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DIGITALIS STRUCTURE–ACTIVITY RELATIONSHIP ANALYSES

CONCLUSIONS FROM INDIRECT BINDING STUDIES WITH CARDIAC $(Na^+ + K^+)$-ATPase

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Abstract—We have performed direct and indirect binding studies with [^{3}H]ouabain or [^{3}H]digitoxin on beef or guinea pig cardiac $(Na^+ + K^+)$-ATPase to measure the potencies of a broad range of cardiotonic steroids for structure–activity relationship (SAR) studies for comparison with previously determined positive inotropic potencies. The positive inotropic potencies of twelve compounds on contracting guinea pig left atria correlated well with the equilibrium dissociation constants (K_D values) from the inhibition of [^{3}H]ouabain binding to guinea pig cardiac $(Na^+ + K^+)$-ATPase ($r = 0.98$ for seven 5β-compounds, $r = 0.95$ for five 5α-compounds). Further we calculated K_D values from the inhibition of [^{3}H]ouabain binding data for a total of 33 digitalis derivatives on the digitalis-sensitive beef cardiac $(Na^+ + K^+)$-ATPase. For the 27 compounds tested on both beef cardiac $(Na^+ + K^+)$-ATPase and guinea pig left atria, the potencies showed a significant correlation ($r = 0.92$ for 22 5β-compounds, $r = 0.96$ for five 5α-compounds. For seven compounds, K_D values were measured on beef cardiac $(Na^+ + K^+)$-ATPase using inhibition of binding of [^{3}H]digitoxin. These values correlated well ($r = 0.99$) with the K_D values from the [^{3}H]ouabain studies.

These results show that: (1) The significant correlation observed between K_D values on guinea pig cardiac $(Na^+ + K^+)$-ATPase and positive inotropic potency in guinea pig left atria is further evidence that the pharmacological receptor for inotropy is part of the enzyme, (2) Inhibition of the binding of [^{3}H]ouabain or [^{3}H]digitoxin can be used to determine the relative potencies of unlabelled digitalis derivatives. The similar relative potencies on beef and guinea pig cardiac $(Na^+ + K^+)$-ATPase of a broad range of digitalis derivatives indicate that the binding site is similar for both species; and (3) SAR studies indicate that functional groups on these steroids have the same influence on potency on either the positive inotropy or cardiac $(Na^+ + K^+)$-ATPase studies.

Although the pharmacological effects of digitalis are well-known, the mechanism of action is still subject to controversy [1]. The only cellular system found to be affected by low concentrations of cardiac glycosides is $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) [2,3]. This enzyme system, which represents the sodium pump of intact cells, has thus been proposed to contain the pharmacological receptor for digitalis. [^{3}H]Ouabain and [^{3}H]digoxin, the first labelled cardiac glycoside studied [4], have been used to characterize binding to $(Na^+ + K^+)$-ATPase. Studies with labelled ligands can also indirectly characterize the interactions of unlabelled compounds with a receptor, using the methods reviewed by Weiland and Molinoff [5].

Structure–activity relationship (SAR) studies can either be carried out on intact tissue, measuring positive inotropy or toxicity, or on partially purified $(Na^+ + K^+)$-ATPase preparations. Studies measuring the inhibition of binding to $(Na^+ + K^+)$-ATPase of labelled ligands by unlabelled analogues give the potency of these analogues as their equilibrium dissociation constants (K_D values) [6]. The positive inotropic potencies of cardiotonic steroids have been determined on isolated hearts of several species, especially guinea pig [7, 8]. A broad range of cardiotonic steroids, including most of the compounds in this study, have previously been tested on the isolated guinea pig left atria preparation [9, 10]. There should be a good correlation between both sets of potency values, if $(Na^+ + K^+)$-ATPase contains the receptor which mediates the positive inotropic effects.

The potency values determined by positive inotropy studies or by enzyme displacement studies will allow us to compare SAR conclusions. The aim of SAR studies is to determine which functional groups are binding to the receptor, in order to lead to more selective drugs. Because equilibrium conditions can be obtained in isolated left atria studies, in contrast to the cat toxicity studies used for the classical digitalis SAR studies [11], it is valid to compare these results with the equilibrium K_D values obtained with cardiac $(Na^+ + K^+)$-ATPase.

In the present study, we have determined the indirect K_D values of the drug receptor complex using cardiac$(Na^+ + K^+)$-ATPase from beef and guinea pig heart. The inhibition of [^{3}H]ouabain binding to beef heart $(Na^+ + K^+)$-ATPase by 33 unlabelled cardiotonic steroids was measured to calculate

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the K_D values for these derivatives. Seven 5β-compounds were further studied by calculation of indirect K_D values in two systems: [^{3}H]digitoxin displacement with beef cardiac ($Na^+ + K^+$)-ATPase and [^{3}H]ouabain displacement with guinea pig cardiac ($Na^+ + K^+$)-ATPase. The indirect K_D values for five 5α-compounds on guinea pig cardiac ($Na^+ + K^+$)-ATPase were also determined. A correlation between indirect K_D values obtained from [^{3}H]digitoxin and [^{3}H]ouabain binding studies would indicate that either compound can be used to characterize the interactions of unlabelled cardiotonic steroids. The absolute potencies of these unlabelled cardiotonic steroids will be much higher on beef heart than on guinea pig cardiac ($Na^+ + K^+$)-ATPase.

