Biochemical Pharmacology, Vol. 33, No. 1, pp. 47–53, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

HETEROGENEITY OF OUABAIN SPECIFIC BINDING SITES AND $(Na^+ + K^+)$-ATPase INHIBITION IN MICROSOMES FROM RAT HEART

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(*Received* 14 *March* 1983; *accepted* 12 *July* 1983)

Abstract—Cardiac glycoside binding to microsomes prepared from rat heart ventricles and enriched in $(Na^+ + K^+)$-ATPase was measured by a rapid filtration technique. The relation between ouabain binding to microsomes and $(Na^+ + K^+)$-ATPase activity has also been examined. Data were statistically analysed by means of two different non linear regression methods.

The experimental results were fitted the most closely by a model describing that ouabain specific binding occurred at two classes of independent sites. High affinity sites were characterized by a dissociation constant of 0.21 ± 0.01 μM and a low capacity (9.4 ± 1.4 pmoles/enzymatic unit). Low affinity sites were characterized by a dissociation constant equal to 13 ± 3 μM and a capacity equal to 87 ± 15 pmoles/enzymatic unit.

Similar results were obtained with the more lipophilic glycoside digoxin. It was also observed that dihydroouabain, a ouabain derivative with a saturated lactone ring, competes with ^{3}H-ouabain for the binding to the two classes of sites.

Binding to these two classes of sites appeared to be associated with a corresponding inhibition of $(Na^+ + K^+)$-ATPase activity.

The mechanism of the positive inotropic effect of cardiac glycosides is a matter of controversy. A casual relationship between inhibition of Na–K pump and increase in contractile force of the heart has been proposed to be the only mechanism of action [1–3] but some authors do not support such an hypothesis [4–6]. Recently, it has been suggested that more than one mechanism could be responsible for ouabain inotropic effect because this glycoside evokes an increase in contractility much higher than the one observed when the Na–K pump is inhibited by lowering K_0 or by treatment with dihydroouabain [7]. Furthermore, there are several reports in the literature showing that ouabain binding to microsomal preparations obtained from various tissues and species could occur at more than one specific binding site [8–17]. The nature of such sites and their relation to $(Na^+ + K^+)$-ATPase has been the subject of several hypothesis [9, 10, 13, 15, 18].

The present experiments were designed to further study the characteristics of cardiac glycoside binding to microsomes prepared from rat heart ventricles and enriched in $(Na^+ + K^+)$-ATPase. We have also compared binding to inhibition parameters.

The results confirm the existence of two classes of binding sites in the rat heart and indicate that both could be related to the inhibition of $(Na^+ + K^+)$-ATPase activity. A preliminary report on some of these results has already been published [18].

MATERIALS AND METHODS

$(Na^+ + K^+)$-ATPase preparation. Atria and ventricles were dissected from hearts of female Wistar rats (2.5–3.5 months old). A fraction enriched in $(Na^+ + K^+)$-ATPase was prepared as described previously for guinea-pig heart [19] with slight modifications. A sulfhydryl group protecting agent (1,4-dithioerythritol 30 mM) was added during homogenization. After a prolonged NaI-treatment (45 min), the homogenate was diluted and centrifuged at 50,000 *g* for 90 min. The resultant pellet was twice resuspended in a solution containing 0.25 M sucrose and 5 mM Tris/HCl (pH 7.4) and then centrifuged for 1 hr at 100,000 *g*. The final pellet was resuspended in 0.25 M sucrose containing 0.1% deoxycholate, 20 mM maleate/Tris (pH 7.4) and stored overnight at −30°. After thawing, the supernatant from a 30 min spin at 20,000 *g* was centrifuged for 30 min at 100,000 *g* to separate a microsomal fraction. The $(Na^+ + K^+)$-ATPase activities in this fraction were determined as previously described by measuring the inorganic phosphate (Pi) liberated [19]. These activities were checked during each binding experiment. The protein content was determined by the method of Lowry *et al.* [20].

