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HETEROGENEITY OF OUABAIN BINDING SITES IN *SCHISTOSOMA MANSONI*

FIRST EVIDENCE FOR THE PRESENCE OF TWO (Na^++K^+)-ATPase ISOFORMS IN PLATYHELMINTHS

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Abstract—Binding experiments with [^{3}H]ouabain were performed to investigate the presence of (Na^++K^+)-ATPase (EC 3.6.1.3) isoforms in adult male *Schistosoma mansoni*, the trematode responsible for human schistosomiasis. Non-linear regression analysis of equilibrium experiments performed with homogenates in a Mg–P_i medium indicated the presence of about 10% (B_{max} = 223 ± 67 fmol/mg protein) high-affinity sites (K_D = 0.285 ± 0.045 μM) and 90% (B_{max} = 2117 ± 348 fmol/mg protein) sites with a 20-fold lower affinity (K_D = 4.9 ± 1.28 μM). This was confirmed by the bi-exponential decay of [^{3}H]ouabain dissociation. Furthermore, determination of association and dissociation rate constants indicated that the two classes of binding sites differed by their dissociation rate constants for ouabain (k_{-1} = 0.0185 ± 0.0019 min^{-1} and 0.0997 ± 0.0528 min^{-1} for high- and low-affinity sites, respectively). Surprisingly, the association rate constant measured for ouabain binding to *S. mansoni* homogenate (0.038 $\mu M^{-1} \cdot min^{-1}$) was lower (25- to 80-fold) than the one usually observed for mammalian enzymes. This is the first direct evidence for the existence of (Na^++K^+)-ATPase isoforms in platyhelminths, invertebrates of great importance from the phylogenetic point of view.

Key words: (Na^++K^+)-ATPase, ouabain, *Schistosoma mansoni*, isoforms, phylogeny

(Na^++K^+)-ATPase (EC 3.6.1.3), the enzyme responsible for active transport of Na^+ and K^+, is found in the plasma membrane of virtually all animal cells [1]. The first direct demonstration of the existence of α-subunit isoforms of this enzyme came independently from SDS–PAGE† studies of rat brain [2] and brine shrimp nauplii [3]. Molecular cloning and sequencing of cDNA revealed the existence of three major α-subunit isoforms in the rats [4–6]. Detection of a set of related α-subunit isoforms was reported in humans at about the same time [7–9]. Using site-directed polyclonal antibodies generated against oligopeptides derived from rat α-isoforms, Pressley [10] recently suggested that at least three α-isoforms are present in all mammals, including both placental and marsupial mammalian species. Studies with birds (chicken [11]) and teleost fishes (catfish [10]) suggested that the presence of multiple α-isoforms extends to all vertebrate classes [10]. On the other hand, the possibility that isoforms occur in invertebrates has been insufficiently explored [10]: with the classical exception of brine shrimp (a crustacean) [3], no heterogeneity of (Na^++K^+)-ATPase has been reported until now [10]. Studying the biochemical properties of (Na^++K^+)-ATPase from *Schistosoma mansoni*, the trematode responsible for human schistosomiasis, we reported earlier that preparations from the tegument showed different ouabain sensitivies than preparations from the rest of the body [12]. Because a direct correlation between ouabain affinity and isozyme type has been found within a given species [2], these results suggested the existence of (Na^++K^+)-ATPase isoforms in *S. mansoni*.

The objective of the present work was to investigate the presence of isoforms of the (Na^++K^+)-ATPase in homogenate of adult male *S. mansoni*, using a [^{3}H]ouabain binding assay, as this has proved to be very efficient in our characterization of rat heart isoforms [13–15].

The results indicated the existence of two classes of independent ouabain binding sites in *S. mansoni* and provided the first direct evidence for the existence of (Na^++K^+)-ATPase isoforms in platyhelminths, a fact which is of great interest from the phylogenetic point of view [16].

MATERIALS AND METHODS

Infection of mice and harvesting of adult male S. mansoni. Male adult worms were obtained from mice infected with male cercariae of *S. mansoni* (BH strain), as previously described [17].

