Biochemical Pharmacology, Vol. 32, No. 3, pp. 423–435, 1983.
Printed in Great Britain.

0006-2952/83/030423-13 $03.00/0

CONSEQUENCES OF SPECIFIC [^{3}H]OUABAIN BINDING TO GUINEA PIG LEFT ATRIA AND CARDIAC CELL MEMBRANES

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(*Received* 21 *December* 1981; *accepted* 10 *August* 1982)

Abstract—An analysis of [^{3}H]ouabain binding to electrically stimulated, contracting guinea pig left atria gave the following results.

(1) A non-linear Scatchard plot with at least two binding sites: a high-affinity site (K_D 1.1×10^{-6} M) with about 430 receptors/μm^2 related to positive inotropy, and a low-affinity site (K_D' 2.1×10^{-4} M) with about 18,000 receptors/μm^2, possibly related to ($Na^+ + K^+$)ATPase inhibition. A crude left atrial homogenate gave about 530 receptors/μm^2.

(2) Half-maximal positive inotropic effects occurred at about 4×10^{-7} M.

(3) $^{86}Rb^+$-uptake was significantly increased at all inotropic ouabain concentrations (10^{-7}–10^{-6} M). Toxic concentrations (above 2×10^{-6} M) inhibited $^{86}Rb^+$-uptake (half-maximal inhibition at about 5×10^{-6} M).

[^{3}H]Ouabain binding to partly purified guinea pig cardiac cell membranes showed: (a) linear Scatchard plots for (Mg^{2+}, Pi)- and (Na^+, ATP, Mg^{2+})-supported binding (K_D 1.18×10^{-7} M and 1.49×10^{-7} M, respectively); (b) non-linear Scatchard plots for (Tyrode + ATP)-supported binding (K_D 4.7×10^{-7} M; K_D' 6×10^{-6} M); and (c) half-maximal [^{3}H]ouabain binding occurred at a lower concentration (about 3.2×10^{-7} M) than half-maximal inhibition of ($Na^+ + K^+$)ATPase activity (about 7.2×10^{-7} M).

Thus, we conclude that there may be more than one type of ouabain binding site in guinea pig left atria, and that measurable inhibition of ($Na^+ + K^+$)ATPase is not necessarily related to positive inotropy in the guinea pig.

($Na^+ + K^+$)ATPase (EC 3.6.1.3), the enzyme system identified as the biochemical equivalent of the "sodium pump", has been implicated in the actions of cardiac glycosides for many years [1, 2]. *In vitro*, cardiac glycosides are specific inhibitors of this membrane-bound enzyme [3–6]. Many workers now agree with the concept that positive inotropy is caused by an inhibition of the sodium pump [7]. However, other workers have contended that binding of cardiac glycosides to ($Na^+ + K^+$)ATPase causes membrane changes which are responsible for the observed positive inotropy [8, 9] and, therefore, that inhibition of the sodium pump is coincidental rather than causal. Some controversial evidence, reviewed recently by Noble [10], suggests that ($Na^+ + K^+$)ATPase activity is increased by low but positive inotropic concentrations of cardiac glycosides. Okita and his co-workers [11, 12] furthermore have proposed that enzyme inhibition is only related to the toxic effects of cardiac glycosides; not, however, to the positive inotropic effects.

In purified ($Na^+ + K^+$)ATPase preparations, both stimulation of the enzyme [13, 14] and inhibition of the enzyme [2, 15, 16] by low concentrations of cardiac glycosides have been reported. A similar discrepancy has been reported in intact cardiac preparations when $^{86}Rb^+$-uptake or Na^+/K^+-concentrations have been measured as indicative of the "sodium pump" [17–19].

Thus, this wide and inconsistent spectrum of opinions regarding the role of ($Na^+ + K^+$)ATPase in the pharmacological effects of cardiac glycosides makes it important to analyse in the same species under identical conditions: (1) the amount of ouabain specifically bound to contracting cardiac tissue in relation to the pharmacological effects (positive inotropy, arrhythmia, contracture); (2) concomitant changes in $^{86}Rb^+$-uptake; (3) the [^{3}H]ouabain–receptor binding in isolated cell membranes; and (4) concomitant changes in ($Na^+ + K^+$)ATPase activity. A previous investigation performed in this way [18] has shown a considerable difference (about 50–100-fold) in the concentrations required to elicit positive inotropy and sodium pump inhibition in the relatively digitalis-insensitive rat. Experiments with sensitive species such as the cat and dog have shown good correlations between cardiac glycoside binding to tissues, the ensuing positive inotropy and sodium pump inhibition [20, 21], in contrast to the results with the rat [18]. However, if a dissociation between the cardiac glycoside concentrations needed to give positive inotropy and sodium pump inhibition is shown in one species, then the whole hypothetical mechanism of cardiac glycoside inhibition of the ($Na^+ + K^+$)ATPase eliciting the increase in the force of contraction is seriously challenged. However, the rat shows considerable electrophysiological differences [22] and is relatively digitalis-insensitive. The guinea pig, a species often used in cardiac glycoside research, is moderately digitalis-sensitive; its cardiac

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electrophysiology is similar to mammals; and the digitalis-induced inotropy can be quantified. Therefore, in the present study, we have determined under the same conditions the ouabain binding and its effects on contracting left atria and cardiac cell membranes of the guinea pig. These results can then be compared with the results of the same experiments previously carried out in the digitalis-sensitive cat and digitalis-insensitive rat.

MATERIALS AND METHODS

Materials. [^{3}H]Ouabain (sp. act. 16 Ci/mmole, lot No. 1162–062), was purchased from New England Nuclear (Dreieich, F.R.G.). ^{86}RbCl (sp. act. 6 mCi/mg, lot No. 10M13J3) and ^{42}KCl (sp. act. 16 μCi/mg K) and [^{14}C]inulin (sp. act. 2.02 μCi/mg, CSA.399 batch 66) were purchased from Amersham Buchler (Braunschweig, F.R.G.). All other chemicals were of analytical grade and obtained from Boehringer-Mannheim (Mannhein, F.R.G.) or E. Merck (Darmstadt, F.R.G.).

Electrically-stimulated guinea pig left atria. Guinea pigs (both sexes, 250–400 g, about 6 weeks old) were killed by a blow to the back of the neck. The left atrium was rapidly freed from ventricular and fatty tissue and suspended under a resting tension of about 1 g (maximal preload) in an organ bath (75 ml) maintained at 35° containing a medium of the following composition (mM): Na^+, 149.3; K^+, 5.4; Ca^{2+}, 1.8; Mg^{2+}, 1.05; Cl^-, 148.0; HCO_3^-, 11.9; $H_2PO_4^-$, 0.43; glucose, 10; pH 7.4; continuously gassed with 95% O_2 and 5% CO_2 as reported earlier [18]. The atrium was stimulated by two platinum electrodes with field stimulation. The developed-force was measured isometrically with an inductive force displacement transducer (W. Fleck, Mainz, F.R.G.) attached to a Hellige recorder. The preparations were allowed to equilibrate for 60 min. The bath solution was changed once at about 45 min. Electrical stimulation was from a Grass SD9 stimulator (frequency 1 Hz, duration 10 msec, intensity 10–20% above threshold).

Each atrial preparation was used for only one ouabain concentration.

[^{3}H]*Ouabain binding to contracting guinea pig left atria.* Atria were prepared as described earlier. After equilibration, [^{3}H]ouabain (about 10^7 cpm = about 5×10^{-9} M) was added together with differing amounts of unlabelled ouabain. Force of contraction was measured continuously, and after 30 min (effect of added ouabain was stable) the atria were rinsed for about 30 sec with distilled water, blotted and weighed. Atria rinsed with Tyrode solution showed no differences in the amount of [^{3}H]ouabain bound. Further studies on the washout of [^{3}H]ouabain or [^{14}C]inulin were performed. Ouabain (1×10^{-4} M) was added for 30 min, followed by [^{3}H]ouabain or [^{14}C]inulin (about 10^7 cpm) for 30 min. The atria were then rinsed for varying times. Extracellular space as measured by [^{14}C]inulin was 33.1 ± 1.3% (N = 4), which is similar to previous results [23, 24]. The amount of radioactivity was assayed in a scintillation counter (Betaszint BF 5000) after dissolving the atria in NaOH (1 M, 1 ml) at 60° for 120 min and addition of 10 ml scintillation fluid [Unisolve 1 (Koch-Light Laboratories, W. Zinsser, Frankfurt am Main, F.R.G.)].

All values were measured as counts per minute, against an external standard. The efficiency of the scintillation counter for tritium was about 45%.

