Biochemical Pharmacology, Vol. 40, No. 12, pp. 2611–2616, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
Pergamon Press plc

A COMPARISON OF THE AFFINITIES OF RAT ($Na^+ + K^+$)-ATPase ISOZYMES FOR CARDIOACTIVE STEROIDS, ROLE OF LACTONE RING, SUGAR MOIETY AND KCl CONCENTRATION

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(*Received* 21 *March* 1990; *accepted* 1 *August* 1990)

Abstract—Binding experiments at equilibrium were performed to study pharmacological properties of isozymes of ($Na^+ + K^+$)-ATPase from rat tissues. Experiments were performed on brain (α_3 isozyme), kidney (α_1 isozyme) and heart microsomes (α_1 and α_2 isozymes). Affinity of series of ouabain and digoxin derivatives was studied in competition experiments. It was observed that: (i) ouabain and digoxin had higher affinity ($P < 0.01$) for α_3 isozyme (K_d of 0.071 ± 0.004 and 0.066 ± 0.001 μM, respectively) than for α_1 isozyme (K_d of 15.9 ± 0.8 and 1.78 ± 0.46 μM, respectively) and α_2 isozyme (K_d of 0.26 ± 0.04 and 0.15 ± 0.06 μM, respectively); (ii) saturation of the C_{20}—C_{22} bond on the $C_{17\beta}$ lactone ring present in ouabain and digoxin markedly decreased the drug affinity for all isozymes ($P < 0.01$); and (iii) suppression of the $C_{3\beta}$ osidic chain decreased the affinity of ouabain and digoxin to a higher extent for α_2 and α_3 than for α_1 ($P < 0.01$). The presence of 10 mM KCl in the incubation medium decreased ouabain affinity for the α_1 isozyme to a much higher extent (K_d increase of about 20-fold) than for the other isozymes (K_d increase of about 2-fold). The results show that the isozymes of ($Na^+ + K^+$)-ATPase from rat tissue are differently sensitive to changes in the substituents of the cardioactive steroids and to the presence of 10 mM KCl.

The existence of different isozymes of the ($Na^+ + K^+$)-ATPase catalytic subunit was firstly reported by Sweadner [1] in rat brain. The two isozymes then identified show different mobilities in sodium dodecylsulfate/polyacrylamide gel electrophoresis. These two forms have been purified independently from axolemma (alpha$_+$ now named α_3) and kidney (α_1). They are controlled by different genes [2, 3] and can be distinguished by their sensitivity to ouabain [4, 5], to trypsin and to various reagents such as *N*-ethyl-maleimide, Cu^{2+}, *O*-phenanthroline, pyrithiamin and erythrosin B [4, 6].

On the other hand, there is now a large body of evidence that ouabain binding to heart microsomes occurs at more than one specific binding sites in various species [7–12]. In rat heart, we have demonstrated the presence of two classes of specific ouabain binding sites and their relation to ($Na^+ + K^+$)-ATPase activity suggests that they were related to two different isozymes [11]. More recently, it has been proposed that the two populations of ouabain binding sites found in dog, ferret and rat heart may be related to two isozymes of ($Na^+ + K^+$)-ATPase similar to the alpha$_+$ and α forms present in the brain [5, 12–14].

The present experiments were designed to further study pharmacological differences between the isozymes of ($Na^+ + K^+$)-ATPase from rat. The apparent affinity of ouabain, digoxin, their dihydroderivatives and their genins, which are known to be inequally active as inhibitors of ($Na^+ + K^+$)-ATPase [15], were estimated in preparations obtained from brain, kidney and heart. The results show that the isozymes were differently sensitive to changes in the substituents of the cardioactive steroids and to the presence of 10 mM KCl. In this paper, we followed the isozymes nomenclature recently proposed by Sweadner [16].

MATERIALS AND METHODS

Preparation of membrane fractions. Heart ventricles, kidneys and forebrains were dissected from the same Wistar rats (about 6 months old). Microsomes enriched in ($Na^+ + K^+$)-ATPase were prepared as described previously for heart [11]. Specific ($Na^+ + K^+$)-ATPase activities were about 165, 176 and 31 μmol P_i/mg protein/hr in microsomes from brain, kidney and heart, respectively and accounted for more than 80% of the total ATPase activities of these preparations.

