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CARDIAC GLYCOSIDE RECEPTOR, $(Na^+ + K^+)$-ATPase ACTIVITY AND FORCE OF CONTRACTION IN RAT HEART*

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Abstract—In order to elucidate the role of $(Na^+ + K^+)$-ATPase and of the ouabain binding sites in the pharmacological effects of ouabain in the digitalis insensitive species rat, specific [^{3}H]ouabain binding and $(Na^+ + K^+)$-ATPase activity were measured simultaneously in a rat heart cell membrane preparation. Specific [^{3}H]ouabain binding, $^{86}Rb^+$-uptake and force of contraction were also measured simultaneously in electrically stimulated contracting ventricular strips of rat heart. The following results were obtained: (1) Rat heart cell membranes exhibit two classes of [^{3}H]ouabain binding sites with apparent dissociation constants (K_D) of the [^{3}H]ouabain–receptor complex $1–2.3 \times 10^{-7}$ M and 2.8×10^{-5} M. (2) $(Na^+ + K^+)$-ATPase activity of rat heart cell membranes is half-maximally inhibited by ouabain at a concentration of 4×10^{-5} M when assayed at the same conditions as [^{3}H]ouabain binding. (3) Specific [^{3}H]ouabain binding to electrically stimulated (1 Hz) contracting ventricular strips of rat heart exhibited only one class of receptors ($K_D = 3 \times 10^{-7}$ M). Force of contraction increased half-maximally at 3×10^{-7} M ouabain when measured simultaneously and $^{86}Rb^+$-uptake was inhibited half-maximally at 3×10^{-5} M ouabain. Thus, there is a serious discrepancy between the effect of ouabain on $(Na^+ + K^+)$-ATPase activity and $^{86}Rb^+$-uptake on one hand and on force of contraction on the other hand, whereas there is a good correlation between [^{3}H]ouabain–receptor binding and increase in force of contraction. These results indicate that inhibition by ouabain of active cation transport is not a mandatory prerequisite of its positive inotropic effect, at least in the rat heart.

The rat heart is known to be rather insensitive to cardiac glycosides [7, 14]. Repke *et al.* [51] found that species variations in susceptibility to cardiac glycosides correspond to variations in the susceptibility of the cardiac $(Na^+ + K^+)$-ATPase to these drugs. Rat heart $(Na^+ + K^+)$-ATPase activity is half-maximally inhibited by ouabain in a concentration of 5.9×10^{-5} M [15] whereas this value for the enzyme from human heart is 2.5×10^{-9} M [22]. The findings of Repke *et al.* [51] have been confirmed and extensified by Allen and Schwartz [4], who furthermore determined [^{3}H]ouabain binding to a rat heart $(Na^+ + K^+)$-ATPase preparation and found an unstable [^{3}H]ouabain–enzyme complex as well as a large amount of [^{3}H]ouabain binding sites unrelated to the enzyme. Akera *et al.* [1] confirmed these findings in rat hearts and concluded that "an attempt to estimate the maximal amount of ATP-dependent [^{3}H]ouabain binding was unsuccessful". Recently, however, Sharma and Banerjee [56] succeeded in measuring specific [^{3}H]ouabain binding to a rat heart cell membrane preparation. Thus, apparently there are specific ouabain binding sites in the rat heart, too.

Experiments quantitating the number of [^{3}H]ouabain binding sites, the affinity of the receptors for [^{3}H]ouabain, and their relation to $(Na^+ + K^+)$-ATPase activity have been successful in cell membranes [18, 23, 32, 41, 63]. Experiments in ox brain and human cardiac cell membranes revealed that [^{3}H]ouabain binding to its receptor causes an inhibition of $(Na^+ + K^+)$-ATPase of the same extent (i.e. 50% of total receptors occupied by [^{3}H]ouabain means 50% of total $(Na^+ + K^+)$-ATPase activity inhibited) [19, 23]. Thus, if the $(Na^+ + K^+)$-ATPase were the receptor enzyme for cardiac glycosides [29, 50, 55] and the insensitivity of the rat heart to cardiac glycosides is reflected on that level, rat heart cell membranes should either contain (a) only a few binding sites or (b) binding sites with low affinity for the drug, or (c) receptors unrelated to $(Na^+ + K^+)$-ATPase activity, or (d) a dissociation between cardiac glycoside binding and cardiac glycoside effects should be measured.

In this study we intended to quantitatively characterize the membrane-bound [^{3}H]ouabain receptors of rat heart, to correlate them to the $(Na^+ + K^+)$-ATPase activity and $^{86}Rb^+$-uptake of contracting cardiac muscle and to measure the ouabain-caused positive inotropy at the very same glycoside concentrations and incubation conditions in order to answer the question: does [^{3}H]ouabain binding to the cell membrane receptor inhibit $(Na^+ + K^+)$-ATPase activity and cause positive inotropy at the same concentrations? A dissociation between these parameters would cast serious doubts on a possible causal relationship between $(Na^+ + K^+)$-ATPase inhibition and positive inotropy.

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MATERIALS AND METHODS

Materials. [^{3}H]Ouabain (=^{3}H-*g*-strophanthin) with a specific activity of 12 Ci/mmole, lot No. 747 186, was purchased from New England Nuclear, Dreieich, Germany. All other chemicals used were of analytical grade and obtained either from Boehringer-Mannheim, Mannheim, Germany or from E. Merck, Darmstadt, Germany.

Preparation of ($Na^+ + K^+$)-*ATPase-containing cardiac cell membranes.* The hearts of 20 young female Sprague–Dawley rats weighing 150–250 g were quickly excised (after short narcotization with ethylether) for each membrane preparation. The hearts were freed from connecting tissue and atria and were homogenized for 2 min in a precooled blender (Fa. Braun-Melsungen, Germany) at top speed in 0.25 M sucrose with 1 mM EDTA neutralized with imidazole to pH 7.0. Then the homogenate was filled up to 200 ml with 0.25 M sucrose and 1 mM EDTA neutralized with imidazole to pH 7.0. Then, after 15 ml 5% (w/v) sodium desoxycholate were added and the homogenate was stirred for 30 min at 0°, it was centrifuged for 30 min at 10,500 rpm (Sorvall RC-5, rotor SS 34). The supernatant was spun down 30 min at 44,000 rpm (Beckmann L-5 50, rotor 60 Ti). The sediment was homogenized in 40 ml 1 mM EDTA neutralized with imidazole to pH 7.0 and frozen at −40° overnight.

The next day it was shaken at 37° until thawed, then 60 ml 1 mM EDTA, neutralized with imidazole to pH 7.0, 0.75 ml 0.1 M $MgCl_2$ and 15 ml 6 M NaJ were added and it was stirred for 5 min at 0°. Thereafter 120 ml 1 mM EDTA pH 7.0 were added and the homogenate was centrifuged for 30 min at 44,000 rpm (Beckman L-5 50, rotor 60 Ti). The sediment was then homogenized and washed three times in 200 ml 1 mM EDTA pH 7.0 each time. The final sediment homogenized in 1 mM EDTA pH 7.0 (40 ml) was used for the experiments. Its ($Na^+ + K^+$)-ATPase activity was determined usually between 0.25–1.0 μmole ATP hydrolysed per min per mg protein at 37°. More than 90 per cent (usually 95–98%) of total ATPase activity was inhibited by 10^{-3} M ouabain.