$^{86}Rb^+$-uptake in contracting guinea pig left atria. Atria were prepared as described earlier. After equilibration, differing amounts of unlabelled ouabain were added. The force of contraction was measured continuously. When the effect of the added ouabain was stable, a tracer amount of ^{86}RbCl (about 3 μCi = about 6×10^6 cpm) was added. After 10 min incubation in the presence of $^{86}Rb^+$, the force of contraction was measured and the atria were rinsed, weighed, dissolved and the radioactivity counted as for the [^{3}H]ouabain binding experiments.

In further experiments, $^{86}Rb^+$-uptake was measured under the following conditions.

(1) ^{86}RbCl (about 3 μCi) was added *with* the unlabelled ouabain. After incubation for 30 min, the radioactivity was measured.

(2) Guinea pigs were treated with reserpine (0.5 mg/kg i.p. on each of 2 days before the experiments). $^{86}Rb^+$-uptake was measured for 10 min after equilibration of the effects of ouabain (4×10^{-7} M, 8×10^{-7} M) as described in (1).

(3) Atria were suspended in bathing solution in which KCl (5.4 mM) had been replaced by RbCl (2 mM) as used by Akera *et al.* [22]. $^{86}Rb^+$-uptake was measured for 10 min after equilibration of the effects of ouabain (2×10^{-7} M) or after contracture occurred with ouabain (1×10^{-5} M, 1×10^{-4} M), as described in (1).

(4) The atria were stimulated at either 3 or 5 Hz. $^{86}Rb^+$-uptake was measured for 10 min after equilibration of the effects of differing concentrations of unlabelled ouabain as described in (1).

$^{42}K^+$-uptake in guinea pig left atria. Atria were prepared as described earlier. After equilibration, differing amounts of unlabelled ouabain were added. The force of contraction was measured continuously. When the effect of the added ouabain was stable, a tracer amount of ^{42}KCl (about 3 μCi = about 6×10^6 cpm) was added. After 10 min incubation in the presence of $^{42}K^+$, the force of contraction was measured, and the atria were rinsed, blotted, weighed and the radioactivity counted as described earlier.

[^{3}H]*Ouabain binding to guinea pig left atria homogenate.* The left atria of 10 guinea pigs were rapidly excised and weighed. The left atria were homogenized in a Potter–Elvehjem homogenizer in 12 ml 0.25 M sucrose–EDTA (pH 7) and centrifuged at 25,000 rpm for 30 min at 0° (Beckmann L-5, rotor 35 Ti). The pellet was resuspended in 5.5 ml EDTA (1 mM) solution. [^{3}H]Ouabain binding to this crude guinea pig left atria homogenate was then measured as described later for guinea pig cardiac cell membranes. Non-specific binding (binding in the presence of 10^{-3} M unlabelled ouabain) was about 6% of total radioactivity bound.

Preparation of ($Na^+ + K^+$)*ATPase-containing guinea pig cardiac cell membranes.* After the left atrium was removed for the experiments described earlier, the rest of the guinea pig heart was frozen

at −40°. About 100 g of this frozen material was used in the preparation of each batch of ($Na^+ + K^+$)ATPase-containing cell membranes. The partial purification procedure has been described previously [18]. The final sediment, homogenized in 1 mM EDTA (pH 7.25) (60 ml), was used for the experiments. The ($Na^+ + K^+$)ATPase activity, determined by the coupled optical assay method [25], was between 0.2 and 0.3 μmoles ATP hydrolyzed/min/mg protein at 37°. About 90–95% of the total activity was inhibited by 10^{-3} M ouabain.

[^{3}H]*Ouabain binding to guinea pig cardiac cell membranes*. The procedures used for these experiments have been described in detail elsewhere [15, 26]. Bound ouabain was quantitated by a rapid filtration method (Whatman GF/C glass filter membranes) to separate free ouabain from membrane-bound ouabain. Non-specific binding (binding in the presence of 10^{-3} M unlabelled ouabain) was less than 5% of total radioactivity bound to the cell membranes. Experiments were performed in duplicate assays and at least twice.

These incubation media were used: (1) Mg^{2+} + Pi supported binding—3 mM $MgCl_2$, 3 mM imidazole/PO_4, about 2×10^{-9} M [^{3}H]ouabain in 50 mM imidazole/HCl buffer (pH 7.25); (2) $Na^+ + Mg^{2+}$ + ATP supported binding—150 mM NaCl, 3 mM $MgCl_2$, 3 mM ATP, about 2×10^{-9} M [^{3}H]-ouabain in 50 mM imidazole/HCl buffer, (pH 7.25); (3) ATP–Tyrode solution supported binding—3 mM ATP, about 2×10^{-9} M [^{3}H]ouabain in Tyrode solution (pH 7.4). The total volume for all binding assays was 2 ml. Incubation time was 2 hr.

[^{3}H]*Ouabain binding to guinea pig cardiac cell membranes and inhibition of* ($Na^+ + K^+$)*ATPase*. Cardiac cell membranes were incubated at 37° in 50 mM imidazole/HCl (pH 7.25), 3 mM $MgCl_2$, 3 mM imidazole/PO_4, 2×10^{-9} M [^{3}H]ouabain and increasing concentrations of ouabain (10^{-9}–10^{-3} M), total volume 2 ml. After 2 hr incubation, 0.5 ml of the incubation mixture were used for determination of [^{3}H]ouabain binding to the membranes (rapid filtration method) and 0.5 ml were used for determination of ($Na^+ + K^+$)ATPase activity (coupled optical assay). In the coupled optical assay, NADH hydrolysis was linear during the duration of the assay. Unlabelled ouabain was added to each ($Na^+ + K^+$)ATPase assay so that the ouabain concentration in each assay was the same as in the incubation mixture.

This experiment was repeated 5 times. All experiments showed similar results. Results of a single experiment are given because each experiment was performed with a different enzyme preparation.

For one experiment, the ($Na^+ + K^+$)ATPase assay mixture was rapidly filtered immediately after the enzyme activity had been measured. The amount of ouabain bound before and after the ($Na^+ + K^+$)ATPase assay could then be compared.

General. All values are given as means ± S.E.M. A surface area of 1000 cm^2/g wet weight has been used [22]. Protein was measured by the procedure of Lowry *et al.* (28) with bovine serum albumin as standard. Statistical analyses were performed by the Student's *t*-test. The criterion for statistical significance was a P value of less than 0.05.

RESULTS

Ouabain-induced positive inotropy was observed within the concentration range of 1×10^{-7}–2×10^{-6} M. At a stimulation frequency of 1 Hz, with a [K^+] of 5.4 mM and [Ca^{2+}] of 1.8 mM, a bath concentration of 4.5×10^{-7} M ouabain produced approximately a 200% increase in the force of contraction. The pre-drug force of contraction was markedly influenced by bath composition and stimulation frequency. The initial force of contraction was 470 ± 25 mg (33.8 ± 1.8 g/g wet weight) (1 Hz, 5.4 mM KCl, N = 85), 960 ± 70 mg (51.6 ± 3.8 g/g wet weight) (3 Hz, 5.4 mM KCl, N = 32), 755 ± 25 mg (38.3 ± 1.3 g/g wet weight) (5 Hz, 5.4 mM KCl, N = 42) and 1200 ± 130 mg (83.3 ± 9.0 g/g wet weight) (1 Hz, 2 mM RbCl replacing 5.4 mM KCl, N = 10). Virtually all atria developing a force greater than 2000 mg, independent of ouabain concentration and bath composition, showed toxicity. For some atria which developed a force greater than 2000 mg, the force of contraction was stable for several minutes before toxicity occurred. Very few atria became toxic at a developed force of less than 1500 mg. Between these values, an increasing percentage of atria developed toxicity. Toxicity was usually observed as ectopic beats, sometimes preceded by a gradual decline in the force of contraction. As the ouabain concentration was further increased, contracture developed after progressively shorter periods of ectopic beats.