[^{3}H]*Ouabain binding assay.* Unless otherwise stated, the incubation medium contained [^{3}H]-ouabain (New England Nuclear), 3 mM $MgCl_2$, 3 mM P_i–Tris, 1 mM EGTA and 20 mM maleate/Tris, pH 7.4 (Mg–P_i medium) at 37°. The non-specific binding was estimated from samples incubated in the absence of Mg^{2+} and P_i and in the presence of 1 mM unlabelled ouabain. In saturation experiments non-specific binding accounted for 11–44% of total binding in absence of KCl, to 57–67% in presence of KCl (kidney). With brain preparation, non-specific binding accounted for 2–16% in absence of KCl to 4–46% in presence of KCl. The incubation was terminated by rapid filtration on Whatman glass fiber filters (GF/F). The filters were washed three

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times with 20 mL chilled sucrose solution (0.25 M sucrose, 5 mM Tris/HCl, pH 7.4 at 4°). When testing the effect of K^+ on ouabain binding to kidney microsomes (KCl = 0 vs KCl = 10 mM), incubation was terminated by diluting the 225 μL reaction mixture with 3 mL of chilled sucrose solution before filtration. Tubes were washed with 3 mL of chilled sucrose solution and filters were further washed with 20 mL portions of washing solutions. Filters were added to 10 mL of Picofluor (Packard)/toluene (1/4, v/v) and the radioactivity counted in a liquid scintillation counter with an efficiency of 40%.

Analysis of results. All $(Na^+ + K^+)$-ATPase preparations used in the present study were characterized by equilibrium binding parameters (K_d, B_{max}), obtained by a computerized non-linear regression program including test of goodness of fit for the model of two classes of independent binding sites [11, 17]. Saturation experiments performed with [^{3}H]ouabain were also graphically represented using Scatchard-plots.

In competition experiments carried out with the unlabelled derivatives of ouabain and digoxin, IC_{50} values (concentration of unlabelled drug displacing 50% of the labelled drug specifically bound) were calculated by a computerized linear regression program [18]. K_i values were calculated according to the method described by Linden [19], which takes into account the receptor concentration. Mean values obtained from three different experiments were compared by Student's *t*-test (two-tailed).

RESULTS

Characterization of ouabain binding sites in rat microsomes from kidney, brain and heart

Saturation experiments performed with [^{3}H]-ouabain (in the absence of K^+) demonstrated the presence of one class of specific binding sites in brain microsomes, with a K_d value (mean ± SE) of 0.067 ± 0.004 μM (N = 3), and in kidney microsomes with a K_d of 16.9 ± 3.9 μM (N = 3). With rat heart microsomes, binding data were best fitted assuming the existence of two independent classes of binding sites: 11% of high affinity sites with K_d equal to 0.2 ± 0.01 μM (N = 3) and 89% of low affinity sites with K_d equal to 15.7 ± 1.8 μM (N = 3).

Structure–activity relationship at the ouabain binding site

We investigated the influence of structural modifications of ouabain and digoxin on their affinity for $(Na^+ + K^+)$-ATPase from rat kidney and rat brain microsomes. Figure 1 shows the competition curves obtained when [^{3}H]ouabain specifically bound to rat kidney and rat brain was displaced by increasing concentrations of ouabain, ouabagenin (which lacks the osidic chain in $C_{3\beta}$) or dihydroouabain (with a saturated lactone ring in C_{20}–C_{22}). The rat brain isozyme was about 200-fold more sensitive to ouabain than the kidney isozyme as shown by the difference ($P < 0.001$) in K_i values (Table 1). Saturation of the lactone ring produced a shift to the right of the ouabain displacement curve with both enzymes. Ratios of dihydroouabain to ouabain K_i values were 17 and 35 for kidney and brain isozymes respectively

