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OUABAIN-RECEPTOR INTERACTIONS IN $(Na^+ + K^+)$-ATPase PREPARATIONS

A CONTRIBUTION TO THE PROBLEM OF NONLINEAR SCATCHARD PLOTS

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SUMMARY

Specific [^{3}H]ouabain binding to rat and guinea pig skeletal muscle (musculus soleus) was studied using a rapid centrifugation and a filtration method. Both assays gave identical results: the incubation of the cell membranes in 50 mM imidazole/HCl buffer pH 7.25 or 7.4, $MgCl_2$, P_i caused a time dependent loss of $(Na^+ + K^+)$-ATPase activity indicating an alteration of the membrane preparation. Ouabain binding properties were changed concomitantly.

If ouabain binding was allowed to proceed until equilibrium was reached (3 min in rat and 10 min in guinea pig) at 37 °C the data plotted according to Scatchard followed a straight line. The dissociation constants of the ouabain-receptor-complexes of the rat cell membrane preparation as calculated from the slope of the plot (K_D = 134 nM) and from the ratio of the dissocation and association rate constants (K_D = 175 nM) agreed within experimental error with that determined by Clausen and Hansen [(1974) Biochim. Biophys. Acta 345, 387–404] in intact soleus muscles (K_D = 210 nM). If ouabain binding was allowed to proceed for a longer period, however, nonlinear Scatchard plots resulted with an identical maximal number of binding sites but inconstant and decreased affinity for the cardiac glycoside.

Experimental evidence is presented that nonlinear Scatchard plots often obtained in hormone (drug)-receptor binding experiments may (among other things) be the result of damaged cell membrane particles in vitro.

INTRODUCTION

The use of radioactively labelled hormones and drugs to study hormone-(drug)-receptor interactions at the molecular level by equilibrium binding experiments has been applied widely in the past few years (for review see refs. 1–3). The usual way to demonstrate and analyse the obtained experimental binding data is the Scatchard plot [4]: the ratio bound/free (B/F) for the ligand is plotted versus the concentration of receptor-bound ligand (B). If this graph yields a straight line,

indicating the same affinity of the hormone (drug) to the receptor irrespective of the hormone concentration in the incubation medium, one single type of receptor may be assumed. However, in most cases of equilibrium binding experiments curvilinear Scatchard plots have been obtained. This has stimulated several theoretical reflections about the value and true meaning of these data [5–9]. It has been found that multiple classes of independent binding sites [10, 11], negative cooperativity in binding [12], classes of cooperatively interacting sites [12], artifacts (nonspecific binding) [13], ligand-ligand interactions (self-aggregation, polymerization, isomerization) [14], heterogeneity of labelled and unlabelled ligand [15], and negatively controlled hormone-receptor interaction [16] etc. may cause nonlinear Scatchard plots. We wish to report experimental evidence for the instability of the cell membrane preparation resulting in curved Scatchard plots.

MATERIALS

[^{3}H]ouabain with a specific radioactivity of 12 Ci/mmol (lot nr. 747 186) was obtained from New England Nuclear, Dreieichenhain, Germany. The liquid scintillation fluids used were Insta-Gel and Insta-Flour (Packard Instruments GmbH, Frankfurt, Germany). Aprotinine (Trasylol®), a proteinase inhibitor, was purchased from Bayer, Leverkusen, Germany. All other chemicals were of analytical grade and obtained through E. Merck A. G. Darmstadt or Boehringer Mannheim GmbH, Mannheim, Germany.

METHODS

Preparation and quantitation of enzyme: ($Na^+ + K^+$)-activated ATPase (EC 3.6.1.3) enriched membrane preparations from rat and guinea pig skeletal muscle (m. scleus) were prepared according to the procedure described by Pitts and Schwartz [17] for cardiac muscle but without the glycerol treatment. ($Na^+ + K^+$)-ATPase activities from rat or guinea pig skeletal muscle were 0.2–0.4 units/mg protein, 85–95 % of total ATPase activity could be inhibited by ouabain (10^{-4} M). ($Na^+ + K^+$)-ATPase activity was measured with the coupled optical assay [18]. One enzyme unit is defined as the amount of enzyme hydrolyzing 1 μmol ATP per min at 37 °C. Protein was quantitated by the procedure of Lowry et al. [19].

