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INTERACTIONS OF CARDIAC GLYCOSIDES WITH CELLS AND MEMBRANES

PROPERTIES AND STRUCTURAL ASPECTS OF TWO RECEPTOR SITES FOR OUABAIN IN ERYTHROCYTES

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Summary

The existence of two types of binding sites for ouabain in human erythrocyte membranes is described. Receptor sites designated as 'type I', which may be identical to the K^+-insensitive sites of intact cells, were detected at concentrations of ouabain as low as 10^{-9} M, but could not be saturated even at 10^{-3} M. This type prevailed at concentrations of glycoside exceeding $2 \cdot 10^{-7}$--$5. 10^{-7}$ M. The 'type II' receptor sites require the inclusion of $Mg^{2+} + P_i$ to form complexes with ouabain; they may be identical to the K^+-sensitive sites of intact cells. These sites were saturated at approx. $5 \cdot 10^{-7}$ M ouabain but could not be detected at higher concentrations. The range of ouabain concentrations at which 'type I' receptors start to predominate (i.e. $5 \cdot 10^{-8}$—$5 \cdot 10^{-7}$ M) was termed 'critical digitalis concentrations'. The process of binding reached equilibrium within 1 and 4 h for 'type I' and 'type II' sites, respectively. The dissociation constant for 'type II' receptor-ouabain complexes was $7.6 \cdot 10^{-9}$ M.

Under similar experimental conditions, rat erythrocyte membranes exhibited only non-saturable sites.

Alterations in the proportions of the two types of receptors were demonstrated by preincubation of the membranes, in the presence or absence of $Mg^{2+} + P_i$, prior to the addition of ouabain. In the first case, 'type II receptor-ouabain' complexes were stabilized at about 50% of the untreated membranes and 'type I-ouabain' complexes slowly approached equilibrium over a period of 24 h. In the latter instance, 'type I' receptors were not detected, and only 'type II-ouabain' complexes prevailed.

Abbreviation: EGTA, ethyleneglycol-bis(β-aminoethylether)-*N*,*N*'-tetraacetic acid.

Differences between two types of receptors for ouabain in membranes of human erythrocytes are shown. Both types were affected by prolonged storage at −20°C or by extracting the membranes with deoxycholate or ether. Addition of liposomes to ether-extracted membranes reactivated 'type I' sites completely and 'type II' sites partially at a phospholipid/protein weight ratio of 0.14 and 0.5, respectively. Monovalent cations (i.e. K^+, Na^+, Cs^+) reduced the binding of ouabain to 'type II' receptor sites only. 'Type II-ouabain' complex could be partially purified by gel filtration and behaved as a very high molecular weight compound.

The data are consistent with two separate, independent receptor types for ouabain in human erythrocyte membranes.

Introduction

Cardiac glycosides have been shown to inhibit active transport of Na^+ and K^+ across the membrane of red cells and other cells [1]. These inhibitors bind to and act on the (Na^+ + K^+)-ATPase in fragmented plasma membrane vesicles as well as on highly purified preparations of the enzyme [2—4]. Hence, certain macromolecules in the plasma membrane of erythrocytes with which cardiac glycosides combine have been termed digitalis receptors, and certain criteria are therefore expected to be satisfied in order to apply in the case of receptor-digitalis interactions [5,6].

Studies with highly purified preparations of (Na^+ + K^+)-ATPase from kidney have shown that the enzyme may acquire different conformations, leading to the binding of cardiac glycosides to certain forms of the enzyme. Ligands such as Mg^{2+}, Na^+, K^+, ATP or P_i are critical in regulating the appropriate form of enzyme and thus controlling the interactions of the enzyme with cardiac glycosides [7]. It was suggested that more than one enzyme-ouabain complex might exist. Eventually a conversion of unstable to stable forms by monovalent cations is possible [8]. Schoner et al. [9] have demonstrated the existence of two sites having different K_D values by plotting the data of ouabain binding to bovine brain (Na^+ + K^+)-ATPase treated with an analogue of ATP applying a Scatchard plot [10]. Experimental evidence points to (Na^+ + K^+)-ATPase as a receptor for cardiac glycosides.

The most purified preparations of this enzyme also behave as fragments of membranes. They contain roughly 50% lipid, tend to form insoluble aggregates and appeared under the electron microscope as membrane structures [4,6]. Whether the lipid component is needed to maintain a strict spatial configuration or participates in the catalytic process remains unsolved as yet. The same uncertainty holds for its role in the inhibitory action of cardiac glycosides. Furthermore, the (Na^+ + K^+)-ATPase has been shown on several occasions to be a latent enzyme, the activity of which was manifested only after special treatment such as the use of detergents [4,11,12].

The lipoprotein nature of the membrane and of the receptor site(s) for digitalis were the premises for applying the experimental approach in this study. It should be emphasized that this approach started with an intact membrane, or a very close derivative, to which ouabain was being bound. This allowed a search

for additional receptor sites, which might be related to the function of the sodium pump, and could be lost during purification of ($Na^+ + K^+$)-ATPase. The problem of whether the membrane ATPase is the only digitalis receptor remains to be resolved, however [6]. The possibility of more than one receptor site for cardiac glycosides in the intact plasma membrane was raised, indirectly, by Godfraind [13] as a general approach to treat the data on digitalis uptake by tissues. Multiple receptor sites for cardiac glycosides might represent different forms of the same entity, or else could mean different entities. Alternatively, different compartments may participate in the events regulating the action of cardiac glycosides. These compartments may not comply with the criteria for specific binding and therefore may not be directly related to ($Na^+ + K^+$)-ATPase. They may, however, be expressed as multiple binding sites not representing conformations of the same receptor.