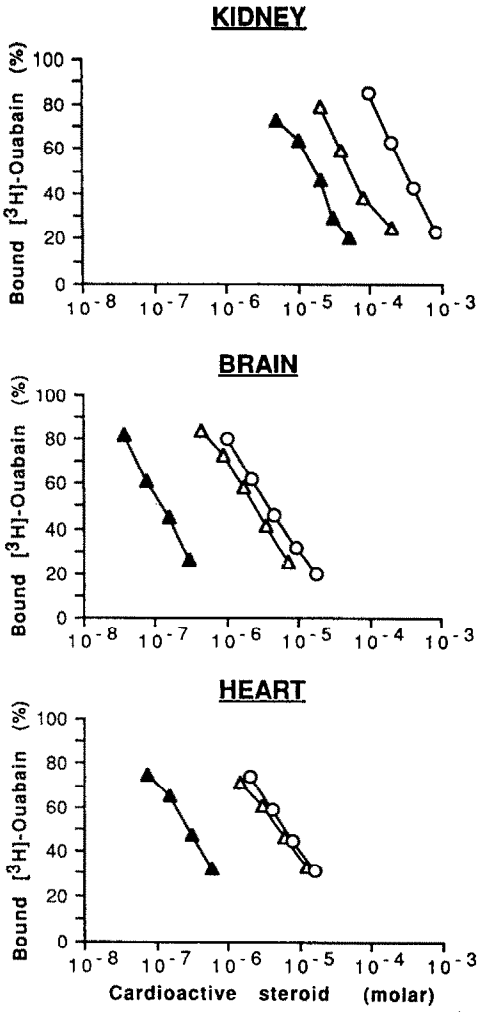

Fig. 1. Displacement of specifically bound [^{3}H]ouabain by ouabain and its derivatives. Microsomes were incubated in Mg–P_i medium in the presence of [^{3}H]ouabain and of various concentrations of ouabain (▲), ouabagenin (△) and dihydroouabain (○). Kidney microsomes (33 μg protein) were incubated for 4 hr in the presence of 1.6×10^{-7} M [^{3}H]ouabain. Brain microsomes (23 μg protein) were incubated for 4 hr in the presence of 10^{-9} M [^{3}H]ouabain. Heart microsomes (61 μg protein) were incubated for 10 min in the presence of 7×10^{-9} M [^{3}H]ouabain. Ordinate indicates the amount of [^{3}H]ouabain specifically bound as per cent of the control value measured in the absence of unlabelled drug (3.82, 4.31 and 0.26 pmol/mg protein for kidney, brain and heart preparations, respectively). Abscissa indicates the concentration of unlabelled drug. Each point is the mean value of assay performed in triplicate in three different experiments. SE of mean did not exceed the size of the symbols.

(Table 1). On the other hand, the lack of osidic chain produced a greater shift to the right of the ouabain displacement curve with brain isozyme than with kidney isozyme: the ratios of ouabagenin to ouabain K_i values were 23 and 5 for brain and kidney enzymes, respectively (Table 1). This indicates a difference in the influence of the osidic chain on ouabain binding to the two $(Na^+ + K^+)$-ATPase isozymes.

Table 1. Dissociation constant values of ouabain, digoxin, and their derivatives

	K_i (μM)		
	Kidney	Brain	Heart (high affinity sites)
Ouabain	15.9 ± 0.8	0.071 ± 0.004	0.26 ± 0.04
Ouabagenin	82.1 ± 3.8*	1.62 ± 0.44†	4.81 ± 0.38*
Dihydroouabain	275 ± 29*	2.46 ± 0.40‡	6.12 ± 0.81‡
Digoxin	1.78 ± 0.46	0.066 ± 0.001	0.15 ± 0.06
Monodigitoxoside digoxin	4.61 ± 0.62†	0.095 ± 0.010†	0.22 ± 0.03
Digoxigenin	6.34 ± 0.63‡	1.24 ± 0.16‡	4.26 ± 0.46*
Dihydrodigoxin	>100	4.47 ± 0.51*	10.3 ± 0.3*

Dissociation constants (K_i) were obtained as described under Methods from competition experiments shown in Figs. 1 and 2. Results are expressed as mean values ± SE. Each value is the mean of assays performed in triplicate in three different experiments.

* Significantly different from ouabain or digoxin in the same tissue ($P < 0.001$).

† Significantly different from ouabain or digoxin in the same tissue ($P < 0.05$).