Preparation of homogenate. About 1500 worms were homogenized in a Dounce homogenizer at 4° in 0.25 M sucrose buffered to pH 7.4 with

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† Abbreviations: POPOP, 1,4-bis[2-(5-phenyloxazolyl)]-benzene; PPO, 2,5-diphenyloxazole; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

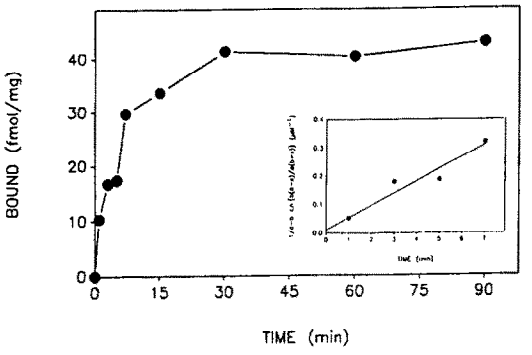

Fig. 1. Time–course of ouabain binding to *S. mansoni* homogenate. Homogenate (300 μg protein) was incubated at 37° in the presence of 3 mM $MgCl_2$, 3 mM P_i-Tris, 1 mM EGTA, 20 mM maleate-Tris (pH 7.4) and 30 nM [^{3}H]-ouabain. The quantity of ouabain specifically bound per mg protein is plotted against the incubation time. Each point is the mean value of triplicate determinations in a typical experiment. Inset: The first four experimental points were plotted according to the simple rate equation for bimolecular association where a and b are equal to the concentration of unoccupied receptor and free ligand, respectively, at time t_0, and x is equal to the concentration of ouabain-binding site complexes after reaction time t. The initial concentration of free binding sites (a) was determined by [^{3}H]ouabain binding under equilibrium conditions according to the experiment of Fig. 2.

5 mM Tris–HCl (sucrose buffer) containing 10 mM dithioerythritol and 0.2 mM phenylmethylsulfonyl fluoride, using 3 sequences of 10 passes of the pestle. The homogenate was stored at −70° until used. The yield of protein in homogenate was about 60–80 μg protein/worm as determined by the method of Lowry *et al.* [18]. No ouabain-sensitive ATPase activity was reliably detected in the homogenate, probably due to the high (basal) Mg^{2+}-ATPase activity.

[^{3}H]*Ouabain binding assay.* The incubation medium contained [^{3}H]ouabain (28.9 Ci/mmol, New England Nuclear, Boston, MA), 3 mM $MgCl_2$, 3 mM P_i-Tris, 1 mM EGTA and 20 mM maleate-Tris, pH 7.4, at 37°. The non-specific binding was estimated by incubating samples in the absence of Mg^{2+} and P_i and in the presence of 0.2 mM unlabeled ouabain [14]. In saturation experiments, non-specific binding accounted for 23–32% of total binding. A rapid filtration technique was used to separate membrane-bound from free ouabain: samples of 500 μL, usually containing 300 μg protein, were diluted rapidly with 5 mL of chilled sucrose buffer and filtered on Whatman glass fiber filters (GF/C). Tubes were washed with 5 mL of chilled sucrose buffer, and filters were further washed twice with 10 mL of the same buffer. Filters were then dessicated and added to 10 mL of the scintillation mixture consisting of toluene containing POPOP (0.1 g/L) and PPO (4 g/L). The radioactivity was counted in a liquid scintillation counter (Beckman LS-150).

Statistical analysis. Binding data were graphically represented using classical plots (e.g. Scatchard plot for saturation experiments and semi-log plot for dissociation kinetics). Parameters were calculated using untransformed data and a computerized non-linear regression analysis based on the steepest descent technique [13]. 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 an experiment was repeated twice, the constant was expressed as the mean value ± SD of the mean from the three different experiments. In this case, SD represents the variation between the experiments and not the "goodness of fit." Two different models (one and two classes of independent binding sites [13]) were discriminated by using the F-test for comparison of total variances [13]. The association rate constant (k_{+1}), was calculated using the simple rate equation for bimolecular association [19]:

$$1/a - b \cdot \ln [b(a-x)/a(b-x)] = k_{+1} \cdot t$$

where a and b represent the concentration of unoccupied receptor and free ligand, respectively, at time t_0, and x the concentration of ouabain-binding site complexes after reaction time t.