Repke *et al.* [12] have proposed that these marked species differences are related to differences in the interaction with ($Na^+ + K^+$)-ATPase. A close correlation between relative potencies in both species, however, would indicate that the actual binding site is very similar, whereas the observed different absolute potency values may be caused by a slightly different structure of the receptor protein.

MATERIALS AND METHODS

Materials. [^{3}H]Ouabain, specific activity 16 Ci/mmole (Lot. No. 1162-062), and [^{3}H]digitoxin, specific activity 13.8 Ci/mmole (Lot. No. 1163-063), were purchased from New England Nuclear (Dreieich, F.R.G.). Several of the cardiotonic steroids used in this study were purchased from Boehringer-Mannheim (Mannheim, F.R.G.) or Serva Biochemicals (Heidelberg, F.R.G.) (compounds **1–9** and **14–17**). Compounds **10**, **12**, **13** and **30** were prepared as previously described [13]. Compounds **18–26** were prepared as previously described [10]. Compounds **27** and **28** were generously supplied by Dr. Karl Thomae GmbH, Biberach a.d. Riß, F.R.G. Uzarigenin (**29**), uzarigenin glucoside (**31**), uzarin (**32**) and uzariside (**33**) were generously supplied by Stada-Arzneimittel AG, Dortelweil/Wetterau, F.R.G. All other chemicals were of analytical grade and were obtained from Boehringer-Mannheim or E. Merck (Darmstadt, F.R.G).

Preparation of ($Na^+ + K^+$)-*ATPase-containing beef cardiac cell membranes.* Beef hearts, obtained fresh from the slaughter house, were divided into portions of about 100 g and frozen at −40°. The partial purification procedure using sodium deoxycholate and sodium iodide extraction has been previously described [14]. 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 [15], was between 0.2 and 0.3 μmole ATP hydrolysed per min per mg protein at 37°. About 90–95% of the total activity was inhibited by 10^{-3} M ouabain.

Preparation of ($Na^+ + K^+$)-*ATPase-containing guinea pig cardiac cell membranes.* Guinea pig hearts (100 g) were kept frozen at −40°. The partial purification procedure was as described for beef heart cardiac cell membranes. The ($Na^+ + K^+$)-ATPase activity was between 0.2 and 0.3 μmole ATP hydrolysed per min per mg protein at 37°. About 85–95% of the total activity was inhibited by 10^{-3} M ouabain.

Binding of [^{3}H]*ouabain or* [^{3}H]*digitoxin to beef or guinea pig cardiac cell membranes.* The procedures used for these experiments have been described in detail elsewhere [6, 16]. Ouabain (1×10^{-2} M) was dissolved in distilled water, and digitoxin (1×10^{-3} M) was dissolved in dimethyl formamide. Further dilutions were made with distilled water. The final concentration of dimethyl formamide in the assays was less than 1%; control experiments showed that 1% dimethyl formamide had no effect on [^{3}H]digitoxin binding. Bound ouabain or digitoxin was quantitated by a rapid filtration method (Whatman GF/C glass filter membranes) to separate free drug from membrane-bound drug. Non-specific binding (binding in the presence of 10^{-3} M unlabelled ouabain or 10^{-5} M unlabelled digitoxin) was very low for ouabain (less than 1%) but higher for digitoxin (about 3–4%). The incubation medium used for all experiments was 3 mM $MgCl_2$, 3 mM imidazole/PO_4, about 2×10^{-9} M [^{3}H]ouabain or about 5×10^{-10} M [^{3}H]digitoxin in 50 mM imidazole–HCl buffer, pH 7.25. Experiments were performed in duplicate assays and at least twice. An incubation time of 3 hr for beef heart membranes or 2 hr for guinea pig heart membranes was used after initial experiments showed that equilibrium was reached within this time.

Measurement of positive inotropic potencies on isolated guinea pig left atria. Inotropic activity was determined using guinea pig left atria suspended in Krebs–Henseleit solution, gassed with 95% O_2 and 5% CO_2, stimulated by rectangular pulses of 10 msec duration at a frequency of 90/min at 32°, as described previously [10]. Each atrium was used for only one cumulative dose–response curve. The drug concentration producing a 75% increase in force of contraction (the ΔF_{75} dose) was read from pooled data of at least ten cumulative dose-response curves.

General. Protein was measured by the procedure of Lowry *et al.* [17], using bovine serum albumin as standard. K_D values for direct binding studies with [^{3}H]ouabain or [^{3}H]digitoxin were calculated from Scatchard plots [18] as described previously [16]. Indirect K_D values, calculated as described previously [6], are given as the mean value, or, when three or more determinations were performed, as the mean ± S.E.M.

