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Quantitative Analysis of the High-Affinity Binding Sites for [^{3}H]Ouabain in the Rat Vas Deferens and Their Immunological Identification as the α_2 Isoform of Na^+/K^+-ATPase

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ABSTRACT. Binding assays were performed with [^{3}H]ouabain to investigate the presence of, and to characterize, a Na^+/K^+-ATPase isoform with high affinity for cardiac glycosides in the rat vas deferens. Nonlinear regression analysis of equilibrium experiments carried out with crude preparations in a Mg-P_i medium indicated the presence of high-affinity sites characterized with good precision (individual coefficients of variation = 11–35%) by their density (B_{max} = 0.42 to 0.72 pmol/mg protein) and dissociation constant (K_d = 0.069 to 0.136 μM) values. The values of the dissociation rate constant (k_{-1}) and the association rate constant (k_{+1}) for these sites were 0.151 to 0.267 min^{-1} and 2.87 to 3.60 $\mu M^{-1} \cdot min^{-1}$, respectively. A higher number of low-affinity sites (K_d around 15 μM), supposed to correspond to the α_1 isoform, was also identified, but their K_d and B_{max} values were not quantified precisely in this crude preparation. Western blot assays indicated hybridization with specific anti-α_1 and anti-α_2 isoform antibodies but not with anti-α_3 isoform antibody. Taken together, the present results indicate the existence of a low proportion of the α_2 isoform of Na^+/K^+-ATPase in the rat vas deferens that can be quantified precisely by [^{3}H]ouabain binding even in a crude membrane preparation that is suitable for studies under conditions of plasticity. BIOCHEM PHARMACOL **55**;9:1531–1535, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. vas deferens; ATPase,Na-K; ouabain; rat; cardiac glycoside

Na^+/K^+-ATPase (EC 3.6.1.37) is the integral plasma membrane enzyme responsible for maintaining the transmembrane gradients of Na^+ and K^+ that are essential for cellular homeostasis. This enzyme consists of α and β subunits, which present distinct isoforms in different tissues and cells [1, 2]. The α_1 isoform was identified in all cells and has been considered as the "housekeeping" enzyme [3]. The α_2 and α_3 isoforms have a more limited distribution. The α_3 isoform is located predominantly in neural tissue, while the α_2 isoform is present in neural tissue and heart ventricles and is also the predominant isoform in adult skeletal muscle [1–4]. Based on mRNA analysis, the α_2 isoform seems to be the main isoform in rat adult smooth muscle as well [2]. This isoform has a high affinity for cardiac glycosides and is sensitive to regulatory mechanisms [3, 5–7]. The presence of α_1 and α_2 isoforms of the Na^+/K^+-ATPase has been demonstrated in smooth muscle of the guinea pig vas deferens [8]. The α_2 isoform in this organ is modulated by autonomic innervation, since there is a specific reduction of this isoform associated with a nonspecific postjunctional supersensitivity to various agonists after denervation [8, 9]. In the rat, denervation of the vas deferens also produces a nonspecific postjunctional supersensitivity [10], although the electrogenic Na^+ transport by the Na^+/K^+ pump does not add much to the membrane potential [11]. Therefore, the present study was performed to investigate the presence of the α_2 isoform of Na^+/K^+-ATPase in the rat vas deferens and to quantify it in a crude membrane preparation suitable for studies on the modulation of receptor expression. Preliminary results of this study were presented at the Eighth International Conference on the Na^+/K^+-ATPase held in August 1996.

MATERIALS AND METHODS

Vas Deferens Preparation

Vasa deferentia of 15–20 adult Wistar rats of the BAW2 colony [12] were cut into small segments and homogenized at 4° in 25 vol. (v/w) of 0.25 M of sucrose buffered to pH 7.4 with 5 mM of Tris-HCl containing 2 mM of dithio-

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threitol and 0.2 mM of PMSF§, using an Ultraturrax homogenizer at 20,500 rpm for 1 min. This operation was repeated twice for 30 sec and followed by 10 up-and-down strokes in a glass Potter homogenizer. After filtration under vacuum through four layers of gauze, the homogenate was centrifuged at 100,000 *g* for 1 hr. The pellet was resuspended in the same buffer but without dithiothreitol and stored at −70°. Such a crude preparation was used because it is more suitable for quantification of sites in muscles under conditions of plasticity since there is a high and reproducible recovery of binding sites, which differs from the variable recovery in more purified membrane preparations [13, 14]. The protein concentration, determined by the method of Lowry *et al.* [15], was about 40–60 mg of protein/g of vas deferens. Alternatively, the homogenate was centrifuged at 3000 *g* for 20 min followed by a 100,000 *g* centrifugation for 30 min in order to obtain a less crude preparation for the Western blot assay.