($Na^+ + K^+$)-ATPase activity was measured with the coupled optical assay [53]. The reaction was continuously recorded and corrected for Mg^{2+}-activated ATPase by inhibition of ($Na^+ + K^+$)-ATPase with 10^{-3} M [^{3}H]ouabain. One enzyme unit (U) is defined as the amount of enzyme hydrolyzing 1 μmole ATP per min at 37°. Protein was measured by the procedure of Lowry *et al.* [42].

[^{3}H]*Ouabain binding experiments.* These have been described in great detail elsewhere [18, 19]. Bound glycosides were quantitated using a rapid filtration technique (Whatman GF/C glass fiber filters) to separate free glycosides from the membrane-bound glycosides. This method with the rapid filtration technique gave the same values as the rapid centrifugation technique, which was used to separate bound from free [^{3}H]ouabain [18].

Unspecific glycoside binding (i.e. [^{3}H]ouabain bound to heat denatured membranes or in the presence of 10^{-3} M unlabeled [^{3}H]ouabain) was usually less than five per cent of total radioactivity bound to the membranes. Experiments were performed in duplicate or triplicate assays and at least twice. Unless otherwise stated, the incubation media were 50 mM imidazole/HCl buffer pH 6.5, 3 mM $MgCl_2$, 3 mM imidazole/PO_4, 3–6 nM [^{3}H]ouabain, total volume 2 ml.

Experiments on electrically stimulated right ventricular strips. Female Sprague–Dawley rats weighing 150–250 g were narcotized shortly with ethylether, the hearts were quickly removed and the ventricles were dissected at room temperature in bathing solution containing (in mM): Na^+ 149.1, K^+ 5.4, Ca^{2+} 1.8, Mg^{2+} 1.05, Cl^- 144.1, HCO_3^- 11.9, $H_2PO_4^-$ 0.42, glucose 10, pH 7.4, continuously gassed with 95% O_2 and 5% CO_2. Precautions were taken to dissect ventricular strips with fibres running in parallel. The strips were less than 1 mm wide, less than 0.5 mm thick, length usually 10 mm, weight about 20 mg. After dissection the preparations were attached to platinum stimulating electrodes and mounted individually in glass tissue chambers as described previously [66]. Developed tension was measured isometrically at the apex of the preload active tension curve of each preparation (preload 0.4–0.5 g) with an inductive force displacement transducer (W. Fleck, Mainz) attached to a Hellige Helco Scriptor recorder. The preparations were allowed to equilibrate for 60 min in bathing solution of the previously described composition at 35°. This solution was exchanged about every 20 min. After equilibration of contraction was attained, the incubation medium (50 ml) was quickly exchanged for that containing the desired [^{3}H]ouabain concentrations. Electrical stimulation during equilibration and experimental periods was carried out by means of rectangular pulses (Grass SD 9 stimulator, frequency 1 Hz, duration 5 msec, intensity 10–20% above threshold). One ventricular strip was used only for one [^{3}H]ouabain concentration.

Determination of ouabain-sensitive $^{86}Rb^+$*-uptake.* This was performed as described by Yamamoto *et al.* [65] with some modifications: the electrically stimulated right ventricular strips were treated exactly as described above for the measurement of force of contraction under the same conditions. After equilibration of the positive inotropic effect at the respective ouabain concentrations, tracer amounts of ^{86}RbCl (1.5 μCi) were added to the incubation medium (50 ml). After incubation for 10 min in the presence of $^{86}Rb^+$, the ventricular strips were rinsed for 15 sec with aqua dest. (4°) and blotted for 3 min on filter paper. After weighing the tissue, the amount of radioactivity in the tissue was assayed after dissolving the cardiac muscle in 1 ml Soluene-350 (Packard Instrument Company, Downers Grove, Ill., USA) at 60° for 60 min and addition of 10 ml scintillation fluid (Unisolve, Packard Instrument Company) in a scintillation counter (Packard Tricarb 2660). $^{86}Rb^+$-uptake was linear for 60 min at least under these conditions. $^{86}Rb^+$ was used as a tracer to calculate the amount of ($^{86}Rb^+ + K^+$)-uptake quantitatively. The amount of ($^{86}Rb^+ + K^+$)-uptake without ouabain calculated by this method was 1160 ± 73 nmoles/g wet weight per min ($\bar{x}$ ± SD). This value is in good agreement with that of Yamamoto *et al.* [65].

RESULTS

Ouabain binding to isolated myocardial cell membranes

The experiments were carried out with [^{3}H]ouabain and ouabain because of its good solubility in water. Binding of [^{3}H]ouabain (4×10^{-9} M) to rat heart cell membranes is shown in Fig. 1. [^{3}H]Ouabain bound in the presence of 10^{-3} M unlabeled ouabain is referred to as "unspecific binding" (i.e. radioactivity bound to the glass fiber filters, trapped between membrane particles, dissolved in the remaining water, etc.). It coincides with the radioactivity "bound" to heat denatured membranes (i.e. membranes heated for 60 min at 60°) and it stays constant for the time observed. It is interesting to note that [^{3}H]ouabain binding to the isolated cell membranes after an initial peak (at about 6 min) declines again. The nature of this apparent dissociation of [^{3}H]ouabain–receptor complex is not quite clear.

A conformational change of the cell membrane protein caused by the incubation and leading to a dissociation of the [^{3}H]ouabain from the binding sites cannot be ruled out [24].

The association rate constant as determined from the initial rate of [^{3}H]ouabain binding [18, 24] (Fig. 2) was calculated as 4.3×10^4 M^{-1} sec^{-1}.

In order to measure the rate of the dissociation, the membranes were allowed to bind [^{3}H]ouabain for 5 min, then the [^{3}H]ouabain-membranes were isolated by rapid centrifugation at 80,000 *g* for 30 min at 0°. The supernatant containing non-bound [^{3}H]ouabain was decanted. Thereafter, the homogenized [^{3}H]ouabain–membrane complex was incubated again in the incubation medium containing 50 mM imidazole/HCl pH 6.5, 3 mM $MgCl_2$, 3 mM imidazole/PO_4 and this time 10^{-3} M unlabeled ouabain (instead of [^{3}H]ouabain) at 37°. The dissociation of the [^{3}H]ouabain–receptor complex thus determined in this "chase experiment" (Fig. 2) does not follow first order kinetics as one should expect if:

$$OR \xrightarrow{k_{-1}} O + R$$

where OR is the ouabain–receptor complex, O = ouabain, R = receptor (=binding protein) and k_{-1} = the dissociation rate constant.