‡ Significantly different from ouabain or digoxin in the same tissue ($P < 0.01$).

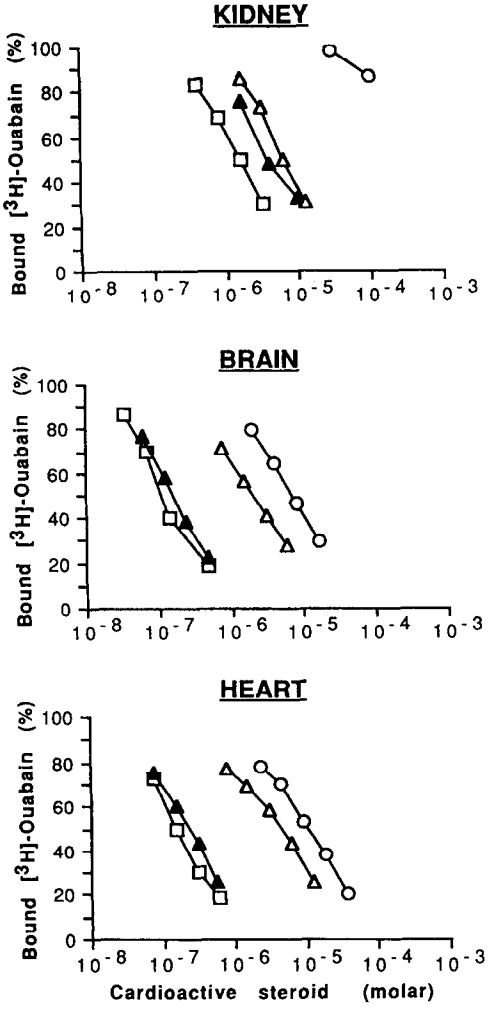

Fig. 2. Displacement of specifically bound [^{3}H]ouabain by digoxin and its derivatives. Microsomes were incubated in Mg–P_i medium in the presence of [^{3}H]ouabain and of various concentrations of digoxin (□), monodigitoxoside digoxin (▲), digoxigenin (△) and dihydrodigoxin (○). (A) Kidney microsomes. (B) Brain microsomes. (C) Heart microsomes. See legend of Fig. 1 for incubation conditions.

In order to study this structure–activity relationship for the high affinity ouabain binding sites of the rat heart, we examined the displacement by unlabelled ouabain, ouabagenin and dihydroouabain of the [^{3}H]ouabain specifically bound in the presence of a very low concentration (7×10^{-9} M) that predominantly labelled the high affinity sites (90% of the total specific binding as calculated from Scatchard data). Figure 1 shows competition of binding by ouabain and its two derivatives on high affinity sites of rat heart. Although K_i values were different, changes in molecular structure evoked similar ratio changes in binding parameters between high affinity sites in rat heart and in rat brain (Table 1).

A similar structure–activity relationship was performed with derivatives of the digoxin family (Fig. 2). Again, brain and kidney isozymes differed mainly by their sensitivity to digoxin (brain isozyme was about 25-fold more sensitive than kidney isozyme; $P < 0.05$) and by the relative effect of removing the osidic chain on $C_{3\beta}$: the ratio of digoxigenin to digoxin K_i values were 19 and 4 for brain and kidney enzymes respectively (see Table 1). Comparing digoxigenin monodigitoxoside and digoxin (a trioside), we observed that the addition of only one osidic residue to the genin molecule increased the affinity as efficiently as the addition of the three osidic residues for brain isozyme (K_i values of 0.095 and 0.066 μM for digoxigenin monodigotoxoside and digoxin, respectively) but not for the kidney isozyme (K_i values of 4.61 and 1.78 μM for digoxigenin monodigitoxoside and digoxin, respectively). Binding of digoxin derivatives to the high affinity binding sites of rat heart microsomes presented a structure–activity relationship similar to that observed with the brain but not the kidney isozyme.