Determination of non-specific binding

The washout of [^{3}H]ouabain and [^{14}C]inulin from contracting guinea pig left atria was measured since

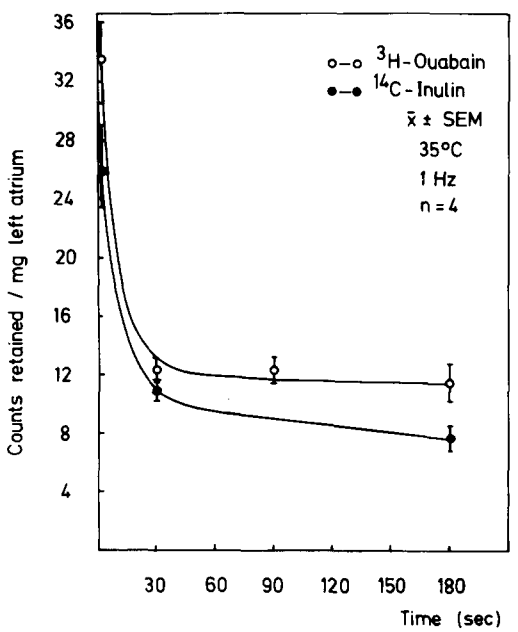

Fig. 1. Washout of [^{3}H]ouabain or [^{14}C]inulin from guinea pig left atria. The left atria were incubated with ouabain (1×10^{-4} M) for 30 min, followed by [^{3}H]ouabain or [^{14}C]inulin (about 10^7 cpm) for 30 min. The [^{3}H]ouabain or [^{14}C]inulin retained after various washing times was then measured.

an incomplete washout of [^{3}H]ouabain from the extracellular space would be represented in a Scatchard plot as a low-affinity site. For these experiments high-affinity ouabain binding sites were occupied by a 30-min incubation with ouabain (1×10^{-4} M) before [^{3}H]ouabain or [^{14}C]inulin were added for 30 min. These results are shown in Fig. 1. Washing for periods longer than 30 sec does not decrease the amount of [^{3}H]ouabain retained by the atrium. However, longer washing times do slightly decrease the amount of [^{14}C]inulin retained, showing that these two compounds have different washout kinetics in guinea pig left atria. Both [^{3}H]ouabain and [^{14}C]inulin values after 180 sec washing have been used as non-specific binding in the Scatchard plot calculations, as discussed later.

Ouabain binding to contracting guinea pig left atria

[^{3}H]Ouabain binding to intact left atria stimulated at 1 Hz was measured to determine the number of active binding sites for ouabain. Fig. 2 shows that the degree of positive inotropy was closely related to the amount of ouabain specifically bound over the ouabain concentration range which gave positive inotropy only (1×10^{-7}–2×10^{-6} M). At a ouabain concentration of 8×10^{-7} M, at which concentration the maximal increase in the force of contraction is measured, only about 25% of the total specific binding is observed. At higher ouabain concentrations, increasing amounts of ouabain are bound, but toxicity, not increased inotropy, is observed.

A Scatchard plot of this ouabain binding is shown in Fig. 3. The curvilinear Scatchard plot could indicate at least two classes of binding sites. Mathematical analysis of the binding shown in Fig. 3A performed according to Weidemann *et al.* [29] with the assumption of only two classes of binding sites, gives the following results: a high-affinity/low-capacity site (K_D 1.1×10^{-6} M, about 0.72 pmoles bound/mg) and a low-affinity/high-capacity site (K_D' 2.1×10^{-4} M, about 30 pmoles bound/mg). The binding to the high-affinity site corresponds to about 430 binding sites/μm^2 left atria tissue. The value for the low-affinity site should be taken as a rough estimate only, since there are insufficient data to determine the homogeneity of these sites. The rapidly increasing value of bound ouabain between 1×10^{-5} and 1×10^{-4} M ouabain may be due either to non-specific binding or to the low-affinity/high-capacity receptor. These binding sites would seem to be unrelated to the positive inotropic effect but may be related to the inhibition of $^{86}Rb^+$-uptake or to the inhibition of the ($Na^+ + K^+$)ATPase. The values for the dissociation constant and the maximum picomoles ouabain bound/mg wet weight vary depending on the value chosen for the non-specific ouabain binding. If the amount of [^{3}H]ouabain bound at a concentration of 1×10^{-4} M ouabain after a 180-sec washing (Fig. 1) is taken as non-specific binding (Fig. 3B), the dissociation constant is calculated as 2.1×10^{-6} M with 1.2 pmoles ouabain bound/mg, or about 700 receptors/μm^2. This Scat-

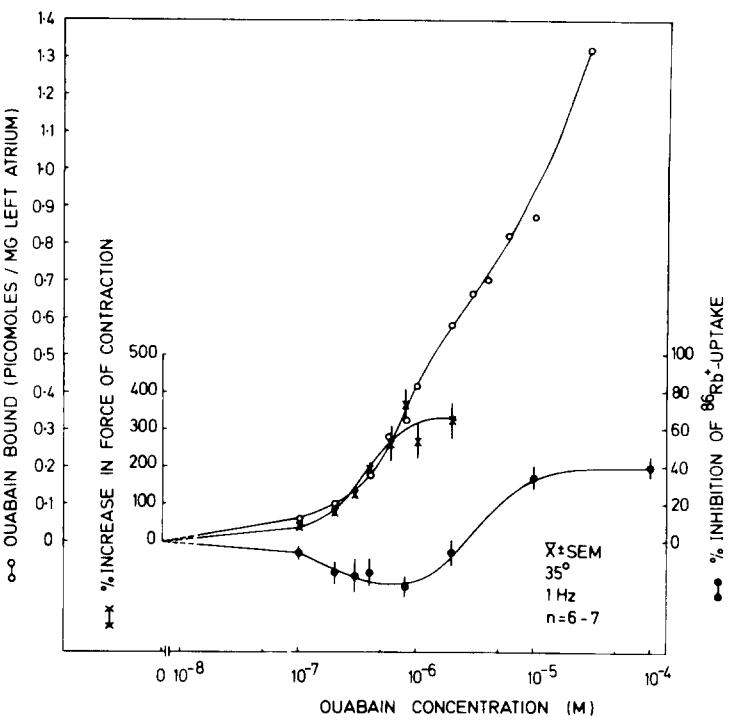

Fig. 2. Concentration–response curves for the positive inotropic effect of ouabain, for specific [^{3}H]-ouabain binding and for the ouabain effect on $^{86}Rb^+$-uptake in contracting guinea pig left atria. The force of contraction was measured after the effect of each ouabain concentration was stable. For the [^{3}H]ouabain binding, ouabain binding in the presence of 1×10^{-4} M ouabain has been defined as non-specific binding. The $^{86}Rb^+$-uptake was measured by adding tracer amounts of $^{86}RbCl$ for 10 min after a stable effect had been achieved with each ouabain concentration.