Instead, two different dissociation rate constants were determined by a computerized model based on a graphical approximation of the theoretical curve for two independent receptors and two independent dissociation rate constants [62]: $k_{-1} = 10^{-2}$ sec^{-1} and $k'_{-1} = 6 \times 10^{-4}$ sec^{-1}. We think that one of the two components of dissociation—probably the slow one—is caused by the same factor as the dissociation in Fig. 1, probably an alteration of the membranes during the preparation procedure. In order to exclude, however, negative cooperative effects in ouabain binding to rat heart membranes, which would be another possible explanation, the dissociation reaction was measured under the condition of dilution ("dilution experiment"). The membranes were allowed again to bind [^{3}H]ouabain (4×10^{-9} M) as in the first experiment for 5 min, then the reaction mixture was diluted 1:100 by rapid addition of the same incubation medium but without any ouabain. As also shown in Fig. 2 the two experimental curves coincide thus demonstrating identical dissociation rates under the two totally different experimental conditions.

The dissociation constant (K_D) calculated from the fast dissociation rate constant and the association rate constant ($k_{-1}/k_{+1} = K_D$) is 2.3×10^{-7} M.

In order to measure [^{3}H]ouabain binding to the membranes at several concentrations and thereby possibly reveal high affinity and low affinity binding sites, a concentration-dependent binding experiment was performed (Fig. 3). Unspecific [^{3}H]ouabain binding was subtracted, time of incubation was 5 min, as this was the time of maximal binding capacity. The amount of ouabain bound to the membranes increases considerably as the concentration

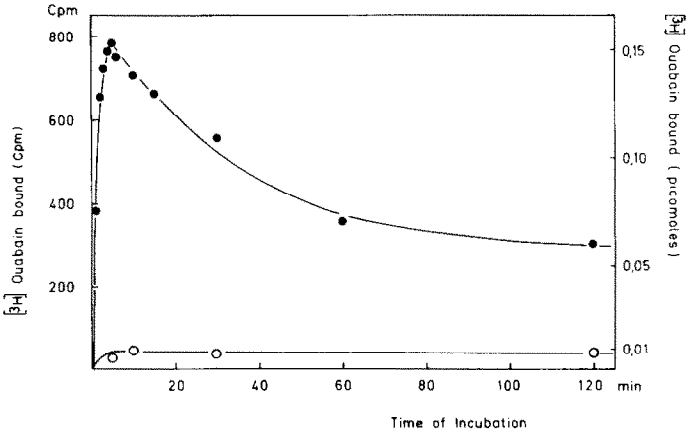

Fig. 1. [^{3}H]Ouabain binding to rat heart cell membranes. Cardiac cell membranes (0.72 mg protein, ($Na^+ + K^+$)-ATPase activity 0.14 U/mg protein) were incubated at 37° in 50 mM imidazole/HCl pH 6.5, 3 mM $MgCl_2$, 3 mM imidazole/PO_4 and 4×10^{-9} M [^{3}H]ouabain. Total vol. 2.0 ml. Radioactivity on the glass fiber filters was determined after rapid filtration (see Materials and Methods). ●——● no further additions = total ouabain binding to the membranes; ○——○ in the presence of 10^{-3} M unlabeled ouabain = unspecific ouabain binding to the membranes. Note that the rapid initial [^{3}H]ouabain binding to the membranes is followed by a dissociation phase (for explanation see text).

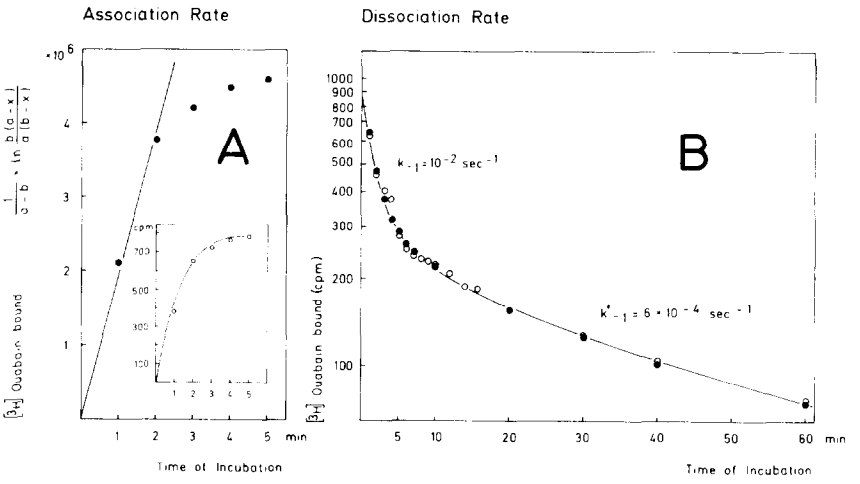

Fig. 2. [^{3}H]Ouabain binding to rat heart cell membranes. Determination of association and dissociation rates. (A) *Association rate:* Cardiac cell membranes (0.72 mg protein, ($Na^+ + K^+$)-ATPase activity 0.14 U/mg protein) were incubated as in Fig. 1, the binding reaction was interrupted by rapid filtration and the radioactivity on the filters was determined (see Materials and Methods). As it can be expected that the association reaction follows second order kinetics, if the concentration of ouabain (a) is similar but not the same as the receptor concentration (b) [18, 20], the initial amount of ouabain bound (x) may be used to determine the association rate constant (k_{+1}). It was calculated as 4.3×10^4 M^{-1} sec^{-1} (B) *Dissociation rate:* Cardiac cell membranes (as in A) were allowed to bind [^{3}H]ouabain for 5 min, then the [^{3}H]ouabain-membranes were isolated by rapid centrifugation at 80,000 *g* for 30 min at 0°. The supernatant containing non-bound [^{3}H]ouabain was decanted. Thereafter the homogenized [^{3}H]ouabain–membrane complex was incubated again in the incubation medium (as in A) but with 10^{-3} M unlabeled ouabain (instead of [^{3}H]ouabain) at 37°. The unlabeled ouabain displaces the membrane-bound [^{3}H]ouabain rapidly ("chase experiment"): ○——○.

In a second experiment (●——●) the cardiac membranes were allowed to bind [^{3}H]ouabain at 37° again as in A. After 5 min the reaction mixture was diluted 1:100 by rapid addition of the same incubation medium but in this case without any ouabain ("dilution experiment"). Still the [^{3}H]ouabain–receptor complex dissociates as rapidly as in the "chase experiment" with identical kinetics. Two dissociation rate constants are determined by a graphical approximation of this experimental curve [62], $k_{-1} = 10^{-2}$ sec^{-1} and $k'_{-1} = 6 \times 10^{-4}$ sec^{-1}.