RESULTS

Time–course of ouabain binding to S. mansoni *homogenate.* Figure 1 illustrates the time–course of 30 nM [^{3}H]ouabain binding to homogenate in the Mg-P_i medium. [^{3}H]Ouabain binding reached a maximum after about 30 min and remained stable over the 90-min incubation period.

[^{3}H]*Ouabain binding as a function of concentration.* Ouabain binding to *S. mansoni* homogenate was measured after a 30-min incubation using concentrations of ligand ranging from 10 to 10,000 nM. As shown in Fig. 2 for a typical experiment, the Scatchard plot was curvilinear and upwardly concave, suggesting the presence of more than one class of specific binding sites. The quantitative analysis of these results is consistent with the existence of two classes of independent binding sites characterized by the following values of B_{max} and K_D: 123 ± 46 fmol/mg protein and 0.241 ± 0.076 μM for the high-affinity sites; 1550 ± 1110 fmol/mg protein and 2.34 ± 0.21 μM for the low-affinity sites. Note that when submitted to an F-test, the total variance obtained when data were analyzed according to the model of two classes of sites (S^2_T = 0.0029; df = 11) was significantly lower ($P < 0.05$) than the total variance obtained when the model of one class of sites was used (S^2_T = 0.0074; df = 13). These results suggest the presence of about 10% high-affinity sites and 90% low-affinity sites.

These data were confirmed in two other experiments performed with different homogenates. Mean values of parameters (±SEM) from the three experiments are reported in Table 1.

Time–course of [^{3}H]*ouabain dissociation from* S. mansoni *homogenate.* In this experiment, homogenate was incubated for 30 min in the presence of 70 nM [^{3}H]ouabain in order to measure the specific

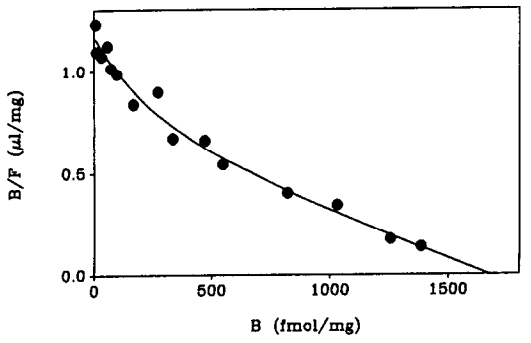

Fig. 2. Scatchard plot for [^{3}H]ouabain specific binding to *S. mansoni* homogenate. Homogenate was incubated for 30 min in Mg-P_i medium as described in the legend of Fig. 1 in the presence of various concentrations of (tritiated + unlabeled) ouabain (from 10 nM to 10 μM). [^{3}H]Ouabain (10 nM; 15.4 Ci/mmol) was diluted with unlabeled ouabain to give final concentrations from 10 to 50 nM. [^{3}H]Ouabain (70 nM) was diluted to give final concentrations from 70 nM to 10 μM. Each point is the mean of triplicate determinations in a typical experiment. The curve was drawn using the parameters fitted by non-linear regression analysis using the model of two classes of independent binding sites. B = ouabain specifically bound. F = free concentration of ouabain.

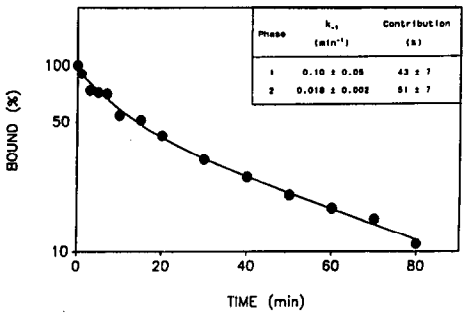

Fig. 3. Time–course of [^{3}H]ouabain dissociation from *S. mansoni* homogenate. Homogenate was incubated in Mg-P_i medium, as described in the legend of Fig. 1, in the presence of 70 nM [^{3}H]ouabain. After a 30-min incubation, an excess of unlabeled ouabain was added. The amount of specifically bound ouabain (BOUND) was determined at different time intervals and expressed as a percentage of specific binding measured just before performing the isotopic dilution. Each point is the mean of triplicate determinations in one experiment. The curve was drawn using the parameters, indicated in the inset, calculated by non-linear regression analysis using the bi-exponential model of decay. k_{-1} is the dissociation rate constant and contribution (%) is the relative amount of low-affinity sites (phase 1) and high-affinity sites (phase 2) that are labeled at this particular concentration of ouabain (70 nM). Note that the ordinate scale is logarithmic.