(^{3}H)-glycoside binding assays. Unless otherwise stated, the incubation medium contained 3 mM $MgCl_2$, 3 mM Pi-Tris, 1 mM EGTA abd 20 mM maleate/Tris, pH 7.4 at 37°C (Mg–Pi medium).

A rapid filtration technique was used to separate membrane-bound from free glycosides: samples of 200 μl usually containing 150 μg protein (130–200 μg) were filtered at 10° on Whatman glass fibers filters (GF/F). The filters were washed three times with 20 ml of chilled solution (0.25 M sucrose, 5 mM Tris/HCl, pH 7.4 at 0°), then added to 10 ml of a scintillation solution (Aqualuma-plus 25%–toluene 75%) and the radioactivity counted in a liquid scintillation counter (Kontron MR 300) with an efficiency determined by internal standardization (about

52%). The non-specific binding was estimated from samples incubated in the absence of Mg and Pi or in the presence of 4 mM unlabelled ouabain. When digoxin was used as a ligand, the dilutions and incubations were performed in glass tubes in the presence of a low concentration of bovine serum albumin (final concentration 7 μg/ml) in order to decrease adsorption to the test-tube. Furthermore, the concentration of ^{3}H-glycoside in solution was checked at the end of the incubation.

$(Na^+ + K^+)$-*ATPase inhibition.* Three assays were used to study the inhibitory effect of ouabain on $(Na^+ + K^+)$-ATPase activity. *Radiochemical assay:* microsomes about 100 g protein corresponding to 0.05 $(Na^+ + K^+)$-ATPase unit were preincubated for 15 min in 1 ml of medium containing 3 mM $MgCl_2$, 100 mM NaCl, 5 mM γ-^{32}P-ATP (originally 4.48 Ci/mmol but diluted with cold ATP in order to get 455000 cpm/ml of final medium), 1 mM EGTA, 5 mM Pi/Tris, 20 mM maleate/Tris (pH 7.4 at 37°) and various concentrations of glycosides. The reaction was started by addition of KCl (final concentration 20 mM) and stopped 20 min later by the addition of 700 μl of a cold solution containing 200 mg carbo adsorbens in 1 ml HCl 1 M. The tubes were then centrifuged at 500 rev/min for 10 min after which 500 μl of the supernatant was taken off, added to 4.5 ml of Aqualuma-plus and counted for radioactivity. These experimental conditions were adjusted in order to hydrolyse less than one fourth of ATP. Samples incubated with 4 mM ouabain in the absence of KCl were used as controls to determine the basal Mg–ATPase activity. When ouabain binding was compared to ouabain inhibition of $(Na^+ + K^+)$-ATPase (Fig. 4), the experimental procedure was that described for the "radiochemical assay" except that γ-^{32}P-ATP was replaced by cold ATP and cold ouabain was replaced by ^{3}H-ouabain. *Fiske and Subbarrow assay:* ATPase activity was determined by measuring the release of inorganic phosphate from ATP at 37° in 1 ml assay medium containing: 100 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 3 mM ATP/Tris, 1 mM EGTA, 20 mM maleate/Tris (pH 7.4). The reaction was started by the addition of microsomes [9–13 μg protein corresponding to 0.006 $(Na^+ + K^+)$-ATPase unit], stopped after 1 hr and the inorganic phosphate liberated was measured [19]. *Coupled optical assay:* $(Na^+ + K^+)$-ATPase activity was measured by continuously recording NADH oxidation in the presence of lactate dehydrogenase, pyruvate kinase and phosphoenolpyruvate [21]. Each cell contained (final volume 2.5 ml): 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 1 mM EGTA, 20 mM maleate/Tris (pH 7.4), 0.3 mM NADH, 2.5 mM phosphoenolpyruvic acid, 40 units pyruvate kinase, 40 units lactate dehydrogenase and 26 μg microsomal protein corresponding to 0.019 $(Na^+ + K^+)$-ATPase unit. After 2 min enzyme preincubation, the reaction was started by the addition of ouabain (sample) or water (control), and the corresponding value of NADH oxidation was recorded at each minute. Inhibition percentage was calculated by comparing the activity in the sample with that in the control after correcting for the small amount of ouabain-insensitive activity measured in the presence of ouabain 4 mM.