RESULTS

Many researchers have studied the interaction of labelled cardiac glycosides with ($Na^+ + K^+$)-ATPase from various sources [2, 6, 19–21]. Erdmann and Schoner [6] showed that beef and dog heart bound about 130 picomole [^{3}H]ouabain/unit ($Na^+ + K^+$)-ATPase activity (1 unit activity = 1 μmole ATP hydrolysed per min per mg protein at 37°). Our results confirm these findings: beef heart ($Na^+ + K^+$)-ATPase bound 127.1 ± 4.1 picomole ouabain/unit activity (n = 40), while guinea pig heart ($Na^+ + K^+$)-ATPase bound 126.1 ± 3.8 picomole ouabain/unit activity (n = 10). In the [^{3}H]digitoxin studies, beef heart ($Na^+ + K^+$)-ATPase bound

Table 1. Comparison of positive inotropic potencies determined with guinea pig left atria (ΔF_{75} values) and equilibrium dissociation constants (K_D values) for the binding of cardiotonic steroids to guinea pig heart ($Na^+ + K^+$)-ATPase at 37°, determined by [^{3}H]ouabain displacement

Compound	K_D ($\times 10^{-9}$M)	Relative potency* K_D values	Relative potency* ΔF_{75} values†
Digitoxigenin (**1**)	310	1.0	1.0
Ouabain (**2**)	128 ± 10 (n = 10)	2.5	4.8
Dihydroouabain (**3**)	2370 ± 500 (n = 3)	0.13	0.10
Digitoxigenin glucoside (**10**)	79 ± 3 (n = 3)	4.0	2.8
3α-Methyldigitoxigenin glucoside (**11**)	1280 ± 150 (n = 3)	0.25	0.14
Digitoxigenin thevetoside (neriifolin) (**14**)	20 ± 5 (n = 3)	15.0	27.0
Digitoxin (**16**)	34 ± 4 (n = 3)	9.2	8.8
Uzarigenin (**29**)	1550	0.20	1.2
Uzarigenin rhamnoside (**30**)	69	4.5	9.3
Uzarigenin glucoside (**31**)	1450 ± 270 (n = 3)	0.21	0.44
Uzarigenin diglucoside (uzarin) (**32**)	4330 ± 900 (n = 3)	0.07	0.12
Uzarigenin triglucoside (uzaroside) (**33**)	2460	0.13	0.22

* Potency relative to digitoxigenin which has been given a value of 1.0.

† The ΔF_{75} value is the drug concentration effecting a 75% increase in force of contraction, as interpolated from cumulative dose–response curves.

155.8 ± 14.0 picomole digitoxin/unit activity (n = 14).

Only a limited number of cardiotonic steroids are available as labelled compounds suitable for direct measurements of K_D values by methods reviewed by Weiland and Molinoff [5]. Labelled ligands such as [^{3}H]ouabain and [^{3}H]digitoxin can be used to characterize indirectly the interactions of unlabelled compounds.

The mathematical procedures for calculating the equilibrium dissociation constant (K_D) of an unlabelled analogue from [^{3}H]ouabain or [^{3}H]digitoxin displacement data have been described by Erdmann and Schoner [6], according to Jensen and Nørby [22]. These calculations assume that the analogues bind to the same receptor as [^{3}H]ouabain and [^{3}H]digitoxin. The data from the inhibition of [^{3}H]ouabain binding by ouabain can be used to calculate the K_D value directly (by Scatchard plot analysis [18]) or indirectly (by the procedures of Erdmann and Schoner [6]). Scatchard plot analysis of [^{3}H]ouabain binding to beef cardiac ($Na^+ + K^+$)-ATPase gave an average K_D value of 2.6 ± 0.10 $\times 10^{-9}$M (n = 40). The average indirect K_D value for ouabain calculated from the same experiments was 2.9 ± 0.15 $\times 10^{-9}$M. For guinea pig cardiac ($Na^+ + K^+$)-ATPase, the K_D values were 1.29 ± 0.08 $\times 10^{-7}$M (Scatchard) and 1.28 ± 0.10 $\times 10^{-7}$M (indirect) (n = 10). The values for digitoxin binding to beef cardiac ($Na^+ + K^+$)-ATPase were 1.7 ± 0.2 $\times 10^{-9}$M (Scatchard) and 2.5 ± 0.3 $\times 10^{-9}$M (indirect) (n = 14). Further [^{3}H]ouabain displacement experiments were carried out to determine whether the K_D value was influenced by the number of receptors present. Using the same beef heart ($Na^+ + K^+$)-ATPase batch, K_D values for digitoxin and digitoxigenin were measured using 0.1 or 0.2 ml of enzyme suspension (final volume 2.0 ml). The K_D values with 0.2 ml of enzyme for digitoxin (1.0×10^{-9}M) and digitoxigenin (13.2×10^{-9}M) were similar to those given in Table 2 (measured with 0.1 ml enzyme). The maximal number of picomole of [^{3}H]ouabain bound in these two series of experiments was 5.0 picomole (0.1 ml enzyme suspension) and 9.9 picomole (0.2 ml enzyme suspension). The K_D values show that the calculations are independent of receptor number present.