Assay of [^{3}H]ouabain binding. The methods have been described in great detail in previous papers [20–23]. In brief: 0.1–1.0 mg membrane protein were incubated in a medium containing 50 mM imidazole/HCl buffer 7.25, 3 mM $MgCl_2$, 3 mM imidazole/phosphate and various amounts of [^{3}H]ouabain for the indicated time and temperature in polypropylene tubes. The receptor for cardiac glycosides is membrane-bound [3, 20, 23–25], and it is therefore possible to separate bound from free [^{3}H]ouabain either by rapid centrifugation (Beckman L5, rotor 50 Ti, 80 000 $\times g$) or by filtration technique (Whatman GF/C glas fiber filters). Both methods gave identical results.

The radioactivity in the pellet was counted in a scintillation counter after dissolving the protein by addition of 0.2 ml 1 M NaOH, heating at 50 °C for 20 min and by addition of 2 drops of concentrated HCl and 10 ml of scintillation fluid (Insta Gel). The radioactivity in the membrane protein on the glassfiber filters was counted after

heating for 30 min at 100 °C and addition of 10 ml scintillation fluid (Insta-Fluor).

Specific [^{3}H]ouabain binding is obtained by substracting from the total radioactive uptake the amount that is not displaced by high concentrations (10^{-4} M) of unlabelled ouabain. Unspecific binding in the pellet (rapid centrifugation method) amounted to less than 3 % of total binding and was identical with that of denatured protein (after heating the membranes for 30 min at 90 °C). Unspecific binding with the filtration method amounted to less than 1 % of total radioactivity. There was no displaceable binding to the filters in the absence of native membrane protein.

Each experiment was performed with at least two different membrane preparations, each experimental point is the mean of duplicate or triplicate determinations.

RESULTS

In a great many experiments by several authors it could be established that ouabain binding to its membrane bound receptor in different tissues and species is time and temperature dependent, saturable, readily reversible and follows the law of mass action [3, 20, 23–38].

The kinetics of this drug-receptor interaction have been described in great detail in previous papers [20–22, 28] for heart, kidney, brain-cell membranes and erythrocytes of different species. The equilibrium binding experiments of the above mentioned tissues revealed but one type of receptor with high affinities for the cardiac glycoside. Dissociation constants between $2.5 \cdot 10^{-9}$ M and $1.7 \cdot 10^{-7}$ M have been determined depending on the species.

Recently, when examining the ouabain-receptor interactions of skeletal muscle cell membranes from rats at 37 °C, a time-dependent behaviour of ouabain binding was found with an initial increase followed by a rather quick decrease in binding capacity (Fig. 1). If the membranes are incubated at lower temperatures, they appear to lose the ouabain binding property less rapidly (not shown). The ATPase activity of the membranes incubated at identical conditions but without ouabain decreases in a time dependent manner, too (Fig. 2). The ($Na^+ + K^+$)-ATPase activity seems to

