Biochimica et Biophysica Acta, 1021 (1990) 148–156
Elsevier

BBAMEM 74713

Two active Na^+/K^+-ATPases of high affinity for ouabain in adult rat brain membranes

Isabelle Berrebi-Bertrand [1,*], Jean-Michel Maixent [2], Georges Christe [3] and Lionel G. Lelièvre [1,*]

[1] *INSERM U127, Hôpital Lariboisière, Université Paris 7, Paris,* [2] *Laboratoires Nativelle, Longjumeau, and* [3] *INSERM U 121, Hôpital Cardiovasculaire, Lyon (France)*

(Received 15 June 1989)
(Revised manuscript received 18 September 1989)

Key words: ATPase, Na^+/K^+-; Ouabain; High-affinity site; (Rat brain)

The degree of heterogeneity of active Na^+/K^+-ATPases has been investigated in terms of ouabain sensitivity. A mathematical analysis of the dose-response curves (inhibition of Na^+/K^+-ATPase) at equilibrium is consistent with the putative existence of three inhibitory states for ouabain two of high (very high plus high) and one of low affinity. The computed IC_{50} values are: 23.0 ± 0.15 nM, 460 ± 4.0 nM and 320 ± 4.6 μM, respectively. The relative abundance of the three inhibitory states was estimated as: 39%, 36% and 20%, respectively. Direct measurements of [^{3}H]ouabain-binding at equilibrium carried out on membrane preparations with ATP, Mg^{2+} and Na^+ also revealed two distinct high affinity-binding sites, the apparent K_d values of which were 17.0 ± 0.2 nM (very high) and 80 ± 1 nM (high), respectively. Dissociation processes were studied at different ouabain concentrations according to both reversal of enzyme inhibition and [^{3}H]ouabain release. The reversal of enzyme inhibition occurred at three different rates, depending upon the ouabain doses used (10 nM, 2 and 100 μM). When the high-affinity sites were involved (ouabain doses lower than 2 μM) the dissociation process was biphasic. A similar biphasic pattern was also detected by [^{3}H]ouabain-release. The time-course of [^{3}H]ouabain dissociation (0.1 μM) was also biphasic. These data indicate that the three catalytic subunits of rat brain Na^+/K^+-ATPase α1, α2 and α3 (Hsu, Y.-M. and Guidotti, G. (1989) Biochemistry 28, 569–573) are able to hydrolyse ATP and exhibit different affinities for cardiac glycosides.

Introduction

The evidence for more than one kind of functional Na^+/K^+-ATPase in several tissues including brain and heart has arisen independently from different lines of research in particular, gel electrophoresis, ouabain sensitivity, and cDNA cloning experiments. The conclusions from these experiments favor a multigenic expression of Na^+/K^+-ATPase.

In brain, it was shown that there were two distinct molecular forms of the catalytic subunit of the Na^+/K^+-ATPase. They were resolved by gel electrophoresis and detected by [γ-^{32}P]phosphorylation or immunoblotting [1,2]. These two isoforms were designated α2 and α1 (previously called α(+) and α, respectively). In rat [1,2] and ox [3,4] brain membranes, differences in affinity for cardiac glycosides in particular ouabain also reflect the existence of different molecular forms of the enzyme: α2 has a high-affinity and α1 has a low-affinity for ouabain. In cardiac myocytes, these two isoforms have been related to two distinct positive inotropic and toxic responses in rats [5–8] and in mongrel dogs [9].

Very recently, in rat brainstem axolemma membranes, Urayama et al. [10] isolated, together with α2, an other isozyme which he termed α3. Immunoblot analysis of trypsin-treated Na^+/K^+-ATPase using isozyme-specific antibodies showed that α3 was significantly more resistant to trypsin digestion than α2. This α3 isozyme would be of relatively high affinity for ouabain: IC_{50} values of either 0.13 μM [10] or 1.0 ± 0.14 μM [11]. (For a review, see Ref. 12).

However, in brain, the physiological role of these isoforms has not yet been established. By means of cDNA technology, the existence of three distinct forms of the Na^+/K^+-ATPase α subunit from adult rat brain

* Present address: Hall de Biotechnologies, Tour 54–64, Université Paris 7, 2, Place Jussieu, 75251 Paris Cedex 05, France.

Correspondence: I. Berrebi-Bertrand, Hall de Biotechnologies, Tour 54–64, Université Paris 7, 2, Place Jussieu, 75251 Paris Cedex 05, France.

has been demonstrated by Shull et al. [13]. According to these authors, the three forms α1, α2 and α3 are the products of separate genes.

Furthermore, by photoaffinity labeling, Lowndes et al. [14] have identified in rat brain a polypeptide which represents a saturable, high affinity cardiac glycoside binding site distinct from the previously reported α1 and α2 sites. This polypeptide is refered to as α(−) since it has a slightly lower apparent molecular weight than α subunits by SDS-PAGE. In contrast Hsu and Guidotti [11] reported that α3 had a lower mobility than α1 and α2.

Thus, in adult rat brain, there exists several (i.e., more than two) functional Na^+/K^+-ATPases. From a pharmacodynamical point of view, this greater heterogeneity would be suspected if one considers the wide variabilities of the sensitivity of α2 form to ouabain. Indeed, the K_d values for the α2 high-affinity sites for ouabain vary by 10-fold, from: 16 [15], 60 [16], 100 [1,17–21], to 160 nM [22]. This isoform has been shown to be present in high quantities in the newborn rat brain. The wide variability for the α2 isoforms contrasts the high reproducibility of the K_d values for the low-affinity forms as measured by Na^+/K^+-ATPase activity: 100 [1,19] or 310 μM [22].