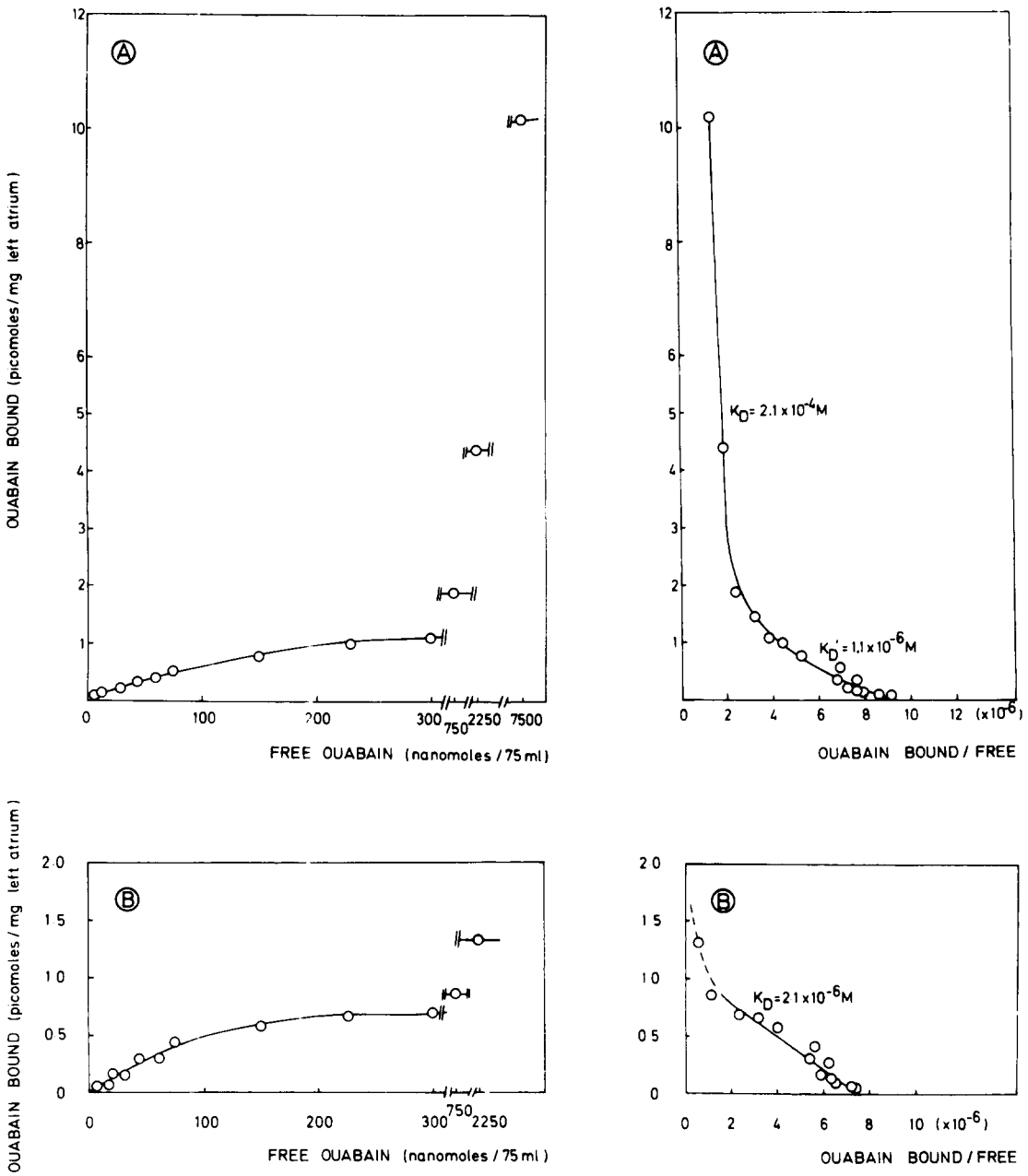

Fig. 3. [^{3}H]Ouabain binding to contracting guinea pig left atria. Each point is the mean of six or seven experiments. [^{3}H]Ouabain bound is given as picomoles bound/mg wet weight left atria in 75 ml. The data were plotted according to Scatchard [50]. (A) The amount bound to the atria and the corresponding Scatchard plot when non-specific binding is defined as zero. Mathematical analysis [29] for two binding sites gave a high-affinity site (K_D 1.1 × 10^{-6} M, about 0.72 pmoles bound/mg) and a low-affinity site (K_D' 2.1 × 10^{-4} M, about 30 pmoles bound/mg). (B) The amount bound to the atria and the corresponding Scatchard plot when the [^{3}H]ouabain bound at a bath concentration of 1 × 10^{-4} M ouabain is defined as non-specific binding. The amount specifically bound under this definition has been used in Fig. 2.

chard plot is linear, up to a value of 1 × 10^{-5} M ouabain. Maximal inhibition of $^{86}Rb^+$-uptake occurs at a concentration of about 1 × 10^{-5} M (Fig. 2), much lower than the ouabain concentration used for the non-specific binding value (Fig. 3B). However, if the amount of [^{14}C]inulin retained after a 180-sec washing (Fig. 1) is taken as non-specific binding, then the resulting Scatchard plot will be curvilinear since this non-specific binding value is less than the amount of [^{3}H]ouabain bound at 1 × 10^{-4} M ouabain.

A Scatchard plot of ouabain binding to a crude guinea pig left atria homogenate in the presence of Mg^{2+} and Pi, can be used to estimate the binding site number in the atria. This Scatchard plot (Fig.

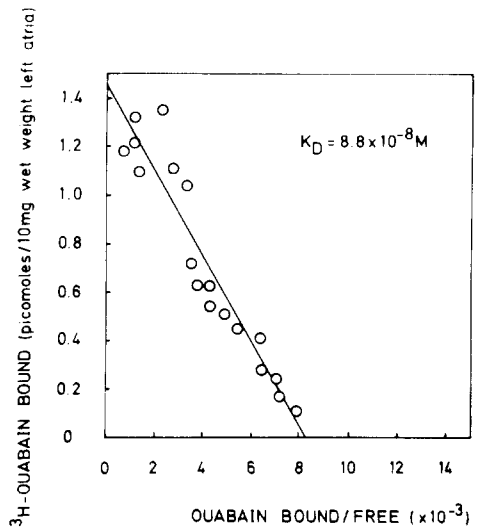

Fig. 4. [^{3}H]Ouabain binding to a crude homogenate of guinea pig left atria. The crude homogenate [1 mg wet weight guinea pig left atria, ($Na^+ + K^+$)ATPase activity 0.17 μmoles ATP hydrolyzed per min at 37°] was incubated at 37° in 3 mM $MgCl_2$, 3 mM imidazole/PO_4, about 2×10^{-9} M [^{3}H]ouabain and 50 mM imidazole/HCl (pH 7.25) and increasing amounts of unlabelled ouabain for 2 hr. The data have been plotted according to Scatchard [50]. The dissociation constant (K_D) of the ouabain-receptor complex was 8.8×10^{-8} M. The number of specific receptors per g wet weight left atria was calculated as 8.7×10^{13}.

4) shows one type of binding sites with a dissociation constant of 8.8×10^{-8}M, a value similar to the dissociation constant in partially purified ($Na^+ + K^+$)ATPase-containing cell membranes under optimal binding conditions. The number of binding sites was calculated as about 8.7×10^{13} sites/g wet weight or about 530 binding sites/μm^2. Lüllmann *et al.* [30] have given a value of 5×10^{14} binding sites/g wet weight in guinea pig left atria, although these workers measured "tissue uptake" rather than specifically bound ouabain. Other researchers have given values of 660 sites/μm^2 in rat ventricular slices [18], and about 760 sites/μm^2 in cat ventricular muscle [31]. Thus, the number of high-affinity binding sites in contracting guinea pig left atria is similar to the number of binding sites in the crude left atria homogenate, and to the literature values for other species.

Influence of ouabain on the $^{86}Rb^+$- or $^{42}K^+$-uptake of electrically stimulated guinea pig left atria

Fig. 2 shows the $^{86}Rb^+$-uptake measured when $^{86}RbCl$ was added for 10 min after a stable effect had been achieved at each ouabain concentration. The $^{86}Rb^+$-uptake was consistently, slightly increased at concentrations which are purely inotropic. Inhibition of uptake was only seen at a concentration which caused contracture (1×10^{-5} M). The $^{86}Rb^+$-uptake was significantly increased over the pre-drug value at all inotropic ouabain concentrations except the maximum inotropic concentration (2×10^{-6} M). The $^{86}Rb^+$-uptake at toxic ouabain concentrations (1×10^{-5} and 1×10^{-4} M) was significantly less than at all inotropic ouabain concentrations. The effect of mildly toxic doses (between 2×10^{-6} and 1×10^{-5} M) could not be observed as a stable inotropic or toxic effect cannot be achieved within 30 or 40 min. At these concentrations, at least 90% of the total ouabain binding has occurred after 20 min (results not shown). Although $^{86}Rb^+$-uptake values for the first 30 min after ouabain has been added (Fig. 5) will be non-equilibrium values, these values will include effects due to the maximal ouabain binding without the problem of defining stable effects of differing degrees of toxicity. Under these non-equilibrium conditions, inotropic concentrations also caused a slight increase in $^{86}Rb^+$-uptake while

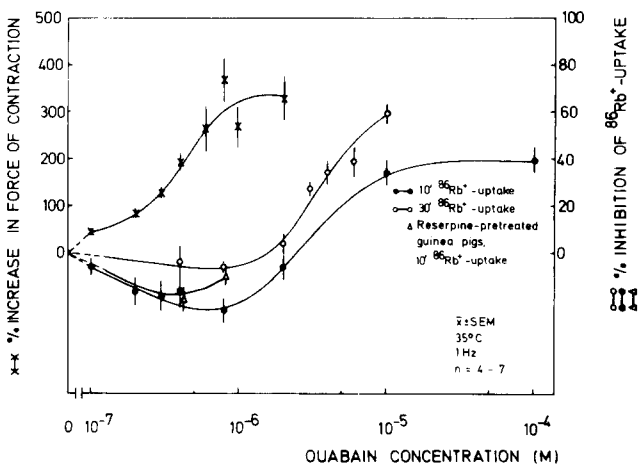

Fig. 5. Effect of ouabain on $^{86}Rb^+$-uptake in contracting guinea pig left atria. The force of contraction measurements are the combined results of [^{3}H]ouabain binding and $^{86}Rb^+$-uptake experiments at 1 Hz. (●—●) $^{86}Rb^+$-uptake when measured for 10 min after a stable force of contraction had been achieved at each ouabain concentration. (○—○) $^{86}Rb^+$-uptake when a tracer amount of $^{86}RbCl$ was added *with* each ouabain concentration for 30 min. (△—△) $^{86}Rb^+$-uptake in reserpine-pretreated guinea pigs when measured for 10 min after a stable force of contraction had been achieved at each ouabain concentration.

increasingly toxic concentrations showed a stepwise inhibition of $^{86}Rb^+$-uptake.

The results of these experiments show that, in contracting guinea pig left atria, $^{86}Rb^+$-uptake is inhibited only by toxic concentrations and not by inotropic concentrations of ouabain. $^{86}Rb^+$-uptake was measured in the atria of reserpine-pretreated guinea pigs at purely inotropic ouabain concentrations (Fig. 5). The force of contraction was increased to a similar extent as in non-treated animals ($160 \pm 36\%$ at 4×10^{-7} M ouabain, $190 \pm 25\%$ at 8×10^{-7} M ouabain). As in non-treated guinea pigs, $^{86}Rb^+$-uptake was slightly increased at these ouabain concentrations. Thus, the lack of inhibition of $^{86}Rb^+$-uptake at inotropic ouabain concentrations is not due to interference from catecholamine release from the atria.