Statistical methods. Assays were repeated on separate membrane preparations, each assay on a particular membrane fraction being performed in triplicate. The binding data were graphically represented using classical plots, e.g. Scatchard and Log concentration–effect plots. The parameters were calculated using a computerized non-linear regression program, based on the steepest descent technique [22, 23] which adjusts the parameters to minimize the sum of relative squared errors (= Method I).

The parameters are given with approximates of standard deviations that represent the "goodness of fit" of the parameter with respect to the model and the data. When a model of two separate saturable processes was used, the data were analyzed according to the following general equation:

$$V = \frac{V_{M1} \cdot F}{K_1 + F} + \frac{V_{M2} \cdot F}{K_2 + F}. \quad (1)$$

For binding experiments, v = ^{3}H-glycoside specifically bound (B), V_M = capacity (B_{max}), K = dissociation constant (K_d) and F = free concentration of glycoside. For inhibition experiments, v = % of inhibition, V_M = % of maximal inhibition, K = IC_{50}. When the model of Hill [24] was used to analyse the binding data, the regression was performed with respect to the following equation:

$$V = \frac{V_M \cdot F^h}{K' + F^h} \quad (2)$$

where V, V_M and F have the same meaning as above.

K' is a constant equal to F^h when $V = V_M/2$ and h is the Hill coefficient.

For the model of Demeyts and Waelbroeck [25] the following equation was used for the regression analysis:

$$B = [RL] = \frac{-(\alpha \cdot [R_T]_{+[L]} \cdot \alpha \cdot [R_T] \cdot \bar{K}_e) \pm \sqrt{(\alpha \cdot [R_T] + [L] \cdot \alpha \cdot [R_T] \cdot \bar{K}_e)^2 + 4(1-\alpha)([R_T]^2 \cdot \alpha \cdot \bar{K}_e \cdot [L])}}{2 \cdot (1-\alpha)} \quad (3)$$

Equation (3) is a mathematical form obtained by substituting K_d derived from Demeyts and Waelbroeck [25, equation 11] into the classical Michaelis–Menten equation. MLAB, a non-linear least-squares curve fitting program [26] was also used to analyse the data transformed according to Scatchard (= Method II). The model for two classes of independent sites corresponded to the following equation [27]:

$$\frac{B}{F} = \frac{1}{2}\left[\frac{B_1 - B}{K_1} + \frac{B_2 - B}{K_2} + \sqrt{\left[\frac{B_1 - B}{K_1} - \frac{B_2 - B}{K_2}\right]^2 + 4\frac{B_1 B_2}{K_1 - K_2}}\right] \quad (4)$$

where B_1 and B_2 are the capacities of sites 1 and 2 respectively and K_1 and K_2 are the dissociation constants. B is the amount of bound ligand at a concentration F of free ligand. This analysis cannot provide the true standard deviation of the parameters such as can be obtained in the case of a linear equation but only a "rough guide" [26].

Different models were discriminated by using a F-test for comparison of total variances [28, 29]; the model with the lowest variance has been assumed to be the most representative of the experimental data.

Drugs. ^{3}H-Ouabain (11.6 Ci/mmol) was purchased from New England Nuclear (Boston, U.S.A.) and ^{3}H-digoxin (55 Ci/mmol) from the Radiochemical Center, (Amersham, U.K.).

The high counting efficiency scintillator Aqualuma-plus was supplied by Lumac B.V. (AC Schoesberg, The Netherlands). All other chemicals were of analytical grade.