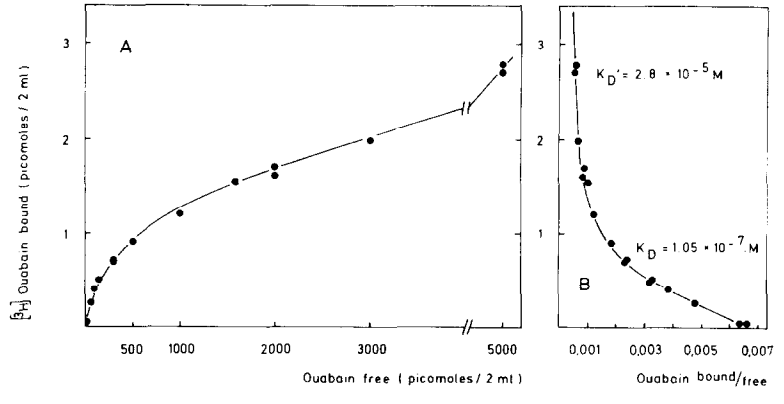

Fig. 3. [^{3}H]Ouabain binding to isolated rat heart cell membranes. Effect of increasing [^{3}H]ouabain concentrations. Cardiac cell membranes (0.3 mg protein, ($Na^+ + K^+$)-ATPase activity 0.25 U/mg protein) were incubated at 37° for 5 min in 50 mM imidazole/HCl, pH 6.5, 3 mM $MgCl_2$, 3 mM imidazole/PO_4, 4 nM [^{3}H]ouabain and increasing ouabain concentrations (10^{-9}–10^{-3} M, as indicated). Unspecific [^{3}H]ouabain binding (in the presence of 10^{-3} M ouabain) was substracted. After rapid filtration (Whatman GF/C glass fiber filters) the radioactivity on the filters was determined. (A) This plot shows that ouabain binding to the membranes increases steadily up to very high free ouabain concentrations. (B) The Scatchard analysis demonstrates at least two components of ouabain binding sites. A high affinity/low capacity receptor ($K_D = 1.05 \times 10^{-7}$ M/10% of binding sites) and a low affinity/high capacity receptor ($K_D' = 2.8 \times 10^{-5}$ M/90% of binding sites). The mathematical analysis was performed according to Weidemann *et al.* [62].

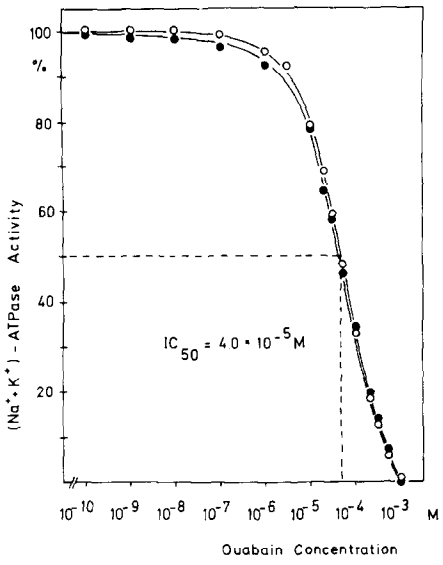

Fig. 4. Inhibition of rat heart $(Na^+ + K^+)$-ATPase activity by ouabain. ●——● Cell membranes (0.3 mg protein, $(Na^+ + K^+)$-ATPase activity 0.25 U/mg protein) were incubated at 37° in the coupled optical assay mixture [53] with the indicated ouabain concentrations. In each experiment activity was continuously recorded for 20 min. After 5–8 min there was no further inhibition of ATPase activity, this "apparent equilibrium value" was used for calculation. ○——○ Cell membranes (0.64 mg protein, $(Na^+ + K^+)$-ATPase activity 0.25 U/mg protein) were incubated for 8 min (maximum of [^{3}H]ouabain binding, as determined before) at 37° in 50 mM imidazole/HCl pH 6.5, 3 mM $MgCl_2$, 3 mM imidazole/PO_4 and the indicated ouabain concentrations. Then 0.5 ml of this incubation medium were placed into the coupled optical assay mixture [53] containing identical ouabain concentrations. Again ATPase activity was recorded continuously for 20 min. It stayed constant throughout this time.

of the glycoside is raised. An analysis of this drug–receptor binding according to Scatchard [52] shows a high affinity binding site (dissociation constant $K_D = 1.05 \times 10^{-7}$ M) and a low affinity binding site ($K_D' = 2.8 \times 10^{-5}$ M). These data were again calculated from the plot (Fig. 3) according to the mathematical method described by Weidemann *et al.* [62] based on a graphical approximation of the theoretical curve for two independent binding sites. The binding capacity of the high affinity receptors was calculated as about 10 per cent of the total number of receptors.

According to these [^{3}H]ouabain binding experiments showing specific and reversible ouabain binding following saturation kinetics and one type of ouabain receptor with high affinity in the rat heart as well as one type of ouabain receptor with low affinity, the $(Na^+ + K^+)$-ATPase inhibition and the pharmacological effects of the cardiac glycoside had to be measured at different drug concentrations, preferably at those concentrations where [^{3}H]ouabain binding had occurred to the membranes.

Ouabain-caused inhibition of myocardial $(Na^+ + K^+)$*-ATPase activity*

The inhibition of $(Na^+ + K^+)$-ATPase activity caused by ouabain is shown in Fig. 4. The experiments were performed by two different methods: at first the membranes were assayed for ATPase activity in the coupled optical assay [53], then ouabain was added and the enzyme activity was determined continuously. Usually after 5–8 min the ouabain-caused inhibition was greatest. This was thought to reflect equilibrium conditions and was therefore taken into account. This method gave a 50 per cent inhibition of the enzyme activity at 4×10^{-5} M. In order to eliminate possible artifacts caused by the ATPase-assay mixture (containing NH_4Cl etc.), we followed

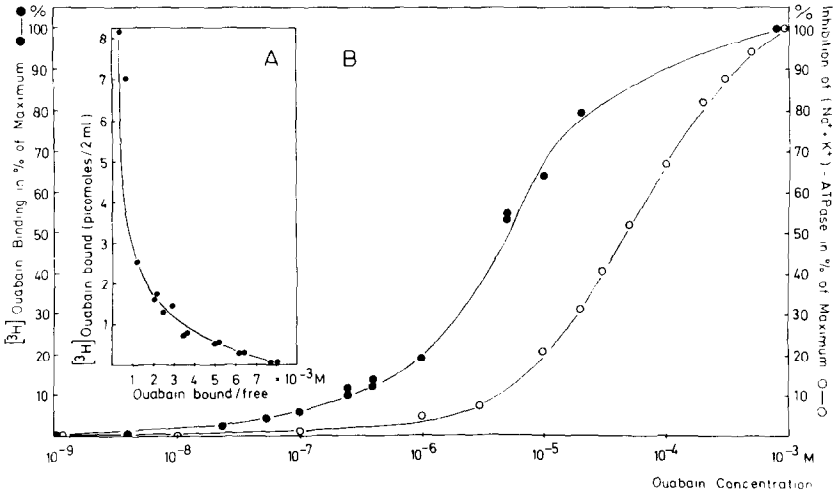

Fig. 5. [^{3}H]Ouabain binding to rat heart cell membranes and inhibition of $(Na^+ + K^+)$-ATPase. Cardiac cell membranes (1.25 mg protein, $(Na^+ + K^+)$-ATPase activity 0.1 U/mg protein) were incubated at 37° in 50 mM imidazole/HCl pH 6.5, 3 mM $MgCl_2$, 3 mM imidazole/PO_4, 4×10^{-9} M [^{3}H]ouabain and increasing amounts of ouabain (10^{-9} M–10^{-3} M), total volume 2.0 ml. After 5 min (time of peak [^{3}H]ouabain binding to these membranes) 0.5 ml of the incubation mixture were used for determination of [^{3}H]ouabain binding to the membranes (rapid filtration method) and 0.6 ml were used for determination of $(Na^+ + K^+)$-ATPase activity (coupled optical assay). Unspecific [^{3}H]ouabain binding (in the presence of 10^{-3} M ouabain) was subtracted.