In the present communication, we have attempted to establish, in part, the existence of more than one type of receptors for ouabain in the plasma membrane of erythrocytes, and to describe some of their basic properties. Although interconversions could exist between the different sites or forms, they may represent different compartments within the membrane. The differences between these two types of receptors were further characterized through the use of detergents, solvents or their stabilities. Preliminary reports have been previously published [14,15].

Materials

Fresh blood was obtained from healthy human donors by venesection or from adult rats into heparinized tubes; alternatively, human outdated blood in acidified citrated dextrose was kindly supplied by the Hadassah Hospital Blood Bank, Jerusalem. Ouabain (octahydrate), *p*-nitrophenylphosphate, ATP, Tris and Dextran T-110 were purchased from Sigma, Israel (Ramat-Gan). Insta gel, Dimilume and Soluene-100 or Soluene-350 were from Packard (Israel). [^{3}H]-Ouabain (specific radioactivity 12—13 Ci/mmol) or deoxy[^{14}C]cholate were purchased from either New England Nuclear (Boston, Mass.) or the Radiochemical Centre (Amersham, England) and were stored at −20°C in ethanolic solutions. Their stability was ascertained by thin-layer chromatography in which 0.5 μCi was developed with chloroform/methanol/water (60 : 30 : 5, by vol.). The radioactive scan showed less than 2.5% contamination or degradation products during a period of 5 years storage.

Methods

Erythrocytes were separated by centrifugation, washed several times and suspended in an isotonic medium. Alternatively, they were hemolyzed in a hypotonic medium. Permeable, porous ghosts or frozen and thawed membrane vesicles were prepared and used immediately or stored at −20°C [16]. The activities of ($Na^+ + K^+$)-ATPase and K^+-activated *p*-nitrophenylphosphatase and the amounts of protein were determined by methods described elsewhere [16]. Cholesterol was assayed according to Zlatkis et al. [17].

Binding of [^{3}H]ouabain

(A) Intact cells. Freshly prepared cells incubated either in isotonic choline medium, pH 7.4, or else part of the choline was replaced by K^+ [18], with 10^{-9}—10^{-5} M ouabain and with a constant amount of radioactivity (i.e. [^{3}H]-ouabain, 0.5 μCi) per experiment. The reaction was terminated by cooling the tubes and the cell-bound radioactivity was examined either by extracting the labelled cells with hot ethanol (or methanol [19]) or by 10% $HClO_4$ [20]. Alternatively, cell-bound [^{3}H]ouabain was counted directly using H_2O_2 or in combination with isopropanol plus Soluene-350 or Dimilume as bleaching agents to reduce quenching due to hemoglobin (cf. booklet by Packard on Soluene-350). Using 10^6—10^9 human red cells and 10—20 μCi [^{3}H]ouabain in pilot experiments, the three procedures gave recoveries of bound radioactivity of 96%, less than 90% and approx. 100%, respectively. The radioactivity of [^{3}H]ouabain used in these experiments but without erythrocytes was taken as 100%.

(B) Membranes. The binding of [^{3}H]ouabain to membrane preparations was measured as follows: (a) Total binding: 0.5—2 mg membrane protein were incubated in 1 ml containing 10 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 2 mM Mg^{2+} and 1 mM phosphate ($Mg^{2+} + P_i$). Alternatively, the three last ligands were replaced by 40 mM Na^+, 1.25 mM Mg^{2+} and 2 mM ATP, 0.25 mM EGTA [21]. (b) 'Type I' receptors: Procedure (a) was followed except that $Mg^{2+} + P_i$, or the alternative ligands, were omitted. (c) 'Type II' receptors: This was obtained by subtraction of 'type I' values from 'total', i.e. a — b. (d) 'Background' binding: In certain instances it was felt that another parameter was needed to emphasize 'type I' binding sites. We therefore measured background values which were obtained by adding a very large excess of unlabelled ouabain (i.e. 10^{-3}—10^{-2} M) when either procedures (a) or (b) were followed. In a control in which the membranes were added last, under zero binding conditions, the values obtained were approx. 3.5% of the 'total' binding and 15% of 'type I' binding.

Each tube contained 10^{-9}—10^{-5} M unlabelled ouabain plus a fixed amount of radioactivity as [^{3}H]ouabain, ranging from 0.3 to 0.6 μCi per tube. Following incubation at 37°C for 60 min (unless otherwise stated) the tubes were placed in ice (all subsequent processing was done below 4°C) and centrifuged at $10^5 \times g$ for 10—30 min. Carrying out the procedure at low temperatures ensured minimum dissociation of the drug for reproducible results.

The pellets were rinsed four times with ouabain-free medium, suspended in hypotonic buffer, dissolved in a mixture of Insta-gel and Soluene-100 at a final ratio of 2 : 33 : 5 (v) then counted. Storing the complex in ice or rinsing with 1 mM unlabelled ouabain had no effect on the final binding values. Protein and K^+-activated phosphatase values were not changed by the cycle of rinsing.

If the rinsing was not repeated four times and instead, the walls of the tube were wiped carefully and completely with a cotton swab after removal of the supernatant by suction [22,23], the radioactivity was almost 10 times higher than that obtained following rinsing. In the latter case it represented as much as 5% of the initial radioactivity whereas following rinsing, the final value was stabilized at about 0.3% of the initial dpm. The amounts of bound ouabain were calculated from the specific radioactivity used in each experiment, and

expressed per mg of membrane protein. In cases where the membranes were pink due to incomplete removal of hemoglobin, the [^{3}H]ouabain binding was expressed by the amount of cholesterol equivalents of membrane protein, assuming 0.23 mg cholesterol = 1 mg membrane protein [24].