Preparations from Heart, Kidney, and Brain

Heart ventricles, kidneys, and brainstems were homogenized in a Potter homogenizer with a motor-driven teflon pestle at 4° in 2–3 vol. of 0.25 M of buffered sucrose containing 0.1 mM of PMSF/g organ. After filtration through gauze (only in the case of heart tissue), the homogenates were centrifuged at 100,000 *g* for 1 hr, and the pellets were resuspended and stored as described for the vas deferens.

Binding Assay

The incubation medium contained [^{3}H]ouabain (New England Nuclear), 3 mM of $MgCl_2$, 3 mM of P_i-Tris, 1 mM of EGTA, and 20 mM of maleate-Tris, pH 7.4, at 37°. The nonspecific binding was estimated by incubating samples in the presence of 1 mM of unlabeled ouabain. A rapid filtration technique was used to separate membrane-bound from free ouabain: samples of 500 μL usually containing 250 μg of protein (crude preparation of vas deferens) were rapidly diluted with 5 mL of chilled sucrose buffer and rapidly filtered on Whatman glass fiber filters (GF/C) under vacuum. Tubes were washed with 5 mL of chilled 5 mM of Tris-HCl buffer (pH 7.4 at 4°), and filters were further washed twice with 10 mL of the same buffer. In these conditions, about 95% of the specific binding sites for ouabain were retained by the filters. Filters were then dried and added to a scintillation mixture consisting of toluene containing 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP, 0.1 g/L) and 2,5-diphenyloxazole (PPO, 4 g/L). The radioactivity retained in the filters was measured in a liquid scintillation counter.

§ *Abbreviations:* PMSF, phenylmethylsulfonyl fluoride; and TBS, Tris-buffered saline.

Statistical Analysis

Binding data were represented graphically using classical plots (i.e. Scatchard plot for saturation experiments and semi-log plot for dissociation kinetics). Parameters were calculated using untransformed data and a computerized nonlinear regression analysis based on the steepest descent technique [4]. The parameters were given with approximates of standard deviations that represent the "goodness of fit" of the parameter with respect to the model and the data. Two different models (one and two classes of independent specific binding sites) were discriminated by using the *F*-test for comparison of total variances [4]. The model of two independent specific binding sites was based on the following equation:

$$B = \frac{B_{max1} \times F}{K_{d1} + F} + \frac{B_{max2} \times F}{K_{d2} + F} \quad (1)$$

where B is the amount of ligand bound at a free concentration of ouabain (F); B_{max1} and B_{max2} are the capacities of sites 1 and 2, and K_{d1} and K_{d2} are their respective dissociation constants. The observed association rate constant, k_{obs}, was calculated by nonlinear regression using the general rate equation for bimolecular association when performed under pseudo-first-order conditions, i.e. when free ligand may be considered constant during the experiment [16]:

$$B = B_{eq}(1 - e^{-k_{obs} \cdot t}) \quad (2)$$

where B and B_{eq} are the amounts of ligand bound at time t and at equilibrium, respectively. The association rate constant, k_{+1}, is related to k_{obs} through the following equation:

$$k_{+1} = (k_{obs} - k_{-1})/[L] \quad (3)$$

where k_{-1} is the dissociation rate constant and [L] is the radioligand concentration.

Western Blot Analysis

SDS–PAGE was carried out according to Laemmli [17]. After heating at 80° for 5 min, samples were separated on a 6% polyacrylamide gel and transferred to nitrocellulose filter papers [18]. After incubation for 1 hr in TBS containing 5% nonfat dry milk, nitrocellulose sheets were washed three times with TBS containing 0.1% Tween 20 and then incubated for 2 hr at room temperature with anti-rabbit Na^+/K^+-ATPase α_1 isoform (mouse monoclonal immunoglobulin G, diluted 1/2600), anti-rat Na^+/K^+-ATPase α_2 isoform (rabbit antiserum, diluted 1/900), or anti-rat Na^+/K^+-ATPase α_3 isoform (rabbit polyclonal immunoglobulin G, diluted 1/1000) from Upstate Biotechnology Inc. Blots were rinsed and incubated for 1.5 hr with anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase

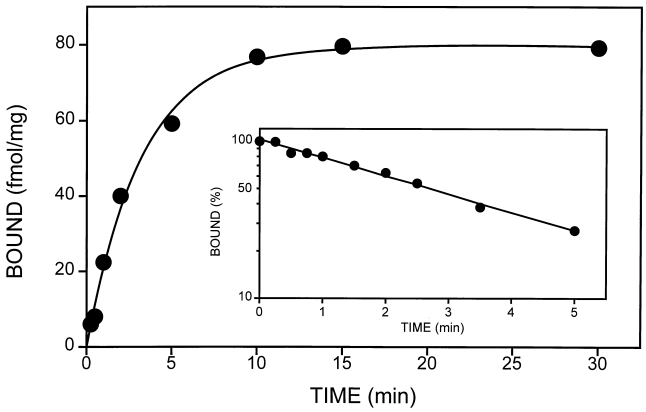

FIG. 1. Time course of [^{3}H]ouabain binding. A crude preparation of rat vas deferens (250 μg of protein) was incubated at 37° in the presence of 3 mM of Mg-P_i medium and 15 nM of [^{3}H]ouabain. The concentration of ouabain specifically bound (BOUND), expressed in fmol/mg of protein, was plotted against the incubation time (TIME). Each point is the mean value of triplicate determinations in a typical experiment. Nonspecific binding accounted for 11% of total binding at equilibrium. *Inset*: Time course of the dissociation of [^{3}H]ouabain binding from rat vas deferens. After 30 min, a large excess of nonradioactive ouabain (0.4 mM) was added to stop the association process. The amount of specifically bound ouabain (BOUND) was determined at different time intervals and is expressed as the percentage of specific binding measured just before performing the dilution. Each point is the mean of triplicate determinations. The curve was drawn using the value of k_{-1} calculated by nonlinear regression analysis using the mono-exponential model of decay. Note that the ordinate scale is logarithmic.

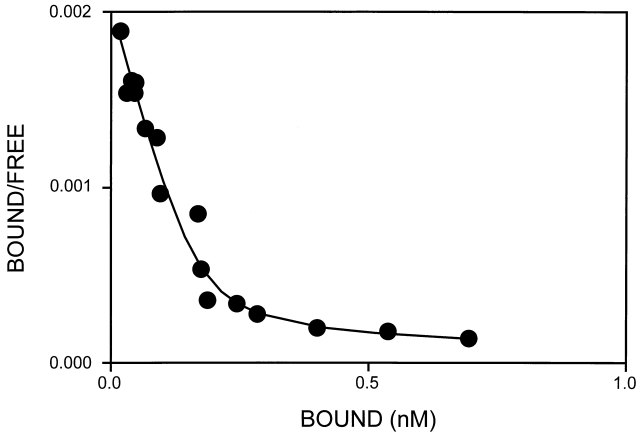

FIG. 2. Scatchard plot for [^{3}H]ouabain specific binding. Protein (0.42 mg/mL) of the crude preparation of rat vas deferens was incubated for 30 min in 3 mM of Mg-P_i medium in the presence of various concentrations of (tritiated + unlabeled) ouabain (from 10 to 5000 nM). Nonspecific binding accounted for 15% (at 10 nM of ouabain) to 63% (at 5000 nM of ouabain) of total binding. Each point is the mean of triplicate determinations in an experiment performed with the same preparation used in the experiments of Fig. 1. The curve was drawn using the parameters fitted by nonlinear regression analysis using the model of two classes of independent binding sites (Equation 1, see Materials and Methods). Bound = ouabain specifically bound; Free = free concentration of ouabain.

(1/11,000). Immunoreactivity was detected by enhanced chemiluminescence (ECL, Amersham).

RESULTS

Time Course of [^{3}H]Ouabain Binding

Figure 1 illustrates the time course for the binding of [^{3}H]ouabain (15 nM) to a crude membrane preparation of the rat vas deferens. The binding reached a maximum after about 15 min and remained stable, thereafter, during the 30-min incubation period.