$^{86}Rb^+$-uptake studies were performed at a stimulation frequency of 3 Hz (Fig. 6). An increased stimulation frequency should increase sodium influx [32, 33]. The increased sodium flux can be observed as an increase in the pre-drug force of contraction [22]. Toxicity was seen at a lower ouabain concentration than with 1-Hz stimulation, as expected from earlier studies [17]. However, as in the 1-Hz studies, increased $^{86}Rb^+$-uptake was seen at inotropic concentrations while inhibition of $^{86}Rb^+$-uptake was seen only at toxic ouabain concentrations.

The pre-drug force of contraction was slightly lower with 5-Hz stimulation than with 3-Hz stimulation. Pre-drug $^{86}Rb^+$-uptake was similar under both

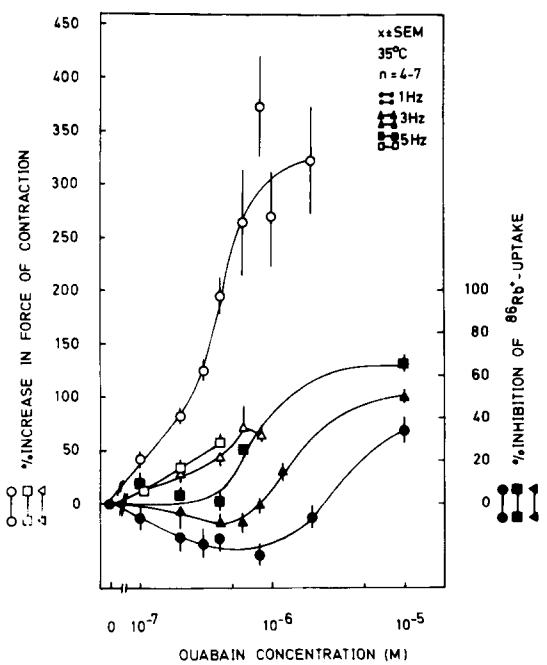

Fig. 6. Effect of ouabain on $^{86}Rb^+$-uptake in contracting guinea pig left atria at different stimulating frequencies. Increase in force of contraction at 1 Hz (○—○), 3 Hz (△—△) and 5 Hz (□—□) and $^{86}Rb^+$-uptake at 1 Hz (●—●), 3 Hz (▲—▲) and 5 Hz (■—■) are plotted against ouabain concentration. $^{86}Rb^+$-uptake was measured for 10 min after a stable effect had been achieved at each ouabain concentration.

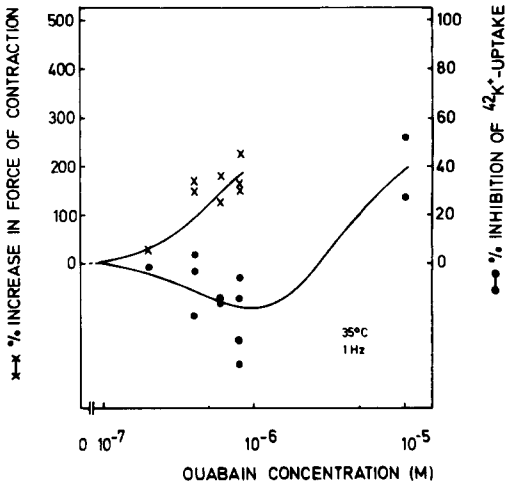

Fig. 7. Effect of ouabain on $^{42}K^+$-uptake in contracting guinea pig left atria. The increase in force of contraction (×) and change in ^{42}K-uptake (●) are given for individual experiments at different ouabain concentrations. $^{42}K^+$-uptake was measured for 10 min after a stable effect had been achieved at each ouabain concentration.

stimulating frequencies and higher than with 1-Hz stimulation. With 5-Hz stimulation, inotropic concentrations of ouabain produced no change in $^{86}Rb^+$-uptake (Fig. 6). As in the 1- and 3-Hz studies, inhibition of $^{86}Rb^+$-uptake was seen only at toxic ouabain concentrations.

A further increase in the pre-drug force of contraction and $^{86}Rb^+$-uptake was observed in the studies where RbCl (2 mM) replaced KCl (5.4 mM). Under these conditions, the highest non-toxic ouabain concentration was 2×10^{-7} M, which is much lower than in the studies with KCl (5.4 mM). The increase in the force of contraction at this concentration was $32 \pm 7\%$. As in the previous studies at 3 and 5 Hz, there was no significant change in $^{86}Rb^+$-uptake at this concentration while toxic concentrations of ouabain (1×10^{-5} M, 1×10^{-4} M) gave a significant inhibition of $^{86}Rb^+$-uptake (64.3 ± 6.8 and $73.8 \pm 1.9\%$ inhibition, respectively).

$^{42}K^+$-uptake was measured at several inotropic concentrations of ouabain and after contracture had occurred with 1×10^{-5} M ouabain. The uptake of $^{42}K^+$ observed after a stable effect had been achieved at each ouabain concentration was increased at inotropic concentrations (Fig. 7), as was $^{86}Rb^+$-uptake at these concentrations. As in the $^{86}Rb^+$-uptake studies, $^{42}K^+$-uptake was inhibited by a toxic concentration of ouabain (1×10^{-5} M).

Ouabain binding to isolated guinea pig cardiac cell membranes

Binding of [^{3}H]ouabain to guinea pig cardiac cell membranes in the presence of 3 mM $MgCl_2$ and 3 mM phosphate reached a stable maximum value within 5 min. The association rate constant as determined from the initial rate of [^{3}H]ouabain binding [26] was calculated as $4.9 \times 10^4\ M^{-1}\ sec^{-1}$.

The dissociation rate for ouabain was measured

in a "chase experiment" as described previously [18]. The dissociation followed first-order kinetics at least for the first 5 min giving a dissociation rate constant at 37° of 1.01×10^{-2} sec^{-1} ($t_{1/2}$ dissociation 69 sec). The dissociation constant (K_D) calculated from these results ($K_D = k_{-1}/k_{+1}$) was 2.06×10^{-7} M. The dissociation constant can also be calculated from a Scatchard analysis of the amount of [^{3}H]ouabain bound at equilibrium in the presence of increasing amounts of unlabelled ouabain. The Scatchard plot is shown in Fig. 8. This plot shows only one type of ouabain binding site with K_D values of 1.18×10^{-7} M (Mg^{2+} + Pi supported binding) and 1.49×10^{-7} M ($Na^+ + Mg^{2+}$ + ATP supported binding). However, binding supported by Tyrode solution + 3 mM ATP gave a curved Scatchard plot (Fig. 8). Mathematical analysis performed according to Weidemann *et al.* [29] with the assumption of two binding sites gave these results: a high-affinity/low-capacity site (K_D 4.7×10^{-7} M, about 15% of binding sites) and a low-affinity/high-capacity site (K_D' 6.0×10^{-6} M, about 85% of binding sites).

[^{3}H]*Ouabain binding to cardiac cell membranes and inhibition of* ($Na^+ + K^+$)*ATPase activity*

These two parameters were measured in samples taken from the same incubation mixture which contained [^{3}H]ouabain, different amounts of unlabelled ouabain and cardiac cell membranes. As can be seen in Fig. 9, ouabain binds to the enzyme at lower concentrations than cause an equivalent inhibition of the enzyme. Half-maximal [^{3}H]ouabain binding occurs at about 3.2×10^{-7} M, a concentration similar to the Scatchard plot dissociation constant for this experiment (2.6×10^{-7} M). However, half-maximal inhibition of ($Na^+ + K^+$)ATPase activity occurs at about 7.2×10^{-7} M. This is a much smaller difference than was observed in rat cardiac cell membranes (about 100-fold difference) [18] but no difference was observed in cat cardiac cell membranes [20].

Because of the relatively fast dissociation rate of [^{3}H]ouabain bound to guinea pig cardiac cell membranes ($t_{1/2}$ 69 sec), the ($Na^+ + K^+$)ATPase assay mixture was rapidly filtered after the enzyme activity had been measured. The results are presented in Fig. 9. A similar dissociation of effects is observed. The Scatchard plot dissociation constant of the binding measured after the ($Na^+ + K^+$)ATPase assay was 2.2×10^{-7} M.