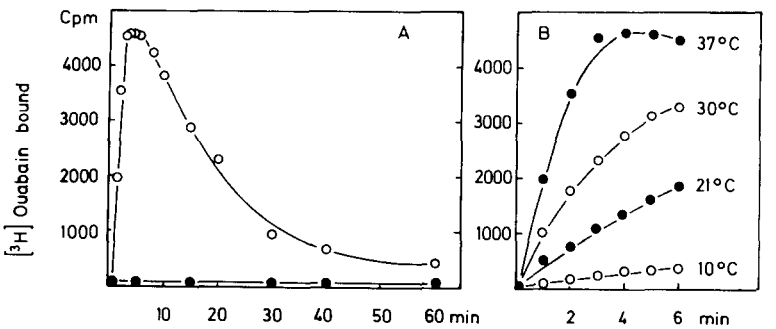

Fig. 1. Temperature dependent [^{3}H]ouabain binding to rat skeletal muscle cell membranes. Cell membranes (1.58 mg protein) were incubated in the presence of 50 mM imidazole/HCl pH 7.25, 3 mM $MgCl_2$, 3 mM imidazole/phosphate and $4 \cdot 10^{-9}$ M [^{3}H]ouabain for the indicated time. Total volume 2 ml. The radioactivity on the filters was determined after rapid filtration. (A) ○ — ○, no additions; ● — ●, in the presence of an excess of unlabelled ouabain (10^{-4} M). All experiments at 37 °C. (B) Initial binding at different temperatures.

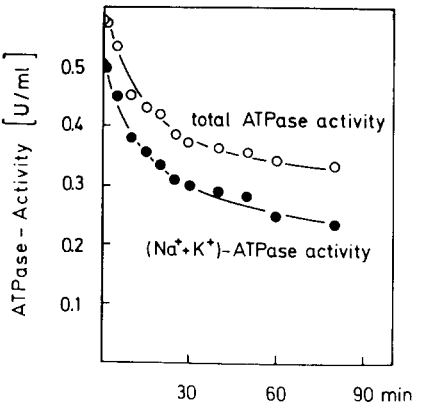

Fig. 2. Decrease of ATPase activity of rat skeletal muscle cell membranes during incubation at 37 °C. Cell membranes (1.6 mg protein) were incubated in the presence of 50 mM imidazole/HCl pH 7.25, 3 mM $MgCl_2$, 3 mM imidazole/phosphate at 37 °C. At the indicated time 0.05 ml of the incubation medium were withdrawn and assayed for total ATPase as well as for $(Na^+ + K^+)$-ATPase activity in the coupled optical assay according to Schoner et al. [18].

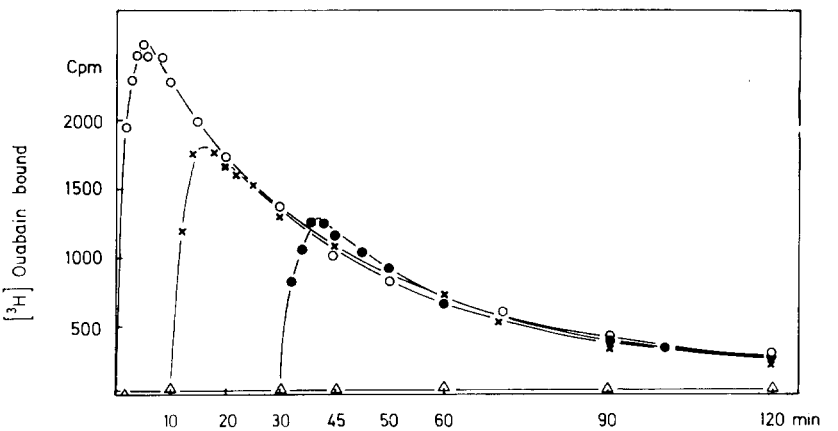

Fig. 3. Time dependent [^{3}H]ouabain binding to rat skeletal muscle cell membranes. Cell membranes (0.5 mg protein) were incubated in 50 mM imidazole/HCl pH 7.25, 3 mM $MgCl_2$, 3 mM imidazole/phosphate and $4 \cdot 10^{-9}$ M [^{3}H]ouabain at 37 °C for the indicated time. × — ×, experiments performed after preincubation without [^{3}H]ouabain for 10 min; ● — ●, 30 min preincubation period without [^{3}H]ouabain △ — △, in the presence of 10^{-4} M unlabelled ouabain. These experiments clearly show that the decrease in ouabain binding is not caused by ouabain itself.

be only slightly more sensitive than Mg^{2+}-ATPase activity. At the conditions tested the activity of $(Na^+ + K^+)$-ATPase decreases less rapidly than the ouabain binding capacity of the membranes. In order to rule out a glycoside-induced degradation of the receptor the membrane particles were preincubated for 10 and 30 min at identical conditions but without ouabain and then the binding experiment was started (Fig. 3). The result shows that the ouabain binding property of the membranes is lost successively during the incubation time.