The guinea pig heart has been widely used for digitalis SAR studies. We have measured the [^{3}H]ouabain displacement K_D values (Table 1) for twelve compounds on guinea pig heart ($Na^+ + K^+$)-ATPase. As shown in Fig. 2, there is a good correlation (r = 0.98 for seven 5β-compounds, r = 0.95 for five 5α-compounds) between these ouabain displacement K_D values and positive inotropy potencies determined on guinea pig left atria. Indirect K_D values for 33 digitalis analogues calculated from [^{3}H]ouabain displacement results on beef heart ($Na^+ + K^+$)-ATPase are given in Table 2, while the structures of these compounds are given in Fig. 1.

For 27 compounds, the [^{3}H]ouabain displacement K_D values on beef cardiac ($Na^+ + K^+$)-ATPase (Table 2) can be compared with inotropic potencies determined on isolated guinea pig left atria [9, 10] (Fig. 3). For the 22 5β-compounds tested in both systems, linear regression gave a slope of 1.18. The slope for the five 5α-compounds tested was 0.73.

Table 2. Comparison of positive inotropic potencies determined with guinea pig left atria (ΔF_{75} values) and equilibrium dissociation constants (K_D values) for the binding of cardiotonic steroids to beef heart ($Na^+ + K^+$)-ATPase at 37°, determined by [^{3}H]ouabain displacement

Compound	K_D ($\times 10^{-9}$M) (± S.E.M.)	Relative potency* K_D values	Relative potency* ΔF_{75} values†
Digitoxigenin (**1**)	10.3 ± 0.43 (n = 3)	1.0	1.0
Ouabain (**2**)	2.9 ± 0.15 (n = 40)	3.6	4.8
Dihydroouabain (**3**)	89	0.12	0.1
Digoxin (**4**)	3.6 ± 0.40 (n = 3)	2.9	2.1
β-Methyldigoxin (**5**)	3.7	2.8	—
Gitoxin (**6**)	15.0 ± 3.0 (n = 3)	0.69	1.4
Gitaloxin (**7**)	2.5 ± 0.5 (n = 3)	4.1	—
Convallatoxin (**8**)	0.86	12.0	3.8
Cymarin (**9**)	3.3	3.1	2.4
Digitoxigenin glycosides			
Digitoxigenin glucoside (**10**)	2.5 ± 0.5 (n = 3)	4.1	2.8
3α-Methyldigitoxigenin glucoside (**11**)	71.0 ± 7.6 (n = 4)	0.14	0.14
Digitoxigenin galactoside (**12**)	11.0 ± 0.9 (n = 3)	0.93	1.0
Digitoxigenin rhamnoside (**13**)	0.79	13.0	22.0
Digitoxigenin thevetoside (neriifolin) (**14**)	0.59 ± 0.08 (n = 3)	17.4	27.0
Digitoxigenin monodigitoxoside (**15**)	1.6	6.3	15.3
Digitoxin (**16**)	1.1 ± 0.2 (n = 4)		8.8
Dihydrodigitoxin (**17**)	27 ± 4 (n = 4)	0.38	—
C17-modified compounds			
(a) From digitoxigenin			
Methyl ester glucoside (**18**)	15.2 ± 1.3 (n = 3)	0.68	1.4
Methyl ester rhamnoside (**19**)	4.6	2.2	—
Ethyl ester glucoside (**20**)	12.7 ± 1.4 (n = 3)	0.81	0.20
n-Propyl ester glucoside (**21**)	19.1 ± 3.2 (n = 3)	0.54	0.085
iso-Propyl ester glucoside (**22**)	16.3 ± 2.3 (n = 4)	0.63	0.21
n-Butyl ester glucoside (**23**)	79 ± 9.2 (n = 3)	0.13	0.015
iso-Butyl ester glucoside (**24**)	89 ± 15 (n = 3)	0.12	0.016
Nitrile glucoside (**25**)	7.4	1.4	2.3
Ketone glucoside (**26**)	14.0	0.74	0.85
(b) From digoxigenin			
Nitrile tridigitoxoside (**27**)	12.5	0.82	—
Nitrile tridigitoxoside 3‴,4‴-carbonate (**28**)	9.1	1.1	—
5α-Cardenolides			
Uzarigenin (**29**)	14.3	0.72	1.2
Uzarigenin rhamnoside (**30**)	6.8	1.5	9.3
Uzarigenin glucoside (**31**)	116	0.089	0.44
Uzarigenin diglucoside (uzarin) (**32**)	1500	0.0068	0.12
Uzarigenin triglucoside (uzaroside) (**33**)	420 ±80 (n = 3)	0.024	0.22

* Potency relative to digitoxigenin which has been given a value of 1.0.