RESULTS

Six separate $(Na^+ + K^+)$-ATPase enriched preparations were used. Each one was obtained from homogenate of ventricles prepared from 50 rat hearts. Specific activities and yield values for both $(Na^+ + K^+)$-ATPase and Mg-ATPase are reported in Table 1. The $(Na^+ + K^+)$-ATPase activity accounted for more than 80% of the total ATPase activity of the preparations.

Time–course of ouabain binding of rat heart microsomes. Figure 1 illustrates the time–course of ^{3}H-ouabain binding to microsomes in the (Mg–Pi) medium. ^{3}H-Ouabain binding reached a maximum after 5–10 min and remained stable over a 120 min incubation period. This is at variance with Erdmann *et al.* [16] who have observed that the amount of ouabain initially bound decreased with the prolongation of the incubation.

Cardiac glycoside binding to microsomes from ventricles as a function of concentration. Ouabain binding to ventricular microsomes was measured after 10 min incubation in the (Mg–Pi) medium. The amount of glycoside bound as a function of free concentrations was represented graphically according to Scatchard. As shown in Fig. 2, the plot was curvilinear and upwardly concave; this could be due either to binding to multiple independent binding sites, or to negative co-operativity between binding

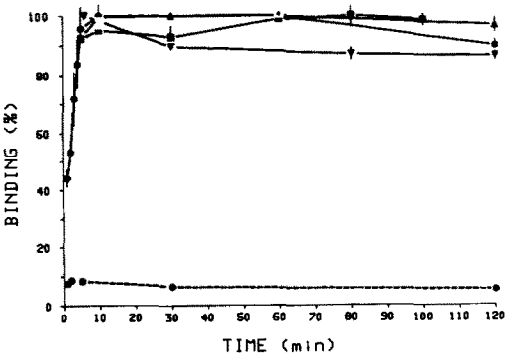

Fig. 1. Time–course of ^{3}H-ouabain binding to rat heart microsomes. After prewarming (4 min at 37°), the microsomes [about 150 μg protein, 0.08 $(Na^+ + K^+)$-ATPase unit] were incubated at 37° in 200 μl of medium containing: 3 mM $MgCl_2$, 3 mM Pi/Tris, 1 mM EGTA, 20 mM maleate/Tris (pH 7.4) and 5.5 to 7 nM of ^{3}H-ouabain (11.6 Ci/mmol). One hundred percent binding represents from 0.14 to 0.43 pmol/mg protein, a value about 10-fold above background (non specific binding). Samples were filtered after various incubation times. (———) Specific binding expressed as percentage of maximum specific binding. (---) Non-specific binding expressed as percentage of maximum specific binding. Points are means (±S.E.M.) of duplicate determinations. The different symbols represent different preparations.

sites [10, 30, 31]. These two possibilities were examined by an analysis of the results according to three different models: we assumed either the existence of 2 classes of independent sites (as the sum of two saturation equations) or the existence of negative co-operativity according to the model of Hill [24] or the model of Demeyts and Waelbroeck [25].

As shown in Fig. 2, the model based on the existence of two classes of sites fitted the data better than the others. Furthermore, when submitted to a F-test (see details in Materials and Methods) the total variance obtained when the data were analysed according to the model of two classes of sites ($S_T^2 = 0.0022$; $d.f. = 13$) was significantly lower ($P \ll 0.05$) than the total variance obtained with the model of Demeyts and Waelbroeck ($S_T^2 = 0.0083$, $d.f. = 14$) and the model of Hill ($S_T^2 = 0.0159$; $d.f. = 14$). Therefore, the quantitative analysis of the results was based on the first model using two statistical methods (see details in Materials and

Table 1. Characterization of microsomes prepared from ventricles

	Ventricles (N = 6)	
	$(Na^+ + K^+)$-ATPase	Mg-ATPase
Specific activity (μmoles Pi mg^{-1} protein hr)	31.3 ± 1.5	6.4 ± 1.0
Yield (EU g wet wt)	0.43 ± 0.04	0.10 ± 0.02

The specific activities were determined as described in Materials and Methods. Yield values are expressed as enzymatic unit (EU) per g of tissue (wet wt). One unit represents the amount of enzyme that hydrolyse 1 μmoles ATP per min. N = number of microsomal preparations.

