Biochemical Pharmacology, Vol. 28, pp. 3051–3056

0006-2952/79/1015-3051 $02.00/0

INTERACTION OF OUABAIN WITH ($Na^+ + K^+$)ATPase FROM HUMAN HEART AND FROM GUINEA-PIG HEART

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(*Received* 26 *January* 1979; *accepted* 10 *April* 1979)

Abstract—($Na^+ + K^+$)ATPase (ATP phosphohydrolase, EC 3.6.1.3.) has been prepared from human heart and guinea-pig heart, with respective specific activities of 10–15 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$ and of 25–30 μmol $P_i \cdot mg^{-1} \cdot h^{-1}$. Residual Mg^{2+}—ATPase activities were about 5 per cent. The parameters of ($Na^+ + K^+$)ATPase activity and of ouabain-interaction have been compared: (1) Half-maximal activity concentrations and Hill coefficients of Na^+, K^+, Mg^{2+} and ATP were similar for the two species. The apparent activation energies calculated from Arrhenius plots were also similar. A transition was observed at about 23°C. (2) Human heart ($Na^+ + K^+$)ATPase was 10 times more sensitive to ouabain-inhibition than that of guinea-pig. Hunter–Downs plots showed a competitive inhibition for K^+ at low K^+ concentration and noncompetitive inhibition at high concentration. (3) The Scatchard plot for [^{3}H]ouabain binding was upward-concave with human heart and linear with guinea-pig heart. (4) The dissociation kinetics of [^{3}H]ouabain from human preparations studied by an isotopic dilution technique indicated two classes of binding sites with k_d of 0.058 min^{-1} and 0.0092 min^{-1}. The dissociation kinetics with guinea-pig heart indicated one single class of binding sites with a k_d of 0.43 min^{-1}. (5) The time-course of 0.2 μM [^{3}H]ouabain binding showed pseudo-first order association kinetics in man and in guinea-pig. k_a for the two classes of binding sites in man were therefore similar, respectively equal to 3.4×10^6 $min^{-1} \cdot M^{-1}$ and to 3.7×10^6 $min^{-1} \cdot M^{-1}$. k_a for guinea-pig heart was equal to $2.3.10^6$ $min^{-1} \cdot M^{-1}$. (6) In guinea-pig heart, K_D calculated from Scatchard plot and from k_d/k_a ratio were equal to the inhibition constant K_i calculated from Hunter–Downs plot indicating that the binding sites were closely related to ($Na^+ + K^+$)ATPase inhibition. (7) In human heart, K_D of the low affinity binding sites was close to K_i, whereas K_D of the high affinity binding sites was several times lower. This suggests that only low affinity binding sites might be involved in ($Na^+ + K^+$)ATPase inhibition by ouabain.

INTRODUCTION

It is widely accepted that ($Na^+ + K^+$)ATPase [ATP phosphohydrolase, EC 3.6.1.3.] plays a key role in the active transport of Na^+ and K^+ across cell membranes [1].

The inhibition of the Na^+ pump by cardiac glycosides is due to the inhibition of this enzyme [1, 2]. Repke had proposed that the positive inotropic effect of these drugs might be related to this inhibition [3]. However, the positive inotropic effect is not always associated with Na^+ pump inhibition [4–7]. Furthermore, at therapeutic concentrations cardiac glycosides evoke a Na^+ pump stimulation [8]. Therefore, the understanding of the therapeutic mode of action of cardiac glycosides requires the study of the properties of human heart ($Na^+ + K^+$)ATPase, especially as the interaction between these drugs and ($Na^+ + K^+$)ATPase shows quantitative as well as qualitative variations among animal species [9–15].

In this paper, we report some properties of a human heart ($Na^+ + K^+$)ATPase preparation, in comparison with the one obtained from the guinea-pig. We have observed that the sensitivity to ouabain and the kinetics of [^{3}H]ouabain binding were not similar for the two cardiac tissues.

