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A methodical pitfall: saturable binding of digitoxin to glass-fibre filters

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In vitro radioligand binding studies have become a standard procedure in experimental pharmacology for the investigation of drug–receptor interactions. Concerning the interpretation of experimental results revealing saturable binding of a radioligand to biological membranes, reservation is recommended, since saturable binding does not necessarily mean binding to pharmacologically relevant receptors [1]. Furthermore, it has been shown that radioligands even may bind to non-biologic materials, e.g. Cuatrecasas and Hollenberg [2] demonstrated a high affinity binding of [^{125}J]insulin to talc powder and Synder *et al.* [3] communicated on a stereoselective binding of opiates to glass-fibre filters.

In investigations on the characteristics of the interaction of cardiac glycosides with their receptor, i.e. the sarcolemmal Na^+/K^+-ATPase (ATP phosphohydrolase, E.C. 3.6.1.3), binding studies using radioactively labelled cardiac glycosides are a tool often applied [4]. Also in our laboratory the measurement of [^{3}H]ouabain-binding to cardiac membranes is a well established method [5, 6]. However, when employing our routine procedure to the measurement of [^{3}H]digitoxin-binding, we were initially troubled by the finding that the characteristics of "specific" digitoxin-binding differed essentially from that of ouabain-binding. Further analysis revealed the reason, namely a saturable, medium-affinity binding of digitoxin to glass-fibre filters.

We consider it worthwhile to communicate this finding in order to demonstrate that the above-mentioned precautions are also valid in experiments on the specific binding of cardiac glycosides, since otherwise misleading interpretations of experimental data and of Scatchard analyses may result.

Materials and Methods

[^{3}H]Digitoxin (13.8 Ci/mmol) and [^{3}H]ouabain (32 Ci/mmol) were obtained from NEN (Dreieich, F.R.G.) and Amersham Buchler (Braunschweig, F.R.G.), respectively. Digitoxin (puriss.) and Digoxin (purum) were purchased from Fluka (Neu-Ulm, F.R.G.). Ouabain and the other chemicals were obtained from E. Merck (Darmstadt, F.R.G.) unless otherwise indicated.

The procedure of the binding experiments has been described earlier in detail [5, 6]. A crude suspension of cardiac membranes from guinea pig cardiac ventricles was prepared at a temperature of 4° as follows. The ventricles were homogenized in a 0.32 M sucrose solution (20 ml/g wet weight); the homogenate was centrifuged at 2000 *g* for 10 min; the supernatant was centrifuged at 30,000 *g* for 18 min; the pellet was resuspended in 50 mM Tris–HCl buffer pH 7.4 (4 ml/g w.w.), frozen in liquid nitrogen and stored at −20°.

The binding assays were performed in triplicate in glass vials (filtration experiments) or in thick-wall polyallomer centrifugation tubes (Beckman, Hannover, F.R.G.). In a

total volume of 1.5 ml 250 µl of the membrane suspension were incubated together with the tritium-labelled cardiac glycoside in a medium composed of 80 mM NaCl, 16 mM $MgCl_2$, 50 mM Tris–HCl and 2.5 mM ATP-Na_2; final pH 7.3. [^{3}H]digitoxin was present in concentrations of 1–2 nM, [^{3}H]ouabain at 3 nM. Unlabelled digitoxin and digoxin were dissolved in a mixture of dimethyl sulfoxide/propylene glycol (1 + 9) and added in a volume of 50 µl, this procedure allowing assay concentrations of 10^{-4} M. Ouabain was added dissolved in buffer; for the sake of comparability the assays were mixed up with 50 µl of the DMSO/PG mixture. Incubation was performed in a water-bath at 37°; after 90 min, when a stable equilibrium binding had been attained, the incubation was terminated. In the filtration experiments membranes were separated by vacuum-filtration of 1 ml aliquots of the incubation medium through pre-wetted glass-fibre filters (Glasfaser-Rundfilter Nr. 6, 25 mm ∅, Schleicher + Schüll, Dassel, F.R.G.) which were then rinsed with two 5 ml aliquots of ice-cold distilled water, the whole procedure being performed in less than 15 sec. The filters were placed in scintillation vials (Packard, Frankfurt, F.R.G.); 2 ml of Soluene® 350 (Packard) were added to dissolve organic material. After addition of 10 ml Dimilume® 30 (Packard) the radioactivity was counted by a Tricarb 460 CD (Packard) at a counting efficiency of about 35%.

