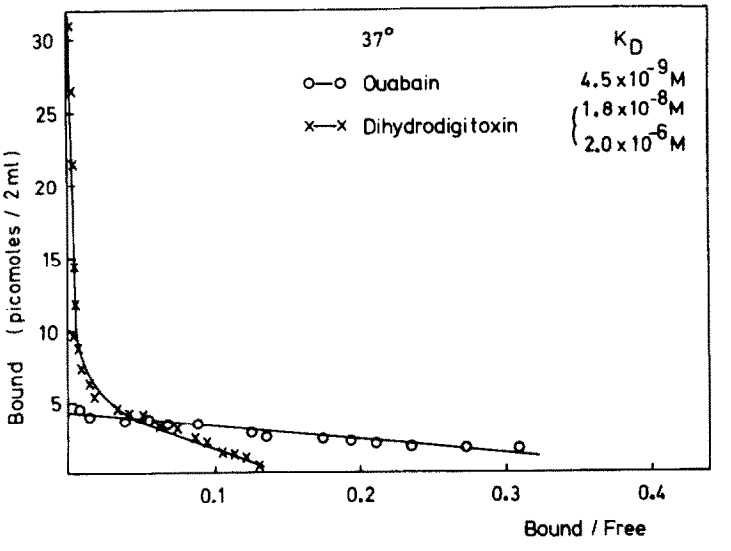

Fig. 6. Binding of ^{3}H-dihydrodigitoxin and ^{3}H-ouabain to human cardiac (Na^- + K^-)-ATPase, plotted according to Scatchard [26]. Cardiac cell membranes (0.48 mg protein, (Na^+ + K^-)-ATPase activity 0.07 μmole ATP hydrolysed/mg protein per min at 37°) were incubated in Mg^{2+},P_i-medium (3 mM $MgCl_2$, 3 mM imidazole/PO_4 in 50 mM imidazole/HCl buffer pH 7.25, final volume 2.0 ml) with 2×10^{-9} M of either ^{3}H-ouabain or ^{3}H-dihydrodigitoxin at 37° for 4 hr. The curvilinear Scatchard plot for ^{3}H-dihydrodigitoxin binding was calculated for two binding sites according to Weidemann *et al.* [27]; a high affinity site ($K_D = 1.8 \times 10^{-8}$ M, about 5.2 pmole bound/2 ml) and a low affinity site ($K_D = 2.0 \times 10^{-6}$ M, about 50 pmole bound/2 ml).

straight lines in Fig. 5 indicate one type of high affinity dihydrodigitoxin binding site.

Human cardiac $(Na^+ + K^+)$-ATPase was used for direct binding studies with ^{3}H-ouabain and ^{3}H-dihydrodigitoxin. ^{3}H-Ouabain showed one high affinity site ($K_D = 4.5 \times 10^{-9}$M, capacity about 15 pmole/mg protein) while ^{3}H-dihydrodigitoxin showed at least two binding sites (Fig. 6). Analysis for two binding sites by the method of Weidemann *et al.* [27] gave a high affinity site ($K_D = 1.8 \times 10^{-8}$ M) of similar capacity to ouabain, and a low affinity site ($K_D = 2.0 \times 10^{-6}$ M) of about 10-fold greater capacity

DISCUSSION

The results presented in this paper show that there are at least two different types of dihydrodigitoxin binding sites in beef and human cardiac $(Na^+ + K^+)$-ATPase. Calculations assuming two binding sites show that these two sites have about a 100-fold different affinity for dihydrodigitoxin in both species. In beef cardiac $(Na^+ + K^+)$-ATPase, the high affinity site (K_D about $2–5 \times 10^{-8}$ M) is supported by either Mg^{2+},P_i or Na^+,ATP,Mg^{2+} as ligands and is heat-sensitive. The low affinity site is an additional binding site which is not occupied by ouabain or digitoxin and is also present in heat-denatured membranes. Neither site binds in the absence of ligands. In the presence of an Na^+-concentration of 200 mM, Mg^{2+},P_i-supported ouabain binding is markedly reduced and specific digitoxin binding is not observed. Under these conditions, dihydrodigitoxin binding shows a straight Scatchard plot, with an affinity similar to the low affinity site seen under maximal binding conditions. Mg^{2+},P_i-supported dihydrodigitoxin binding to human cardiac $(Na^+ + K^+)$-ATPase gave similar results to the beef cardiac $(Na^+ + K^+)$-ATPase.

The sample of dihydrodigitoxin is likely to be a mixture of the stereoisomers, 20(*R*)- and 20(*S*)-dihydrodigitoxin. Unfortunately, chemical attempts to separate the stereoisomers were not successful. Fullerton *et al.* [28], however, have shown that several 20(*R*)- and 20(*S*)-isomeric pairs of cardanolides have similar inhibitory potencies on rat brain $(Na^+ + K^+)$-ATPase. Further, the marked differences between the two types of dihydrodigitoxin binding sites (sensitivity to heat, amount of dihydrodigitoxin bound and the binding characteristics of ouabain and digitoxin) seem to be too large to be due only to the binding of different stereoisomers. However, this possibility cannot be definitely excluded.

The high affinity site can be further characterized by the measurement of the association and dissociation rate constants (Fig. 5). These results indicate that there is only one type of high affinity dihydrodigitoxin binding site.

The results are evidence that, in sensitive species, there are at least two different types of specific digitalis binding sites, as previously found in the less sensitive guinea pig and rat. Further, the low affinity compound, dihydrodigitoxin, binds to the different binding sites, in contrast to the high affinity compounds, ouabain and digitoxin.

Two different ouabain binding sites have been demonstrated under various artificial conditions in digitalis-sensitive species by several researchers. De Pover and Godfraind [6], using Mg^{2+},ATP,Na^+-supported ouabain binding to human cardiac $(Na^+ + K^+)$-ATPase, showed two binding sites, in marked contrast to an earlier report [14]. Wellsmith and Lindenmayer [15] showed two binding sites with different kinetics in canine ventricular sarcolemma and suggested that the second site was $(Na^+ + K^+)$-ATPase in a low affinity conformation. The results of Heller and Beck [29] led them to propose that there are two types of ouabain binding sites in human red blood cells. However, one type was not saturable and binding only approached equilibrium after 24 hr. Two distinct molecular forms of $(Na^+ + K^+)$-ATPase have been shown by Sweadner [30] in the brain of several species but not in other organs, possibly because of technical difficulties. These *in vitro* experiments, including the experiments reported in this paper, do not indicate whether these apparently distinct receptors for digitalis glycosides have significance for the biological effects of digitalis. The biological significance can only be determined in whole animal or in isolated organ studies. However, our results show that with further studies on semisynthetic digitalis derivatives it may be possible to differentiate between those receptors and possibly between the wanted and unwanted effects of digitalis.

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