This scattering of K_d values of α2 might be interpreted as variable contributions of two types of sites with relatively close ouabain affinities as recently shown by Hsu and Guidotti [11]. To investigate the putative presence of a third active Na^+/K^+-ATPase form in adult rat brain membranes, three discriminatory criteria have been used: the direct measurement of [^{3}H]ouabain-binding at equilibrium and the dissociation processes at different drug concentrations according to both reversion of enzyme inhibition and [^{3}H]ouabain release. Our data, consistent with the mathematical analysis of the dose-response curves of Na^+/K^+-ATPase to ouabain, show that there are three active forms, two of high affinity and one of low affinity for ouabain.

Thus, the three subunits α1, α2, α3 [11] of rat brain Na^+/K^+-ATPase are able to hydrolyse ATP.

Materials and Methods

Isolation of membranes from rat brain

Membrane fractions enriched in Na^+/K^+-ATPase activity were purified from newborn (6 h after birth) and adult (6 weeks) Wistar rat brains according to the method of Jørgensen [23] used by Sweadner [1]. The only modification to this protocol was buffering of all the solutions with 30 mM imidazole-HCl (pH 7.2). Moreover, for complete tissue homogenization and maintenance of enzyme activity, five strokes of a Teflon-glass homogenizer were necessary for the adult rat brain whereas only one stroke was required for the neonatal brain. In the two cases the last pellet (130000 × *g* (Beckman SW54 Ti) for 30 min) constitute the microsomal fraction.

The protein content was determined by the method of Lowry et al. [24].

Enzyme assays

(a) Sensitivity of Na^+/K^+-ATPase to ouabain. Enzyme activities were measured at 37 °C as a function of time and amount of microsomal proteins (from 0.1 to 2 μg). The relationships were linear. The enzymatic assays were carried out either with native vesicles or with vesicles permeabilized by SDS (sodium dodecyl sulfate) treatment (0.3 mg SDS/mg of proteins for 30 min at 20 °C [25,26]. Before and after an SDS treatment the ouabain-insensitive activity was, in adult rat brain, less than 35% and 3%, respectively. The specific activity of the Na^+/K^+-ATPase was 135 ± 15 μmol inorganic phosphate liberated per hour per mg of protein (units) in adult rat brain. The yield in enzyme activity was eighty percent, it was similar to that found in ox brain membranes [27]. In newborn (6 h after birth) rat brain preparations the specific activity of the Na^+/K^+-ATPase was 24 ± 3 units. In homogenates from newborn rat brain, the specific activity of the ouabain-sensitive Na^+/K^+-ATPase represented about 10–20% of the basal Mg^{2+}-ATPase activity. This was too low to allow any calculation of the yield in Na^+/K^+-ATPase activity in our membrane fractions.

The Na^+/K^+-ATPase activity was determined using the coupled assay method as previously described [28,29]. The activity was measured in an ATP regenerating medium by continuously recording NADH oxidation, using a Varian DMS70 spectrophotometer.

Each cell contained (final volume 0.6 ml) 100 mM NaCl, 2 mM of phospho*enol*pyruvate, 10 mM KCl, 4 mM ATP, 4 mM $MgCl_2$, 30 mM imidazole-HCl (pH 7.2), 0.4 mM NADH, 3.5 units pyruvate kinase, and 5 units of lactate dehydrogenase. The enzymatic reaction was initiated by the addition of microsomal proteins. When the decrease in absorbance was linear versus time (about 5 min), various amounts of ouabain were added (ouabain from Calbiochem and Nativelle laboratories). The corresponding values of NADH oxidation were continuously monitored for up to 35 minutes. The linearity versus time indicated that a new steady-state level between digitalis and Na^+/K^+-ATPase had been reached. All the results presented here have been obtained from experiments in which enzyme activity was linear versus time, i.e. from 10 to 30 min. The inhibition levels induced by a single dose of ouabain remained stable during at least 30 min. In the assay, the maximum final concentrations of digitalis receptors vary from 0.01 to 1 nM, with final concentrations of ouabain varying from 0.1 nM to 2 mM. Inhibition percentage was calculated by comparing the activities in the presence or absence of ouabain after correcting for the ouabain-

insensitive ATPase activity measured in the presence of 2 mM ouabain.

(b) Release of ouabain from the enzyme. Dissociation of ouabain from the enzyme was initiated by a 100–120-fold dilution of the incubation medium in the same buffer but without ouabain. 30 μg of membrane vesicles were incubated for 30 min at 37° C in 30 μl of the assay medium containing either 10 nM, 2 μM or 100 μM ouabain. To ensure the equilibrium ATPase–ouabain interaction had been reached, the preincubation time was 30 min (see Results). By diluting 5 or 6 μl of this medium with 600 μl of the ouabain-free assay mixture, the final concentrations of the drug dropped from 10 nM to 0.08 nM, from 2 μM to 0.02 μM and from 100 μM to 0.8 μM, respectively. Ouabain bound to the enzyme dissociated via a pseudo-first-order process when chased by a 100 or 120-fold dilution. The apparent dissociation rate constants k_{-1} were calculated from a plot of log percent of maximum activity inhibited versus time.

[^{3}H]Ouabain binding

Ouabain binding studies were carried out using [^{3}H]ouabain (DuPont-New England Nuclear) with a specific radioactivity ranging from 18 to 20 Ci/mmol. The binding medium consisted of 4 mM $MgCl_2$, 4 mM ATP, 100 mM NaCl, and 40 mM imidazole-HCl (pH 7.4). These ligands plus [^{3}H]ouabain were incubated at 37° C for 10 min in a final volume of 10 ml.

To study the equilibrium binding and dissociation processes, two different protocols have been designed.

(a) Equilibrium binding. In the presence of increasing concentrations of labeled ouabain from 10 nM to 600 nM (specific radioactivity varying from 19 to 1.6 Ci/mmol), the reaction was initiated by the addition of 40 μg of microsomal fractions permeabilized by the same SDS treatment as described above. The final receptor concentration was 0.8 nM. After 60 min, duplicate aliquots of 4.5 ml were removed and filtered.