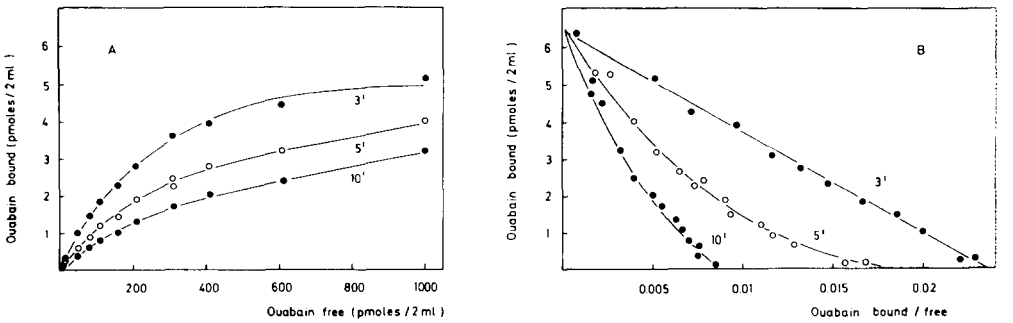

Fig. 4. [^{3}H]ouabain binding to rat skeletal muscle. Cell membranes (0.55 mg protein) were incubated for the indicated time in 50 mM imidazole/HCl pH 7.25, 3 mM $MgCl_2$, 3 mM imidazole/phosphate and $5.5 \cdot 10^{-9}$ M [^{3}H] ouabain and increasing concentrations of unlabelled ouabain. Total volume 2 ml. At the indicated time the incubation period was interrupted by rapid filtration. The radioactivity on the filters was determined. The data of A are plotted according to Scatchard [4] in B.

From the above mentioned experimental data one would expect a time- and temperature-caused damage of the muscle cell membrane preparation resulting in a decreased ouabain binding capacity. A decreased affinity of the receptor for the drug, however, cannot be excluded. To test this possibility a binding experiment with increasing ouabain concentrations was performed with different incubation times (Fig. 4). The data of Fig. 4A (bound ouabain versus free ouabain) are plotted according to Scatchard [4] in Fig. 4B.If the binding process is interrupted after 3 min, a straight line for ouabain binding in the Scatchard plot is obtained. The dissociation constant (K_D) calculated from the slope of the plot is 134 ± 5 nM ($n = 4$). This value agrees within experimental error with the dissociation constant calculated from the ratio of the association (k_{+1}) and dissociation rate constants (k_{-1}) at 37 °C from $K_D = k_{-1}/k_{+1}$ [20]. k_{+1} determined from the initial binding rates was $3.3 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ and