In the centrifugation experiments the incubation vials were cooled in crushed ice in order to terminate incubation. Membranes were separated by centrifugation (Kontron ultracentrifuge TGA-65) at 4° for 18 min at 52,000 *g*. After decanting the supernatant and rinsing the pellet twice quickly and carefully with 1 ml of ice-cold buffer, the pellet was resuspended in 1.5 ml of buffer by sonication. An 1 ml aliquot was given together with 10 ml Dimilume® 30 into a scintillation vial and the radioactivity was counted at an efficiency of 35%.

Since total binding of [^{3}H]digitoxin and of [^{3}H]ouabain amounted maximally to about 11% at the lowest concentrations, the free ligand concentrations were assumed to be equal to the concentrations initially added to the incubation medium.

Results and Discussion

To characterize the specific binding of digitoxin to Na^+/K^+-ATPase the amount of [^{3}H]digitoxin being unspecifically bound had to be determined. In general, the unspecific, concentration proportional binding of a radioligand can be measured as the binding not "displaceable" by an excess concentration of unlabelled ligand [1, 7], independent of whether the unlabelled ligand is of the same species as the labelled ligand or not. In [^{3}H]digitoxin binding experiments, however, the amount of filter-bound radioactivity was different whether the assay was performed in the presence of 10^{-4}M digitoxin or 10^{-4} M ouabain (Table 1): in the presence of 10^{-4} M digitoxin unspecific binding amounted to 10% of the total, in the presence of 10^{-4} M ouabain it attained a value of about 30%. These results indicated that there was a saturable [^{3}H]digitoxin-binding which ouabain molecules did not interfere with (in the following called "ouabain-insensitive saturable digitoxin-binding"). In order to characterize the affinity of the ouabain-insensitive saturable [^{3}H]digitoxin-binding, cardiac membranes were incubated with [^{3}H]digitoxin and with increasing concentrations of unlabelled digitoxin in the presence of 10^{-4} M ouabain (Fig. 1(A)). The steep part of the inhibition curve occurred at digitoxin concentrations between 10^{-6} and 10^{-5} M. When the binding of [^{3}H]digitoxin in the presence of 10^{-4} M digitoxin was considered as a concentration proportional, unspecific binding, the half-maximally inhibiting concentration as a measure of the affinity of the ouabain-insensitive saturable digitoxin-binding amounted to $IC_{50} \sim 3 \times 10^{-6}$ M.

When the membranes were, however, separated by centrifugation, [^{3}H]digitoxin-binding was identical whether determined in the presence of 10^{-4} M digitoxin or 10^{-4} M ouabain (Table 1). Since ouabain-insensitive saturable digitoxin-binding was only detected in the filtration experiments, it was checked whether digitoxin was bound to the glass-fibre filters. [^{3}H]digitoxin and increasing concentrations of digitoxin were incubated in assays from which the membranes had been omitted; aliquots of 1 ml were then processed as usual. The amount of [^{3}H]digitoxin retained

Table 1. Fraction of [^{3}H]digitoxin-binding "undisplaceable" by excess concentrations of unlabelled digitoxin resp. ouabain

Filtration		Centrifugation	
Digitoxin 10^{-4} M	Ouabain 10^{-4} M	Digitoxin 10^{-4} M	Ouabain 10^{-4} M
11 ± 1%	28 ± 2%	25 ± 3%	26 ± 2%

Assay composition: [^{3}H]digitoxin 1–2 nM, unlabelled cardiac glycoside as indicated, concentration of receptor sites 3 nM, NaCl 80 mM, $MgCl_2$ 16 mM, Tris–HCl 50 mM, pH 7.3, 37°. After 90 min of incubation membranes were separated either by filtration or by centrifugation. Total [^{3}H]digitoxin-binding in the absence of an unlabelled ligand was set 100%. Presented are mean values ± S.E.M. of 2–6 experiments each performed as a triplicate determination.