METHODS

Preparation and assay of the ($Na^+ + K^+$)ATPase

Human heart samples have been obtained from children operated for right ventricular outflow tract hypertrophy, and immediately frozen at −30°C. Preparation of ($Na^+ + K^+$)ATPase and determination of enzyme activities were performed as described previously for guinea-pig heart [16]. Usually, 5–10 μg proteins were incubated for 60 min at 37°C in a medium containing, unless otherwise stated (final volume, 1 ml): 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 2.5 mM ATP, 1 mM EGTA [ethylene glycol bis (β-aminoethylether)-*N,N'*-tetraacetic acid], 20 mM maleic acid (pH was adjusted at 7.4 with Tris). The reaction was stopped with trichloroacetic acid, and inorganic phosphate (Pi) was measured by the Fiske and Subbarow method [17]. When ATPase assays were performed at substrate concentrations lower than 0.5 mM, Pi was measured by the malachite green method [18].

Determination of [^{3}H]*ouabain binding*

[^{3}H]Ouabain binding was determined by the filtration technique. About 5 mg of enzyme protein were incubated at 37°C in 30 ml medium containing: 100 mM NaCl, 3 mM $MgCl_2$, 2.5 mM ATP, 1 mM EGTA, 20 mM Tris/maleate (pH 7.4) and various concentrations of [^{3}H]ouabain (0.12–12 Ci/mmol). After various incubation periods, 1-ml portions of the medium were filtered at 0°C on 0.45 mm Sartorius filters (5·M·01386) or on Whatman glass fiber filters (GF/F) in a Millipore 3025 Sampling Manifold. After washing with 10 ml of chilled solution (0.25 M sucrose, 5 mM Tris/HCl at pH 7.4) the filters were dissolved in the followed scintillation solution: toluene/Triton X-100/ethylene-glycol-monomethylether (600/250/150, by vol) containing 2,5-diphenyloxazole (6 g/l), *p*-bis-(*O*-methylstyryl)-benzene (1 g/l) and

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naphthalene (75 g/l). The radioactivity of the samples was counted as usual, with appropriate controls, and the efficiency (35 per cent) was determined with internal standards. The non-specific radioactivity retained by the filters was estimated from samples incubated in the presence of 1 mM unlabelled ouabain, it reflected namely the remaining contaminations. As contaminations varied with the concentration of radioactivity in the medium, they are determined for each concentration of [^{3}H]-ouabain. At the highest [^{3}H] ouabain concentration, non-specific radioactivity was 150 c.p.m./ml sample filtered and total binding was at least 760 c.p.m./ml sample filtered. In experiments with high [^{3}H]ouabain concentrations, glass fiber filters were used that gave the lowest contaminations. The specific binding was stable for hours at 0°C in buffered sucrose.

The dissociation of [^{3}H]ouabain from ($Na^+ + K^+$)ATPase was followed with an isotopic dilution procedure. After preincubation of the enzyme in the presence of [^{3}H]ouabain until steady-state binding, an excess of unlabelled ouabain was added to the medium (final concentration: 0.2 mM). Under these conditions, the association of [^{3}H]ouabain to the enzyme was stopped and the amount remaining bound was measured after various incubation times. Apparent dissociation rate constants (k_d) were calculated from the slope of the curves of log bound [^{3}H]ouabain versus time. Apparent association rate constants (k_a) were estimated from experiments in which [^{3}H]ouabain binding to ($Na^+ + K^+$)ATPase followed pseudofirst order kinetics. The amounts of [^{3}H]ouabain bound at various times were subtracted from the steady-state binding and the values obtained were plotted on a semilogarithmic scale (see Fig. 4). The slope of the resulting straight line, k_{obs}, is related to k_a through the following equation [19]:

$$2.303\, k_{obs} = k_a L + k_d \qquad (1)$$

where L is the concentration of [^{3}H]ouabain. The lines of the kinetic plots were fitted by eye.

Reagents

All solutions were prepared using distilled, deionized water. ATP was purchased from Boehringer GmbH, Mannheim, G.F.R. and [^{3}H]ouabain from the Radiochemical Centre, Amersham, U.K. All other chemicals were of analytical grade and purchased from E. Merck, Darmstadt, G.F.R.

Statistical methods

Whenever possible, values are presented as means ± S.E.M.

RESULTS

Kinetic properties of ($Na^+ + K^+$)ATPase.