(b) Dissociation process. Once equilibrium binding had been achieved, 10 ml of the prewarmed binding medium supplemented with 0.2 mM unlabeled ouabain, were added to the same volume of incubation medium containing 100 nM labeled ouabain. The final concentration of unlabeled ouabain was 0.1 mM. At various times, aliquots of 0.9 ml were removed and rapidly filtered.

Whatever the experimental conditions, all the aliquots were filtered under vacuum on HAWP Millipore filters (0.45 μm) and rinsed three times with 4 ml of the same ice-cold buffer. The radioactivity bound to the filters and the specific binding measurements were determined as previously described [30]. Non specific binding, at 0.6 μM [^{3}H]ouabain, accounted for 15% of total radioactivity bound to the membranes.

Modeling of enzyme titration curves

The following model was used:

$$V = \frac{V_{max}}{10^{(I-K_I)}+1} \quad (1)$$

where: V is the observed velocity at a given inhibitor concentration, V_{max} is the maximal velocity observed in the absence of inhibitor, I is the decimal logarithm of inhibitor concentration, K_I is the decimal logarithm of the IC_{50}.

This model may be viewed as a simplified version of the general logistic function used by De Lean et al. [31] which may be written with the above conventions:

$$V = \frac{V_{max}-d}{10^{(I-K_I)b}+1} + d \quad (2)$$

where d is the residual enzyme activity at infinite inhibitor concentration and b is a 'slope factor' that may deviate from 1 if some cooperation or threshold effects or any other phenomenon causes the system to depart from first-order kinetics.

In our model we assumed that $d = 0$ which is justified since ouabain-dependent activities were considered and $b = -1$ thus making the hypothesis that the inhibition is strictly of the first order.

Activities being expressed as relative to the maximal activity in the absence of ouabain (constraint the total activity to 100% of control). The program used did not allow such a constraint, thus V_{max} is itself an output of the fitting procedure.

The experimental data were fitted with a sum of 1 to 3 functions as shown in Table I, i.e., assuming the presence of 1 to 3 sites of different affinity for ouabain. This was achieved by means of a FORTRAN IV program, run on a PDP 11/23 t for the non-linear least-squares iterative adjustment using the method of Marquardt as designed by Bevington [32].

The fit is generally improved when one component (as function (1)) is added to the model. To test whether this improvement is significant, we performed a F test in the following form: a first model with n components (thus $n \times 2$ parameters) yields a X^2 value named X_n with $N-(n\times 2)$ degrees of freedom, a second model having $n+1$ components and thus $(n+1)\times 2$ parameters gives a lower X^2 value (X^2_{n+1}) with $N-(n+1)\times 2$ where N is the number of experimental data points. The F value was calculated as:

$$F = \frac{(X_n^2 - X_{n+1}^2)/2}{X_{n+1}^2/(N-(n+1)\times 2)}$$

The improvement was considered as significant when the above F value exceeded the theoretical F value for 2 and $N-(n+1)\times 2$ degrees of freedom with $P = 0.01$.

Results

In an attempt to determine whether two or three ouabain sensitivities could be discriminated in our preparation, we have privilegiated the highly sensitive enzymatic test.

Sensitivity of Na^+/K^+-ATPase activity to ouabain

The dose-response curves of Na^+/K^+-ATPase activities to ouabain in adult and newborn rat brain preparations are depicted in Fig. 1 and Fig. 2, respectively. These curves exhibited a complex pattern. Up to 70% of the activity was inhibited with about 1 μM ouabain, and a complete inhibition occurred with 1–2 mM drug. The curves spanned over at least six logarithmic units indicating the presence of more than one class of enzyme.

The experimental data were fitted with a sum of one to three functions assuming the presence of one, two (high and low) affinity, or three, two high (very high, high) and a low affinity, independent, non interconvertible, saturable inhibitory processes exhibiting different affinities for ouabain.

As shown in Table IA, in adult rat brain, the F value for testing the significance of the improvement brought about by the two-site model versus the one site model was $F = 35$ with 2 and 51 degrees of freedom. Thus, the improvement was highly significant. The F Table value for $p = 0.01$ was 5.18 for 2 and 40 degrees of freedom. The improvement of fit for the three site-model versus the two site-model was $F = 6.6$ with 2 and 49 degrees of freedom. Thus, the addition of a third, high-affinity, binding component seems statistically valid.

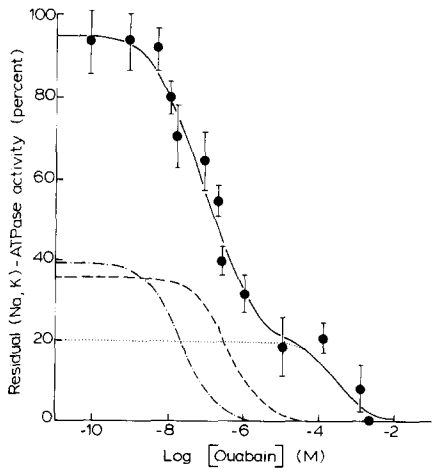

Fig. 1. Dose-response curve of adult rat brain Na^+/K^+-ATPase to ouabain (●). Three-site model fit. The theoretical titration curve for each site was plotted as ·–·–· for very high affinity sites, — — — for high-affinity and ····· for low-affinity sites. The F values for the improvements of fit are listed in Table IA. The dose-response curve remained unaffected when the enzyme preparations were treated by SDS in order to render the vesicles leaky to ligands. Each point represents the mean of 21 to 42 observations with bars denoting S.E. computed from percentage inhibition in each experiment. Each experiment was performed at least three times with at least seven different membrane preparations. For clarity, the 42 values found with 2 mM ouabain are represented by one symbol.