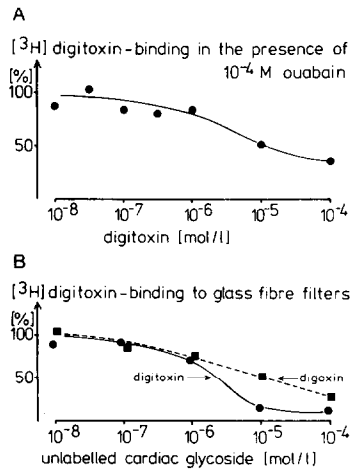

Fig. 1. (A) Inhibition of ouabain-insensitive [^{3}H]digitoxin-binding by increasing concentrations of unlabelled digitoxin measured in a filtration experiment. [^{3}H]digitoxin-binding in the presence of 10^{-4} M ouabain, which represented about 30% of the total [^{3}H]digitoxin-binding (the latter representing binding in the absence of ouabain, cf. Table 1), was set 100%, Abscissa: concentration of unlabelled digitoxin. Indicated are mean values obtained in two experiments performed as triplicate determinations. (B) Inhibition of [^{3}H]digitoxin-binding to glass-fibre filters by increasing concentrations of unlabelled digitoxin or digoxin. One ml aliquots of an incubation medium from which the membranes had been omitted were filtered under reduced pressure through glass-fibre filters, which were then rinsed with 2 × 5 ml of ice-cold distilled water. The amount of [^{3}H]digitoxin retained by the filters in the absence of an unlabelled ligand was set 100% (ordinate); this value represented 5% of the total amount of [^{3}H]digitoxin subjected to filtration. Abscissa: concentration of unlabelled digitoxin resp. digoxin present in the incubation medium. Indicated are the mean values of experiments performed in triplicate.

by the filters was found to be reduced in the presence of unlabelled digitoxin (Fig. 1(B)). Consequently, saturable digitoxin-binding has to be assumed occurring at sites existing in a limited number within the glass-fibre material. The small fraction of [^{3}H]digitoxin-binding remaining "undisplaced" in the presence of 10^{-4} M digitoxin could perhaps indicate the presence of additional concentration proportional digitoxin-binding. The concentration of unlabelled digitoxin reducing [^{3}H]digitoxin binding by 50% as an estimate of the affinity of the saturable digitoxin-binding to the filter material amounted to $IC_{50} \sim 3 \times 10^{-6}$ M. This value was similar to the IC_{50} of the ouabain-insensitive saturable digitoxin-binding, suggesting that the latter binding was caused by the saturable binding of digitoxin to the glass-fibre filters. Also digoxin molecules competed with [^{3}H]digitoxin for the binding sites on the glass-fibre (Fig. 1(B)), the IC_{50} was about 10^{-5} M, thus indicating a lower affinity of digoxin compared with digitoxin. Ouabain in concentrations up to 10^{-4} M did not reduce the [^{3}H]digitoxin-binding demonstrating ouabain being devoid of any affinity to these sites.