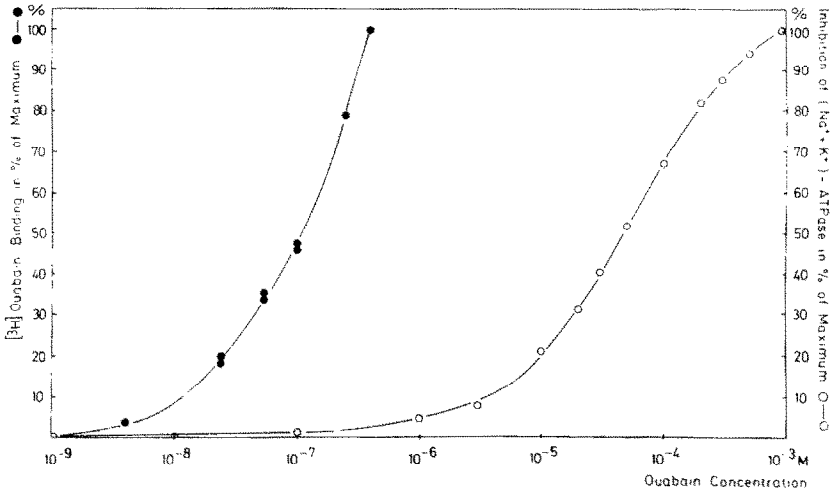

Fig. 6. High affinity [^{3}H]ouabain binding to rat heart cell membranes and inhibition of ($Na^+ + K^+$)-ATPase. The experiment was performed as in Fig. 5. This time, however, only the high affinity [^{3}H]ouabain binding is plotted versus the inhibition of ($Na^+ + K^+$)-ATPase.

another experimental procedure: the enzyme was incubated in the same medium and at the very same conditions as those used for the ouabain binding experiments (Figs. 1–3). After 8 min some of this medium including enzyme protein was quickly analyzed for ATPase activity with the coupled optical assay containing the same ouabain concentration as the incubation mixture. Again ouabain-caused 50 per cent inhibition of enzyme activity was measured at 4×10^{-5} M as both experimental results coincided in spite of the different experimental conditions.

In order to compare binding and inhibition directly under identical conditions, we incubated the enzyme in another experiment with radioactively labeled ouabain (concentration range 4×10^{-9}–10^{-3} M) in 50 mM imidazole/HCl pH 6.5, 3 mM $MgCl_2$, 3 mM imidazole/PO_4 at 37° for 5 min, total vol. 2 ml. 0.5 ml of this medium then was used to measure ^{3}H activity and 0.5 ml was used to determined the ($Na^+ + K^+$)-ATPase activity in the coupled optical assay. Thus, there were truly identical conditions for ouabain binding and inhibition of ($Na^+ + K^+$)-ATPase activity of the membranes. Again, however, we found a distinct dissociation between both parameters (Fig. 5). Apparently ouabain being bound to the membranes does not inhibit ($Na^+ + K^+$)-ATPase activity to the same extent. If only the high affinity binding of ouabain is plotted (Fig. 6) instead of total concentration-dependent ouabain binding, the two curves dissociate even further.

Effect of ouabain on force of contraction of electrically stimulated rat heart ventricular strips

The time courses of the positive inotropic effect of ouabain are shown in Fig. 7.

In the absence of ouabain, force of contraction decreased by about 20 per cent within 60 min. Ouabain increased the force of contraction in a concentration-dependent manner. Threshold and maximally effective concentrations were 10^{-7} and 3×10^{-5} M, respectively. Higher concentration of ouabain led to arrhythmias and/or contractures as a sign of toxicity. It is also apparent from Fig. 7 that the positive inotropic effect of ouabain developed the faster the higher the concentration of the drug. After peaking, the positive inotropic effect declined slightly in all cases.

The concentration–response curve for the positive inotropic effect of ouabain is shown in Fig. 8. The half-maximal increase in force of contraction under

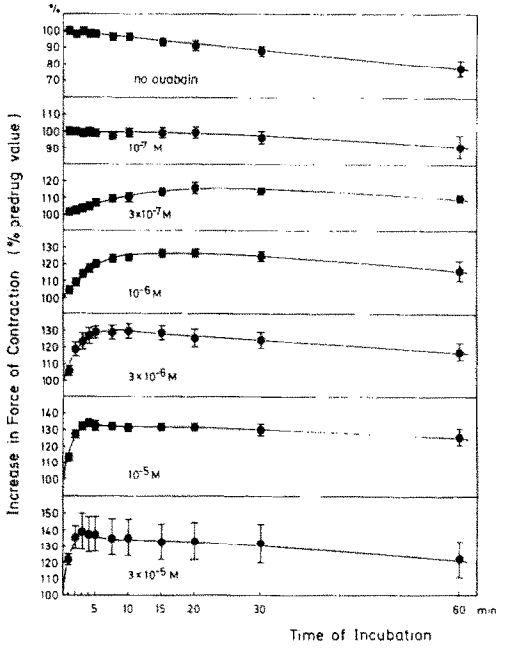

Fig. 7. Timecourse of ouabain effect on force of contraction in electrically stimulated rat ventricular strips. Ventricular strips incubated for 60 min with the indicated ouabain concentrations were electrically stimulated at 1 Hz. Force of contraction was continuously recorded. Each time-response curve is the mean ± S.E.M. of 5–8 experiments. Muscles showing arrhythmias or contractures were discarded. Predrug values before addition of ouabain: 23.3 ± 1.8 g/g wet weight (n = 52).

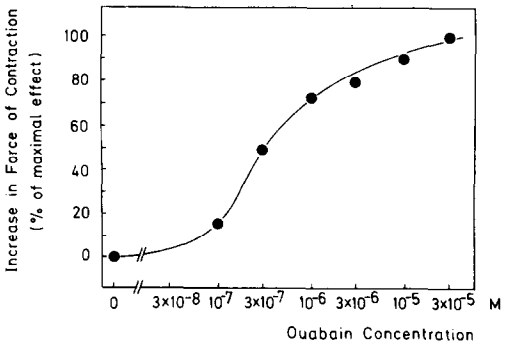

Fig. 8. Concentration–response curve for the positive inotropic effect of ouabain in rat heart ventricular strips. Maximal inotropic effects at each ouabain concentration (same experiments as in Fig. 7) are plotted versus the ouabain conentrations used. The increase in force of contraction after 3 min at 3×10^{-5} M ouabain is arbitrarily set as 100 per cent, because further increases in ouabain concentration invariably led to arrhythmia and contracture. Half-maximal positive inotropic effect occurred at 3×10^{-7} M ouabain.

the conditions tested occurred at an ouabain concentration of 3×10^{-7} M. Thus, this concentration agrees well with that of half-maximal binding (1.05×10^{-7} M or 2.3×10^{-7} M), if one takes the high affinity binding site (Fig. 3). It does not, however, agree with 50% $(Na^+ + K^+)$-ATPase inhibition (4×10^{-5} M).