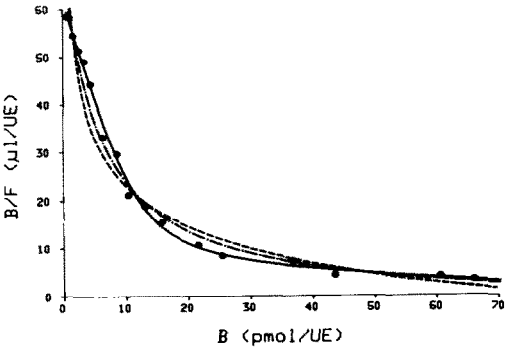

Fig. 2. Scatchard plot for ^{3}H-ouabain binding to $(Na^+ + K^+)$-ATPase from ventricles. The microsomes [128 µg protein, 0.071 $(Na^+ + K^+)$-ATPase unit] were incubated at 37° for 10 min in the presence of 3 mM $MgCl_2$, 3 mM Pi/Tris, 1 mM EGTA, 20 mM maleate/Tris (pH 7.4) and various concentrations of (tritiated + cold) ouabain (from 10^{-8} M to 2×10^{-5} M). ^{3}H-Ouabain 10^{-8} M (11.6 Ci/mmoles) was diluted with cold ouabain to give final concentration from 10^{-8} to 10^{-7} M. ^{3}H-Ouabain 4×10^{-8} M was diluted to give final concentrations from 10^{-7} to 3×10^{-6} M and ^{3}H-ouabain 9×10^{-8} M was diluted to give concentrations from 3×10^{-6} to 2×10^{-5} M. The non-specific binding measured in the presence of 4 mM unlabelled ouabain was subtracted from corresponding values of total binding, to obtain the specific binding. Each point is the mean of triplicate determinations in a typical experiment (preparation 2, Table 2). A computerized mathematical analysis was applied to the untransformed data (method I). Three models were used: 2 independent classes of sites and co-operativity according to Hill and Demeyts (see details in the text). The curves were drawn using the calculated parameters for each model. (——) Model of two classes of independent sites (equation 1). (---) Model of Hill (co-operativity) (equation 2). (-·-) Model of negative co-operativity according to Demeyts (equation 3). *B* Ouabain specifically bound. *F* Free concentration of ouabain (calculated).

Methods). Results of such an analysis with three different preparations are reported on Table 2 where binding capacities and affinities for the high and low affinity sites were compared. Similar results were obtained using the two statistical methods, indicating that about 10% of binding sites displayed a high affinity for ouabain ($K_d = 2 \times 10^{-7}$ M) whereas 90% of binding sites were characterized by a lower affinity ($K_d = 1 \times 10^{-5}$ M).

In other experiments, we have compared the binding of ouabain with the binding of digoxin, a more lipophilic glycoside. Data reported on Table 3 show that the binding capacities were not different for the two glycosides. That was not the case for affinity values; when compared with ouabain, digoxin affinity was higher at low affinity sites ($P < 0.01$) and lower at high affinity sites ($P > 0.05$) (Table 3). As a result, the ratio of the dissociation constant of the low affinity sites to the dissociation constant of the high affinity sites was significantly lower ($P > 0.05$) with digoxin (ratio = 14 ± 5) than with ouabain (ratio = 39 ± 6), indicating that ouabain allowed a better differentiation of the two sites than did digoxin.