To characterize the saturable digitoxin-binding to cardiac membranes, the binding of [^{3}H]digitoxin was measured in the presence of increasing concentrations of unlabelled digitoxin in a filtration experiment. The amount of digitoxin saturably bound was calculated from the difference between the total [^{3}H]digitoxin-binding and (a) the [^{3}H]digitoxin-binding in the presence of 10^{-4} M digitoxin = total saturable digitoxin-binding, (b) the [^{3}H]digitoxin-binding in the presence of 10^{-4} M ouabain = ouabain-sensitive digitoxin-binding. The binding data were analyzed by means of a Scatchard plot (Fig. 2). The points concerning total saturable digitoxin-binding ran curvilinear formally indicating either negative cooperativity or distinct populations of independent binding sites with different affinities and capacities. The points for the ouabain-sensitive digitoxin-binding could be connected by a straight line indicating binding to one population of independent sites with a dissociation constant K_D of 4×10^{-8} M and a number of binding sites of $B_{max} = 3$ pmol/ml. The difference between the data for the total saturable digitoxin-binding and the data for the ouabain-sensitive digitoxin-binding, i.e. the ouabain-insensitive saturable digitoxin-binding, is also shown in Fig. 2. When the first points were connected by a straight line, a K_D of about 1×10^{-6} M could be calculated, which was in the same order of magnitude as the IC_{50} value for this type of binding derived from the experiments shown in Fig. 1(A) and the IC_{50} value for the affinity of saturable digitoxin-binding to filter-material derived from the results shown in Fig. 1(B). The last point with an abscissa near 42.5 pmol/ml deviated from the straight line, possibly indicating the presence of additional ouabain-insensitive saturable digitoxin-binding with lower affinity. However, this value was less reliable than the other data, since its calculation was based on only a small difference between total and unspecific [^{3}H]digitoxin-binding.

When [^{3}H]ouabain was used as the radioactive ligand, binding in the presence of 10^{-4} M ouabain or 10^{-4} M digitoxin was similarly about 3% of the total. The data obtained for the saturable ouabain-binding in a filtration-experiment are depicted in Fig. 2; binding was characterized by a K_D of 1.7×10^{-7} M and a B_{max} of 3 pmol/ml. Thus the number of ouabain-binding sites agreed with the number of ouabain-sensitive digitoxin binding sites. Since binding of ouabain under the applied conditions occurred probably at Na^+/K^+-ATPase [8], it can be concluded that the ouabain-sensitive binding of digitoxin represents the binding of digitoxin to its proper receptor sites. Hence, the additional saturable digitoxin binding was an unspecific saturable binding, namely the binding to the glass-fibre filters.

In summary, this report demonstrates that

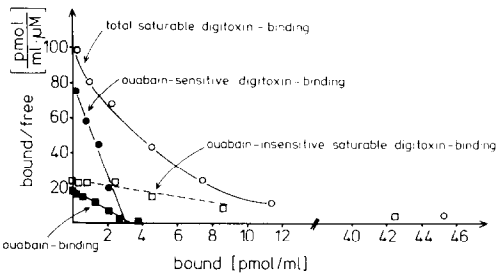

Fig. 2. Concentration-dependent saturable binding of digitoxin and ouabain presented in form of a Scatchard plot. (a) The data concerning total saturable digitoxin binding were calculated considering as the unspecific binding of [^{3}H]digitoxin its binding in the presence of 10^{-4} M digitoxin. (b) Ouabain-sensitive digitoxin-binding was calculated assuming [^{3}H]digitoxin-binding in the presence of 10^{-4} M ouabain to represent unspecific binding. (c) Ouabain-insensitive saturable digitoxin-binding was the difference between the binding data of (a) and (b). (d) Specific ouabain-binding was measured in an assay containing [^{3}H]ouabain (3 nM) was unlabelled ouabain in concentrations up to 10^{-4} M, this concentration indicating unspecific [^{3}H]ouabain-binding. The points represent mean values of experiments performed in triplicate.

[^{3}H]digitoxin-binding experiments can be complicated by a saturable medium-affinity binding of digitoxin to glass-fibre filters. The entirely different Scatchard curves for total saturable digitoxin-binding and specific, ouabain-sensitive digitoxin-binding resulted from the fact that different amounts of unspecific [^{3}H]digitoxin-binding were subtracted from the data for total [^{3}H]digitoxin-binding. This finding underlines that the correct analysis of binding data critically depends on the exact determination of true unspecific binding.

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