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Characterization of the Fourth α Isoform of the Na,K-ATPase

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Abstract. The Na,K-ATPase is a major ion transport protein found in higher eukaryotic cells. The enzyme is composed of two subunits, α and β, and tissue-specific isoforms exist for each of these, α1, α2 and α3 and β1, β2 and β3. We have proposed that an additional α isoform, α4, exists based on genomic and cDNA cloning. The mRNA for this gene is expressed in rats and humans, exclusively in the testis, however the expression of a corresponding protein has not been demonstrated. In the current study, the putative α4 isoform has been functionally characterized as a novel isoform of the Na,K-ATPase in both rat testis and in α4 isoform cDNA transfected 3T3 cells. Using an α4 isoform-specific polyclonal antibody, the protein for this novel isoform is detected for the first time in both rat testis and in transfected cell lines. Ouabain binding competition assays reveal the presence of high affinity ouabain receptors in both rat testis and in transfected cell lines that have identical K_D values. Further studies of this high affinity ouabain receptor show that it also has high affinities for both Na^+ and K^+. The results from these experiments definitively demonstrate the presence of a novel isoform of the Na,K-ATPase in testis.

Key words: Na,K-ATPase — α4 isoform — testis — ouabain — 3T3 cell expression system

Introduction

The Na,K-ATPase is a heterodimeric, membrane spanning enzyme that is responsible for the ATP-coupled translocation of Na^+ and K^+ ions across the plasma membrane of higher eukaryotic cells. This activity produces an electrochemical gradient that is essential for the proper functioning of several cellular processes including maintenance of the resting membrane potential, control of cell volume, and active transport of many essential nutrients into the cell (Lingrel & Kuntzweiler, 1994). In addition to its critical role in maintaining cellular homeostasis, the Na,K-ATPase is also the molecular receptor for a class of drugs known as cardiac glycosides which includes ouabain, digoxin and digitoxin (Thomas, Gray & Andrews, 1990; Schwartz, Lindenmeyer & Allen, 1975).

The Na,K-ATPase functions as a heterodimer consisting of the catalytic α subunit and the glycosylated β subunit. The catalytic α subunit contains the ATP, Na^+ and K^+ binding sites, as well as the cardiac glycoside binding site (Lingrel & Kuntzweiler, 1994), whereas the β subunit is important for proper folding and transport to the plasma membrane of the enzyme (Lingrel & Kuntzweiler, 1994) and may also influence its substrate affinities (Blanco, Sanchez & Mercer, 1995; Malik et al., 1996). Several isoforms of each of these subunits have been cloned and characterized in this and other laboratories (Sweadner, 1989; Lingrel & Kuntzweiler, 1994). There are three characterized α isoform subunits (α1, α2 and α3) and three different β isoform subunits (β1, β2, and β3), each having distinct tissue-specific expression patterns (Orlowski & Lingrel, 1988; Sweadner, 1989; Lingrel & Kuntzweiler, 1994; Blanco et al., 1995; Malik et al., 1996; Arystarkhova & Sweadner, 1997). For example, the α1 isoform is expressed ubiquitously, the α2 isoform is predominantly expressed in heart, skeletal muscle and brain and the α3 isoform is expressed in neural tissues and in ovary (Orlowski & Lingrel, 1988; Sweadner, 1989; Lingrel & Kuntzweiler, 1994).

Our laboratory, through screening of a human genomic library for Na,K-ATPase α isoforms, identified a gene ATP1AL2 whose sequence was similar to but did not correspond to any previously identified isoforms of the Na,K-ATPase (Shull & Lingrel, 1987). Subsequent studies of the ATP1AL2 gene resulted in the cloning of the corresponding rat cDNA (Shamraj & Lingrel, 1994).

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Sequence comparison analysis using the rat ATP1AL2 deduced amino acid sequence showed identity highest to the rat Na,K-ATPase α1, α2 and α3 isoforms (78, 78 and 76% amino acid identity, respectively) and at a lower level to the gastric and distal colon H,K-ATPases (62 and 59%, respectively) (Shamraj & Lingrel, 1994). Further analysis showed that the amino acid sequence of its putative transmembrane domains is 79–100% identical to the sequences of the other Na,K-ATPase isoforms and that the region of most dissimilarity lies in the amino terminus, a characteristic common to the other three isoforms (Shamraj & Lingrel, 1994). From these sequence analyses, the ATP1AL2 gene was postulated to encode a novel isoform of the Na,K-ATPase α subunit and designated α4. However, the existence of a functional protein encoded for by the α4 isoform gene was not shown. In the work presented here, we demonstrate that the fourth α isoform subunit exists in testis and investigate its biochemical characteristics.