Table 1. Capacity and dissociation constant values for the two classes of ouabain binding sites present in *S. mansoni* homogenate

	B_{max} (fmol/mg)	K_D (μM)
High-affinity sites	223 ± 67	0.285 ± 0.045
Low-affinity sites	2117 ± 348	4.90 ± 1.28

Capacity (B_{max}) and dissociation constant (K_D) values were calculated on untransformed data (obtained in equilibrium experiments like the one described in Fig. 2) by non-linear regression analysis using the model of two classes of independent binding sites. Values shown are the means ± SEM from three different experiments performed with homogenates obtained from about 1500 worms each.

binding at equilibrium (100%), after which a large excess (0.2 mM) of unlabeled ouabain was added to promote the dissociation process without altering the volume (<1%) (= isotopic dilution). The dissociation curve was curvilinear in a semi-log scale (Fig. 3), indicating that the dissociation did not follow first-order kinetics. Indeed, a bi-exponential model of decay fit the data better (see Materials and Methods). As calculated from the best fit to the dissociation curve, 43% of binding occurred at a low-affinity site (high k_{-1} value) and 51% at a high-affinity site (low k_{-1} value), at this particular concentration of ouabain, in good agreement with the theoretical values expected from K_D and B_{max} reported in Table 1.

Association rate constant. Figure 1 shows how the association rate constant (k_{+1}) was calculated for ouabain binding to *S. mansoni* homogenates. Data obtained at 1-, 3-, 5- and 7-min incubations (i.e. relatively far from equilibrium) were plotted (Fig. 1, insert) according to the simplified form of the second-order equation (see Materials and Methods). The value of k_{+1} (equal to the slope) in this typical experiment was $0.043 \pm 0.005\ \mu M^{-1} \cdot min^{-1}$ as calculated by linear regression. As this concentration of ouabain (30 nM) binds to both types of sites and as the difference between high- and low-affinity sites is classically due to the difference in the k_{-1} and not the k_{+1} values, we assumed that the k_{+1} measured here was valid for both high- and low-affinity sites. The lack of any evidence for heterogeneity in the association curves was also a reason to conclude that the sites had similar association rate constants. The k_{+1} value corresponding to the mean ± SEM from three different experiments is reported in Table 2.

Comparison between dissociation constants measured at equilibrium (K_d) *and kinetically* (k_{-1}/k_{+1}). Table 2 summarizes data of [^{3}H]ouabain binding to *S. mansoni* homogenate. Ratios of k_{-1} and k_{+1} values for both classes of sites were roughly in good agreement with K_D values calculated from independent equilibrium experiments, further validating our evidence for the existence of two isoforms of $(Na^+ + K^+)$-ATPase in *S. mansoni* differing by their dissociation rate constants for ouabain, as is the case for the different isoforms present in mammals.

Table 2. [^{3}H]Ouabain binding to *S. mansoni* homogenate: Comparison between rate constants and equilibrium constants measured for both classes of sites

	k_{+1} ($\mu M^{-1} \cdot min^{-1}$)	k_{-1} (min^{-1})	k_{-1}/k_{+1} (μM)	K_D (μM)
High-affinity sites	0.038 ± 0.002	0.0185 ± 0.0019	0.487 ± 0.056	0.285 ± 0.045
Low-affinity sites	0.038 ± 0.002	0.0997 ± 0.0528	2.62 ± 1.40	4.90 ± 1.28

The association rate constant (k_{+1}) and dissociation constant (measured in equilibrium experiment, K_D) values are the means ± SD of the mean of three different experiments. The dissociation rate constant (k_{-1}) is the parameter ± SD calculated by non-linear regression analysis from one experiment. The approximate standard deviation represents here the "goodness of fit." k_{-1}/k_{+1} is the dissociation constant calculated from the rate constants. SD was obtained by calculating the propagation of errors.