Effect of KCl *on ouabain binding*

The effect of KCl on ouabain binding to $(Na^+ + K^+)$-ATPase from brain and kidney was examined by comparing [^{3}H]ouabain binding at equilibrium in presence and absence of 10 mM KCl. In preliminary kinetic experiments, we observed that equilibrium was reached after 4 hr (KCl = 0) or 5 hr (KCl = 10 mM) with brain preparation and after

Table 2. Binding parameters of ouabain to $(Na^+ + K^+)$-ATPase from rat brain, kidney and heart microsomes in the presence and absence of 10 mM KCl

	$[K^+] = 0$ (1)	$[K^+] = 10$ mM (2)	(2)/(1)
Rat heart			
High affinity sites			
B_{max} (pmol/mg)	4.9 ± 0.7	4.4 ± 0.2	0.88 ± 0.13
K_d (μM)	0.21 ± 0.01	0.35 ± 0.10	1.67 ± 0.48
Low affinity sites			
B_{max} (pmol/mg)	45 ± 8	45*	1*
K_d (μM)	13 ± 3	250 ± 78	19.2 ± 7.5
Rat brain			
B_{max} (pmol/mg)	201 ± 5	214 ± 6	1.06 ± 2.98
K_d (μM)	0.072 ± 0.003	0.18 ± 0.01	2.5 ± 0.17
Rat kidney			
B_{max} (pmol/mg)	186 ± 4	186*	1*
K_d (μM)	17 ± 0.6	422 ± 19	24.8 ± 1.4

Microsomes from rat brain and kidney were incubated with 9 (kidney) or 10 (brain) different concentrations of ouabain in the presence and absence of 10 mM KCl. Incubation times were 4 hr and 20 min (no KCl), and 5 hr and 30 min (with KCl) for brain and kidney preparations, respectively. Rat heart microsomes were incubated in the presence of 15 different concentrations of ouabain for 13 min in the absence of KCl or for 1 hr in the presence of 10 mM KCl. Binding parameters (K_d and B_{max}) were calculated as described in Materials and Methods by computerized non-linear regression of untransformed data from three experiments (triplicate determinations). Each parameter is characterized by a standard deviation representing the goodness of fit. Standard deviation values of (2)/(1) were calculated as indicated in Materials and Methods.

* B_{max} fixed at the control (KCl = 0) value.

20 min (KCl = 0) or 30 min (KCl = 10 mM) with the kidney preparation. Saturation experiments were then performed with both preparations in the Mg–P_i medium in the presence and absence of 10 mM KCl. Scatchard-plot anlaysis showed that K^+ did not modify the B_{max} of ouabain binding to rat brain microsomes but decreased the affinity: K_d for ouabain binding was enhanced 2.5-fold (±0.17) by 10 mM KCl (Table 2).

Since the affinity of ouabain for rat kidney $(Na^+ + K^+)$-ATPase was low in the absence of KCl (K_d = 17–24 μM) and since KCl further lowered the affinity of ouabain, it was not feasible to use the high concentrations necessary to saturate the binding sites of kidney enzyme. Thus, to calculate the K_d in the presence of KCl, we assumed that the B_{max} value was not modified by KCl in this tissue. This procedure has already been used by Hansen [20] and is further warranted by the fact that KCl did not modify the B_{max} of ouabain in rat brain microsomes (Table 2). This analysis showed that the affinity of ouabain for rat kidney microsomes was markedly decreased by KCl since K_d value increased 25-fold (±1.4) (Table 2).

To determine the effect of KCl on ouabain binding to rat heart microsomes, we performed saturation experiments with [^{3}H]ouabain in the Mg–P_i medium. Data of specific binding were analysed using a model of two classes of independent binding sites, computed parameters are shown in Table 2. Data obtained in the absence of KCl are consistent with previous observations [10, 11]. In the presence of 10 mM KCl, we observed a 1.7-fold (±0.5) increase of K_d for high-affinity sites and a 19-fold (±7.5) increase of K_d for low-affinity sites.

DISCUSSION

Heterogeneity of ouabain binding sites in rat microsomes from kidney, brain and heart

A single class of binding sites with low affinity for ouabain was found in rat kidney microsomes, in good agreement with other studies [21–23]. This is consistent with the report that α_1 mRNA is the predominant isoform transcript in rat kidney [24].