† The ΔF_{75} value is the drug concentration effecting a 75% increase in force of contraction, as interpolated from cumulative dose—response curves.

There was a good correlation between the relative potencies in the two systems (5β-compounds, r = 0.92; 5α-compounds, r = 0.96). The results show that guinea pig cardiac ($Na^+ + K^+$)-ATPase and isolated guinea pig left atria are about 30 times less sensitive to digitalis than is beef cardiac ($Na^+ + K^+$)-ATPase.

The K_D values for beef brain ($Na^+ + K^+$)-ATPase for twelve of the compounds in Table 2 have previously been given [6]. As shown in Fig. 4, there is a good relationship (r = 0.89) between the results with ($Na^+ + K^+$)-ATPase from different sources, except for uzarigenin (**29**). Digitoxigenin and uzarigenin, the 5α-isomer of digitoxigenin, had similar relative potencies on both beef cardiac ($Na^+ + K^+$)-ATPase and guinea pig left atria but uzarigenin was shown to be about 35 times less potent than digitoxigenin on beef brain ($Na^+ + K^+$)-ATPase.

Although [^{3}H]ouabain has been the most widely used labelled cardiac glycoside in studies of the digitalis receptor, equilibrium dissociation constants may be accessible from displacement studies of other labelled cardiotonic steroids, if all compounds are

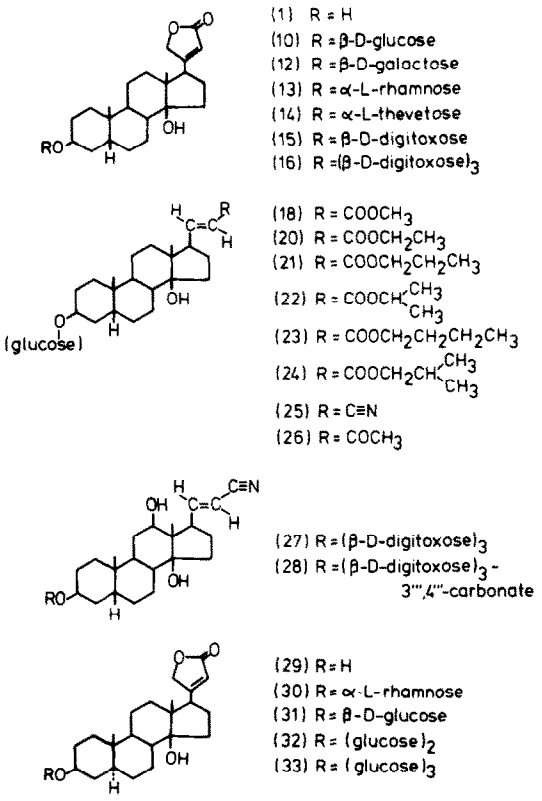

Fig. 1. Structural formulae of compounds used in this study. Compounds are numbered as in Tables 1–3.

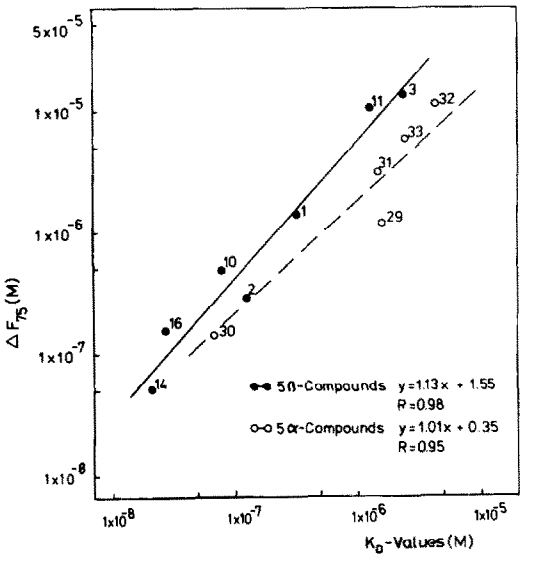

Fig. 2. K_D values and inotropic potencies on guinea pig heart. The K_D values determined on guinea pig heart ($Na^+ + K^+$)-ATPase are taken from Table 1. Inotropic potency on the isolated guinea pig left atria has been defined as the ΔF_{75} value [9, 10]. The ΔF_{75} value is the concentration, interpolated from at least ten cumulative dose–response curves, which increases the force of contraction by 75%. The compounds are numbered as in Table 1.

binding to the same site. The K_D values for seven compounds obtained from [^{3}H]digitoxin displacement studies with beef cardiac ($Na^+ + K^+$)-ATPase are given in Table 3. A comparison of these values with the values from [^{3}H]ouabain displacement studies (Table 2) shows that the [^{3}H]digitoxin displacement K_D values were consistently higher. However, potency ratios relative to digitoxigenin (Tables 2 and 3) were similar for both methods ($r = 0.99$), indi-