DISCUSSION

Heterogeneity of ouabain binding sites in S. mansoni. Analysis of the curvilinear Scatchard plot obtained for ouabain binding to *S. mansoni* homogenate indicated the presence of two classes of ouabain binding sites. This was confirmed by kinetic studies. The dissociation process followed a bi-exponential decay, and the ratio of dissociation to association rate constants was similar to the dissociation constant obtained from equilibrium experiments. Such a conclusion is contrary to that of Fetterer *et al.* [20], based on competition experiments with [^{3}H]ouabain in the homogenate of *S. mansoni*. However, as their competition curve appeared somewhat shallow (Hill coefficient of less than 1), as expected in the presence of multiple classes of binding sites [21], we carefully re-analyzed their data by non-linear regression and found that they would be more consistent with the existence of 19% high-affinity sites and 81% low-affinity sites. Furthermore, present results confirmed our previous hypothesis based on differences in sensitivity to ouabain exhibited by preparations from the tegument and carcass of schistosomes [12].

(Na^++K^+)-ATPase isoforms and evolution. In mammals, heterogeneity of ouabain binding sites has generally been ascribed to different α-subunit isoforms of (Na^++K^+)-ATPase [22–27]. Therefore, the results in this paper could be considered as the first evidence of heterogeneity of isoforms of (Na^++K^+)-ATPase in platyhelminths, a group of particular phylogenetic importance since it has been proposed that multicellular animals could have originated from some primitive noncoelomate platyhelminth [16]. The presence of two isoforms of (Na^++K^+)-ATPase in *S. mansoni* suggests that the multiplication of the α-subunit gene occurred very early in the evolutionary process, and not just 200 million years ago when mammals separated from birds [11]. Alternatively, *S. mansoni* (and platyhelminth as a whole?) isoforms could evolve independently. Regardless of the evolutionary mechanism, the presence of two isoforms of (Na^++K^+)-ATPase in a second nonvertebrate class could further strengthen the argument that isoforms of the Na^+-K^+ pump confer some selective advantage [10].

Since little is known about the ATPase in invertebrates, we can not exclude alternative explanations, such as differences in local environment (e.g. lipid composition), the presence of extrinsic modulatory factors, or even the presence of distinct isoforms of the β-subunit, recently implicated as a contributor to glycoside binding [28].

Localization and physiological role of (Na^++K^+)-*ATPase in* S. mansoni. As previously reported, (Na^++K^+)-ATPase preparations from the tegument and other somatic tissues of *S. mansoni* exhibit differences in sensitivity to ouabain [12]. We propose here that the two isoforms present in the homogenate have a distinct tissue localization, which could be related to a characteristic physiologic role. In the tegument, the (Na^++K^+)-ATPase is present exclusively in the basal membrane of the cell [29]. This polarized distribution is typical of epithelial cells and is a prerequisite for the uptake of solutes and nutrients like amino acids and glucose by Na^+-coupled transport. In *S. mansoni*, where the tegument is cellular and apparently modified for absorptive purposes [30], glucose and methionine were shown to be transported through the tegument, at least in part, by a Na^+ gradient-dependent transport [30]. On the other hand, (Na^++K^+)-ATPase present in the somatic tissue should have a uniform, i.e. not polarized, distribution in the plasma membrane of excitable cells, where it would be responsible for the maintenance of ionic gradients. Since adult male worms possess an important muscular mass [31], the carcass (Na^++K^+)-ATPase should be found, mostly, in the muscle cells. This is in agreement with the large tension increase of the longitudinal musculature observed by Fetterer *et al.* [32] when ouabain was applied to adult male *S. mansoni*, indicating a significant role for active Na^+-K^+ transport in muscle contraction.

Particularity of S. mansoni (Na^++K^+)-*ATPase.* The association rate constant measured for ouabain binding to *S. mansoni* homogenate ($0.038\ \mu M^{-1} \cdot min^{-1}$) was surprisingly lower (25- to 80-fold) than the one usually observed for mammalian enzymes from different sources [33, 34], indicating a species difference between parasite and mammalian enzymes. This extends our previous report of differences in vanadate sensitivity between (Na^++K^+)-ATPase from *S. mansoni* carcass and lamb kidney, used as a typical source of mammalian (Na^++K^+)-ATPase [35].

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