Materials and Methods

Tissue Culture and Cotransfection of NIH 3T3 cells

Establishment of stable NIH 3T3 cell lines expressing rat α4 cDNA was performed as described previously (O'Brien, Lingrel & Wallick, 1994; Kuntzweiler et al., 1995a, Kuntzweiler, Argüello & Lingrel, 1996). Rat α4 cDNA was inserted into the pKC4 mammalian expression vector. Cotransfection of 18 μg of α4/pKC4 expression vector and 1 μg of pSVneomycin expression vector was by the calcium phosphate precipitation method. Cells were selected 48 hr later in 200 μg/ml of G418 (Geneticin, GibcoBRL). Seven different transfections were performed. Each transfection was approximately 16–25% efficient in producing rat α4 isoform expressing, neomycin resistant cells.

Northern Blotting

Total cellular RNA was isolated using the guanidine thiocynanate method (Tri-Reagent, Molecular Research Center). For isolation of total RNA from transfected NIH 3T3 cells, 1 ml of Tri-reagent was applied directly to confluent 100-mm tissue culture dishes. Cells were then harvested by scraping using a sterile plastic cell scraper (Fisher). For isolation of total RNA from mouse testis, tissues were homogenized in 5 ml of Tri-reagent using a Polytron on high speed for 30 sec.

Total RNA samples (10 μg/sample) were denatured in 1 M glyoxal, 54% DMSO and 0.01 M sodium phosphate buffer (pH 6.8), analyzed using a 1% agarose gel in 0.01 M sodium phosphate buffer, and then transferred to nylon membrane (Sure Blot Hybridization Membrane, Oncor). Northern blots were screened using a 338 bp rat α4 specific DNA probe (Shamraj & Lingrel, 1994). Levels of GAPDH were analyzed in order to assess equal loading of total RNA in each lane.

Microsome Preparations

Approximately 2.0 g of rat testes tissue was homogenized in Na^+-free homogenization buffer (250 mM sucrose, 30 mM histidine (pH 7.2), 2 mM Na^+ free EDTA) and then centrifuged at 6,800 × *g* at 4°C for 10 min. The supernatant from this initial centrifugation was saved, the pellet were resuspended in homogenization buffer, rehomogenized and centrifuged as described above. Cells from 30 confluent plates of transfected NIH 3T3 cells or wild-type NIH 3T3 cells were harvested by scraping with a sterile plastic cell harvester (Fisher), combined and washed in 250 mM sucrose. The sucrose was removed by centrifugation at 3,000 × *g*, cells were resuspended in lysis buffer (1 mM $NaHCO_3$, 2 mM $CaCl_2$, 5 mM $MgCl_2$), homogenized using a glass dounce and teflon coated pestle, and then centrifuged at 3,000 × *g* for 2 min. The supernatant from this initial centrifugation was saved, the pellet was resuspended in lysis buffer, re-homogenized and centrifuged as described above. The protocol for the preparation of membranes from both rat testis and 3T3 cells is the same from this point forward. The combined supernatants from these two initial centrifugation steps were centrifuged at 39,200 × *g* for 30 min at 4°C. The pellet from this centrifugation was resuspended in 5 ml of 1 mM imidazole, 1 mM EDTA (pH 7.4), homogenized and then centrifuged at 39,200 × *g* for 30 min at 4°C. The pellet from this last centrifugation was resuspended in 1–2 ml of 1 mM imidazole, 1 mM Na^+-free EDTA, pH 7.4, homogenized and stored at −80°C. The 1 mM imidazole, 1 mM Na^+-free EDTA was brought to pH 7.4 using a solution of concentrated tris. Protein concentrations were determined using the BCA Protein Assay by Pierce.

SDS-PAGE

SDS-PAGE was performed as previously described (Laemmli, 1970). Briefly, 10–20 μg of microsome preparations were incubated for 1 hr at 37°C in 50 mM Tris (pH 6.8), 5% SDS, 1% β-mercaptoethanol (BME), 10% glycerol and bromophenol blue. Samples were electrophoretically separated on a 10% polyacrylamide gel using a running buffer composed of 0.192 M glycine, 0.025 M tris and 0.10 % SDS. The molecular size of separated proteins were determined using BenchMark prestained protein ladder (GibcoBRL) on individual gels.