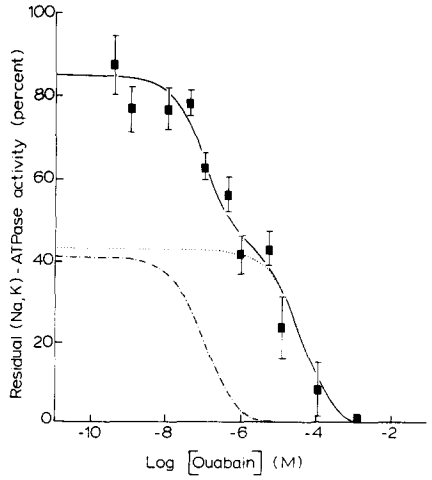

Fig. 2. Dose-response curve of newborn rat brain Na^+/K^+-ATPase to ouabain (■). Two-site model fit. The theoretical titration curve for each site was plotted as ·–·–· for high affinity sites and ····· for low-affinity sites. The F values for the improvements of fit are listed in Table IB. The improvement when including a third component was not significant. Results are means ± S.E. of 4–10 determinations on seven membrane preparations (with 30–38 animals per preparation).

According to the computed parameters presented in Table IA the three sites would be:

(i) a very high affinity site with an apparent IC_{50} value of 23 nM and a contribution of 39.4%,

(ii) a second high-affinity site exhibiting an apparent IC_{50} equal to 460 nM and associated with 35.7% of the total enzyme activity, and

(iii) a low-affinity site (apparent $IC_{50} = 320$ μM) representing 20.1% of the total activity.

Although the statistical analysis shows that it is very unlikely that a third component would have appeared by chance, these data could only suggest the existence of a third active enzyme site of either high or very high affinity. The dose-response curve found here was similar to that observed with genins.

As a control, we have tested the validity of the assertion: three possible sites in adult brain by analyzing the dose-response curve in newborn rat brain (Fig. 2, Table IB) known to have two active sites [33].

According to the F values, it is clear (Table IB) that the best fit corresponded to a two-site model with:

(i) a high-sensitivity enzyme form associated with 41.4% inhibition of activity and an apparent affinity IC_{50} of 100 nM, and

(ii) a low-sensitivity enzyme form with an IC_{50} value equal to 40 μM and associated with 43.4% inhibition.

Including a third site did not cause a significant improvement of fit at $P = 0.01$ (Table IB).

Direct [^{3}H]ouabain-binding measurements

Since in adult rat brain, there would probably coexist two high affinity active sites for ouabain, they could be

TABLE I

Mathematical analysis of inhibition versus dose curves

Results of model adjustments and comparisons on the plots of ouabain-sensitive activity versus dose for ouabain inhibition. The data were those of Figs. 1 and 2. Final values of inhibition constants (IC_{50}) are expressed in mole/liter (M), the percentage of blockage at saturation is given in brackets for every site. The value of the F parameter calculated to test the significance of the improvement of fit when passing from 1 to 2 sites ($F1 \rightarrow 2$) or from 2 to 3 sites ($F2 \rightarrow 3$) are given with the associated couple of degrees of freedom (see Methods for F computation). The theoretical F value for degrees of freedom nearest to the experimental ones was read out of F-tables in Bevington (1969) for $P = 0.01$.

TABLE IA

Adult rat brain ATPase inhibition

The fit was significantly improved when introducing a second site and also when assuming the presence of a third site for ouabain inhibition. Note that the significance is ascertained for $P < 0.01$.

Model	1 site	2 sites	3 sites
IC_{50} (M) and proportional contribution	$3.1 \cdot 10^{-7}$ M (88%)	$7.95 \cdot 10^{-8}$ M (67.2%) $1.25 \cdot 10^{-4}$ M (25.5%)	$2.3 \cdot 10^{-8}$ M (39.4%) $4.6 \cdot 10^{-7}$ M (35.7%) $3.2 \cdot 10^{-4}$ M (20.1%)
F value	$F(1 \rightarrow 2) = 35$ (2,51)		$F(2 \rightarrow 3) = 6.6$ (2,49)
Theoretical F value $P < 0.01$		F (2,40) = 5.18	

TABLE IB

Newborn rat brain ATPase inhibition

The fit was largely improved when a second site was introduced into the model, whereas including a third site did not cause a significant improvement of fit at $P = 0.01$. However, the improvement would have been considered significant if P is chosen at 0.05, since the corresponding F value is $F(2,60) = 3.05$.

	1 site	2 sites	3 sites
IC_{50} (M) and proportional contribution	$2 \cdot 10^{-6}$ M (76.8%)	$1 \cdot 10^{-7}$ M (41.4%) $4 \cdot 10^{-5}$ M (43.4%)	$2 \cdot 10^{-8}$ M (24.6%) $9.3 \cdot 10^{-7}$ M (32.9%) $8.3 \cdot 10^{-5}$ M (30.9%)
F value	$F(1 \rightarrow 2) = 37$ (2,60)		$F(2 \rightarrow 3) = 3.1$ (2,58)
Theoretical F value $P < 0.01$		$F(2,60) = 4.98$	

TABLE II

Apparent affinities for ouabain (IC_{50}) and K_d values, respective contributions and binding capacity, dissociation rate constants (enzymatic and binding assays) of very high-, high- and low-affinity digitalis receptor forms

	Enzyme form		
	very high affinity	high-affinity	low-affinity
IC_{50} (M)	$(2.30 \pm 0.01) \cdot 10^{-8}$	$(4.6 \pm 0.04) \cdot 10^{-7}$	$(3.2 \pm 0.046) \cdot 10^{-4}$
K_d (M)	$(1.70 \pm 0.02) \cdot 10^{-8}$	$(8 \pm 0.1) \cdot 10^{-8}$	
Contribution (%)	39.4	35.7	20.1
B_{max} (pmol/mg protein)	123 ± 3	87.5 ± 2.2	
Dissociation rate constant (k_{-1} in 10^{-3} s^{-1}) determined by:			
Enzymatic assay	1.1 ± 0.1	7 ± 1 **	39 ± 4 ***
Binding assay	0.22	1.4	