Ether extraction

(A) 'Wet'. A suspension of membranes in 0.8 ml buffer were mixed with 7 ml cold diethylether for 5 min, then centrifuged. The pellets were either prepared for binding or were counted, depending on whether the extraction was done before or after interaction with ouabain.

(B) 'Dry'. Membrane pellets either before or after a binding experiment, were gassed with N_2, 4 ml dry, cold ether was added. Samples were mixed for 5 min and then centrifuged. The extracts were analyzed for cholesterol and phospholipids. Total amounts of lipids in these membranes were obtained following exhaustive extraction with chloroform and methanol according to Folch et al. [25]. Relipidation was done with liposomes prepared from either human erythrocytes or rat liver microsomal lipids following ultrasonic irradiation, added to the ether-extracted pellets at a weight ratio of phospholipid/protein as indicated in the text. Incubation was carried out for 15 min at 37°C. The delipidated or relipidated pellets were processed either for binding or counting.

Extraction with deoxycholate

Following binding, the rinsed pellets were suspended in 10 mM Tris buffer containing deoxycholate at a final concentration which yielded a detergent/protein weight ratio of 0.8. These tubes were either shaken at 4°C for 60 min or mixed and left overnight at 4°C. Following centrifugation at $10^5 \times g$ for 30 min the supernatant was separated from the precipitate which was subsequently suspended in 10 mM Tris buffer pH 7.4. Control experiments without detergent showed that up to 20% of radioactivity could come off during shaking and centrifugation.

The supernatant was either filtered through PM-10 or UM-30 ultrafiltration membranes (Amicon) to about 1/10th of its volume or applied to a Sephadex G-25 (coarse) column (dimensions: radius, 0.5 cm; height, 5 cm; bed vol., 4 ml). The free, unbound [^{3}H]ouabain was separated from the bound glycoside quantitatively. Counting of radioactivity was done by using 7 volumes Insta-gel (Packard) to 1 volume of the aqueous medium.

Results

(A) Sites of ouabain binding

Human erythrocytes accumulate ouabain molecules and the uptake was attributed only to binding of the glycoside to the plasma membrane without any uptake across the membrane into the cell [20]. We have also observed that [^{3}H]ouabain-labelled erythrocytes, rinsed and then hemolyzed with a cold hypotonic buffer, yielded labelled ghosts which retained all the radioactivity of the intact cells. No radioactivity was associated with the membrane-free hemolyzate (i.e. hemoglobin). The number of ouabain molecules bound at equilib-

rium to intact human erythrocytes suspended in isotonic choline and containing 'therapeutic' concentrations of oubain (i.e. 10^{-9} M) was progressively reduced but not totally abolished when 1—20 mM K^+ were added (data not shown) in agreement with previously published data [18—20,22,26]. Even then, the number of glycoside molecules retained was always greater than the 'background radioactivity'. Furthermore, the effects of K^+ were only apparent at concentrations of ouabain not exceeding 10^{-6} M. These results suggested the existence of more than one type of binding site for ouabain in the intact cell. These sites may be empirically referred to as 'potassium-sensitive' and 'potassium-insensitive' sites.

(B) Distinction between receptor sites in membranes

Assuming the existence of at least two types of binding sites in human erythrocytes, it was desirable to study these interactions, using isolated membranes. The experimental conditions included incubation of the membranes without or with Mg^{2+} plus P_i. Consequently, the binding sites in the membranes were referred to as 'type I' and 'type II' receptors, respectively. Comparison between the two types is shown in Fig. 1. Results similar to those shown in Fig. 1 were also obtained using [^{3}H]ouabain with a constant specific radioactivity. These findings suggest that the 'K^+-sensitive' and 'K^+-insensitive' sites in intact cells could be identical with the 'type II' and 'type I' receptor sites of the isolated membranes, respectively.

'Type I' receptors could not be saturated even at 10^{-3} M ouabain although higher concentrations were not measured, whereas 'type II' sites became saturated at concentrations from $5 \cdot 10^{-7}$ M. At higher concentrations,

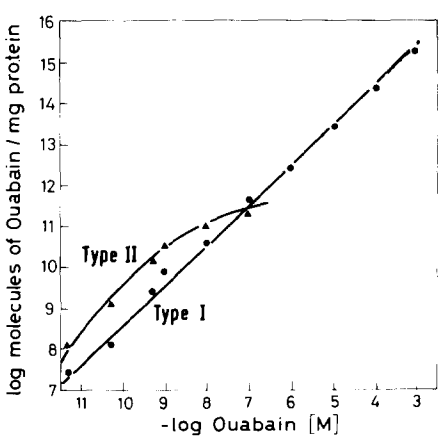


Fig. 1. Effect of initial ouabain concentrations on its binding to membranes of human erythrocytes. Increasing concentrations ($5 \cdot 10^{-12}$—$5 \cdot 10^{-3}$ M) of unlabelled ouabain and a fixed amount of [^{3}H]-ouabain (either 10^4 or $5 \cdot 10^5$ dpm) were incubated with frozen and thawed membranes (0.2—2 mg protein) to equilibrium binding. The final volume of 1 ml contained 10 mM Tris, pH 7.4, 1 mM P_i and 1.25 mM Mg^{2+} according to conditions. Termination of the reaction and processing for counting are given in the Methods. The range of ouabain concentrations at which the receptors of 'Type II' became indistinguishable from those of 'type I' (i.e. approx. 10^{-7} M) was referred to as critical digitalis concentration. Above this concentration the line obtained in the presence of Mg^{2+} + P_i coincided with the line obtained in the absence of these ligands. For definition of 'type I and II' receptors, see Methods.

the two curves became indistinguishable. Scatchard plots of the binding data (results not shown) indicated that K_D values cannot be obtained for 'type I' sites supporting the non-saturable behavior of these sites or alternatively, sites with very low affinity for ouabain. On the other hand, 'type II' sites exhibited a $K_{D(II)}$ value of $7.6 \cdot 10^{-9}$ M (ref. 10 and cf. Erdman and Schoner [27]). We therefore suggest the term 'critical digitalis concentration' as the range of ouabain concentrations which allows the distinction between the two types of receptor sites (e.g. about 10^{-7} M).