Polyclonal Antibody Production and Western Blotting

A polyclonal antibody to the rat α4 isoform, designated αb4, was produced in rabbits by Zymed Laboratories, Inc. A nineteen amino acid peptide corresponding to amino acids 16–33 of the deduced rat cDNA sequence (Shamraj & Lingrel, 1994) was used as an antigen for polyclonal antibody production. Serum from injected rabbits were affinity-purified using a SulfoLink Kit column (Pierce) containing a 6% crosslinked agarose support conjugated to the peptide. Preimmune serum was affinity purified using an ImmunoPure® Plus Immobilized Protein A column (Pierce). The concentration of purified αb4 antibody and preimmune IgG was quantified spectrophotometrically at 280 nm.

SDS-polyacrylamide gels were transferred to PVDF membrane (Amersham) at 20 V overnight at 4°C in 25 mM Tris (pH 8.3). Blots were incubated for 1 hr in Blocking Reagent (Boerhinger Mannheim) at room temperature then transferred to a solution of 0.5% Blocking reagent containing either αb4 or α1 isoform-specific monoclonal α6F (University of Iowa Developmental Hybridoma Bank) for 1 hr at room temperature. Blots were washed twice in TBST (50 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween) for 10 min, twice in 0.5% Blocking Reagent for 10 min, and then placed in a solution containing either peroxidase conjugated anti-rabbit or anti-mouse (Calbiochem-Novabiochem), for detection of α4 and α1 antibodies respectively, and 0.5% blocking solution for 1 hr at room temperature. Detection of signal was accomplished using an ECL system (Amersham).

The Measurement of [^{3}H]-Ouabain Binding to Membrane Preparations

All ouabain binding competition assays were performed under these assay conditions: 50 mM tris-HCl (pH 7.4), 5 mM tris-phosphate (pH 7.4), 5 mM $MgCl_2$, and 250 μg protein in a total volume of 0.5 ml (Kuntzweiler et al., 1995*a,b;* Kuntzweiler et al., 1996). The affinity for ouabain was determined using unlabeled ouabain in a self-competition

assay with [^{3}H] ouabain, while cation affinities were examined using varying concentrations of either NaCl or KCl to compete with [^{3}H] ouabain binding, as described previously (Kuntzweiler et al., 1995*a,b;* Kuntzweiler et al., 1996). A minimum of three curves in triplicate were performed to characterize each protein-ligand interaction. Analysis of testis membranes included at least three different sets of membrane preparations, while analysis of α4 isoform expressing 3T3 cell membranes included three different clonal lines. All ouabain binding competition curves shown in this paper are the results from one representative experiment of several assays performed. The results have been normalized using the calculated Bmax and Bmin as 100 and 0% total binding, respectively, as described previously (Kuntzweiler et al., 1996). The errors between triplicate samples within each experimental curve were calculated to be smaller than the symbol size on normalized graphs.

Raw data were fit and plotted using the KaleidaGraph program by Abelbeck Software and the K_D for ouabain binding and IC_{50} values for Na^+ and K^+ competition were determined as described previously (Kuntzweiler et al., 1995*a,b;* Kuntzweiler et al., 1996). K_D values reported in this paper are averages from at least four curves, including various clonal lines and membrane preparations. IC_{50} values for Na^+ and K^+ competition are averages from at least three curves, also including various clonal lines and membrane preparations. Errors associated with each K_D and IC_{50} value are the standard deviations from the mean of all incorporated values.

Results

Generation of rat α4 Isoform cDNA Expressing 3T3 cells

The ability of the rat α4 isoform cDNA to code for a functional protein was examined using a 3T3 cell expression system (Kuntzweiler et al., 1995*a,b;* Kuntzweiler et al., 1996). The rat cDNA was cotransfected along with a vector coding for neomycin resistance into 3T3 cells, a mouse fibroblast cell line that only expresses the α1 isoform. Cells able to survive G418 selection were screened for α4 isoform expression by northern analysis using an α4 isoform specific probe (Shamraj & Lingrel, 1994) (Fig. 1). Multiple clones expressing the α4 isoform mRNA were obtained from each of several transfections performed. The slightly larger size of the rat α4 isoform mRNA compared to the endogenous α4 isoform expressed in mouse testis was expected due to the positioning of the polyadenylation site in the pKC4 expression vector. Positive clones from multiple transfections were chosen for further analysis.