Inhibition by ouabain of the microsomal $(NA^+ + K^+)$*-ATPase activity.* In order to estimate

Table 2. ^{3}H-Ouabain binding to rat microsomes isolated from ventricles

	Method I				Method II			
	Prep. 1 (N = 17)	Prep. 2 (N = 17)	Prep. 3 (N = 17)	$\bar{X}$ (± S.E.M.)	Prep. 1 (N = 17)	Prep. 2 (N = 17)	Prep. 3 (N = 17)	$\bar{X}$ (± S.E.M.)
High affinity sites								
B_{max} (pmoles/EU)	7.0 ± 0.7	9.5 ± 0.7	11.8± 0.9	9.4 ± 1.4	6.9	7.5	10.9	8.4 ± 1.2
K_d (µM)	0.19 ± 0.02	0.22 ± 0.02	0.21 ± 0.02	0.21 ± 0.01	0.19	0.18	0.19	0.19 ± 0.00
Low affinity sites								
B_{max} (pmoles/EU)	56 ± 3	102 ± 7	102 ± 9	87 ± 15	56	83	93	77 ± 12
K_d (µM)	7.6 ± 0.8	14 ± 2	18 ± 2	13 ± 3	7.3	8.8	14.2	10 ± 2

Three microsomal fractions from 50 rats each were prepared and assayed as in Fig. 2.

Method I. For each experiment, 4 parameters (B_{max1}, K_{d1}, B_{max2}, and K_{d2}) were calculated on untransformed data by an iterative program based on the steepest descent technique (equation 1). Each parameter is characterized by a standard deviation representing the goodness of fit. N is the number of ouabain concentrations used.

Method II. The MLAB iterative program was used to analyse the data transformed according to the equation of Scatchard (equation 4). With both methods, the standard deviations of the mean parameters (S.E.M.) represent the variation between the three preparations.

Table 3. ^{3}H-Ouabain and ^{3}H-digoxin binding to a ventricular microsomal preparations

	Ouabain (N 17)	Digoxin (N = 18)
High affinity sites		
B_{max} (pmoles/EU)	7.0 ± 0.7	6.3 ± 2.5
K_d (μM)	0.19 ± 0.02	0.27 ± 0.08
Low affinity sites		
B_{max} (pmoles/EU)	56 ± 3	55 ± 3
k_d (μM)	7.6 ± 0.8	3.7* ± 0-7

* $P < 0.01$ (two-tailed Student's t-test).

One preparation (prep. 1, Table 2) was used for ouabain and digoxin assay (see legend Fig. 2 for procedure). The standard deviations of the parameters represent the goodness of fit. The number of determinations corresponds to the number of different ouabain concentrations used in the assay.

the degree of inhibition of $(Na^+ + K^+)$-ATPase achieved when ouabain binding had reached steady state level, binding of ouabain was allowed to equilibrate before starting ATP hydrolysis (Radiochemical Assay).

Microsomes were preincubated in the presence of Na, Mg ATP and Pi, but in the absence of K, in order to allow fast binding of ouabain. After 15 min preincubation, ATPase reaction was initiated by adding KCl to reach 20 mM. As Fig. 3 illustrates, the inhibition–effect curve has a complex pattern when it is compared to a theoretical curve established assuming the existence of one sigmoidal concentration–effect curve with an IC_{50} value equal to the ouabain concentration found experimentally to inhibit 50% of the $(Na^+ + K^+)$-ATPase activity. Therefore, results were analysed assuming that the experimental curve could be the sum of two separate inhibitory processes (equation 1). The continuous line shown in Fig. 3 has been obtained according to this model which apparently fits the experimental data. This indicates the presence of a high affinity process corresponding to an inhibition of 13.2 ± 1.0% of $(Na^+ + K^+)$-ATPase activity with an IC_{50} value equal to 0.18 ± 0.02 μM and of a lower affinity process with an IC_{50} value equal to 89 ± 21 μM. The results were not significantly different when the fitting was performed on untransformed data, i.e. on values of total activity containing an ouabain insensitive term.