Below the critical digitalis concentration (e.g. $4.2 \cdot 10^{-8}$ M) equilibrium was reached within 60 min and 4 h for 'types I and II' sites, respectively. However, at the 60 min incubation period, more than 90% of the equilibrium binding values of 'type II' sites were obtained. Above the critical digitalis concentrations (e.g. $2.4 \cdot 10^{-7}$ M), only one site exists (i.e. 'type I') and equilibrium was reached within 60 min.

The binding of ouabain to 'type II' receptors at equilibrium is dependent on both the concentration of the receptors (expressed as mg protein) and on the initial concentration of the drug (Fig. 2). By contrast, 'type I' receptors bind, at equilibrium, smaller amounts of the drug; moreover, although the binding seemed to be independent of the protein concentration, it is more likely an artifact due to counting rates too close to the background values. In their studies, Erdman and Hasse [22] indicated that both specific and non-specific binding of ouabain to human erythrocyte membrane occurs [22]. Since they

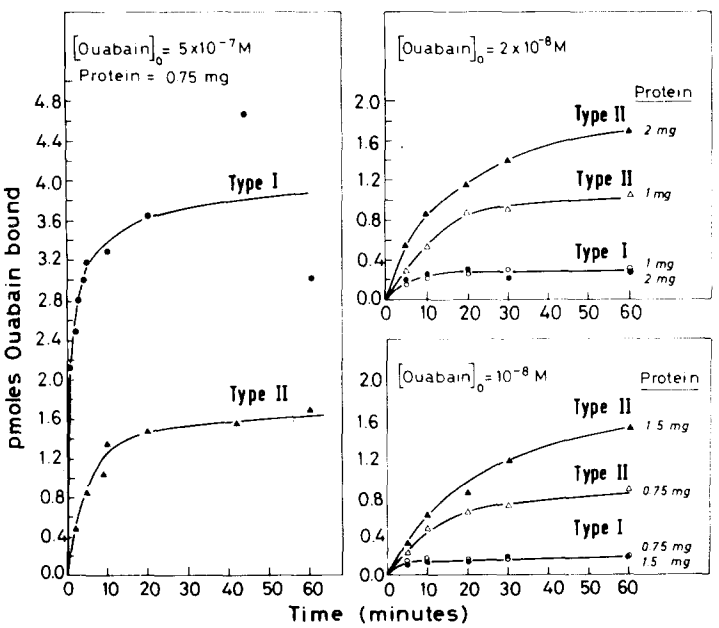


Fig. 2. Kinetics of binding. [^{3}H]ouabain at concentrations below the critical digitalis concentration (i.e. $1 \cdot 10^{-8}$—$2 \cdot 10^{-8}$ M, right panel) or above critical digitalis concentration (i.e. $5 \cdot 10^{-7}$ M, left panel) was incubated with 0.75—2 mg protein of human erythrocyte membranes. All other details are described in Methods and Fig. 1.

used concentrations of ouabain (i.e. $4.25 \cdot 10^{-9}$ M) far below the critical digitalis concentration, the values calculated from their data correspond to 0.044 and 0.247 pmol ouabain bound/mg protein to sites, termed in our study 'type I' and 'type II', respectively. Calculated from Fig. 2, at membrane protein concentrations ranging from 0.5 to 2 mg/ml, the amounts of ouabain bound to 'type I' receptor sites were 0.18, 0.25 and 4 pmol, corresponding to initial concentrations of $1 \cdot 10^{-8}$, $2 \cdot 10^{-8}$ and $5 \cdot 10^{-7}$ M ouabain, respectively. These data indicate that the contribution of 'type I' receptors to the total binding becomes much more significant above the critical digitalis concentration. At an initial ouabain concentration of $5 \cdot 10^{-7}$ M (Fig. 2) 'type I' receptors bound at equilibrium 4 pmol whereas 'type II', only 1.6 pmol.

(C) Membranes from digitalis-sensitive and -resistant species

Humans are considered digitalis-sensitive whereas adult rats behave as resistant species. Specifically, higher concentrations of a cardiac glycoside are needed to show positive ionotropic action or K_i values for inhibition of either the sodium pump or the activity of the $(Na^+ + K^+)$-ATPase, respectively, by ouabain, in rat than in human tissues. It could also mean that distinct differences may exist in the stability of the digitalis-receptor complexes obtained from these species [23].

Comparison of the binding of ouabain to erythrocyte membranes prepared from blood of humans or rats is shown in Fig. 3. The binding of ouabain to rat receptors under the conditions for either 'type I or II' showed a linear response over a wide range of drug concentrations. In the range at which the human 'type II' receptors were saturated by ouabain (at approx. $2 \cdot 10^{-8}$ M; Fig. 4), the predominant receptors in the rat were those of 'type I', indicating that the rat membranes probably exhibit only site(s) of low affinity. Similar results were obtained when we also attempted to measure the binding of ouabain to these membranes in the presence of ATP, Mg^{2+} and Na^+.