[^{3}H]*Ouabain binding to electrically-stimulated ventricular strips*

Although previous experiments [24] have shown that ouabain binding to isolated cell membranes follows the same kinetics as ouabain binding to intact contracting skeletal muscle (musculus soleus) of the rat [9, 10], there always remains some doubt about the validity of results obtained from a cell membrane preparation. Therefore, we measured [^{3}H]ouabain binding to the intact ventricular strips during the above-described measurements of the positive inotropic effects of ouabain (Fig. 9). The incubation period was interrupted when maximal force of contraction at the different [^{3}H]ouabain concentrations was reached as can be seen from Fig. 7. The amount of [^{3}H]ouabain bound to the myocardial tissue was then measured. When the data are plotted as in Fig. 3, it becomes obvious that ouabain binding follows saturation kinetics (Fig. 9). The Scatchard plot analysis shows that there is only one type of specific ouabain binding receptor with a dissociation constant of 3×10^{-7} M. This result and that of Fig. 8 clearly demonstrates that half-maximal ouabain binding and half-maximal positive inotropic effect occur at an identical ouabain concentration. From this plot the number of ouabain receptors apparently coupled to the positive inotropic effect can easily be determined as 0.66×10^{14}/g wet wt or as about 660 receptors/μm^2 cell surface, if the cell surface is assumed as 1000 cm^2/g wet wt [64]. From these data we may assume that the low affinity/high capacity binding sites for ouabain as determined in the cell membrane experiments (Fig. 3, $K_D = 2.8 \times 10^{-5}$ M) probably are artifacts or binding sites unrelated to the measured positive inotropic effect. They do not appear to exist in the contracting intact muscle strips.

Influence of ouabain on the $^{86}Rb^+$*-uptake of the electrically stimulated, contracting ventricular strips*

Figure 10 shows that ouabain does inhibit the $^{86}Rb^+$-uptake in contracting cardiac tissue in a con-

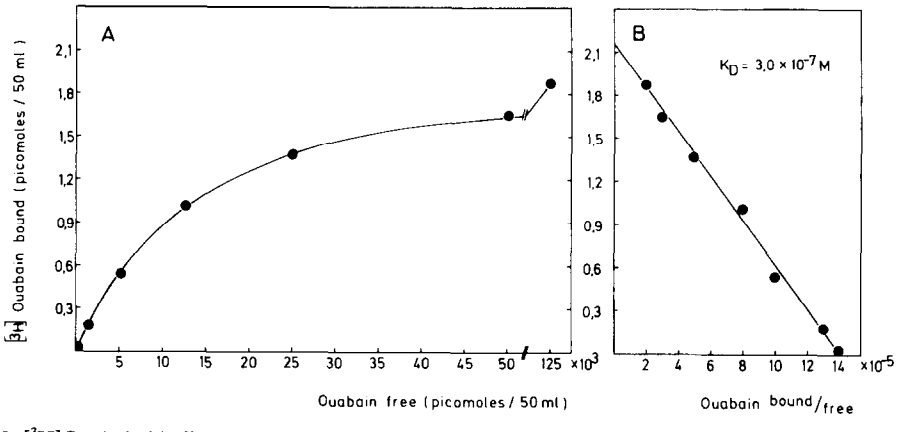

Fig. 9. [^{3}H]Ouabain binding to contracting rat heart ventricular strips. Ventricular strips (approximately 20 mg wet weight, details see Materials and Methods) were incubated at 37° in Tyrode solution containing [^{3}H]ouabain (4×10^{-9} M, 2.5×10^{-8} M, 1×10^{-7} M, 2.5×10^{-7} M, 5×10^{-7} M, 1×10^{-6} M, 2.5×10^{-6} M, 1.5×10^{-4} M = unspecific binding value) and electrically stimulated (1 Hz). Incubation volume 50 ml. Incubation time was interrupted at the time of peak inotropic effect (10–20 min; see Fig. 7), and the ventricular strips were blotted and assayed for radioactivity. Each experimental point is the mean of 4 experiments. Radioactivity was calculated per mg of wet tissue weight in 50 ml. (A) The amount of [^{3}H]ouabain bound to the muscles is plotted versus [^{3}H]ouabain free (in the incubation medium). (B) The same data as in A plotted according to Scatchard [52] clearly demonstrate only one type of binding sites for ouabain with saturation. Dissociation constant (K_D) of the [^{3}H]ouabain–receptor complex is 3×10^{-7} M. The number of specific receptors per g wet weight was estimated as 0.66×10^{14}.

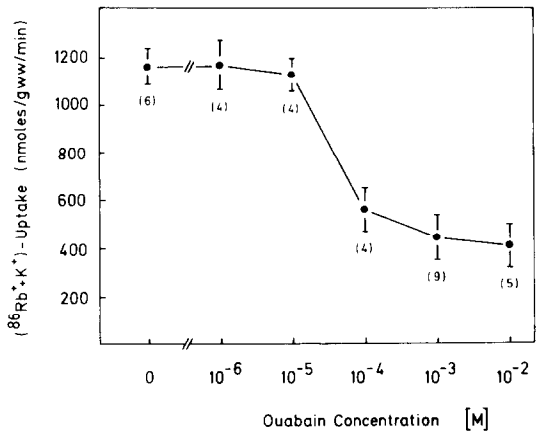

Fig. 10. Effect of ouabain on ($^{86}Rb^+ + K^+$)-uptake of electrically stimulated contracting rat heart ventricular strips. Ventricular strips were incubated under identical conditions as in Fig. 7 and Fig. 9. Force of contraction was continuously recorded after addition of the indicated concentrations of ouabain. At the time of peak inotropic effect (10^{-7}–10^{-5} M) or after 5 min (10^{-4}–10^{-2} M ouabain) 1.5 μCi ^{86}RbCl (about 3×10^6 cpm) were added to the incubation medium. After 10 min of incubation in the presence of $^{86}Rb^+$, incubation was interrupted and the strips were blotted, weighed and assayed for radioactivity.

centration-dependent manner with a half-maximal effect at about 3×10^{-5} M. This value is in good correlation with the half-maximal inhibition of ($Na^+ + K^+$)-ATPase activity of rat heart cell membranes (4×10^{-5} M) (Fig. 4).

DISCUSSION

The present investigation was designed to possibly answer the following questions:

(1) Are the cell membrane-bound ouabain receptors (high and/or low affinity receptors) coupled to the positive inotropic effects of the drug?
(2) Does inhibition of ($Na^+ + K^+$)-ATPase coincide with ouabain binding and is it necessary for the positive inotropic effect of ouabain?
(3) What are the possible reasons for the insensitivity of rat heart to cardiac glycosides?