DISCUSSION

There is a wide and inconsistent spectrum of opinions regarding the role of the (Na^+ +

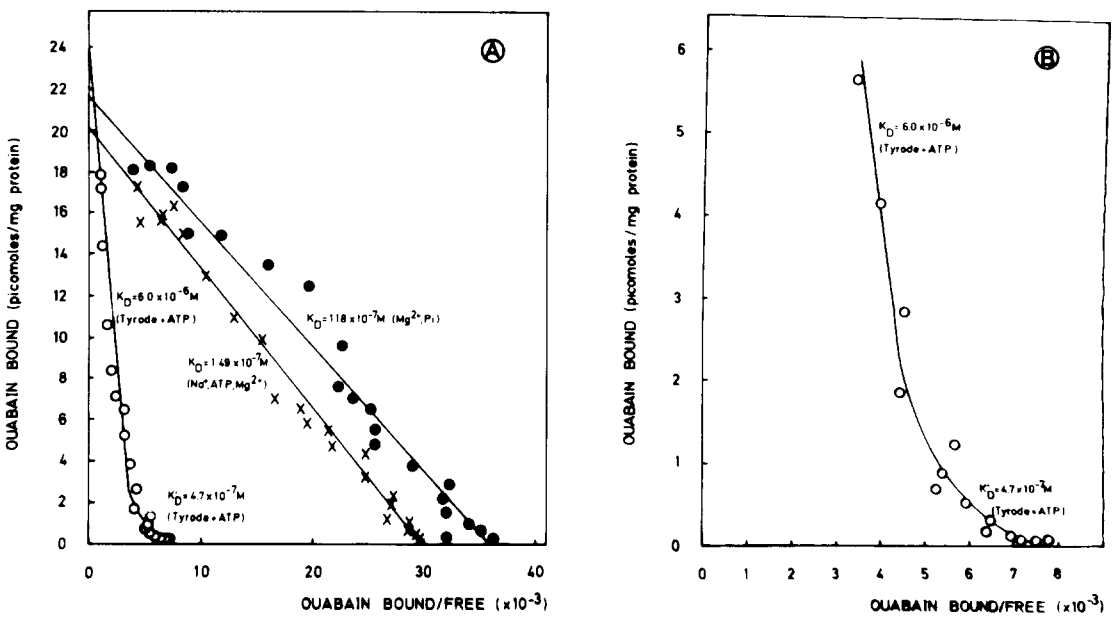

Fig. 8. [^{3}H]Ouabain binding to guinea pig cardiac cell membranes, plotted according to Scatchard [50]. Cardiac cell membranes [0.47 mg protein, ($Na^+ + K^+$)ATPase activity 0.21 μmoles ATP hydrolyzed per mg protein per min at 37°] were incubated in either a (Mg^{2+}, Pi)-medium [3 mM $MgCl_2$, 3 mM imidazole/PO_4, 2×10^{-9} M [^{3}H]ouabain, 50 mM imidazole/HCl (pH 7.25), final volume 2 ml] or a (Na^+, ATP, Mg^{2+})-medium [150 mM NaCl, 3 mM ATP, 3 mM $MgCl_2$, 2×10^{-9} M [^{3}H]ouabain, 50 mM imidazole/HCl (pH 7.25), final volume 2 ml] at 37° for 2 hr. The dissociation constants (K_Ds) were 1.18×10^{-7} M [(Mg^{2+}, Pi)-supported binding] and 1.49×10^{-7} M [(Na^+, ATP, Mg^{2+})-supported binding]. Cardiac cell membranes [0.93 mg protein, ($Na^+ + K^+$)ATPase activity 0.21 μmoles ATP hydrolyzed per mg protein per min at 37°] were incubated in Tyrode solution + 3 mM ATP, with 2×10^{-9} M [^{3}H]ouabain, final volume 2 ml, at 37° for 2 hr. The Scatchard plot of this binding was curvilinear. Mathematical analysis [29] for two binding sites gave a high-affinity site (K_D 4.7×10^{-7} M, about 15% of binding sites) and a low-affinity site (K_D' 6×10^{-6} M, about 85% of binding sites). The binding of [^{3}H]ouabain to guinea pig cardiac cell membranes supported by Tyrode solution + 3 mM ATP is shown in more detail in 8B.

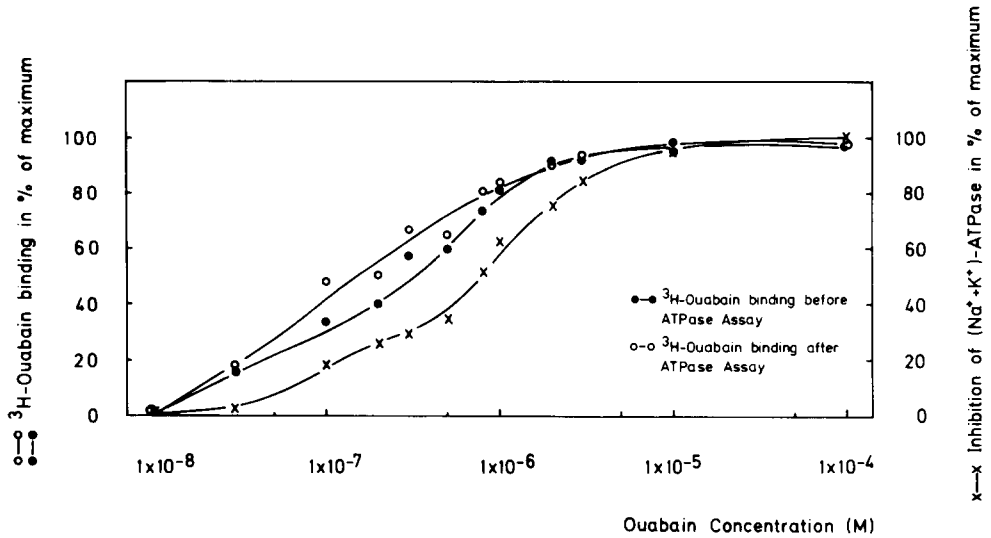

Fig. 9. [3H]Ouabain binding to guinea pig cardiac cell membranes and inhibition of ($Na^+ + K^+$)ATPase. Cardiac cell membranes [0.47 mg protein, ($Na^+ + K^+$)ATPase activity 0.21 μmoles ATP hydrolyzed per mg protein per minute at 37°] were incubated at 37° in 3 mM $MgCl_2$, 3 mM imidazole/PO_4, 2×10^{-9} M [3H]ouabain, 50 mM imidazole/HCl (pH 7.25) and increasing amounts of ouabain (10^{-8}–10^{-4} M), total volume 2 ml. After 2 hr, 0.5 ml of the incubation mixture were used for determination of [3H]ouabain binding to the membranes (rapid filtration method) (maximal ouabain binding was set as 100%) and 0.5 ml were used for determination of ($Na^+ + K^+$)ATPase activity (coupled optical assay [25]) (maximal ($Na^+ + K^+$)ATPase activity was set as 100%). It is important to note that the determination of ($Na^+ + K^+$)ATPase activity was performed in the presence of the same ouabain concentration as in the incubation medium. Thus, any dissociation of the [3H]ouabain–receptor complex will be followed by rebinding of unlabelled ouabain to the receptor. Non-specific [3H]ouabain binding (in the presence of 1×10^{-4} M ouabain) was subtracted. Half-maximal effects were seen at 3.2×10^{-7} M (ouabain binding) and 7.2×10^{-7} M [inhibition of ($Na^+ + K^+$)ATPase activity]. After determination of the ($Na^+ + K^+$)ATPase activity, the contents of the cuvette were rapidly filtered. The amount of [3H]ouabain bound was determined again. Maximal [3H]ouabain bound was set as 100%. These resulting values of [3H]ouabain bound to the membranes, after the ($Na^+ + K^+$)ATPase assay had been peformed, are still to the left of the enzyme inhibition curve. Thus, this figure shows that ouabain is bound to the cardiac cell membranes at concentrations lower than those which inhibit ($Na^+ + K^+$)ATPase activity.

K^+)ATPase in the pharmacological effects of digitalis. Many workers have proposed that inhibition of ($Na^+ + K^+$)ATPase leads to positive inotropy [27]. As an alternative hypothesis, Lüllmann and co-workers [9, 34] have proposed that digitalis binding to ($Na^+ + K^+$)ATPase releases calcium bound to phospholipids near the inner face of the enzyme without inhibition of the enzyme at non-toxic concentrations. Okita and co-workers [11, 12] have contended that ($Na^+ + K^+$)ATPase is only involved in the toxic effects of digitalis. Because of these differences, we have measured ouabain binding and its effects on the force of contraction and $^{86}Rb^+$-uptake under the same conditions, in the isolated left atria of the moderately digitalis-sensitive guinea pig.