When assayed under standard conditions (see Methods), the ATPase activities of the human heart and guinea-pig heart preparations amounted to 10–15 and 25–30 μmol Pi·mg^{-1} protein·h^{-1}, respectively. In the absence of Na^+ or K^+, or in the presence of 1 mM ouabain, the activities were lowered about 20-fold. No enzymic hydrolysis was detected in the absence of Mg^{2+}. ($Na^+ + K^+$)stimulated ATP hydrolysis increased linearly with incubation time, at least up to 240 min.

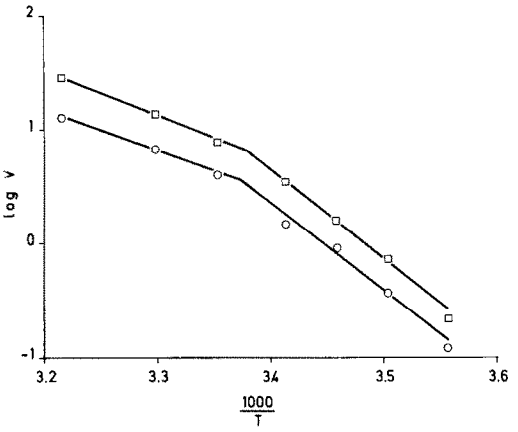

Fig. 1. Arrhenius plots for ($Na^+ + K^+$)ATPase activity. Human heart (O) and guinea-pig heart (□) preparations were incubated for 30 min at 25, 30 and 37°C and for 240 min at 8, 12, 16 and 20°C in the medium containing 100 mM Na^+, 10 mM K^+, 3 mM Mg^{2+}, 2.5 mM ATP, 1 mM EGTA and 20 mM Tris/maleate (pH 7.4). Each point is the mean of 4 values. Standard error of the mean did not exceed the diameter of the symbol.

The temperature-dependence of enzyme activities was studied between 8 and 37°C. The Arrhenius plots (Fig. 1) showed a transition temperature at 23°C (man) and 22°C (guinea-pig). The calculated apparent activation energies were equal to 17 and 35 kcal/mol for human heart and to 18 and 36 kcal/mol for guinea-pig heart. These data are similar to those reported for other homeotherms [13].

The dependence of ($Na^+ + K^+$)ATPase activity on ATP and cations concentrations is summarized in Table 1. Half-maximal activation concentrations ($K_{0.5}$) and Hill coefficients (n) were similar for the two species. n was equal to one for ATP and Mg^{2+} as previously shown with purified preparations from rat brain in contrast with non-purified preparations [21]. n was higher than one for Na^+ and K^+. Sigmoïdal activation curves for Na^+ and K^+ have been reported for a number of species [22–24] except in crab nerves where n for K^+

Table 1. ($Na^+ + K^+$)ATPase activation parameters

Activation by	Species	$K_{0.5}$ (mM)	n
Na^+	man	4.8	1.7
	guinea-pig	5.2	1.7
K^+	man	1.2	1.4
	guinea-pig	1.0	1.5
Mg^{2+}	man	0.35	0.9
	guinea-pig	0.50	1.0
ATP	man	0.081	1.0
	guinea-pig	0.10	1.0

The enzyme (5–10 μg/ml) was incubated at 37°C for 60 min in the presence of 100 mM Na^+, 3 mM K^+, 3 mM Mg^{2+} 2.5 mM ATP, 1 mM EGTA, 20 mM Tris/maleate (pH 7.4). Na^+, K^+, Mg^{2+} or ATP concentration was varied keeping the other ligands constant. $K_{0.5}$ and n values were estimated from Hill plots [20] by linear regression. The curves showed a good linearity for values of log (v/V-v) between + 0.5 and −0.5. At least 6 concentrations (in quadruplicate) of ATP or cations stood within these limits.