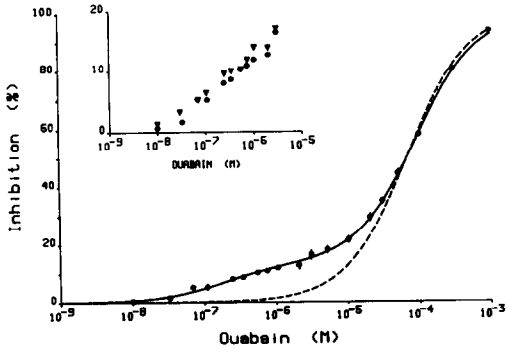

Fig. 3. Ouabain inhibition of $(Na^+ + K^+)$-ATPase activity in microsomes from ventricles: relation to ouabain binding. The microsomes [0.05 $(Na^+ + K^+)$-ATPase unit] were incubated in absence of K for 15 min at 37°. KCl 20 mM was then added to promote the activation of $(Na^+ + K^+)$-ATPase during 20 min. Each point is the mean of three experiments. Each experiment was performed on a different preparation and in triplicate. The data were analysed statistically according to the model of 2 independent processes by using a computerized regression analysis (equation 1, method I). The full line represents the fitted curve drawn according to the model and the calculated parameters. For the high affinity process: %Inh. Max. = 13.2 ± 1.0, IC_{50} (μM) = 0.18 ± 0.02. For the low affinity process: % Inh. Max. = 86 ± 10, IC_{50} (μM) = 89 ± 21. The dotted line is the theoretical curve for a simple inhibitory process (simple sigmoid) that should have a IC_{50} equal to the apparent IC_{50} for the experimental curve (6.65×10^{-5} M).

Inset. Inhibition of $(Na^+ + K^+)$-ATPase activity (●) and ouabain binding (▼) as functions of ouabain concentration in the low doses range (high affinity process). The binding was measured in exactly the same conditions as the ATPase inhibition. The binding data are expressed as percentage of maximal binding estimated from Table 2.

The amount of ouabain bound under those experimental conditions has been measured on the same microsomal preparations and expressed as percentage of maximum binding. As inset of Fig. 4 shows for the lowest concentrations, binding and inhibition curves are superimposed.

$(Na^+ + K^+)$-ATPase activity was also studied using the Fiske and Subbarow Assay and the Coupled Optical Assay (see Materials and Methods) and these results were compared to those obtained with the Radiochemical Assay (Fig. 4). When ouabain action on $(Na^+ + K^+)$-ATPase activity was studied by the Fiske and Subbarow assay, no shoulder appeared in the inhibition curve so that the high affinity process was not detected. When the inhibitory effect of ouabain was measured at equilibrium using the Coupled Optical Assay, the experimental data were similar to those obtained with the Radiochemical Assay.

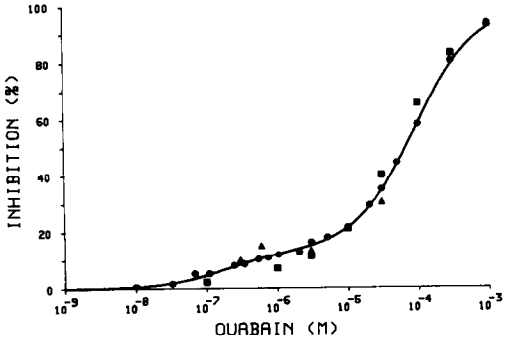

Fig. 4. Comparison of different assays for the inhibitory action of ouabain on $(Na^+ + K^+)$-ATPase activity. $(Na^+ + K^+)$-ATPase activity was measured in microsomes from rat heart ventricles using 3 different assays; the inhibitory effect of ouabain was expressed as percentage of maximal inhibition (see Materials and Methods). ● Radiochemical Assay: see legend Fig. 4. ■ Fiske and Subbarow Assay. Each point is the mean of two experiments, each being performed on a different microsomal preparation and in triplicate. ▲ Coupled Optical Assay. Each point represents one experiment. The maximal inhibitory effect was measured after 2 min, a time sufficient to reach equilibrium.