Studies on partially purified cardiac ($Na^+ + K^+$)ATPase have shown both an inhibition (2, 15, 16) and a stimulation [13, 14] at digitalis concentrations considered to be in the therapeutic range. We have therefore measured ouabain binding and concomitant changes in ($Na^+ + K^+$)ATPase activity in partially purified ($Na^+ + K^+$)ATPase from the guinea pig heart.

Ouabain binding to contracting guinea pig left atria

Several researchers have studied the correlation between the amount of cardiac glycosides bound and positive inotropy. A good relationship was shown [35] between the myocardial content of digoxin and the observed inotropy in dogs. A good correlation was observed between the positive inotropic effects of digoxin and per cent inhibition of ($Na^+ + K^+$)ATPase in dogs [36]. A mathematical model was developed [31] to use [3H]ouabain binding to estimate digitalis receptor occupancy in cat papillary muscle. There was a good correlation between the number of receptors occupied and the increase in contractile force achieved. Studies with the relatively insensitive rat [18] showed also that half-maximal ouabain binding and half-maximal positive inotropic effects occur at the same ouabain concentration. The present study similarly shows a good correlation between the amount of ouabain bound to guinea pig left atria and the observed increase in the force of contraction.

Ghysel-Burton and Godfraind [37] have determined the dissociation constant and the capacity of the saturable binding site in guinea pig left atria at

a $[K^+]$ of 6 mM. The dissociation constant was 3.89×10^{-7} M with a binding capacity of 3.72×10^{-7} moles/kg wet weight. This binding capacity is lower than our results, as expected, since we have used a lower $[K^+]$ of 5.4 mM. Godfraind [38] gave a value of 9.5×10^5 ouabain molecules/cell as the receptor capacity in guinea pig atria (about 300 binding sites/μm^2). A binding capacity of about 9×10^{-7} moles of ouabain/kg (about 540 binding sites/μm^2) has been determined for the saturable binding compartment by Busse *et al.* [23] in guinea pig left atria. However, these authors used only one distinctly inotropic concentration of ouabain (4×10^{-7} M). The cardiac glycoside receptor density has been estimated at about 800/μm^2 in guinea pig papillary muscle [30], about 660/μm^2 in rat heart ventricular slices [18], about 760/μm^2 in cat ventricular muscle [31] and about 1000/μm^2 in the human heart [39]. As shown in the present study, the number of binding sites varies, depending on the value chosen for non-specific binding. If there is assumed to be no non-specific binding, the Scatchard plot is curvilinear (Fig. 3A) with about 430 high-affinity receptors/μm^2. However, when the value of [^{3}H]-ouabain bound in the presence of 1×10^{-4} M ouabain after 180 sec washing (Fig. 1) is defined as non-specific binding, the Scatchard plot is almost linear, giving about 700 receptors/μm^2. When the [^{14}C]-inulin retained after a 180-sec washing is used as non-specific binding, the Scatchard plot is curvilinear, with about 380 high-affinity binding sites/μm^2.

A Scatchard plot of ouabain binding to a crude guinea pig left atria homogenate (Fig. 4) gives a value of about 530 receptors/μm^2 of high affinity (K_D 8.8×10^{-8} M). Since care was taken so that no membranes were lost in the preparation of this crude homogenate, we think that the number of receptors calculated from this Scatchard plot (Fig. 4) is a reasonable estimate of the number of high-affinity receptors in the contracting left atria. This estimate is similar to both the number of high-affinity receptors [no non-specific binding (Fig. 3A)] and the number of receptors calculated when the binding at 1×10^{-4} M is defined as non-specific (Fig. 3B). These values are comparable to the literature values, except for the lower values obtained by Godfraind [38] and Ghysel-Burton and Godfraind [37] at higher $[K^+]$.

The Scatchard plot of ouabain binding to contracting guinea pig left atria (Fig. 3A) would appear to show two types of binding sites: one higher-affinity site which is saturable and corresponds to the positive inotropic effect (and possibly partly to the inhibition of $^{86}Rb^+$-uptake), and secondly, a lower-affinity site which may be either non-specific binding or binding leading to inhibition of $(Na^+ + K^+)$ATPase or $^{86}Rb^+$-uptake. This low-affinity binding is probably not an artifact due to incomplete removal of [^{3}H]-ouabain from the extracellular space during washout since the amount of [^{3}H]ouabain, unlike [^{14}C]inulin, does not decrease after prolonged washing (Fig. 1). We cannot determine whether this binding is non-specific or to a low-affinity site because, at present, there are no compounds that selectively bind to the possibly different receptors. Further, the dissociation constant (about 1.1×10^{-6} M) for the high-affinity site is similar to the maximum inotropic ouabain concentration. Thus, at maximum inotropic concentrations, not all high-affinity receptors will be occupied. However, some of the low-affinity sites will be occupied at these concentrations. These low-affinity sites may be involved in the toxic effects and in the inhibition of $^{86}Rb^+$-uptake seen at these doses.

Influence of ouabain on the $^{86}Rb^+$- and $^{42}K^+$-uptake of contracting guinea pig left atria

In order to clarify the effects of ouabain binding on the force of contraction and $^{86}Rb^+$- or $^{42}K^+$-uptake, it is necessary to measure both parameters in the same preparation under the same conditions. $^{86}Rb^+$-uptake has been widely used as an estimate of sodium pump activity [40, 41]. However, Akera *et al.* [22] have recently shown that $^{86}Rb^+$- or $^{42}K^+$-uptake may not reflect changes in transmembrane Na^+-movement when Rb^+ or K^+ are in excess.

The effects of cardioactive glycosides on the $^{86}Rb^+$-uptake have been studied in several species. Digoxin gave a decrease in $^{86}Rb^+$-uptake of similar magnitude to the increase seen in dP/dt in the dog heart [21]. Studies with cat papillary muscles [20] have shown a good correlation between the half-maximal increase in the force of contraction and half-maximal inhibition of active $^{86}Rb^+$-uptake. Studies with the relatively insensitive rat [18], however, showed a considerable difference between the concentrations of ouabain causing a half-maximal increase in the force of contraction and half-maximal inhibition of active $^{86}Rb^+$-uptake. In studies with the moderately sensitive guinea pig [42], increases in the binding of digitoxin and ouabain and an increase in the force of contraction were concomitant with inhibition of the sodium pump, measured as $^{86}Rb^+$-uptake inhibition. During the washout of the glycosides, the loss of the positive inotropy paralleled the recovery of the sodium pump activity. However, these workers used doses (1.2×10^{-6} M ouabain, 4×10^{-7} M digitoxin, 1.5 Hz stimulation) that probably would be ultimately toxic in guinea pig heart. $^{86}Rb^+$-uptake was measured in ventricular slices in a K^+-free solution containing RbCl (2 mM) after Na^+-preloading at 0°. Thus, it is difficult to correlate the force of contraction measurements with the $^{86}Rb^+$-uptake inhibition studies. Later work [40, 41] omitted the Na^+-preloading but revealed no force of contraction measurements for the muscles used in the $^{86}Rb^+$-uptake studies.

Other studies have not observed this parallelism. Noack *et al.* [24] noted an increase in cellular K^+ and a decrease in cellular Na^+ under the influence of moderately inotropic ouabain and digitoxigenin concentrations in guinea pig left atria. This activation was transient and occurred before maximum inotropy was observed. Godfraind and Ghysel-Burton [37, 43] showed that "therapeutic" levels of ouabain produced an increase in cellular K^+ and a decrease in cellular Na^+ in guinea pig left atria. Ouabain concentrations which increased the diastolic tension reversed these effects.

Thus, the literature results on the effect of therapeutic levels of cardiac glycosides on the sodium pump in guinea pig atria are contradictory. Our results show that, at purely inotropic ouabain concentrations, no inhibition of $^{86}Rb^+$- or $^{42}K^+$-uptake

was seen, but at toxic concentrations increasing inhibition was observed as the concentration was increased. This implies that, in guinea pig atria as in rat ventricular slices [18], there is a dissociation between these two effects of ouabain, positive inotropy and inhibition of the sodium pump measured by $^{86}Rb^+$- or $^{42}K^+$-uptake. If there is a true dissociation of these two effects in guinea pig left atria, then at least two different receptors might be involved, as suggested by the Scatchard plot of ouabain binding to contracting left atria (Fig. 3). However, we must question the assumption that $^{86}Rb^+$- or $^{42}K^+$-uptake values are valid measurements of the $(Na^+ + K^+)$ATPase in contracting guinea pig left atria. Akera *et al.* [22] have suggested that ouabain-sensitive $^{86}Rb^+$-uptake is a measure of sodium pump activity provided that neither Rb^+ nor K^+ are in excess compared to the Na^+ available to the pump. These workers consider that a continuous enhancement of sodium influx by means of a high-frequency electrical stimulation may provide conditions under which the specific $^{86}Rb^+$- or $^{42}K^+$-uptake represents the capacity of the sodium pump. However, our results show that at both 3 and 5 Hz, no inhibition of $^{86}Rb^+$-uptake is seen with inotropic ouabain concentrations. Stimulating frequencies of 3 or 5 Hz may still be insufficient to adequately preload the muscle cell with sodium although the initial $^{86}Rb^+$-uptake is similar under both conditions and higher than at a 1-Hz stimulation. Thus, although we are not certain that we can assume that $^{86}Rb^+$- and $^{42}K^+$-uptake are valid measurements of $(Na^+ + K^+)$ATPase under the experimental conditions used, the conclusions from the $^{86}Rb^+$-uptake studies and from the Scatchard plot of [^{3}H]ouabain binding to contracting guinea pig left atria agree that there may be two types of ouabain receptor.