Concentration-Dependency of [^{3}H]Ouabain Binding at Equilibrium

Ouabain binding was measured at equilibrium, after 30 min of incubation, using concentrations ranging from 10 to 5000 nM. As shown in Fig. 2 for the best experiment, the Scatchard plot was curvilinear and upwardly concave, suggesting the presence of more than one class of specific binding sites. The total variance obtained when data were analyzed according to the model of two classes of sites was significantly lower ($P < 0.05$, F-test) than the total variance obtained when the model of one class of sites was used. The high-affinity sites were characterized very precisely by their B_{max} and K_d values (Table 1). On the other hand, the low-affinity sites were not quantified with precision as the coefficients of variation were about 50% for both B_{max} and K_d (Table 1). These data were confirmed in two other experiments performed with the same preparation and also in one experiment performed with a different preparation. In all of these experiments, the B_{max} (0.42 to 0.72 pmol/mg protein) and K_d (0.069 to 0.136 μM) values for the high-affinity sites were very precise (coefficients of variation from 11 to 35%) and similar to those measured from the experiment of Fig. 2. On the other hand, B_{max} and K_d values for the low-affinity sites were less precise (coefficients of variation from 95 to 210%) than in the above-mentioned experiment.

Kinetics of [^{3}H]Ouabain Dissociation

In this experiment, the crude preparation was incubated for 30 min in the presence of a low concentration of [^{3}H]ouabain (15 nM) in order to measure the specific binding to the high-affinity sites at equilibrium (100%); thereafter, a large excess of nonradioactive ouabain (0.4 mM) was added to stop the association process in order to measure the rate of dissociation of the receptor–ligand complex. In a typical experiment shown in Fig. 1 (inset), the dissociation curve was linear in a semi-log scale, indicating that the dissociation followed first-order kinetics. Indeed, these data were well fitted when a mono-exponential model of decay was used to calculate the dissociation rate constant (Table 1). Similar kinetics were observed in an independent experiment performed with a different preparation ($k_{-1} = 0.151 \pm 0.016$ min^{-1}).

TABLE 1. Equilibrium and kinetic constants for ouabain binding to the rat vas deferens

Ouabain binding sites	B_{max} (pmol/mg protein)	K_d (μM)	k_{+1} ($\mu M^{-1} \cdot min^{-1}$)	k_{-1} (min^{-1})	k_{-1}/k_{+1} (μM)
High affinity	0.42 ± 0.04	0.090 ± 0.013	2.87	0.267 ± 0.008	0.093
Low affinity	5.39 ± 2.34	16.4 ± 9.6			

Capacity (B_{max}) and dissociation constant (K_d) values were calculated by nonlinear regression analysis using the model of two classes of independent specific binding sites (Equation 1, see Materials and Methods) from the experiment of Fig. 2. Association and dissociation rate constants (k_{-1} and k_{+1}) were calculated, as indicated in Materials and Methods, from the experiments of Fig. 1. Note that the parameters were calculated from single experiments performed with the same preparation and are given with their standard deviation as an indication of the "goodness of fit."

Association Rate Constant

The observed association rate constant (k_{obs}) calculated from the typical experiment shown in Fig. 1 was 0.310 ± 0.021 min^{-1}. The actual association rate constant (k_{+1}) based on this value of k_{obs} and on the k_{-1} calculated from Fig. 1 (inset) is shown in Table 1. A similar value of k_{+1} (3.60 $\mu M^{-1} \cdot min^{-1}$) was calculated in an independent experiment performed with a different preparation.

Comparison Between Dissociation Constants Measured at Equilibrium (K_d) and Kinetically (k_{-1}/k_{+1})

In accord with theoretical expectation [16], the ratio of dissociation to association rate constants was in very good agreement with the dissociation constant calculated from an independent experiment performed at equilibrium, using the same preparation (Table 1).

Western Blot

Figure 3 shows that the anti-α_1 isoform antibody hybridized with a protein characterized by an apparent molecular mass between 97 and 116 kDa in the four samples used (kidney, heart, vas deferens, and brainstem), as expected for the ubiquitous α_1 isoform of Na^+/K^+-ATPase. The anti-α_2 isoform antibody labeled the vas deferens, as well as the heart and brainstem samples that were used as positive controls. Note that the protein labeled in the vas deferens exhibited a slightly higher electrophoretic mobility than the ones present in heart and brainstem. Furthermore, the specificity of the antibody was checked by the lack of hybridization with the kidney. The anti-α_3 isoform antibody was unable to hybridize with the vas deferens, although a strong reaction was observed with the brainstem (positive control). Even using a high exposure time that allowed the visualization of a discrete band in the heart (Fig. 3), where very little α_3 isoform is present in the conductive tissue [19], we were not able to detect any reaction with the vas deferens.