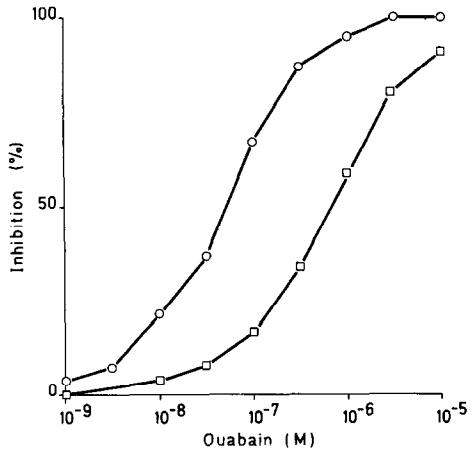

Fig. 2. $(Na^+ + K^+)$ATPase inhibition by ouabain. The human (○) and guinea-pig (□) enzymes were incubated at 37°C for 60 min in the presence of 100 mM Na^+, 3 mM K^+, 3 mM Mg^{2+}, 2.5 mM ATP, 1 mM EGTA, 20 mM Tris/maleate (pH 7.4) and various concentrations of ouabain. Each point is the mean value from 6 experiments. Standard error of the mean did not exceed the diameter of the symbol.

was equal to 1 [25]. Such curves are usually interpreted on the basis of allosteric or multiple 'independent'-site models [26].

$(Na^+ + K^+)$ATPase inhibition by ouabain.

$(Na^+ + K^+)$ATPase inhibition was estimated after 60 min incubation in the presence of ouabain (Fig. 2). The human enzyme was sensitive to concentrations as low as 10^{-9} M. The I_{50} values were 0.05 and 0.7 μM respectively for the human and guinea-pig ATPases. As K^+ is known to antagonize ouabain inhibition, the ouabain inhibition constants (K_i) and the K^+ apparent dissociation constant (K_s) were estimated according to Hunter and Downs [27]. As shown in Fig. 3, the I_{50} values increased linearly with K^+ concentration up to 5 mM, which indicated a competitive inhibition in the low range of K^+ concentrations. The K_i for ouabain estimated from these graphs amounted to 0.013 μM

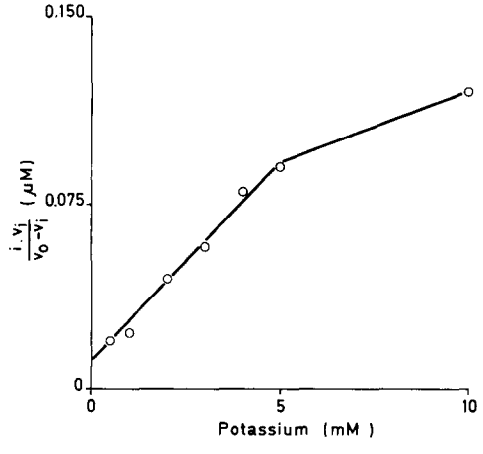

Fig. 3. Hunter–Downs plot (20) for ouabain inhibition (human heart). $iv_i/v_o - v_i$ vs KCl concentration. v_o is the uninhibited velocity, v_i the inhibited velocity, i the ouabain concentration Data reported here were obtained for $i = I_{50}$ (n = 6).

(man) and 0.14 μM (guinea-pig). The apparent K_s for K^+ was equal to 1 mM in the two species. This value is close to that obtained from Hill plots (Table 1). These results are in good agreement with those reported by Akera *et al.* for five other animal species [9].

Ouabain binding to $(Na^+ + K^+)$ATPase.

As illustrated in Fig. 4, the binding of [^{3}H]ouabain was maximal after 10 min incubation at 37°C when the ligand concentration was 0.2 μM. When the amounts bound at various times were subtracted from the steady-state binding and the resulting values plotted on a semi-logarithmic scale, straight lines were obtained with half-times of 56 sec for human heart ATPase and 48 sec for guinea-pig heart ATPase. These observations indicate that, under these conditions, the binding followed a pseudo-first order kinetics. The association rate constants (k_a) calculated according to equation (1) (see Methods) are listed in Table 2. As shown in Fig. 5a, the dissociation of [^{3}H]ouabain from guinea-pig preparations was a first order process, with a half-life of 1.6 min, independent of the [^{3}H]ouabain concentration in the preincubation medium. In contrast, the dissociation from human preparations was markedly slower and was not a simple exponential process (Fig. 5b). Besides, the half-life of the [^{3}H]ouabain–enzyme complex was dependent on the [^{3}H]ouabain concentration in the preincubation medium. Analyses of the curves according to Choi and Akera [29] gives two exponentials. The slow process represents 76 per cent of the total number of sites at 0.002 μM [^{3}H] ouabain ($t_{1/2}$ = 81 min) and 52 per cent at 0.2 μM ($t_{1/2}$ = 74 min). The fast process represents 26 per cent of the total number of sites at 0.002 μM ($t_{1/2}$ = 11 min) and 49 per cent at 0.2 μM ($t_{1/2}$ = 11 min). The dissociation rate constants calculated from these curves are shown in Table 2.