We thought that a quantitative and qualitative evaluation of [^{3}H]ouabain binding to isolated cardiac cell membranes and to intact contracting myocardial tissue as well as that of ouabain-caused inhibition of ($Na^+ + K^+$)-ATPase activity, of $^{86}Rb^+$-uptake and of ouabain-induced positive inotropic effect in heart muscle might reveal some of the answers.

Are the cell membrane-bound ouabain receptors coupled to the positive inotropic effects of the drug?

Measuring the exact amount of [^{3}H]ouabain binding sites in rat heart (isolated heart, isolated membrane preparations) has been described as extremely difficult, mainly because of a large amount of unspecific binding [1, 4, 25]. Quantitative data have, however, been obtained on ouabain binding sites in other species [2, 18, 61]. Most authors found a close correlation between [^{3}H]ouabain binding and positive inotropic effects (for review see [33, 44, 54]). Our present experimental data also agree well with these findings.

Ouabain binding to the cell membrane preparation from rat heart shows, in contrast to human heart or calf heart, two different types of binding sites, a high affinity/low capacity binding site with an apparent dissociation constant (K_D) = 1.05×10^{-7} M and about 10 per cent of total [^{3}H]ouabain binding, and furthermore a low affinity/high capacity binding site $K_D' = 2.8 \times 10^{-5}$ M, 90 per cent of total [^{3}H]ouabain binding. From these data it cannot be decided whether the low affinity binding site is an artifact [24], a second receptor [58] or an expression of negatively cooperating receptors [13, 37, 39, 40]. Several other possibilities being discussed recently (mobile receptors, conformational changes of the membranes, aggregation–disaggregation of protein) [5, 6, 35, 38, 49] may, we think, be excluded, as specific [^{3}H]ouabain binding to intact contracting ventricular tissue exhibits only one type of receptors, $K_D = 3 \times 10^{-7}$ M. Half-maximal positive inotropic effect of ouabain was found under identical conditions at the same ouabain concentration (3×10^{-7} M). Thus, we would like to answer the first question by stating that the high affinity/low capacity ouabain binding site in the cell membrane preparation is the same as that one in the contracting intact myocardial tissue. Within experimental error their K_D-values (a reciprocal measure of the receptor affinity for the drug) agree. The rather slight difference (1.05×10^{-7} M versus 3×10^{-7} M) can be explained by the different incubation media (e.g. without and with potassium!). K_D as determined from the initial association and dissociation rate constants in the cell membrane preparation was 2.3×10^{-7} M and thus agrees very well with half-maximal positive inotropic effect. In accordance with these results, Sharma and Banerjee [56] have reported a K_D-value of 3.2×10^{-7} M for the [^{3}H]ouabain–receptor complex of rat cardiac cell membranes.

Recently, it has been reported that brain ($Na^+ + K^+$)-ATPase (but not that of other organs) exists in two different molecular forms exhibiting non-linear ouabain binding kinetics. In cardiac cell membranes, however, only one type of this enzyme was found [57]. However, it might have been possible that, due to the experimental procedure, a low affinity ouabain binding site was not detected in the contracting intact cardiac tissue. Thus, it cannot be completely ruled out that a low affinity ouabain binding site exists in rat heart. It is, however, not related to positive inotropy.

[^{3}H]Ouabain binding to its receptor causes an increase in contractile force of the same extent when measured at identical conditions. We did not find an "unspecific binding site" or so called negatively cooperative effects in the contracting ventricular strips. The similarity between the dissociation reaction of the [^{3}H]ouabain–receptor complex in the "dilution experiment" and in the "chase experiment" (Fig. 2) also excludes negative cooperativity [40]. Therefore, we would like to suggest that the measured low affinity/high capacity binding site ($K_D' = 2.8 \times 10^{-5}$ M) in the membrane preparation is an artifact, or sites unrelated to positive inotropy [24].

Critically, however, we would like to note that the concentration-dependent [^{3}H]ouabain binding experiments (Fig. 3, Fig. 9) ought to be equilibrium binding experiments. It is impossible, however, to determine equilibrium, as Fig. 1 demonstrates clearly. Therefore, we have always taken "maximal" [^{3}H]ouabain binding or "maximal" ouabain effects and the time thereof as "apparent equilibrium". The similar initial peak in the increase of contractile force followed by a distinct decrease has been also found by Fricke *et al.* [25]. They did not give an explanation for this phenomenon; we do not want to speculate either.

Does inhibition of ($Na^+ + K^+$)*-ATPase activity coincide with ouabain binding and is it necessary for the positive inotropic effect of ouabain?*

This question has been considered for some time, and we would therefore like to consider some controversial points. If investigators find a close correlation between ($Na^+ + K^+$)-ATPase inhibition and positive inotropic effect, it does not necessarily mean that ($Na^+ + K^+$)-ATPase inhibition in fact causes increased contractile force. Even one example of a true dissociation between both parameters would make this hypothesis [29, 33, 44, 54] very dubious. It is, however, certainly not enough to apply cardiac glycosides, measure increased contractile force, then isolate the ($Na^+ + K^+$)-ATPase-containing cell membranes and assay enzyme activity—because during the usually long course of ($Na^+ + K^+$)-ATPase preparation (homogenization, centrifugation procedure, extraction of cations, etc.) the formerly bound cardiac glycosides may have dissociated totally or partly. Even in crude homogenates of cardiac muscle formerly exposed to cardiac glycosides, the exact assessment of specific [^{3}H]ouabain binding is almost impossible. After homogenizing the tissue, the ouabain receptor may have different properties, be exposed to a different environment (pH, cation concentration, etc.) and may thus change its affinity or binding capacity for the cardiac glycoside. Recently, Huang *et al.* [33] have nevertheless analyzed the inhibition of ($Na^+ + K^+$)-ATPase in the dog and rabbit drug-exposed heart. They have seen a "sustained positive inotropic effect of ouabain . . . under conditions in which no inhibition of the enzyme is detectable". Although very sensitive new methods for assaying ($Na^+ + K^+$)-ATPase had been developed, the homogenization of cardiac tissue in the presence of 1 M KCl, Tris/HCl and Tris/EDTA may have caused a dissociation of the ouabain–enzyme complex. We therefore have assayed ATPase activity in those membranes that had bound [^{3}H]ouabain. Although we did measure the [^{3}H]ouabain–enzyme complex at a [^{3}H]ouabain concentration of 10^{-7} M for instance, there was no detectable ($Na^+ + K^+$)-ATPase inhibition (Fig. 5). At higher glycoside concentrations there was always a greater extent of [^{3}H]ouabain bound than enzyme activity inhibited. This was probably not due to an artifact or an experimental error, since in previous experiments with isolated cell membranes from human cardiac tissue and ox brain, as well as from ox heart, we did find [^{3}H]ouabain binding and ($Na^+ + K^+$)-ATPase inhibition coinciding at the same concentrations when analyzed under identical conditions [19, 23]. Recent investigations (unpublished) show that in frog heart cell membrane preparations (rana esculenta) a dissociation between [^{3}H]ouabain binding and ($Na^+ + K^+$)-ATPase inhibition is found as well. Half-maximal [^{3}H]ouabain binding was measured at 3.5×10^{-9} M and half-maximal inhibition of the enzyme activity at 5×10^{-6} M (both assayed under identical conditions).