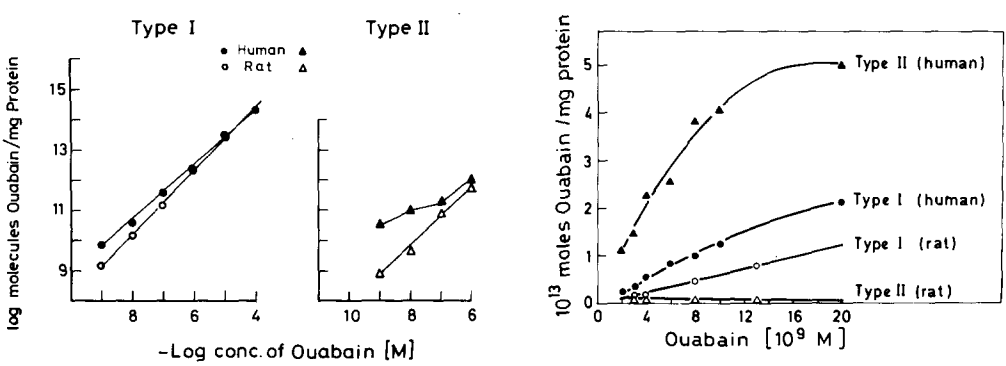


Fig. 3. Binding of ouabain to human and rat erythrocyte membranes. The conditions of binding are similar to those in Fig. 1.

Fig. 4. Binding of 'therapeutic' concentrations of ouabain to human and rat erythrocyte membranes. Binding of $3 \cdot 10^{-9}$—$20 \cdot 10^{-9}$ M [^{3}H]ouabain to human and rat erythrocyte membranes was done under conditions as described in Fig. 1.

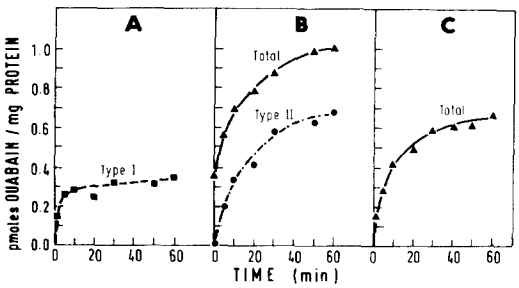


Fig. 5. Stabilization of 'type II' receptors by ligands. (A) 42.4 nM [^{3}H]ouabain ($5 \cdot 10^5$ dpm) were incubated with 1 mg membrane protein under 'type I' conditions, at 37°C, for a total period of 60 min. (B) Membranes were first incubated with [^{3}H]ouabain for different periods of time, then $Mg^{2+} + P_i$ were added, and the incubation period was completed to a total of 60 min. (C) Incubation of membranes first with $Mg^{2+} + P_i$ for different periods of time followed by addition of [^{3}H]ouabain and completion of incubation up to a total of 60 min. The rest of the details are described in Methods and Fig. 1.

(D) Stabilization of 'type II' receptors in human erythrocyte membranes

The role of $Mg^{2+} + P_i$ in distinguishing between the two types of receptors, in either supporting interconversions between the receptors or stabilizing one of them (i.e. 'type II') is demonstrated in Fig. 5. Below the critical digitalis concentration in the absence of $Mg^{2+} + P_i$, ouabain receptors in human erythrocyte membranes may be stabilized as 'type I' sites at an equilibrium value of 0.34 pmol/mg protein (Fig. 5A). The amount of free P_i in the suspension of the membranes was too low (i.e. 5 nmol/mg protein) to support binding of ouabain to 'type II' receptors. The simultaneous presence of both types of receptors was demonstrated by incubating the membranes first with ouabain alone for different periods of time up to 60 min, $Mg^{2+} + P_i$ was only then added at various periods of time and the period of incubation was completed for a total of 60 min. Under these conditions, the 'type I' sites, with an equilibrium value of 0.34 pmol, and the 'type II' sites exhibiting a binding of 0.67 pmol/mg protein, were obtained (Fig. 5B). The possibility of eliminating one of the two types was also examined by reversing the conditions. If the membranes were first incubated with $Mg^{2+} + P_i$ for various lengths of time (up to 60 min) and [^{3}H]ouabain was only added later and the incubation was continued for a total of 60 min, the kinetic behavior of binding was that of 'type II' receptors only, with an equilibrium value of 0.68 pmol/mg protein (Fig. 5C). No binding to 'type I' receptors could be detected.

Although this experiment could be interpreted as a shift or a conversion of the binding sites completely to 'type II' receptors, it must be emphasized that these sites were stabilized in that form only by the presence of ouabain. In order to support this hypothesis, a similar experiment was done (data not shown) in which the membranes were first incubated with $Mg^{2+} + P_i$ for periods of 2—30 min, ouabain was then added and the incubation continued after the addition of ouabain for 5, 10 and 20 min. Only 'type II' receptors were formed and the amounts of ouabain bound were entirely dependent on the time in which the membranes were in contact with ouabain (second incubation period). It would appear that the complex formation is independent of the length of the preincubation time with $Mg^{2+} + P_i$.

It may be concluded that although the formation of an appropriate conformation requires a preliminary exposure of the receptors to $Mg^{2+} + P_i$, the 'fixation' of this conformation (designated here as 'type II') is accomplished only by the subsequent addition of ouabain. On the other hand, if all three ligands were simultaneously present, the 'fixation' of 'type II' receptors occurs together with other receptors which have been designated 'type I'.