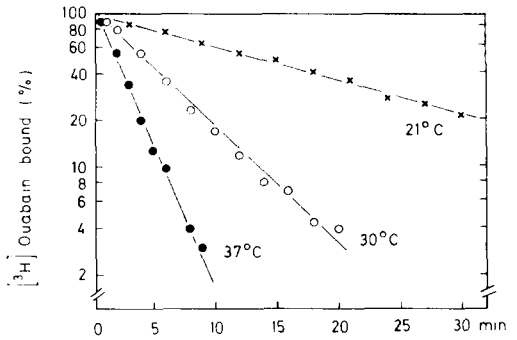

Fig. 5. Dissociation of the [^{3}H]ouabain-receptor-complex from rat skeletal muscle. Cell membranes were allowed to bind [^{3}H]ouabain in the presence of 50 mM imidazole/HCl pH 7.25, 3 mM $MgCl_2$, 3 mM imidazole/phosphate and $5.5 \cdot 10^{-9}$ M [^{3}H]ouabain for 3 min at 37 °C. After rapid centrifugation (80 000 × g for 30 min. at 0 °C) the pellet was homogenized in 0.01 M imidazole/HCl pH 7.25. 0.6 mg protein of this [^{3}H]ouabain-receptor-complex [20] was again incubated in 50 mM imidazole/HCl pH 7.25, 3 mM $MgCl_2$, 3 mM imidazole/phosphate and 10^{-4} M unlabelled ouabain at the indicated temperatures and time. The radioactivity after rapid filtration was determined on the filters.

TABLE I

KINETIC CONSTANTS OF [^{3}H]OUABAIN BINDING TO CELL MEMBRANES FROM RAT SKELETAL MUSCLE

The rate constants were determined from the initial binding and the dissociation of bound [^{3}H]-ouabain from the receptor in the presence of high concentrations of unlabelled ouabain respectively as described in detail in a previous paper [20].

Temperature (°C):	4	10	15	21	30	37
Association rate constant k_{+1} ($M^{-1} \cdot s^{-1}$)	$0.61 \cdot 10^3$	$1.3 \cdot 10^3$	$2.4 \cdot 10^3$	$0.6 \cdot 10^4$	$1.8 \cdot 10^4$	$3.3 \cdot 10^4$
Dissociation rate constant k_{-1} (s^{-1})	$0.7 \cdot 10^{-4}$	$1.5 \cdot 10^{-4}$	$3.5 \cdot 10^{-4}$	$0.85 \cdot 10^{-3}$	$2.9 \cdot 10^{-3}$	$0.58 \cdot 10^{-2}$
Dissociation constant from $k_{-1}/k_{+1} = K_D$ (M)	$1.14 \cdot 10^{-7}$	$1.2 \cdot 10^{-7}$	$1.45 \cdot 10^{-7}$	$1.4 \cdot 10^{-7}$	$1.6 \cdot 10^{-7}$	$1.8 \cdot 10^{-7}$
From Scatchard plots	–	–	–	–	–	$1.3 \cdot 10^{-7}$

k_{-1} was $0.58 \cdot 10^{-2}$ s^{-1} (Fig. 5) giving K_D as 175 nM (177 ± 5 nM, $n = 3$) (Table I). After 5 and 10 min a curved Scatchard plot with the same intercept on the ordinate indicates an inconstant, decreased affinity of the receptor for the drug, but a constant total number of maximal binding sites.

The same experiments were repeated with guinea pig skeletal muscle cell membranes (Fig. 6). Again, the time dependent binding curve shows a decrease after 10 min, which is slower, however, than in rat skeletal muscle. The Scatchard plot of the binding shows a linear line if the incubation period was 10 min and a curved line for 60 min of incubation (Fig. 7). As in rat skeletal muscle the two lines meet when intercepting with the ordinate. This indicates a constant total number of receptors, too, but decreased and inconstant affinity for the drug. The dissociation constant at 10 min as calculated from the slope of the Scatchard plot was 29 nM. Thus the affinity

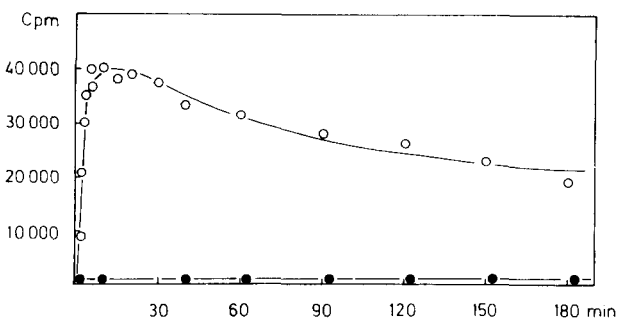

Fig. 6. Time-dependent binding of [^{3}H]ouabain to guinea pig skeletal muscle cell membranes. Cell membranes (1.4 mg protein) were incubated in 50 mM imidazole/HCl pH 7.25, 3 mM $MgCl_2$, 3 mM imidazole/phosphate and $4 \cdot 10^{-9}$ M [^{3}H]ouabain at 37 °C for the indicated time. Total volume 2 ml. ● — ●, in the presence of additional 10^{-4} M unlabelled ouabain.