Procedures which increase the pre-drug force of contraction, presumably by an increased sodium influx rate, give increased pre-drug $^{86}Rb^+$-uptake. In these procedures, the initial $^{86}Rb^+$-uptake is increased and less ouabain is required to cause toxicity and $^{86}Rb^+$-uptake inhibition. These observations would suggest that the stimulation of sodium uptake [37, 43] observed at positive inotropic ouabain concentrations in guinea pig atria is an artifact caused by control conditions under which the sodium influx rate is submaximal (3.3 Hz at 30°, initial force of contraction about 0.75 g/100 mg tissue). Procedures which produce positive inotropy will increase the sodium influx rate and thereby increase the sodium content of the atria, at least for part of each cycle [44]. Akera *et al.* [22] have noted that, among the substrates for $(Na^+ + K^+)$ATPase, only the sodium ion concentration [45] was possibly lower than its K_M value.

Lee *et al.* [46] showed a good correlation between onset of positive inotropy with dihydroouabain and an increase in sodium ion activity in Purkinje fibres of the digitalis-sensitive sheep and also between washout of non-toxic dihydroouabain inotropy and decrease in sodium ion activity. Glitsch *et al.* [47] found that the internal concentration of sodium and calcium in guinea pig left atria were far below saturation for the sodium–calcium carrier system. Increasing the sodium concentration gave a positive inotropic contractile response. Thus, the lack of inhibition of $^{86}Rb^+$- and $^{42}K^+$-uptake by inotropic concentrations of ouabain in guinea pig left atria may have two explanations: (1) sodium content is low and the sodium influx rate is submaximal so that $^{86}Rb^+$-uptake is not a valid measurement of $(Na^+ + K^+)$ATPase activity, or (2) there are two receptors involved in producing the pharmacological effects of ouabain. It must be emphasized that force of contraction and $^{86}Rb^+$- or $^{42}K^+$-uptake must be measured in the same preparation under the same conditions, so that a comparison can be made between these two parameters.

The results presented earlier show that, in the moderately digitalis-sensitive guinea pig, there is a dissociation between the ouabain concentrations necessary to give positive inotropy and inhibition of $^{86}Rb^+$-uptake when both these parameters are measured in the same preparation. The dissociation of these effects is smaller than that observed in the digitalis-insensitive rat [18]. However, the digitalis-sensitive cat showed no dissociation between these two parameters [20]. Lüllmann and Peters [9, 34] have proposed that effective pump inhibition in intact cells only becomes evident when all surplus $(Na^+ + K^+)$ATPase molecules are occupied by ouabain. The species differences seen with our studies could be explained by this concept only if we assume that the cat has no surplus $(Na^+ + K^+)$ATPase molecules, while, in the rat, only a very small proportion of $(Na^+ + K^+)$ATPase molecules are necessary to maintain Na^+/K^+-gradients. The guinea pig would occupy an intermediate position. However, there is no evidence to support this. The number of surplus $(Na^+ + K^+)$ATPase molecules should be markedly reduced at higher stimulation frequencies. However, our studies at 3 and 5 Hz still show this dissociation of positive inotropy and pump inhibition measured by $^{86}Rb^+$-uptake (Fig. 6).

Ouabain binding to guinea pig cardiac cell membranes

A Scatchard plot of the binding of ouabain to $(Na^+ + K^+)$ATPase-containing cardiac cell membranes (Fig. 8) shows that there is only one type of binding under maximal binding conditions (Mg^{2+} + Pi, or $Na^+ + Mg^{2+}$ + ATP). Binding supported by (Mg^{2+} + Pi) shows a slightly higher affinity for ouabain than the ($Na^+ + Mg^{2+}$ + ATP)-supported binding. This result has been shown earlier with digoxigenin [49]. De Pover and Godfraind [49], using ($Na^+ + Mg^{2+}$ + ATP)-supported binding, showed only one type of binding site in a guinea pig cardiac $(Na^+ + K^+)$ATPase preparation. These workers also measured the association and dissociation rate constants for ouabain (k_{+1} $2.3 \times 10^6\,min^{-1}\,M^{-1}$, which is about $3.8 \times 10^4\,sec^{-1}\,M^{-1}$; k_{-1} $0.43\,min^{-1}$, which is about $7.2 \times 10^{-3}\,sec^{-1}$) and their values are very similar to those obtained in the present study (k_{+1} $4.9 \times 10^4\,sec^{-1}\,M^{-1}$, k_{-1} $1.01 \times 10^{-2}\,sec^{-1}$). The dissociation constant calculated from the association and dissociation rate constants (2.06×10^{-7} M) in this study is similar to the dissociation constant calculated from the Scatchard plot (1.18×10^{-7} M) (Fig. 8).

A Scatchard plot of the binding of ouabain in the

presence of Tyrode solution and ATP (3 mM), conditions we think similar to those used with contracting left atria, consistently gave a curved Scatchard plot (Fig. 8). Mathematical analysis performed according to Weidemann *et al.* [29] showed the presence of a high-affinity/low-capacity site (K_D 4.7×10^{-7} M, about 15% of binding sites) and a low-affinity/high-capacity site (K_D 6.0×10^{-6} M, about 85% of binding sites). The total number of binding sites was similar with all binding media.

Curved Scatchard plots may have multiple interpretations [4] and may not be taken as a sole argument for the existence of more than one binding site. However, these results, when considered together with the [^{3}H]ouabain binding and $^{86}Rb^+$-uptake results with contracting left atria presented earlier, present a strong case for the possible existence of at least two different types of ouabain receptors in the guinea pig heart under physiological conditions.

[^{3}H]*Ouabain binding to cardiac cell membranes and inhibition of* ($Na^+ + K^+$)*ATPase activity*

Both these parameters were measured in samples taken from the same incubation mixture. [^{3}H]Ouabain was bound to the enzyme at lower concentrations than those concentrations needed to give enzyme inhibition (Fig. 9). This difference was relatively small when compared with the large difference seen previously [18] with rat cardiac cell membranes (about 100-fold).

However, no difference was seen with cat cardiac cell membranes [20]. The results with guinea pig cardiac cell membranes show that, in guinea pig heart as in rat heart, ouabain binding does not correlate with the inhibition of ($Na^+ + K^+$)ATPase when both are measured in the same incubation mixture.

Thus, we have analyzed in the guinea pig the pharmacological effects of ouabain binding. The results can be summarized as follows.

(1) The amount of ouabain specifically bound to contracting guinea pig left atria is proportional to the pharmacological effects elicited, as was seen earlier in the cat [20, 31], rat [18] and dog [21].

(2) $^{86}Rb^+$- and $^{42}K^+$-uptake, apparent measures of ($Na^+ + K^+$)ATPase activity in contracting muscle, are not inhibited by inotropic concentrations of ouabain. However, toxic concentrations cause an increasing inhibition of $^{86}Rb^+$- and $^{42}K^+$-uptake.

(3) Under maximal binding conditions, there is apparently only one type of binding site on isolated guinea pig cardiac cell membranes.

However a curved Scatchard plot is seen when ouabain binding is measured and analyzed in Tyrode solution + 3 mM ATP.

(4) In guinea pig cardiac cell membranes, ouabain binding occurs at a lower concentration than that which gives an equivalent inhibition of ($Na^+ + K^+$)ATPase activity. This difference is much smaller than that observed with rat cardiac cell membranes [18] but no difference was seen with the digitalis-sensitive cat [20].

Therefore, we conclude that: (1) inhibition of ($Na^+ + K^+$)ATPase is not necessarily related to positive inotropy in the guinea pig, and (2) there is the possibility that there is more than one type of ouabain binding site in the contracting guinea pig left atria.

Acknowledgements—The skilful technical assistance of Miss Susanne Döhl is appreciated. This study was supported by the Deutsche Forschungsgemeinschaft (Er 65/4–3).

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