In order to test the possibility that a certain equilibrium between the two types of receptors could exist in these membranes, we have attempted to shift the equilibrium by incubating the membranes for 2 h in the absence of $Mg^{2+} + P_i$. The membranes were then rinsed and incubated afresh with ouabain (either below or about the critical digitalis concentration) in the absence or presence of $Mg^{2+} + P_i$, to examine which type of receptor remained. The results of this experiment (data not shown) clearly indicated that both types of receptor sites still existed and their properties were, in principle, similar to those of untreated membranes. This means: 'type II' receptors below the critical digitalis concentration, and only one type (i.e. 'type I') above it since the presence or absence of $Mg^{2+} + P_i$ had no effect on total binding. However, the incubation of the membranes in buffer only, for 24 h, caused changes in the binding capacity of both types of receptors. Although 'type I' receptors apparently reached an equilibrium value with ouabain which is similar to that of untreated membranes (e.g. 80%), 'type II' sites were more affected by the preincubation in the absence of the ligands and reached equilibrium at a value only 50% of the untreated membranes. These results strongly point to the presence of two different types of receptor sites for ouabain in the membrane of human erythrocytes.

(E) Effects of cations and ionic strength on ouabain binding

Fig. 6 shows the effects of Cs^+ on the binding of ouabain, below the 'critical digitalis concentration', to the receptor sites of human erythrocyte membranes. The cation affected only 'type II' sites. Similarly, Na^+ and K^+ affected the equilibrium binding values of 'type II' receptors when present during the binding

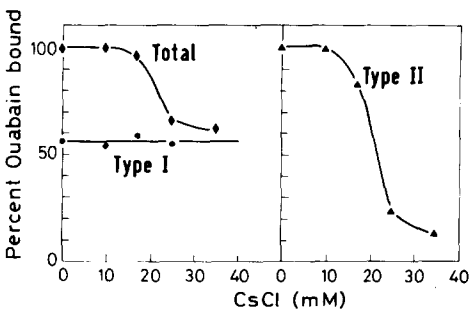


Fig. 6. Effect of CsCl on the equilibrium binding of ouabain to 'type I and II' receptors. 2 mg membrane protein were incubated at 37°C for 60 min with $3 \cdot 10^{-8}$ M, [^{3}H]ouabain in a final volume of 1 ml either with 1.25 mM $MgCl_2$ and 1 mM phosphate in 10 mM Tris-HCl, pH 7.4 ('total binding') or in buffer only ('type I'); 'type II' values were caclulated by subtracting 'type I' from 'total'. CsCl was added to give final concentrations varying from 10 to 35 mM. 100% binding to 'type I' and to 'type II' was 0.5 and 0.43 pmol/mg protein, respectively.

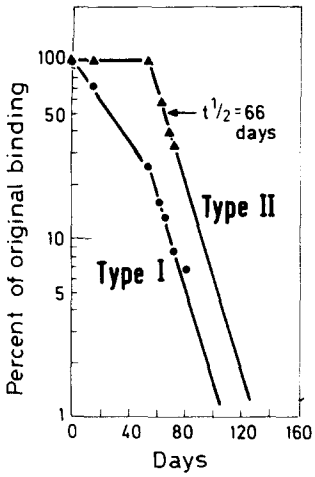


Fig. 7. Stability of membranes to storage at low temperatures. Erythrocyte membranes were stored in aliquots at −20°C in 10 mM Tris-HCl, pH 7.4, and binding to the receptors of both types was measured at the indicated days.

process. High concentrations of all three cations reduced to zero the binding of ouabain to 'type II' receptors only. However, the concentrations at which these cations reduced the maximal amount of bound digitalis to half its value, were 1, 7.5 and 22 mM for K^+, Na^+ and Cs^+, respectively. Isotonic Tris reduced by 25% only the amounts of bound ouabain to 'type II' receptors whereas isotonic sucrose had no effect.

(F) Stability of receptor sites

(1) Storage at low temperatures. Storing membranes in 10 mM Tris, pH 7.4, at −20°C for at least 56 days had no effect on 'type II' receptors, but caused a considerable decrease in the binding capacity of 'type I' sites. Additional storage resulted in an exponential reduction of binding to both types with a half-life time of about 10 days (Fig. 7).

(2) Extraction with ether. In order to show differences in the receptor types originating from differences in their molecular architecture, the possibility of a selective removal of lipid components by mild agents was examined.

Membranes were treated with 'wet' or 'dry' ether, either before or after formation of the ouabain-receptor complex. The more severe conditions with 'wet'-aqueous ether extracted about 50% of both cholesterol and total phospholipids. Consequently, all the radioactivity from 'type II' complexes was removed, and about 80% from 'type I' sites.

Extraction of membrane pellets with 'dry' ether prior to the binding process, removed 50% of the membrane cholesterol but only 26% of the total phospholipids (cf. ref. 28). This prevented binding of ouabain to 'type I' receptors, and removed 73% of the radioactivity if the ether was applied following the formation of the complex of drug-receptor. Under similar conditions, 'type I' receptors were only partially affected, and exhibited approx. 60% of the unextracted values.

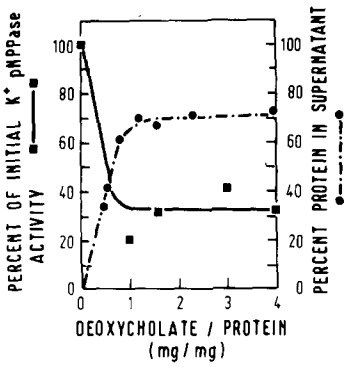
Restoration of the damaged binding capacity may be achieved by adding

liposomes prepared from rat liver microsomal lipids, devoid of cholesterol, to the ether-extracted membranes. When the liposomes were incubated with delipidated membranes at a ratio of 0.14 (by weight) 'type I' receptor sites regained their original activity. On the other hand, increasing the ratio up to 0.5 corrected only partially (i.e. 35%) the damaged 'type II' receptors. With liposomes from erythrocytes the restoration of binding was much higher (data not shown).