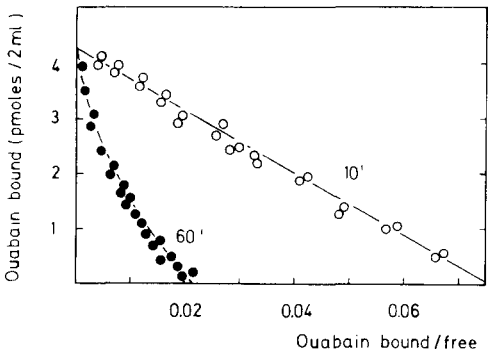

Fig. 7. Ouabain binding to guinea pig skeletal muscle cell membranes. Cell membranes (0.15 mg protein) were incubated for the indicated time at 37 °C in 50 mM imidazole/HCl, pH 7.25, 3 mM $MgCl_2$, 3 mM imidazole/phosphate, $4 \cdot 10^{-9}$ M [^{3}H]ouabain and increasing concentrations of unlabelled ouabain. Total volume 2 ml.

for ouabain of the receptor from guinea pig skeletal muscle is several fold higher than that from rat skeletal muscle.

Identical results were obtained with different incubation mediums (at pH 7.4, with 5 mM $MgCl_2$, with 50 mg/ml bovine serum albumin), in the presence of 8000 units trypsin inhibitor from soybean or 6 mg of aprotinine (Trasylol®, a proteinase inhibitor isolated from beef lung). Thus, the changes of receptor affinity for ouabain are not due to a contamination of the membrane preparations used with proteases. It has, however, repeatedly been observed that the membranes suspended in the incubation medium tended to sediment rather quickly after having been exposed to higher temperatures (i.e. 20–37 °C) for several minutes. We believe, that this reflects a change of the membrane composition caused by the incubation procedure.

DISCUSSION

The receptor for cardiac glycosides being closely connected with membrane-bound $(Na^+ + K^+)$-ATPase [3, 20, 23–26] exhibits properties of a drug receptor similar to those known from some membrane-bound hormone receptors [1, 2]. It shows time – and temperature dependent binding, saturability, reversibility and high affinity [3, 27, 28]. Furthermore, it has been found out that binding of ouabain to this receptor results in an immediate and proportional inhibition of the $(Na^+ + K^+)$-ATPase activity if both are assayed at identical conditions [22]. Recently it could clearly be established that ouabain binding correlates directly with inhibition of erythrocyte potassium influx [29] and with positive inotropy in the hearts of several species [30]. Species differences in the sensitivity to the inotropic effects of cardiac glycosides can be correlated with the affinity of cardiac glycosides to their membrane bound receptors and with the glycoside sensitivity of the $(Na^+ + K^+)$-ATPase preparations [20, 31–34]. Thus this binding site for cardiac glycosides serves as a model of a membrane bound drug-receptor-effector(-enzyme) system being related to the inotropic effect.

In different tissues of several species tested at optimal binding conditions, until now we could find only one type of ouabain binding sites [20, 28]. The data of

the concentration dependent binding experiments when analyzed according to Scatchard [4] gave linear plots. There have been some reports on nonlinear Scatchard plots, however. Taniguchi and Iida [35] performing their experiments on ox brain microsomes at pH 6.1 determined a high affinity binding site ($K_D = 0.18$ μM) and a low affinity binding site ($K_D = 20$ μM). At pH 7.25 and 7.4 we could detect only one type of ouabain binding sites in ox brain cell membranes with a 10 fold lower dissociation constant ($K_D = 13$ nM), though. In a previous paper [21] we have presented evidence that the ouabain receptor from ox brain cell membranes exists in two different conformational states.