Detection of the α4 Isoform Protein in Rat Testis and in α4 Isoform Expressing 3T3 Cells

The production of the α4 isoform protein in both rat testis and in transfected cell lines was examined by western blot analysis using an α4 isoform-specific polyclonal antibody, αb4. The αb4 polyclonal antibody was designed to recognize a unique peptide sequence based on the rat α4 isoform deduced amino acid sequence. This peptide antigen sequence is derived from the 5′ region of the protein that is the least conserved between the isoforms. Many of the epitopes for other isoform-specific Na,K-ATPase α subunit antibodies are also located in the corresponding isoform's 5′ sequence (Arystarkhova & Sweadner, 1996). Using the αb4 antibody, a protein was detected in microsomal samples of both rat testis and transfected cell lines that was not detected in wild type 3T3 cells (Fig. 2*a*). The binding of this antibody was specific for the α4 isoform since (1) immunoreactivity was not detected in untransfected 3T3 cells (Fig. 2*a*), (2) immunoreactivity could be competed by purified peptide epitope and (3) no specific bands were detected using purified preimmune IgG (*data not shown*). To directly compare the size of the α4 isoform with other α isoforms of the Na,K-ATPase, expression of the ubiquitous α1 isoform was assessed using an α1 isoform-specific monoclonal antibody α6F (Takeyasu et al., 1988). As expected, the α1 isoform was detected in all samples and both the α1 and α4 isoforms appear to have similar molecular sizes of 99 kDa (Fig. 2*b*). The data from these western blots show for the first time expression of a protein in transfected cell lines from the rat α4 isoform cDNA. Furthermore, the protein expressed from the α4 isoform cDNA is identical in size to a protein expressed in rat testis.

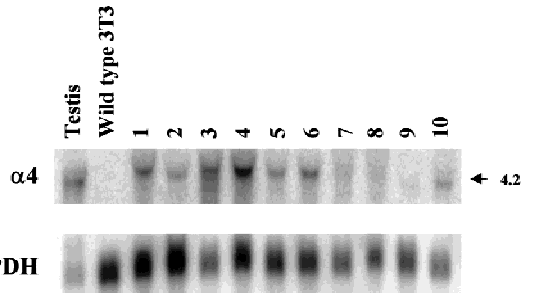

Fig. 1. Stable expression of the α4 isoform cDNA in 3T3 cells. Northern analysis of G418 resistant cells to screen for the expression of rat α4 isoform cDNA using a 338 bp rat α4 isoform specific DNA sequence as a probe. Lane 1, mouse testes; lane 2, wild-type 3T3 cells; lanes 3-12, rat α4 isoform cDNA transfected cells.

[^{3}H] Ouabain Binding Self-Competition Assays Using Rat α4 3T3 Cells and Rat Testes Membrane Preparations

Because the Na,K-ATPase is known to be the molecular receptor for cardiac glycosides, the first biochemical characteristic of this novel protein we examined was its affinity for the cardiac glycoside ouabain. The presence of a high affinity ouabain receptor was examined using [^{3}H] ouabain binding self-competition assays on membrane preparations. This type of competitive binding experiment measures the amount of [^{3}H] ouabain specifically bound to the Na,K-ATPase but can only detect the presence of ouabain receptors whose K_D for ouabain binding is less than 4.5 μM, and will therefore not detect

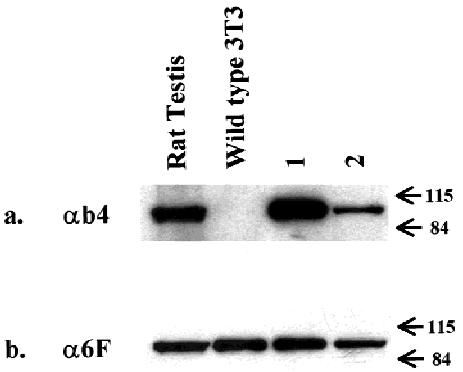

Fig. 2. Protein expression of the α4 isoform in rat testis and in α4 isoform cDNA transfected 3T3 cells. Western analysis of microsomal proteins probed with (*a*) α4 isoform-specific polyclonal antibody αb4, and (*b*) α1 isoform-specific monoclonal antibody α6F (University of Iowa Developmental Hybridoma Bank). Lane 1, rat testes; lane 2, wild-type 3T3 cells; lanes 3-4, rat α4 isoform cDNA transfected cell lines.

the endogenous 3T3 cell α1 isoform whose affinity for ouabain is in the millimolar vicinity (Kuntzweiler et al., 1995*a,b;* Kuntzweiler et al., 1996; Price & Lingrel, 1988). Ouabain binding self-competition assays reveal specific binding of nanomolar quantities of [^{3}H] ouabain to rat testis and to each of the cell lines tested (Fig. 3*a*). No specific binding was observed in assays using wild type 3T3 cells (Fig. 3*a*). Levels of specific ouabain binding were calculated by subtracting the amount of [^{3}H] ouabain binding in the presence of 4.5 μM unlabeled ouabain from the amount of [^{3}H] ouabain binding without unlabeled ouabain present. From this analysis, it is clear that both 3T3 cells expressing the α4 isoform cDNA and rat testis contain a protein that has a high affinity for ouabain binding.