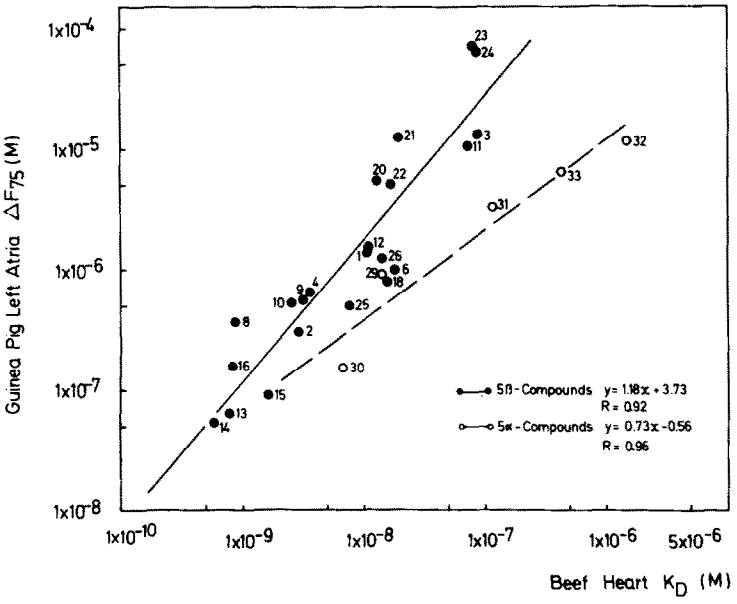

Fig. 3. K_D values with beef heart ($Na^+ + K^+$)-ATPase and inotropic potencies with isolated guinea pig left atria. The K_D values were calculated from [^{3}H]ouabain binding inhibition studies and are given in Table 2. Inotropic potency on the isolated guinea pig left atria has been defined as the ΔF_{75} value [9, 10]. The ΔF_{75} value is the concentration, interpolated from at least ten cumulative dose–response curves, which increases the force of contraction by 75%. The compounds are numbered as in Table 2.

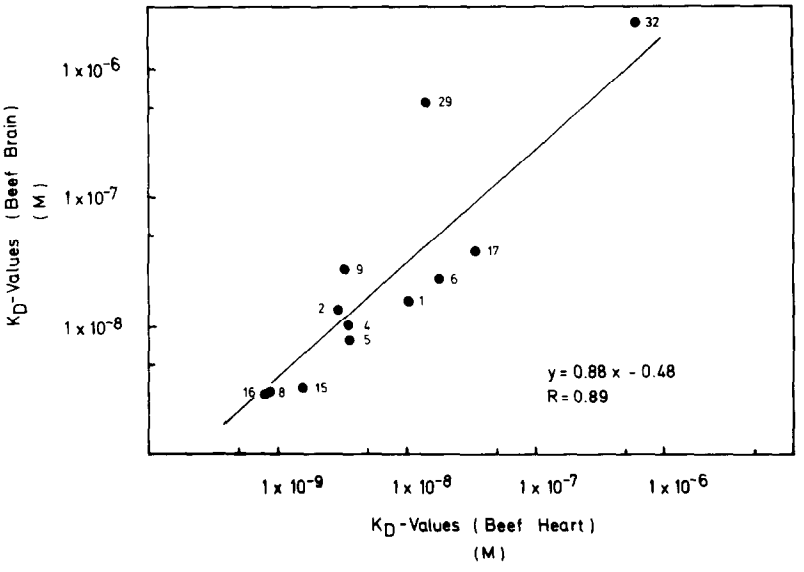

Fig. 4. K_D values with beef heart and beef brain ($Na^+ + K^+$)-ATPase. The K_D values were calculated from [^{3}H]ouabain binding inhibition studies and are given in Table 2 for beef heart ($Na^+ + K^+$)-ATPase. The K_D values with beef brain ($Na^+ + K^+$)-ATPase are taken from Erdmann and Schoner [6]. The compounds are numbered as in Table 2.

cating that SAR conclusions could be derived from either [^{3}H]ouabain or [^{3}H]digitoxin displacement studies.

DISCUSSION

Although the evidence is controversial, many workers have suggested that the digitalis binding site on ($Na^+ + K^+$)-ATPase mediates the pharmacological responses (see [1–3] for reviews). If this enzyme is the pharmacological receptor for inotropy, then potencies of a broad range of cardiotonic steroids determined on the cardiac enzyme should parallel positive inotropic potencies for these compounds determined on an intact heart muscle preparation of the same species. These potency values can be used for SAR studies to determine which functional groups interact with the binding site on the contracting heart muscle or the isolated ($Na^+ + K^+$)-ATPase. Further, if there are species differences in pharmacological effects, these differences should be observed in studies with the respective cardiac ($Na^+ + K^+$)-ATPase preparations.