** $P < 0.01$.

*** $P < 0.005$.

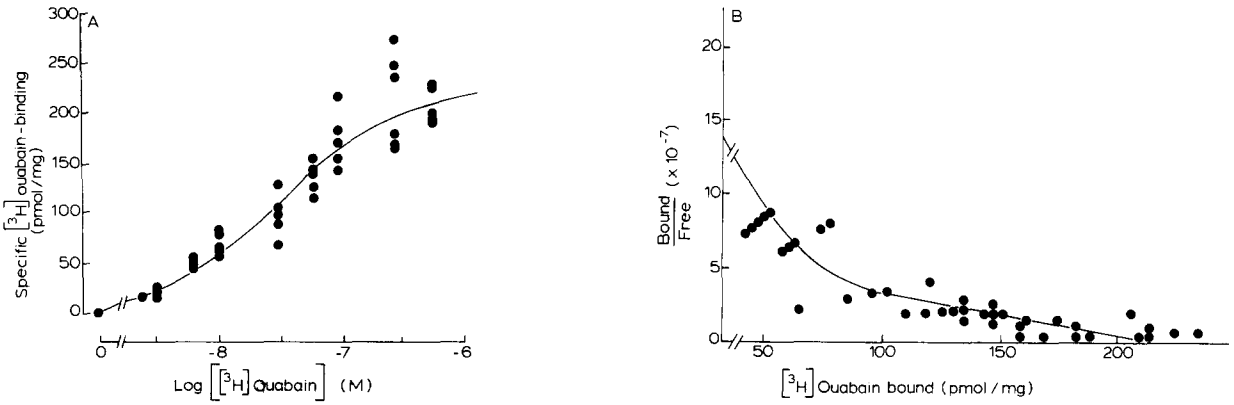

Fig. 3. (A) Dose-response curve for specific [^{3}H]ouabain binding and, (B) Scatchard plot of [^{3}H]ouabain saturation curve with adult rat brain membranes enriched in Na^+/K^+-ATPase activity. Points represent the means of six experiments on three different preparations.

studied by radiolabeled ouabain-binding measurements.

Thus, we have examined [^{3}H]ouabain-binding with the same microsomal vesicles as for Na^+/K^+-ATPase assays, under experimental conditions (ATP, Mg, Na) similar to those used for the enzyme assay, (except that K was omitted).

With drug concentrations varying from 3 to 600 nM, analysis of specific ouabain-binding showed two high affinity binding sites: a very high affinity site (apparent K_d: 17 nM) and a second site of lower affinity so called high (apparent K_d: 80 nM) (Table II, Fig. 3A). The binding capacity was 123 ± 3 and 87.5 ± 2.2 pmoles per mg of proteins for the very high and high affinity sites, respectively (Table II, Fig. 3B). As a result, in the presence of 600 nM [^{3}H]ouabain, the high-affinity sites represent 51% of the sites occupied. Due to the high level of non-specific binding (up to 50%) at higher ouabain concentrations (6 μM), it was not possible to characterize the low-affinity subunit (apparent IC_{50} = 320 μM) detected by the Na^+/K^+-ATPase assays.

Dissociation process

(a) [^{3}H]Ouabain enzyme dissociation. Following a 60 min preincubation with 0.1 μM of drug at the end of which equilibrium had been reached, the dissociation process of [^{3}H]ouabain was initiated by addition of a large excess of unlabeled ouabain (100 μM). As predicted by the apparent IC_{50} and K_d values found here, at this dose of ouabain (0.1 μM) both types of high-affinity sites are involved. If one takes into account both the ouabain concentration and the apparent K_d values found, most (68%) of the occupied sites were of very high affinity and 31.6% of high affinity for ouabain.

The time course of [^{3}H]ouabain dissociation was not a simple exponential process but the sum of two exponentials. The dissociation occurred according to a two phase phenomenon (Fig. 4); a rapid initial phase associated with the sites of the lowest affinity and a second slower phase corresponding to the sites of the highest affinity [34]. Two apparent rate constants could be calculated: the first apparent rate constant representing the initial phase was that of the high affinity sites (computed $k_{-1H} = 1.4 \cdot 10^{-3}\ s^{-1}$). The second apparent rate constant (i.e. the second phase) with ($k_{-1VH} = 0.22 \cdot 10^{-3}\ s^{-1}$) corresponds to the very high affinity sites.

By determination of the intercept for the second slope with the ordinate [35], the contribution of the high-affinity sites represented $21 \pm 4\%$ of the total amount of [^{3}H]ouabain bound at 0.1 μM. According to the enzyme response to ouabain (Fig. 1) at 0.1 μM, the percent of maximal inhibition was 60% and thus, the high-affinity sites represented $((21 \pm 4\%)/60\%) \times 100$, i.e. about 35% of the total process. This is consistent with the data found by mathematical analysis (35.7% see Table IA).

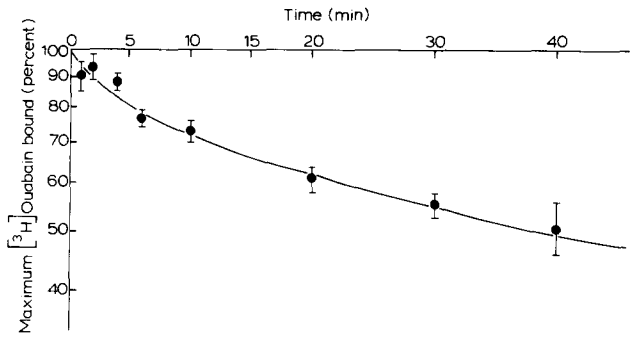

Fig. 4. Dissociation of [^{3}H]ouabain-enzyme complex. Binding in the presence of ATP, Mg^{2+}, Na^+, plus 100 nM [^{3}H]ouabain (●) was carried out for 30 min at 37 °C. At this time (time 0 of dissociation experiment), excess unlabeled ouabain (0.1 mM final concentration) was then added and aliquots were removed, filtered and washed as described under Experimental procedures. Amounts of bound ouabain (log scale) are plotted versus time. Under these conditions the loss of radioactive label bound to the enzyme shows a biphasic first-order kinetic. The two apparent rate constants of this process of dissociation were calculated from the slopes of these representations. Results depicted in this figure are the average of four separate experiments on two different microsomal preparations.