DISCUSSION

1. *Heterogeneity of cardiac glycoside binding sites in rat heart.* Ouabain binding heterogeneity has been reported in microsomal preparations from beef and rat brain [8–11] and cardiac tissues from various species [12–17]. Such heterogeneity could be due to the presence of different $(Na^+ + K^+)$-ATPase conformations with different affinities for ouabain [8, 11, 12, 14] or to the existence of independent binding sites [9, 10, 13, 15, 16]. In this work, we obtained upwardly concave Scatchard plot for ouabain binding to rat heart microsomes, that could be explained by the existence of either multiple binding sites (i.e. independent sites of conformations of the same site not in equilibrium [14]) or of co-operativity [10, 30, 31]. Among the three models tested, the model of two classes of independent sites filled the experimental data for ouabain binding to rat heart microsomes the most closely, as shown by *F*-test analysis. The existence of two classes of independent binding sites in rat heart has been proposed by Erdmann *et al.* [16].

The present results are in agreement with this interpretation if it is assumed that *F*-test for comparison of total variances allows to discriminate between models (see Materials and Methods). However, in view of the experimental variance, negative cooperativity cannot be definitely ruled out with only statistical analysis. The existence of independent binding sites rather than negative cooperatively is also indicated by the superimposition of ^{3}H-ouabain dissociation curves after isotopic or volumetric dilution (data not shown).

The high affinity sites described in this study have an affinity for ouabain similar to the binding sites found by Sharma and Banerjee in microsomes [32] and Adams *et al.* in isolated rat cardiac cells [33].

The evidence for two binding sites has been confirmed with digoxin. In addition, it was found that the affinity of the two sites was not the same for the two glycosides, ouabain allowing a better identification of the two binding sites.

2. *Nature of the two classes of cardiac glycoside binding sites: relation to* $(Na^+ + K^+)$-ATPase *and* Na–K *pump activities.* Experiments illustrated in Fig. 3 suggest the existence of two different $(Na^+ + K^+)$-ATPase inhibitory processes. The proportion of the low and high affinity components of the inhibitory effect was similar to the proportion of low and high affinity sites measured in binding experiments. These results strongly suggest that both classes of binding sites are on $(Na^+ + K^+)$-ATPase. It could be that two isozymes coexist in our preparations with a ratio equal to the one found for inhibition and binding. Indeed, the presence of two distinct molecular forms of the $(Na^+ + K^+)$-ATPase differing in affinity for strophanthidin has been reported in the brain of several mammals [34].

As shown in Fig. 4, the ease of detection of the high affinity process depends greatly upon the experimental conditions. Steady state binding before measurement of the inhibitory effect of ouabain seems to be important. The "Fiske and Subbarow assay" did not reveal the high affinity process; as reported earlier by De Pover and Godfraind [35], measurement of the cumulative amount of inorganic phosphate released during the non steady state period (Fiske and Subbarow Assay) can lead to underestimate of glycoside potency, especially for the low concentrations. The observation of the high affinity process may also depend upon the method of rat heart $(Na^+ + K^+)$-ATPase preparation (see Mansier and Lelièvre [36] and Schwartz *et al.* [37] but not Erdman *et al.* [16]).

In intact tissue, Erdmann *et al.* [16] have demonstrated that ^{86}Rb-uptake was not modified at ouabain concentrations lower than 10^{-5} M, but was nearly completely inhibited at 10^{-4} M, indicating that only low affinity sites are inhibitory *in vivo*. This opens the question of the function of the high affinity sites and of their relation to ouabain action in intact cells.

Acknowledgements—We thank Dr. J. Cumps (Laboratoire de Chimie Thérapeutique) and Dr. P. Demeyts (International Institute of Cellular and Molecular Pathology) for helpful discussion on statistical analysis and on computer programmation.

F. Noël is Chargé de Recherches du F.N.R.S. This work has been supported by grant No. 3.9001.79 of the Fonds de la Recherche Scientifique Médicale.

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