These results emphasize the role played by the lipids, particularly by cholesterol, in the process of binding of cardiac glycosides to the receptors in the intact membrane, and also allow a distinction between the different types of sites present.

(G) Effects of deoxycholate

This bile salt is considered a relatively mild detergent if applied carfefully and sensible [11]. When applying deoxycholate the selection of detergent concentration for solubilization should be based on a weight ratio between detergent and membrane protein, instead of percent concentration of deoxycholate, perferably at final concentrations of deoxycholate above its critical micellar concentrations. We have consequently examined the effects of deoxycholate on the K^+-activated phosphatase and on the complex of ouabain with the membrane receptors. Fig. 8 shows the correspondence between 'solubilization' of the membranes and the decrease in the activity of K^+-activated *p*-nitrophenyl phosphatase by deoxycholate. The residual activity was associated only with



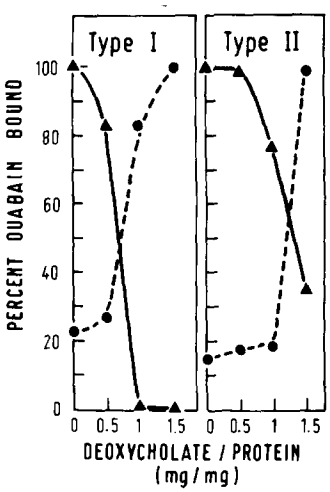


Fig. 8. Effect of deoxycholate on solubility of membrane proteins and activity of K^+-activated phosphatase. The conditions of deoxycholate extraction and the determination of protein and K^+-activated nitrophenol phosphatase (pNPPase) activity are as outlined in Methods.

Fig. 9. Effect of deoxycholate on the stability of the complexes between the membraneous 'type I and II' receptors with ouabain. The complexes were formed as outlined in Fig. 6 for both types and then treated with deoxycholate as outlined in Methods. Some of the radioactivity came off the membranes by rinsing only (15—20%). The radioactivity in the supernatant and precipitate was determined following solubilization with Insta-gel. ▲———▲, percent ouabain in precipitate; ●- - - - -●, percent ouabain in supernatant.

the insoluble pellet. Addition of high concentrations of glycerol (up to 40% by weight) protected only the insoluble portion of the enzyme. The inactivation seems to be irreversible since removal of deoxycholate by dialysis or gel filtration did not restore enzymatic activity. The K^+-activated *p*-nitrophenyl phosphatase activity cannot be taken as a measure of ouabain binding under these circumstances.

Fig. 9 shows the effects of varying deoxycholate/protein weight ratio on the 'solubilization' of the ouabain-membrane complex. At a ratio of 0.75, half the 'type I' receptors-ouabain complex became soluble, whereas a higher ratio (i.e. 1.25) was required for the 'type II' sites. Gel filtration on Sephadex G-25 or Sepharose 4B or membrane ultrafiltration have shown that the detergent dissociated a considerable fraction of the bound ouabain during the process of solubilization at a ratio of 0.8, leaving only a portion bound to 'type II' receptors but not to 'type I'. The fact that all the protein and the bound radioactivity were eluted in the void volume during gel filtration on both Sephadex G-25 and Sepharose 4B and were also retained on the membranes following ultrafiltration supported the idea that the detergent did not reduce the apparent molecular size of the receptor complex during the 'solubilization'. Consequently, deoxycholate did not enable membrane proteins to penetrate polyacrylamide gels of very large pore size during disc gel electrophoresis (data not shown).

Discussion

In this study we have examined the possible existence of more than one binding site for cardiac glycosides in intact, unmodified, isolated membranes from human erythrocytes. Ouabain was chosen because of its greater water solubility, its extremely low permeability across human erythrocyte membranes (in contrast to digoxin) [29] as seen by the distribution values erythrocytes/plasma using cells from different species [30] and our own data indicating that all the ouabain taken up by intact erythrocytes could be detected after hemolysis, in the ghosts. Furthermore, ouabain has low affinity for proteins [31]. Hence, minimum 'non-specific' binding to hydrophobic domains in the erythrocyte membrane should be anticipated. Greenberger and Caldwell [32] have clearly shown lack or partitioning of ouabain into chloroform from a mixture of chloroform and water. This observation was confirmed and extended using [^{3}H]ouabain and the solvent mixture of chloroform, methanol and water according to Folch et al. [25]; all the radioactivity was found in the upper, aqueous-methanolic phase (unpublished observations). These data indicate the elimination, or reduction to minimum of 'non-specific' binding of ouabain, in contrast to less polar glycosides such as digitoxin. Ouabain may be operationally classified as a 'polar, soluble amphiphile, without the ability to form lyotropic mesomorphism' [33]. It probably forms micelles above its critical micellar concentrations, like bile salts or saponins [33,34] and this value seems to be higher than the pharmacological, or even the toxic, concentration range.

Our data are consistent with the results of Baker and Willis [19] showing a biphasic curve of ouabain-binding to cells when the concentrations of the drug

in the medium were increased. Such curves fit the relation formulated by Godfraind and Ghysel-Burton [35] in which the total uptake of ouabain by guinea pig atria is a function of the sum of two components: the one, a linear dependency on the glycoside concentration and the other, a hyperbolic-saturation-relation, as follows:

$$U = aC_m + \frac{bC_m}{C_m + K_b}$$

in which a, a proportionality constant for linear uptake; and b and K_b, capacity and equilibrium constants for the hyperbolic uptake; C_m, the concentration of ouabain in the medium [35]. In their studies, Godfraind and Ghysel-Burton [35] have demonstrated a high affinity and a low affinity ouabain uptake processes, at concentrations ranging from 10^{-9} to 10^{-8} M and from $3 \cdot 10^{-8}$ to 10^{-5} M, respectively. The high affinity process represented only 3% of the capacity of the low affinity process [35].