(b) Dissociation of ouabain from the enzyme-ouabain complex. The interactions between ouabain and the specific high-affinity sites are characterized by fast association and slow dissociation processes. For the low-affinity sites, the association of the drug is slow, whereas the dissociation is fast [34]. By studying the kinetics of enzyme reversal from inhibition, it is possible to accurately determine the dissociation processes for the three types of sites, that is not possible by [^{3}H]ouabain-binding measurements. Indeed, the time course of relief from inhibition parallels the release of ouabain from the enzyme. Since the dissociation is a first-order reaction, the relief from inhibition may be evaluated by the $t_{1/2}$ value, which represents the time required to recover 50% of the Na^+/K^+-ATPase activity available between the two ouabain doses used (Table II).

When ouabain concentration was shifted from 100 μM to 0.8 μM, the semilogarithmic representation of the enzyme recovery was not linear versus time. The k_{-1} values were $(39 \pm 4) \cdot 10^{-3}$ s^{-1} and $(7 \pm 11) \cdot 10^{-3}$ s^{-1} for the low- and high-affinity sites, respectively (Table II). Assuming that the proportional contributions and apparent IC_{50} values of the three putative sites, in adult rat brain, were those presented in Table IA, one can calculate the involvement of each site in the dissociation process 25% and 69% of the total activity recovered were due to low- and high-affinity sites, respectively.

For a shift between 2 μM and 0.02 μM, the kinetic of enzyme recovery was not linear. The relief from inhibition involved 43% of very high and 57% of high-affinity sites. The k_{-1} values were $(1.1 \pm 0.1) \cdot 10^{-3}$ s^{-1} and $(7 \pm 1) \cdot 10^{-3}$ s^{-1}, respectively.

At very low drug concentrations (from 10 nM to 0.08 nM), about 94% of the released ouabain came from the very high affinity sites. In this case, the semilogarithmic representation of enzyme recovery was linear versus time (data not shown), the k_{-1} was $(1.1 \pm 0.1) \cdot 10^{-3}$ s^{-1} (Table II). The $t_{1/2}$ values were 0.3, 1.65 and 60 min, respectively, for the low, high and very high affinity sites. It is noteworthy that, for all the sites, the enzymatic activities measured at the end of the release processes, at either 0.08 nM, 0.02 μM or 0.8 μM ouabain, were similar to those measured directly by the association processes performed at the same respective drug concentrations. For each site type, the enzyme-ouabain equilibrium reached by the association process was similar to that obtained by dissociation.

Discussion

The present paper shows that in membranes isolated from the whole adult rat brain, three active forms of the Na^+/K^+-ATPase exist, two of which being of high affinity for digitalis. The experimental arguments are the following:

(1) Under conditions where the low-affinity [^{3}H]ouabain-binding sites cannot be measured and where one would expect only one high-affinity ouabain-binding site, two ouabain-binding sites were detected: one of very high and one of high affinity, the contributions of which were almost in equal proportions (39 and 36%, respectively) (Table II). The total contribution of both sites (i.e. 75%) represents that previously attributed to the high-affinity form alone (70% in Sweadner [1] and 77% in Matsuda [36]). Curvilinear Scatchard plots may also be explained by a model of anticooperativity among the enzyme subunits. However, so far, this type of reactivity has never been described for ouabain-binding.

(2) At concentrations of ouabain involving the very high (10^{-8} M), the high (10^{-6} M) and the low affinity enzyme forms (10^{-4} M), ouabain dissociates from the Na^+/K^+-ATPase according to three different rate constants. This multiplicity of the dissociation processes was observed by two different independent methods: [^{3}H]ouabain-binding measurements (Fig. 4) and Na^+/K^+-ATPase assays (Table II). The biphasic dissociation curves were not due to the presence of significant amounts of an impurity or to a contamination by dihydro-ouabain. Indeed, the radioactivity present in one lot of [^{3}H]ouabain used in this study was recovered in a single peak by high pressure liquid chromatography. If three dissociation processes were found by two different methods, their rate constants vary with the method used. By [^{3}H]ouabain-binding measurements, the apparent dissociation rate constants of both site types were 5-fold slower than those found by enzymatic assays. According to enzyme activity and binding measurements, the k_{-1} values were $1.1 \cdot 10^{-3}$ s^{-1} and $0.22 \cdot 10^{-3}$ s^{-1}, for the very high affinity sites, $7 \cdot 10^{-3}$ s^{-1} and $1.4 \cdot 10^{-3}$ s^{-1} for the high-affinity sites. This could be explained by the presence (enzyme assays) or absence ([^{3}H]ouabain-binding) of K ions known to interfere with digitalis binding and release [37].