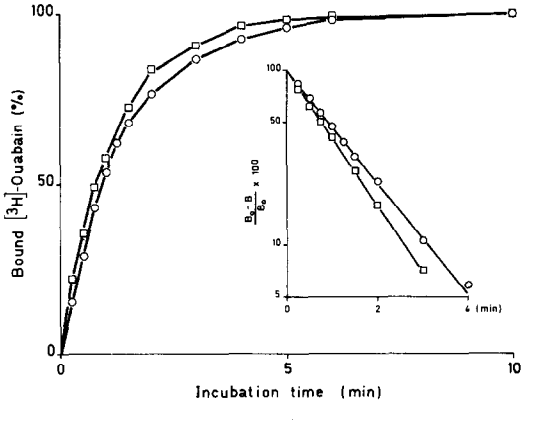

Fig. 4. Time-course of [^{3}H]ouabain binding to $(Na^+ + K^+)$ATPase. The human (○) and guinea-pig (□) preparations (about 160 μg/ml) were incubated at 37°C in the presence of 100 mM Na^+, 3 mM Mg^{2+}, 2.5 mM ATP, 1 mM EGTA, 20 mM Tris/maleate (pH 7.4) and 0.2 μM [^{3}H]ouabain. Each point is the mean value from 4 experiments. The standard error of the mean did not exceed the diameter of the symbol.

Inset: semi-log plot of $(B_0 - B/B_0) \times 100$ vs time where B is the bound [^{3}H]ouabain at each time and B_0 at steady-state.

Table 2. Ouabain-(Na^+ + K^+)ATPase interaction parameters

	k_a ($min^{-1}.M^{-1}$)	k_d (min^{-1})	K_D (k_d/k_a) (μM)	K_D (Scatchard) (μM)	K_i (Hunter–Downs) (μM)
Guinea-pig	$2.3.10^6$	0.43 ± 0.04	0.19	0.17	0.14
Human heart (a)	$3.4.10^6$	0.058 ± 0.003	0.017	—	0.013
(b)	$3.7.10^6$	0.0092 ± 0.0003	0.0025	0.0048	—

K_D calculated from the ratio of rate constants k_d/k_a and from Scatchard plots are compared to the inhibition constants K_i calculated from Hunter–Downs plots. k_d (± S.E.M. from three experiments) have been estimated from dissociation kinetics observed following the termination of the [^{3}H]ouabain reaction. k_a have been calculated by equation (1) from pseudo-first order binding kinetics (see Methods).