The binding of ouabain to erythrocyte membranes, in the present study, could represent either: (a) one type of receptors with multiple sites having each a different dissociation constant which may be saturated at different concentration ranges, or (b) two separate entities, each capable of binding ouabain, which may reflect two receptor sites in the membrane or may be due to membranes obtained from two populations of erythrocytes. The published data on the interactions of ouabain with purified preparations of $(Na^+ + K^+)$-ATPase from different sources are consistent with a single type of high affinity receptors [27]. On the other hand, tissues and isolated cells as well as membrane preparations containing $(Na^+ + K^+)$-ATPase activities exhibited more than one type of binding which were referred to, in certain instances, as 'specific' and 'non-specific' binding [4]. It is possible though, that the lower affinity sites are removed during purification of the $(Na^+ + K^+)$-ATPase or modified by the process. so that they are either undetected or their binding capacity is drastically reduced.

However, for animals having in common a resistance to ouabain and other cardiac glycosides, the criteria for specific binding to 'digitalis receptors' are not satisfied. This relative resistance is reflected in the need for higher concentrations of ouabain to elicit pharmacological or toxic effects, and consequently lower K_D of binding and saturation at unusually high concentrations of ouabain are found [6]. The cells of these 'insensitive' species exhibit active transport of cations, and the hydrolysis of ATP by membrane preparations is activated by Na^+ and K^+, which in turn is inhibited by ouabain, in spite of the requirement for higher concentrations. Therefore, the lower affinity in these cases cannot be explained on the basis of binding to 'non-specific' receptor sites. Relative 'insensitivity' may result from structural or compositional arrangements or factors within the membrane which are mandatory for the effective and stable binding [6]. Thus, similar results in 'digitalis-sensitive' species should be considered as a manifestation of low affinity receptor sites, in addition to the high affinity (or 'specific') ones.

The results present in Fig. 5 and in the text cannot, at this stage, be interpreted as a process of interconversion of the receptor forms, and are more likely consistent with the idea of two separate sites. Additional evidence was

provided by examining various aspects of the binding properties as well as structural parameters. Storing the membranes at —20°C for extended periods caused a dissimilar deterioration for the two sites. However, the half-life time of the final decay period was equal for both sites, i.e. 10 days, although the overall half-life time was 66 days (Fig. 7).

Monovalent cations such as Cs^+ or K^+ were considered to affect the rate of both 'inhibitory' and 'non-inhibitory' binding of ouabain to intact erythrocytes [26]. 'Inhibitory' refers to inhibition of cation transport. This effect was later shown by Gardner and Frantz [18] to be associated only with the 'specific' or 'inhibitory' sites, and the changes in binding resulted from alterations in the apparent affinity with which ouabain is bound. Externally added Na^+, Li^+ and Cs^+ increase while K^+ and Rb^+ decrease this affinity [18]. Unpublished data with membrane vesicles show a decrease in the equilibrium amounts of ouabain bound to 'type II' sites only by cations representing the two groups of Gardner and Frantz [18], i.e Na^+ and Cs^+ as well as by K^+ (Heller, M. and Beck, S., unpublished data).

Although our evidence does not support the idea that 'type I' sites are interconvertible with 'type II' receptors or that they are associated with the same molecules as 'type II' sites, they could probably also be located on the outer surface of the membrane, but may be lost during the purification procedure of the $(Na^+ + K^+)$-ATPase. Most purification procedures for this enzyme inevitably involve the use of detergents due to its particulate nature, and even the final product continues to behave as a membrane fragment [4,12]. Several attempts to solubilize and characterize a stable complex between ouabain or other cardiac glycosides with receptors from membrane preparations using detergents or other means, have been reported [36—38]. Although sodium deoxycholate has been preferred for purification of the $(Na^+ + K^+)$-ATPase [11], sodium dodecyl sulfate was the detergent of choice in the attempts to solubilize the complex [36—38]. Both detergents have been shown to solubilize membranes 'completely' at a detergent/lipid ratio (w/v) of 1.6 with membrane concentration exceeding 2 mg/ml [34]. The critical micellar concentration of both detergents is also very close, being in the range of a few millimolar [34]. At this concentration, the erythrocyte membrane was solubilized with a concomitant inactivation of the membrane-bound K^+-activated phosphatase (Fig. 8) and the complexes of ouabain with both types of receptors underwent almost complete dissociation (Fig. 9). It is therefore obvious that removal of lipid components by the detergent would destroy the complex or probably even prevent its formation. This assumption was confirmed in the experiments, described in the text, in which a mild agent such as ether was employed and caused either decrease in binding capacity or dissociation of the complex of both receptor types. These receptors were in turn reactivated to different degrees by the addition of phospholipid liposomes. The addition of deoxycholate at concentrations below the critical micellar concentration (i.e. 2 mM at a detergent/protein ratio (w/w) of 0.8) was sufficient to cause partial solubilization of 'type II-ouabain' complex but was still inadequate for an efficient separation from other membrane proteins. This ratio was, however, sufficient to dissociate the complex of ouabain with 'type I' sites which was no longer detected afterwards.

These data and the results presented in this study support the idea of two binding sites for ouabain in erythrocyte membranes having different properties, and both could be related to the transport enzyme. Alternatively, only one of these sites is associated directly with the $(Na^+ + K^+)$-ATPase and the other, indirectly.

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