(3) The mathematical and statistical analysis of the dose-response curve of Na^+/K^+-ATPase activity versus ouabain or digitoxigenin, in adult rat brain only, have a better fit with a three-site model than with a two-site model (Fig. 1 and Table IA), two sites being of high affinity. It is worthy of note that all the computed IC_{50} values and proportional contributions found by this model were similar to the experimental K_d values and percentages. The relatively large scattering in the K_d values (from 16 to 160 nM) reported in the literature [1,15–22] could be due to variable percentages of very high and high-affinity sites according to own author enzyme preparation. Another explanation could reside in the short duration of the kinetic assays as compared to the period of time required to reach equilibrium. Indeed, since the high-affinity sites had an experimental IC_{50} value equal to $4.6 \cdot 10^{-7}$ M and an apparent dissociation rate constant of $7 \cdot 10^{-3}$ s^{-1}, the calculated

association rate constant was $1.5 \cdot 10^4$ $M^{-1} \cdot s^{-1}$. Assuming that ouabain (0.1 μM), in large excess over the site concentration, binds to the site according to a pseudo-first-order kinetics, the time required to occupy the half number of available high-affinity sites would be between 6 and 7 min. This period of time (6 min) was that initially used to estimate the dual inhibitory potency of ouabain in rat brain preparations [1]. In the present paper, the kinetic assays were carried out for 30 min. The need of a longer period of incubation is confirmed by the results of Erdmann et al. [15]. By [^{3}H]ouabain binding these authors observed that the K_d values of the high-affinity sites found after a 2-hour incubation correspond to both the computed IC_{50} value ($2.3 \cdot 10^{-8}$ M) and the K_d value ($1.7 \cdot 10^{-8}$ M) found here.

The presence of a second functional Na^+/K^+-ATPase of high affinity for digitalis might result from slight direct or indirect alterations of the preexisting high- or low-affinity sites. Direct alterations of the enzyme itself by the membrane isolation procedure or by SDS treatment and indirect effects via modifications in the phospholipid compositions [38] could change the sensitivity to digitalis. One cannot exclude the possibility that the relative abundance of the three isoenzymes may have changed along with the isolation procedure. An analysis of the dose-response curves of Na^+/K^+-ATPase to ouabain in homogenates could not be carried out since the specific activities of the enzyme in these fractions were too low.

Under the best conditions, i.e., in adult rat brain, the specific activity of the Na^+/K^+-ATPase was 5.0 ± 0.3 units with the basal Mg^{2+}-ATPase activity of 19.8 ± 3.8 units.

Note that with native vesicles, i.e. not permeabilized by SDS, the same triphasic dose-response curve (enzyme assay) was observed with digitoxigenin, a compound which freely penetrates the vesicles (IBB, data not shown).

These alterations cannot be excluded but seem very unlikely in that the phenomenon was observed with (i) a membrane isolation procedure very similar to that used by Sweadner [1] and other laboratories and (ii) specific activities (135 μmol P_i/h per mg proteins) in our preparations very close to that found by Sweadner in whole brain preparations [1].

However, by gel electrophoresis and immunoblot, even after a reduction and alkylation of the SH groups that reveals two forms in rat heart, one could detect only two major bands representing the α subunits [7]. Recently, Schneider et al. [39] reported that the α3 protein synthetized in vitro had '$\alpha(+)$' like electrophoretic mobility on SDS gels. The fact that we could not detect a third band in our preparations might be due to technical limitations when applied to brain membranes since Lowndes [14] by photo-affinity labeling, was able to demonstrate the existence of a third form ($\alpha(-)$) of the α subunit in adult rat brain membranes; although the activity associated with this form remains questionable.

The presence of a second active Na^+/K^+-ATPase of high affinity for digitalis plus the α1 low-affinity sites (i.e., three forms in toto) in whole rat brain membranes, is in full agreement with and complementary to those drawn from cDNA studies: identification in adult rat brain of three classes of messenger RNA [13,39–41]. According to three different experimental approaches: binding measurements, enzyme assays on membranes and Rb^+ uptake by cultured cells transfected with cDNA encoding α3 subunit of rat brain, it appears that the functional rat α3 subunit is of high affinity for cardiac glycosides [42].

Urayama et al. [10] also found a Na^+/K^+-ATPase activity (termed as α3) of high affinity for ouabain. The IC_{50} value reported by this group (0.13 μM) is very similar to that found here (0.46 μM). Hsu [11] reported an apparent affinity of 1 μM in an enzyme population where "more than 96% of the activity behaves as a component with a high affinity for ouabain". However, these latter authors could not be sure that the α3 Na^+/K^+-ATPase is able to hydrolyze ATP.

However, if the low affinity form could be attributed to the α1 form, α3 would have a binding constant of 10 nM [43], 80 nM [42] or 130 nM [10]. Thus, it remains unknown which form (α2 or α3 or $\alpha(-)$) correspond to the functional high (IC_{50} = 460 nM) or very high affinity (IC_{50} = 23 nM) sites described here (Table II). The relative proportional contribution of each active form in isolated membranes was very similar and could not obviously be linked to the relative abundance of the messenger RNA species, in spite of the observation that α3 would be present abundantly in the nervous systems of adult rats [44].

Finally, the physiological relevance and the cellular localization of these functional enzyme forms still remain open questions.

Acknowledgments

The authors thank Dr. D. Charlemagne for helpfull discussions, Dr. J.F. Cloix (INSERM U7) for performing the High Pressure Liquid Chromatography and Dr. Ken Boheler for rereading the manuscript. This work was supported by Grants from C 32R01 and AIP 87 the University Paris VII (L.G.L.) and by INSERM and CNRS (I.B.B.).

References

1 Sweadner, K.J. (1979) J. Biol. Chem. 254, 6060–6067.
2 Sweadner, K.J. and Farshi, S.K. (1987) Proc. Natl. Acad. Sci. USA 84, 8404–8407.