The IC_{50}-values agree with those of other investigators [7, 51] as well as the [^{3}H]ouabain binding data [56] although assayed in a different manner. Thus, we must conclude that ouabain binding to the membranes at least in rat heart does not necessarily cause ($Na^+ + K^+$)-ATPase inhibition and apparently inhibition of that enzyme is also not a mandatory prerequisite for the positive inotropic effect of ouabain. In order to further substantiate these conclusions we determined ($Na^+ + K^+$)-ATPase activity in intact cardiac tissue by assaying $^{86}Rb^+$-uptake in contracting ventricular strips. In these experiments the IC_{50} for ouabain was measured as 3×10^{-5} M. This value does not agree with the half-maximally effective ouabain concentration for positive inotropy (3×10^{-7} M) but rather with the IC_{50} for the ($Na^+ + K^+$)-ATPase (4×10^{-5} M). Thus, it seems quite unlikely that the discrepancy between the effective ouabain concentration for enzyme inhibition and positive inotropy is confined to the broken cell membrane preparation.

There are several groups reporting a dissociation between ($Na^+ + K^+$)-ATPase inhibition and positive inotropic effects [45–48, 59]. These investigators (except Peters *et al.* [48]), however, have found ATPase still inhibited while there was no longer any positive inotropic effect (after a wash-out procedure of cardiac glycosides). Our results rather point into the opposite direction: *positive inotropy but no ATPase inhibition.* Recently, there has been some, mainly electrophysiological, evidence that active cation transport of the cell membrane is activated by low doses of cardiac glycosides rather than inhibited [11]. Similar biochemical evidence showing a distinct stimulation of ($Na^+ + K^+$)-ATPase has been reported [27, 28, 30, 48] (for review see [54]). We did not, however, find an activation of ($Na^+ + K^+$)-ATPase, either in our membrane preparation or in the $^{86}Rb^+$-uptake (Fig. 4). Most other investigators were unable to demonstrate an activation of ($Na^+ + K^+$)-ATPase activity by low concentrations of cardiac glycosides either (for review see [61]). The electrophysiological evidence for such an activation of active cation transport in the presence of low concentrations of cardiac glycosides has been derived from conducting tissue preparations and their interpretation is complex. Other experiments of this kind in cardiac working muscle have rather rendered evidence for an inhibition of active cation transport by concentrations of cardiac glycosides having positive inotropic effects [12].

The half-maximal inhibition of ($Na^+ + K^+$)-ATPase activity by ouabain was measured at an ouabain concentration of 4×10^{-5} M. This value agrees with that found by Dransfeld *et al.* [15] (5×10^{-5} M) and by Repke *et al.* [51] (5.9×10^{-5} M), whereas Inturrisi and Papaconstantinou [34]

measured the IC_{50} as 6.8×10^{-4} M. As our experimental data concerning the ouabain-caused inhibition of this enzyme agree very well with that of other investigators, we must conclude that there is a serious discrepancy between half-maximal ouabain binding to the receptor for cardiac glycosides (3×10^{-7} M), between half-maximal positive inotropic effect (3×10^{-7} M) and half-maximal ($Na^+ + K^+$)-ATPase inhibition (4×10^{-5} M) and half-maximal inhibition of $^{86}Rb^+$-uptake (3×10^{-5} M) in rat heart. In a recent paper, Sharma and Banerjee [56] report in close agreement with our data a dissociation constant (K_D) for specific [^{3}H]ouabain binding to a rat heart microsomal preparation of 3.2×10^{-7} M and an even higher IC_{50} for ($Na^+ + K^+$)-ATPase inhibition of 1.2–1.4×10^{-4} M. Thus, the discrepancy between [^{3}H]ouabain binding and ($Na^+ + K^+$)-ATPase inhibition in rat heart cell membranes appears to be a fact.

We therefore think that our data rather rule out a causal relationship between ($Na^+ + K^+$)-ATPase inhibition and increase of force of contraction in rat heart, as this enzyme is not inhibited at low concentrations of ouabain producing positive inotropy.

What are the possible reasons for the insensitivity of rat heart to cardiac glycosides?

In man, distinct positive inotropic effects are measured in nanomolar serum concentrations of ouabain [31], and half-maximal ouabain receptor binding occurs at 2.5×10^{-9} M [20]. These values show that the rat heart is about 100 times less sensitive to ouabain than hearts from, for example, humans, dogs and cats [14]. However, cardiac glycosides apparently have a higher affinity to nervous tissue in the rat [14].

($Na^+ + K^+$)-ATPase preparations from rat brain are inhibited at lower glycoside concentrations (5×10^{-7} M, [1]) than that from rat heart (4×10^{-5} M). In as yet unpublished ouabain binding experiments we have also found that rat brain membranes contain a cardiac glycoside receptor which is different from that found in rat heart (K_D of the ouabain–receptor complex, 10^{-8} M).

In human heart the cardiac glycoside receptor density was determined as about 1000 receptors/μm^2 [16] versus about 660/μm^2 in rat heart. These values agree very well with those determined by McCall [43] for cultured rat heart cells: 720/μm^2 and 760/μm^2 in cat ventricle [44]. As these numbers of cardiac glycoside receptors are really quite similar, the slightly smaller amount in the rat heart is probably not a reason for the low sensitivity to cardiac glycosides.

Tobin *et al.* [60] and Akera *et al.* (2) have suggested that the known species differences in cardiac glycoside sensitivity may be caused by a rapid dissociation of the drug from its receptor. In fact, the dissociation rate constants at 37° are quite different: human $k_{-1} = 3 \times 10^{-4}$ sec^{-1} [22], rat $k_{-1} = 10^{-2}$ sec^{-1}, while the association rate constants measured under identical conditions are rather similar: human $k_{+1} = 1.2 \times 10^5$ M^{-1} sec^{-1} [22], rat $k_{+1} = 4.3 \times 10^4$ m^{-1} sec^{-1}. The dissociation rate constants of other species fall in between [2, 17, 18, 60]. This does not give a comprehensive explanation, though, because a faster dissociation rate in the presence of an unchanged association rate naturally results in a raised dissociation constant (K_D) or decreased affinity of the receptor for the drug ($K_D = k_{-1}/k_{+1}$).

Our results, in accordance with previous work on other species [8, 26, 36, 44], clearly show that specific ouabain binding to contracting ventricular strips agrees with the ouabain effect on force of contraction measured under identical experimental conditions. The affinity of the receptor for cardiac glycosides in the rat heart is, however, very low—this apparently explains the low sensitivity of the rat heart to cardiac glycosides. This explanation agrees with recent experiments by Akera *et al.* [3] based on ($Na^+ + K^+$)-ATPase determinations and measurements of force of contraction in the presence of several drugs besides ouabain.

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