DISCUSSION

Analysis of the curvilinear Scatchard plots obtained for ouabain binding to crude preparations of rat vas deferens indicated the presence of two classes of ouabain binding sites. The quantification of the high-affinity sites for ouabain, which was our primary goal, was very precise and reproducible. The value of the dissociation constant measured here (K_d = 0.069 to 0.136 μM) was very similar to that reported for the rat α_2 isoform of the hydrolytic peptide of the αβ-protomer of Na^+/K^+-ATPase in either heart ventricles [4] or transfected cells [20]. However, the quantitative analysis of the low-affinity binding sites (K_d around 15 μM) was difficult, due to the imprecision of the parameters estimated by nonlinear regression. These low-affinity sites are supposed to correspond to the ubiquitous α_1 isoform of the Na^+/K^+-ATPase that exhibits a K_d value of about 15 μM in the rat under the same experimental conditions [4]. The difficulty to obtain more precise values for the low-affinity sites can be ascribed to the high concentrations of [^{3}H]ouabain necessary for labeling these

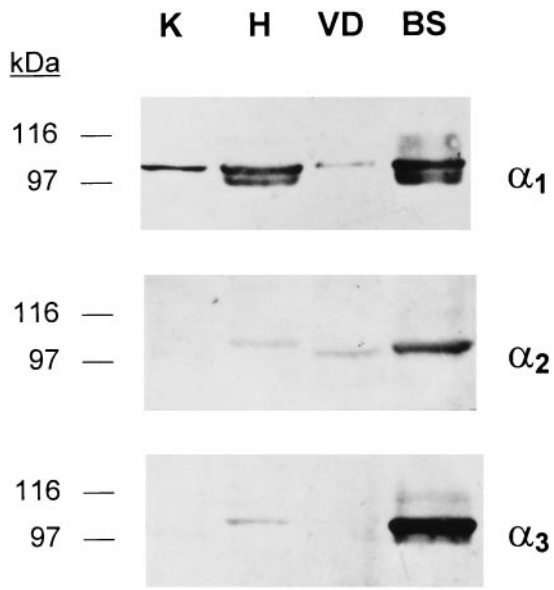

FIG. 3. Western blots of rat vas deferens using specific antibodies against α_1, α_2, and α_3 isoforms of Na^+/K^+-ATPase. K, rat kidney (15 μg of protein/lane); H, rat heart ventricles (40 μg protein/lane); VD, rat vas deferens (60 μg of protein/lane); and BS, rat brainstem (25 μg of protein/lane). Crude preparations were used for all the organs with the exception of the vas deferens (see Materials and Methods). Samples were fractioned by electrophoresis through an SDS-containing 6% polyacrylamide gel, transferred to a nitrocellulose filter, and probed with the specific antibodies indicated at the right. The positions of molecular mass markers are indicated on the left. All these bands were absent after incubation with the secondary antibody and omission of the primary antibody.

sites (micromolar range) in a crude preparation, which is relatively poor in enzyme. With respect to the dissociation assay for the high-affinity sites, the mono-exponential decay of [^{3}H]ouabain indicates that with the concentration of ouabain used (15 nM), only one binding site was involved allowing the precise quantification of the dissociation rate constant. The value of this constant is in very good agreement with the constant measured in other preparations for the rat α_2 isoform, in similar experimental conditions [4, 20]. In addition, the association rate constant for ouabain binding in the rat vas deferens was very similar to the corresponding constant for the rat α_2 isoform [4] and for Na^+/K^+-ATPases of other mammalian species [21]. Finally, the use of antibodies labeling with high specificity the three isoforms of rat Na^+/K^+-ATPase let us identify these high-affinity sites for ouabain as the α_2, and not α_3, isoform of Na^+/K^+-ATPase. The somewhat higher electrophoretic mobility of the protein hybridized with the anti-α_2 antibody present in vas deferens may be due to a different level of phosphorylation or alternative splicing, even if this has never been shown for Na^+/K^+-ATPase.

Taken together, the present data indicate the existence of high-affinity sites for [^{3}H]ouabain in rat vas deferens that share all the characteristics of the α_2 isoform of Na^+/K^+-ATPase. Whether this isoform suffers a specific reduction after denervation, as occurs in the guinea pig vas deferens [8], is now under investigation since we demonstrated here that a precise quantification of this isoform by [^{3}H]ouabain binding is possible in a crude preparation suitable for this kind of study.

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