Depending on the concentration of the ligands magnesium and phosphate, a curved Scatchard plot was obtained indicating the existence of a receptor conformation with a high (R_β) and a low affinity (R_α). This, however, did not affect the total number of binding sites, which remained constant.

In this set of experiments we have measured ouabain binding to rat and guinea pig skeletal muscle. Both species especially the rat are relatively insensitive to cardiac glycosides. A linear Scatchard plot of the experimental binding data is obtained, if the reaction ouabain + receptor $\rightleftharpoons$ ouabain-receptor-complex was allowed to proceed until equilibrium is attained (3 min). The dissociation constant ($K_D = 134$ nM) as calculated from the slope of the plot agrees within experimental error with that determined from the ratio of the rate constants ($K_D = 175$ nM). Clausen and Hansen [36] determined the dissociation constant in isolated rat soleus muscle (not cell membrane preparations!) as 210 nM. Due to the fact that even traces of K^+ in the incubation medium would increase the K_D (Clausen and Hansen allowed K^+ concentration to rise up to 0.8 mM) the slightly higher value can be explained easily. In fact, the data from intact muscle cells with an incubation period of 240 min [36] and our data from cell membrane preparations at optimal conditions agree rather well. This result adds to the validity of such investigations on drug-receptor-interactions in cell membrane preparations. If the membrane particles are incubated in the presence of buffer, $MgCl_2$ and P_i for a longer period, they get apparently damaged (this is indicated by a distinct loss of ATPase activity) and the Scatchard plots of the ouabain binding experiment become nonlinear. The maximal number of glycoside receptors is, however, not decreased. The alteration of the membranes, therefore, causes a change of affinity (change of conformation?) rather than a destruction of the receptors.

Weidemann et al. [10] described a mathematical method, based on a graphical approximation of the theoretical curve for two independent receptors, to calculate the different dissociation constants and number of respective binding sites from nonlinear Scatchard plots. Although we were able to repeat the published calculation of Weidemann et al. [10] with their data, our experimental data of the curved plots did not agree with a reasonable analysis, i.e.: our experimental data did not coincide with any theoretical plot for two independent receptors unless there were one type of receptors with a negative dissociation constant. The Hill plot of our data showed a slope of exactly 1 for linear Scatchard plots and a slope of 0.91 and 0.99 for the experiments in rat skeletal muscle with an incubation period of 5 and 10 min. These data for guinea pig skeletal muscle were 1.01 and 0.80 for the 10 min and 60 min incubation period.

It has been reported that phospholipase A treatment of the membrane particles only decreases the affinity for ouabain, but does not change the binding capacity

[37]. The addition of phospholipids (phosphatidylserine and phosphatidylinositol) to the incubation medium restored the binding properties approximately up to control values again [37, 38]. In a recent paper Kahn et al. [11] described three classes of insuline receptors in rat liver membranes, which lose insuline binding properties quite rapidly within minutes after incubation. This has been termed "receptor degradation" as the binding capacity decreases. In that system, however, a corresponding enzyme has not been determined.

It is possible that the membrane composition of rat and guinea pig skeletal muscle is damaged in a way, which we admittedly cannot specify yet, during the incubation period resulting in the changes described above. In an earlier paper [39] we have described that trypsin as well as SH reagents may decrease $(Na^+ + K^+)$-ATPase activity without changing ouabain binding capacity in ox brain cell membranes. Those experiments, however, showed an unaltered affinity of the receptor for ouabain after treatment (the dissociation constant remained unchanged). The experiments reported in this paper demonstrate that the ouabain receptors from guinea pig and rat skeletal muscle have different properties, though. Furthermore and more important it can be shown that nonlinear Scatchard plots among other things [5–16] may be caused by an alteration of the membrane particles in vitro representing an "artifact" rather than negative cooperativity or different classes of receptors.

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