Direct binding studies of cardiotonic steroids with ($Na^+ + K^+$)-ATPase, as have been performed with [^{3}H]digoxin [4] and [^{3}H]ouabain [20,21], are limited because only a few suitable labelled compounds are available. Indirect binding studies, such as those of Erdmann and Schoner [6] and Bossaller and Schmoldt [23], can be performed using the inhibition of binding of a labelled compound by an unlabelled analogue to characterize indirectly the binding of the unlabelled analogue. The procedures as reviewed by Weiland and Molinoff [5] have been followed to calculate the K_D values from both direct and indirect binding studies.

In the present study, we have performed direct binding studies using[^{3}H]ouabain or [^{3}H]digitoxin

Table 3. Equilibrium dissociation constants (K_D values) for the binding of cardiotonic steroids to beef heart ($Na^+ + K^+$)-ATPase at 37°, determined by [^{3}H]digitoxin displacement

Compound	K_D ($\times 10^{-9}$M) (± S.E.M.)	Relative potency*
Digitoxigenin (**1**)	16.6 ± 1.0 ($n = 3$)	1.0
Ouabain (**2**)	3.3	5.0
Dihydroouabain (**3**)	183	0.09
Digitoxigenin glucoside (**10**)	3.3	4.9
3α-Methyldigitoxigenin glucoside (**11**)	116	0.14
Digitoxigenin thevetoside (neriifolin) (**14**)	0.91	18.2
Digitoxin (**16**)	2.5	6.7

* Potency relative to digitoxigenin which has been given a value of 1.0.

with beef or guinea pig cardiac ($Na^+ + K^+$)-ATPase. Indirect binding studies (inhibition of binding of either [^{3}H]ouabain or [^{3}H]digitoxin) were then performed with a series of unlabelled cardiotonic steroids which have a broad range of potencies. Comparisons were made between these results and those previously obtained with contracting guinea pig left atria [9,10] or with beef brain ($Na^+ + K^+$)-ATPase [6].

The potency values have been calculated as the equilibrium dissociation constants (K_D values) by the mathematical procedures given by Erdmann and Schoner [6]. The K_D values are given in Tables 1–3. In Table 1, the K_D values for twelve compounds on guinea pig cardiac ($Na^+ + K^+$)-ATPase have been given, together with previously reported positive inotropic potencies on guinea pig left atria [9,10]. As seen in Fig. 2, there is a good correlation for these twelve compounds between the K_D values on the isolated cardiac enzyme and positive inotropic potencies on isolated heart muscle ($r = 0.98$ for seven 5β-compounds, $r = 0.95$ for five 5α-compounds). The [^{3}H]ouabain displacement K_D values for 33 analogues tested on beef cardiac ($Na^+ + K^+$)-ATPase are given in Table 2. There is a significant correlation ($r = 0.92$ for 22 5β-compounds, $r = 0.96$ for five 5α-compounds) between these values and the positive inotropic potencies on guinea pig left atria. The different slopes for 5α- and 5β-compounds (Figs. 2 and 3) suggest that the 5α-derivatives interact differently with the receptors in both species.

Simultaneous measurement of binding to ($Na^+ + K^+$)-ATPase and positive inotropy is necessary to determine if this enzyme contains the pharmacological receptor. However, the results given above show that ($Na^+ + K^+$)-ATPase is probably the receptor, because of the same order in relative potencies on guinea pig left atria and beef cardiac ($Na^+ + K^+$)-ATPase, in spite of the large differences in absolute potencies. Similarities in relative potency were also noted by Repke *et al.* [12] and Flasch and Heinz [19], using different derivatives of digitalis. The differences in absolute potencies between various species may be due either to differences in local chemical interactions between the drug and its binding sites or to differences in the allosteric effects consequent to binding. However, the good correlation between relative potencies in Table 2 and Fig. 3 indicates that the glycoside recognizing part of the receptor must be similar in guinea pig and beef heart, interacting with the same functional groups. The differences in absolute potency are possibly due to a different protein structure of the receptor macromolecule.

Comparisons of the [^{3}H]digitoxin displacement K_D values (Table 3) with the [^{3}H]ouabain values in Table 2 show that the relative potencies are similar for both systems ($r = 0.99$). This indicates that, provided the labelled and unlabelled analogues bind to the same receptor, displacement of either labelled cardiotonic steroid can be used for indirect binding studies. The indirect [^{3}H]ouabain displacement K_D values for twelve of the compounds in Table 1 have been previously given [6] for beef brain ($Na^+ + K^+$)-ATPase. Brain and cardiac ($Na^+ + K^+$)-ATPase may be significantly different, as has been demonstrated in the relatively digitalis-insensitive rat [21]. Sweadner [24] showed that brain contains two types of ($Na^+ + K^+$)-ATPase. For most compounds studied on both beef brain and cardiac ($Na^+ + K^+$)-ATPase, relative potencies are similar (Fig. 4). However, uzarigenin (**29**) is relatively more potent (compared to digitoxigenin) on beef cardiac ($Na^+ + K^+$)-ATPase. These changes in relative potency may indicate either structural differences in these two enzymes or differences in the interaction of uzarigenin and digitoxigenin with the enzymes. Uzarigenin, unlike all other aglycones tested on guinea pig left atria, showed a slow rate of inotropy onset (unpublished results). However, this slow onset cannot be the reason for the observed differences between beef cardiac and brain ($Na^+ + K^+$)-ATPase as these studies were performed under equilibrium conditions. Alternatively, the results with uzarigenin may indicate that the receptors for digitalis in the two different organs are very similar, but not identical.