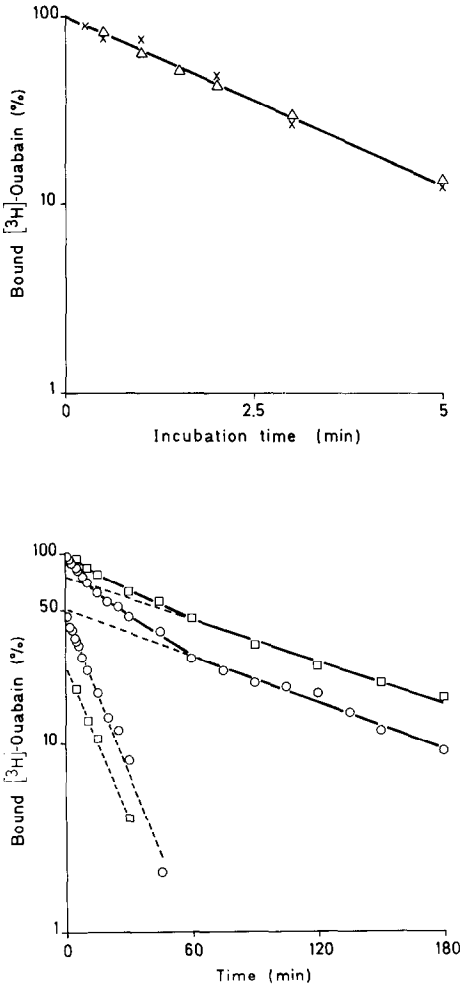

Fig. 5. Time-course of [^{3}H]ouabain dissociation from (Na^+ + K^+)ATPase. The enzyme was incubated at 37°C in the presence of 100 mM Na^+, 3 mM Mg^{2+}, 3 mM ATP, 1 mM EGTA, ^{3}H ouabain and 20 mM Tris/maleate (pH 7.4). After 60 min incubation, 20 mM unlabelled ouabain was added to make the final concentration 0.2 mM. Each point is the mean value from 3 experiments. The standard error of the mean did not exceed the diameter of the symbol.
(a) Guinea-pig heart: [^{3}H]ouabain at 2.10^{-8} M (△) or at 2.10^{-6} M (×)
(b) Human heart: [^{3}H]ouabain at 2.10^{-9} (□) or at 2.10^{-7} M (○)

Figure 6 shows saturation curves of (Na^+ + K^+)ATPase by [^{3}H]ouabain between 0.001 and 10 μM [^{3}H]ouabain. At 10 μM, both preparations bound 150–200 pmol [^{3}H]ouabain per unit of enzyme activity (one unit is the amount of enzyme which hydrolyses 1 μmol ATP per min). The Scatchard plot

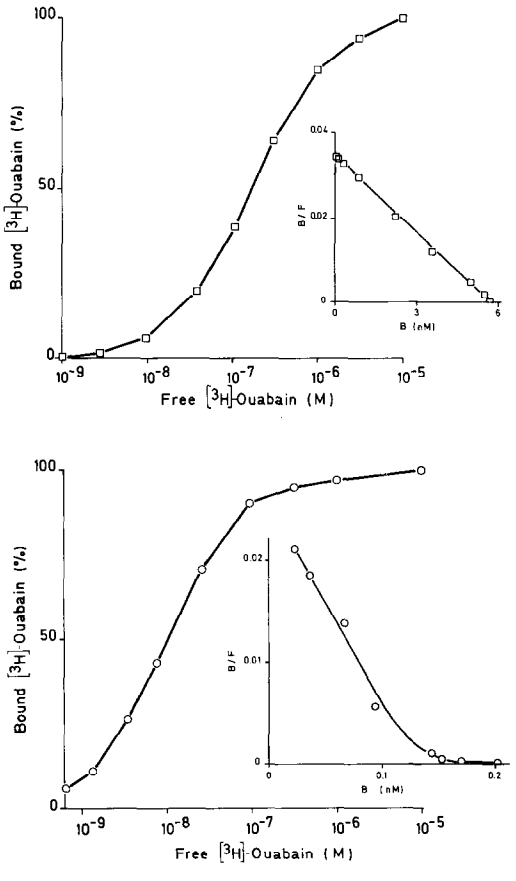

Fig. 6. Ouabain binding to (Na^+ + K^+)ATPase as a function of ouabain concentration. The enzyme (0.16 mg/4 ml for guinea-pig and 0.024 mg/4 ml for human) was incubated at 37°C for 4 h in the presence of 100mM Na^+, 3 mM Mg^{2+}, 3 mM ATP, 1 mM EGTA, [^{3}H]ouabain and 20 Tris/maleate (pH 7.4). Each point is the mean of 3 experimental values. Inset: Scatchard plot.
(a) Guinea-pig heart
(b) Human heart.

was linear with guinea-pig preparations and upward-concave with human preparation. Binding parameters were calculated according to Scatchard [30]. For human heart preparations, the parameters were estimated for the high affinity sites only. Data reported in Table 2 show that with guinea-pig heart apparent K_D from Scatchard plot was in good agreement with K_D calculated from k_d/k_a ratio, and that K_i was close to K_D values. With human heart, K_D for high affinity binding sites calculated from Scatchard plot was close to K_D calculated from k_d/k_a ratio. K_i was several times higher than K_D values for high affinity sites and was close to K_D for low affinity sites. k_a values for human heart were close to that calculated for guinea-pig heart while k_d values were considerably lower.