In rat brain, an heterogeneity of sites is generally reported [1, 21] and related to up to three distinct (α_1, α_2, α_3) forms of the α-subunit, by use of molecular cloning and immunological techniques [2, 25, 26]. In the present binding experiments, only one single class of binding sites with high affinity for ouabain was detected. This discrepancy of results could be accounted for by the age of the rats (6 months old) used in this study, as it has been shown that the high affinity form is predominant in adult neurones [27]. It must furthermore be pointed out that the dissociation constant of ouabain observed in this study was similar to the value found in mouse 3T3 cells transfected with the cDNA encoding α_3 isozyme of rat brain (8×10^{-8} M) [28]. K_i values of ouabain in brain preparation were also consistent with the sensitivity to ouabain of the trypsin-resistant α_3 isozyme [26]. This indicates that rat brain preparation used in this study contained predominantly the α_3 isoform of $(Na^+ + K^+)$-ATPase [28].

In heart microsomes, two classes of ouabain binding sites of respectively high and low affinity were identified, confirming previous results [10, 11]. Orlowski and Lingrel have recently reported [24] that α_1 and α_2 mRNA are the only forms present in

adult rat heart. Therefore, it is likely that the low affinity binding occurred on the α_1 isoform and the high affinity binding on the α_2 isoform. The affinity of ouabain and digoxin for the putative α_2 isoform was slightly lower than for the α_3 isoform. However, the difference was significative only in the case of ouabain ($P < 0.01$).

Importance of the osidic chain and saturation of the lactone ring for binding of ouabain and digoxin to ($Na^+ - K^+$)*-ATPase isozymes of the rat*

Experimental results show that on both brain and heart (high affinity) preparations, the changes in affinities after removal of the sugar chain and the saturation of C_{20}—C_{22} bond of the $C_{17\beta}$ lactone ring were similar for ouabain and digoxin. In addition, it appears that heart (high affinity) preparation had always a lower affinity than brain preparation. This is consistent with the previous conclusion that heart (high affinity) and brain preparations contained two different isozymes, respectively α_2 and α_3. Furthermore, K_i values of ouabagenin and digoxigenin were of the same order of magnitude in respectively brain and heart (high affinity) preparations.

When such a comparison was made with the α_1 isoform contained in the kidney preparation, we observed that the K_i value of ouabagenin was 13-fold higher than that of digoxigenin ($P < 0.001$). This indicates that the α_2 and α_3 isozymes did not distinguish between ouabagenin and digoxigenin but that the α_1 isoform had a much lower affinity for ouabagenin than for digoxigenin.

Antagonistic effect of KCl *on ouabain binding*

Antagonism of ouabain binding by K^+ is a classical feature of ($Na^+ + K^+$)-ATPase that has been described in Mg–P_i as well as in Na–Mg–ATP medium [29]. The decrease of ouabain affinity observed when K^+ is added to Mg–P_i medium is due to decrease of the association rate constant [29]. In the present saturation experiments, at equilibrium, it was observed that when the incubation medium was supplemented with KCl 10 mM, the apparent affinity of ouabain for binding to α_1 isoform (kidney and heart, low-affinity) showed a 20-fold decrease by contrast with the putative α_2 and α_3 isozymes who showed only a 2-fold decrease in affinity. Such observations indicate that the functional characteristics of the ($Na^+ + K^+$)-ATPase isozymes present dissimilarities.

In summary, experimental data reported in this paper show that ouabain discriminated much better than digoxin between the high and low affinity ($Na^+ + K^+$)-ATPases and that the presence of KCl in the medium enhanced the difference in apparent affinities of ouabain for these isozymes. They indicate the possibility of having still more selective inhibitors of the high affinity isozymes of ($Na^+ + K^+$)-ATPase, probably within hydrophilic cardioactive steroids. This may present pharmacological importance since in cardiac preparation, toxicity is related to inhibition of the low affinity isoform [10, 11, 17].

Acknowledgements—F. Noël was on leave from Departamento de Farmacologia Basica e Clinica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil. We thank Dr M. Wibo for helpful suggestions during the redaction of this paper, Mrs A. Lebbe for her technical assistance and Dr J. Cumps for providing us with facilities for computer analysis.

This work has been supported by FRSM Grant No. 3.9006.87 and by Actions de Recherche concertées, Grant No. 89/95-135.

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