Classical digitalis SAR studies (for reviews see [25, 26] relied on data from intact preparations, especially the cat toxicity test [11]. Partially purified ($Na^+ + K^+$)-ATPase has been used by many workers (for examples, see [6, 19, 23, 27–29]) to test a range of cardiotonic steroids. The SAR conclusions have been discussed in detail [6, 25, 26]. In the present study, these SAR conclusions are compared with the results of testing of 33 cardiotonic steroids which have more than 1000-fold potency differences. SAR conclusions can be divided into three sections: (a) changes to the unsaturated lactone ring; (b) changes to the steroid portion; and (c) changes to the sugar portion of the molecule.

(a) Changes to the lactone portion

The present results on beef cardiac ($Na^+ + K^+$)-ATPase are compatible with the SAR model derived from guinea pig left atria studies for the modified C17β-side chain [10, 28]. In this model, the lactone or its isosteres bind through a two point attachment with the electron-deficient C20 atom and the electron-rich heteroatom attached to C22, with both binding sites lying in a cleft of the enzyme. These modified C17-derivatives are useful for defining the structural requirements for binding of the lactone ring portion of the molecule to its receptor site.

(b) Changes to the steroid portion

The results for the A/B-*trans* (5α-) steroids tested in this study (compounds **29–33**) can be used for comparison with the literature studies indicating that 5α-steroids, except the 2,3-di-linked glycoside asclepin [32], have a markedly reduced biological potency [25]. The K_D value of uzarigenin rhamnoside (**30**), a 5α-steroid, shows that these derivatives can have higher biological potency. Uzarigenin (**29**), the 5α-isomer of digitoxigenin, has only a slightly lower K_D value on beef cardiac ($Na^+ + K^+$)-ATPase than digitoxigenin, showing that the change from a 5β-steroid to a 5α-steroid need not greatly affect potency.

Hydroxyl groups on the steroid portion reduce the potency [digitoxin compared with its 12β-hydroxyl derivative, digoxin (**4**) and its 16β-hydroxyl derivative, gitoxin (**6**)]. Formyl substitution of the 16β-

hydroxyl group, as in gitaloxin (**7**), increased potency, as shown by de Pover and Godfraind [30], but gitaloxin was still less potent than digitoxin in our study. Introduction of C3α-methyl group reduced potency by a factor of about 30 (compounds **10** and **11**); this result has been previously noted [31].

Several groups (reviewed in [26]) have proposed that the steroid portion is bound by hydrophobic interactions. The decrease in potency noted in the present study after substitutions on the steroid rings agrees with this proposal. Further, our results suggest that the configuration at the A/B-ring junction does not play the significant role previously assigned.

(c) Changes to the sugar portion

The effects of different sugars on the potency on beef cardiac ($Na^+ + K^+$)-ATPase are similar to the effects previously noted on contracting guinea pig left atria [9] with α-*L*-thevetose being the most effective enhancer of potency. All the most active compounds, of both 5α- and 5β-steroids, are glycosides of 5-deoxy-sugars (β-D-digitoxose, α-L-rhamnose and α-L-thevetose). This indicates either that the 5′-CH_3 group must play an important role in the interaction of the sugar portion with the receptor, or that the 5′-CH_2OH group inhibits the binding of the sugar portion. In the 5α-series, uzarigenin rhamnoside (**30**) is more than twice as potent on beef cardiac ($Na^+ + K^+$)-ATPase as uzarigenin (**29**), but uzarigenin glucoside (**31**) is about eight times less potent. Uzarigenin diglucoside (**32**) is about 13 times less potent than the monoglucoside, while uzarigenin triglucoside (**33**) is intermediate in potency between the mono- and diglucoside.

Several compounds with substitutions on the third sugar unit were tested. β-Methyl-digoxin (**5**) was equipotent with digoxin. The 3‴,4‴-carbonate derivative (**28**) was slightly more potent than its parent compound (**27**). The work of Yoda [23] on the sugar portion of digitalis analogues suggested that the C3′-hydroxyl group binds to the receptor. Our results suggest that the sugar interaction is more complicated, with the C5′-substituent playing an important role. Indirect binding studies with labelled cardiotonic steroids are shown to be useful for characterization of unlabelled cardiotonic steroids with cardiac ($Na^+ + K^+$)-ATPase. The indirect K_D values so obtained can be used for SAR studies.

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