3 Erdmann, E. and Schoner, W. (1973) Biochim. Biophys. Acta 330, 316–324.
4 Hansen, O. (1976) Biochim. Biophys. Acta 433, 383–392.
5 Adams, R.J., Schwartz, A., Grupp, G., Grupp, I., Lee, S.W., Wallick, E.T., Powell, T., Twist, V.W.W. and Gathiram, P. (1982) Nature 296, 167–169.
6 Finet, M., Godfraind, T. and Noel, F. (1983) Br. J. Pharmacol. 80, 751–759.
7 Charlemagne, D., Mayoux, E., Poyard, M., Oliviero, P. and Geering, K. (1987) J. Biol. Chem. 262, 8941–8943.
8 Grupp, I.L., Grupp, G. and Schwartz, A. (1981) Life Sci. 29, 2789–2794.
9 Maixent, J.M., Charlemagne, D., de la Chapelle, B. and Lelièvre, L.G. (1987) J. Biol. Chem. 262, 6842–6848.
10 Urayama, O. and Sweadner, K.J. (1988) Biochem. Biophys. Res. Commun. 156, 796–800.
11 Hsu, Y.-M. and Guidotti, G. (1989) Biochemistry 28, 569–573.
12 Sweadner, K.J. (1989) Biochim. Biophys. Acta 988, 185–220.
13 Shull, G.E., Greeb, J. and Lingrel, J.B. (1986) Biochemistry 25, 8125–8132.
14 Lowndes, J.M., Ruoho, A.E. and Hokin-Neaverson, M. (1988) in Progress in Clinical and Biological Research, The Na^+, K^+ Pump. Molecular Aspects Vol. 268A (Skou, J.C., Norby, J.G., Maunsbach, A.B. and Esmann, M., eds.), pp. 113–118, Alan R. Liss, New York.
15 Erdmann, E., Werdan, K. and Brown, L. (1985) Trends Pharmacol. Sci. 6, 293–295.
16 Akera, T., Ng, Y.C., Shieh, I.S., Bero, E., Brody, T.M. and Braselton, E. (1985) Eur. J. Pharmacol. 111, 147–157.
17 Matsuda, T., Iwata, H. and Cooper, J.R. (1984) J. Biol. Chem. 259, 3858–3863.
18 Specht, S.C. (1984) Biochem. Biophys. Res. Commun. 121, 208–212.
19 Lytton, J. (1985) Biochem. Biophys. Res. Commun. 132, 764–769.
20 Schmitt, C.A. and McDonough, A.A. (1986) J. Biol. Chem. 261, 10439–10444.
21 Schellenberg, G.D., Pech, I.V. and Stahl, W.L. (1981) Biochim. Biophys. Acta 649, 691–700.
22 Felge, G., Leutert, T. and De Pover, A. (1988) in Progress in Clinical and Biological Research, The Na^+, K^+ Pump. Cellular Aspects Vol. 268B (Skou, J.C., Norby, J.G., Maunsbach, A.B. and Esmann, M., eds.), pp. 377–384, Alan R. Liss, New York.
23 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52.
24 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
25 Mansier, P. and Lelièvre, L.G. (1982) Nature 300, 535–537.
26 Mansier, P., Charlemagne, D., Rossi, B., Preteseille, M., Swynghedauw, B. and Lelièvre, L.G. (1983) J. Biol. Chem. 258, 6628–6635.
27 Klodos, I., Ottolenghi, P. and Boldyrev, A. (1975) Anal. Biochem. 67, 397–403.
28 Thomas, R., Allen, J., Pitts, B.J.R. and Schwartz, A. (1979) Eur. J. Pharmacol. 53, 227–237.
29 Lelièvre, L.G., Maixent, J.M., Lorente, P., Mouas, C., Charlemagne, D. and Swynghedauw, B. (1986) Am. J. Physiol. 251, H923–931.
30 Charlemagne, D., Maixent, J.M., Preteseille, M. and Lelièvre, L.G. (1986) J. Biol. Chem. 261, 185–189.
31 De Lean, A., Munson, P.J. and Rodbard, D. (1978) Am. J. Physiol. 235, E97–102.
32 Bevington, P.R. (1969) Data Reduction and Error Analysis for the Physical Sciences. McGraw Hill, New York.
33 Matsuda, T., Iwata, H. and Cooper, J.R. (1984) J. Biol. Chem. 259, 3858–3863.
34 Wellsmith, N. and Lindenmayer, G.E. (1980) Circ. Res. 47, 710–720.
35 Lelièvre, L.G., Charlemagne, D., Mouas, C. and Swynghedauw, B. (1986) Biochem. Pharmacol. 35, 3449–3455.
36 Matsuda, T., Iwata, H. and Cooper, J.R. (1985) Biochim. Biophys. Acta 817, 17–24.
37 Akera, T. and Brody, T.M. (1971) J. Pharmacol. Exp. Ther. 176, 545–557.
38 Abeywardena, M. and Charnock, J. (1983) Biochim. Biophys. Acta 729, 75–84.
39 Schneider, J.W., Mercer, R.W., Gilmore-Hebert, M., Utset, M.F., Lai, C., Greene, A. and Benz, E.J. Jr. (1988) Proc. Natl. Acad. Sci. USA 85, 284–288.
40 Herrera, V.L.M., Emanuel, J.R., Ruiz-Opazo, N., Levenson, R. and Nadal-Ginard, B. (1987) J. Cell. Biol. 105, 1855–1866.
41 Hara, Y., Urayama, O., Kawakami, K., Nojima, H., Nagamune, H., Kojima, T., Ohta, T., Nagano, K. and Nakao, M. (1987) J. Biochem. (Tokyo) 102, 43–58.
42 Hara, Y., Nikamoto, A., Kojima, T., Matsumoto, A. and Nakao, M. (1988) FEBS Lett. 238, 27–30.
43 Schwartz, A., Grupp, G., Wallick, E., Grupp, I.L. and Ball, W.J. (1988) in Progress in Clinical and Biological Research, The Na^+, K^+ Pump. Cellular Aspects Vol. 268B (Skou, J.C., Norby, J.G., Maunsbach, A.B. and Esmann, M., eds.), pp. 321–338, Alan R. Liss, New York.
44 Schneider, J.W., Mercer, R.W., Caplan, M., Emanuel, J.R., Sweadner, K.J., Benz, E.J. Jr. and Levenson, R. (1985) Proc. Natl. Acad. Sci. USA 82, 6357–6361.