DISCUSSION

The specific activity of the $(Na^+ + K^+)$ATPase preparations from human heart was twofold lower than that of the guinea-pig heart preparations. Similar species differences have been reported by others [31–33]. Previous attempts to purify the enzyme from human heart have provided preparations with much lower $(Na^+ + K^+)$stimulated activities and higher Mg^{2+}-dependent basal activities [34, 35]. The dependence of the $(Na^+ + K^+)$ATPase reaction on temperature and on concentration of ATP, Mg^{2+}, Na^+ and K^+ was quite similar in human heart and guinea-pig heart. Our data are in agreement with those obtained on other homeotherms [13, 21–24].

The human $(Na^+ + K^+)$ATPase was 10 times more sensitive to ouabain-inhibition than the guinea-pig enzyme. Akera *et al.* have reported that the heart $(Na^+ + K^+)$ATPases of the dog, sheep and pig were 2–3 times more sensitive than that of the guinea-pig [9]. On the basis of the present results, human heart $(Na^+ + K^+)$ATPase appears to be one of the most sensitive to ouabain. The determination of ouabain I_{50} in the presence of various concentrations of K^+ allowed the analysis of ouabain–K^+ interaction according to Hunter and Downs [27]. It showed that K^+ competed with ouabain for KCl concentrations lower than 5 mM and that K^+ effect tended to a maximum for higher concentrations. This observation is in agreement with reports on K^+-ouabain competition in other tissues [9].

Ouabain K_i was estimated from Hunter and Downs plot and compared to K_D estimates from binding data. The binding data obtained with the guinea-pig heart ATPase are compatible with the existence of a single class of ouabain binding sites involved in $(Na^+ + K^+)$ATPase inhibition by ouabain. Indeed, the dissociation constant K_D calculated from the Scatchard plots was remarkably similar to that derived from the kinetics experiments (k_d/k_a) and to K_i value. The binding of [^{3}H]ouabain to the human heart ATPase was more complex and the experimental data indicated the existence of two classes of ouabain binding sites. This was evidenced by dissociation kinetics showing two dissociation rate constants and by the non-linearity of the Scatchard plot. Association rate constants were apparently not different for the two groups of sites. Studying $(Na^+ + K^+)$ATPase from different species, Erdmann and Schöner [14] have reported that association rate constants of ouabain were similar and that species differences were related to dissociation rates constants as also proposed by others [10, 12, 36]. It is therefore not surprising to make a similar observation for two classes of binding sites in one preparation. The present results are consistent with kinetic studies of Choi and Akera [29] with rat brain. In view of the existence of two phases in the time curve of the release of [^{3}H]ouabain from $(Na^+ + K^+)$ATPase, they proposed that ouabain dissociation might proceed through several separate sequential and parallel pathways. On the other hand, Hansen [37] has observed that Scatchard plots of ouabain binding to beef brain $[Na^+ + K^+]$ATPase resulted in curved lines, the non-linearity being affected by the composition of the medium. Hansen proposed from his results the existence of two (or more) populations of enzymes with different affinities for substrate and ligands affecting ouabain binding. More recently Schöner *et al.* [38] with beef brain enzyme and Erdman *et al.* [39] with human heart enzyme have reported the existence of high and low affinity ouabain binding sites. As the incubation medium were different from ours, a comparison of the quantitative data is not relevant. It might be that the difference between guinea-pig and human enzymes, as shown in this paper, is artifactual and due to a medium composition which did not allow the identification of high affinity sites in guinea-pig preparation.

In human preparations, ouabain low affinity binding sites dissociation constant (K_D) was close to ouabain K_i calculated from Hunter–Down plots. This indicates that low affinity binding sites [5] were involved in $(Na^+ + K^+)$ATPase inhibition by ouabain. As far as the function of ouabain high affinity sites is concerned the question as to whether they are or not involved in the stimulation of the Na pump observed with ouabain in intact cardiac tissue [8] is left open until more information is obtained on this action.

Acknowledgement—This work has been supported by FRSM Grant No. 20 121. We thank M. Wibo for reading the manuscript.

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