[^{3}H] ouabain binding self-competition assays were next used to calculate the K_D for ouabain binding of the cardiac glycoside receptors identified in rat testis and in α4 isoform expressing 3T3 cells. The well-characterized sheep α1 isoform expressed in 3T3 cells (sheep α1 3T3 cells) was used as a positive control for a high affinity ouabain receptor (K_D = 1.5 nM) (Kuntzweiler et al., 1995*a,b;* Kuntzweiler et al., 1996). Data from these assays reveal that the ouabain receptors in both α4 3T3 cells and in rat testis have very similar K_D values for ouabain binding (312.67 ± 93.73 nM and 316.75 ± 134.66 nM, respectively) (Fig. 3*b*). The finding that both receptors have the same ouabain-affinity strongly supports our hypothesis that the previously cloned α4 isoform cDNA codes for the same enzyme found in rat testis.

Na^+ and K^+ competition curves in rat α4 3T3 Cell Membrane Preparations

Analysis of the ion binding affinities of the α4 isoform was performed using α4 isoform expressing 3T3 cells.

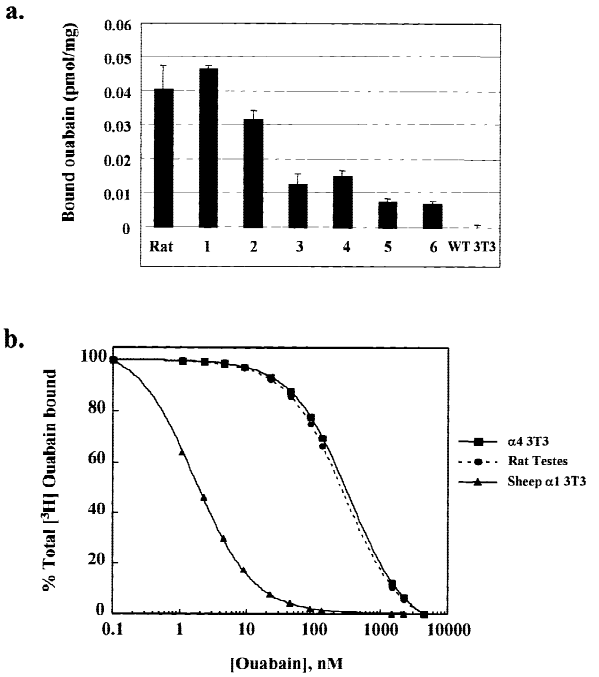

Fig. 3. Ouabain binding self-competition assays. (*a*) Quantitation of the levels of α4 isoform protein expression in membranes of transfected 3T3 cells by calculating the amount of specifically bound [^{3}H] ouabain per mg of protein. Membrane preparations from rat testis, six different α4 isoform transfected 3T3 cell lines and wild-type 3T3 cells were assayed. Error bars represent the difference between at least two different experiments. (*b*) Various concentrations of unlabeled ouabain were used as a competitor for binding to membrane preparations from α4 3T3 cells (--■-), rat testes (--●--) and sheep α1 isoform expressing 3T3 cells (--▲--). Data are fit and plotted as described in Materials and Methods. K_D values for [^{3}H] ouabain binding are as follows: α4 3T3 cells, 312.67 ± 93.73 nM; rat testes membranes, 316.75 ± 134.66 nM; and sheep α1 3T3 cells, 1.87 ± 0.08 nM.

Expression of the α4 isoform in 3T3 cells is not selected for by function but indirectly by neomycin resistance, therefore these cells do not produce the α4 isoform at a level sufficient for measuring Na^+ and K^+ stimulation of enzyme activity. It is necessary to express the highly ouabain-sensitive α4 isoform in a cell line such as 3T3 whose endogenous Na,K-ATPase is relatively ouabain insensitive in order to differentiate the two enzymes, but it is exceedingly difficult to measure the enzyme activity of a ouabain-sensitive isoform in the presence of an insensitive isoform (Kuntzweiler et al., 1995*a,b;* Kuntzweiler et al., 1996). In order to evaluate the α4 isoform's cation affinities, ouabain binding competition assays using varying concentrations of Na^+ or K^+ as competitors for binding were employed (Kuntzweiler et al., 1995*a,b;* Kuntzweiler et al., 1996). This assay provides a very sensitive, indirect measure of the enzyme's ability to bind ions and also examines its ability to change conformation in the presence of different ligands. The principle of this assay is based on the fact that the E_2P

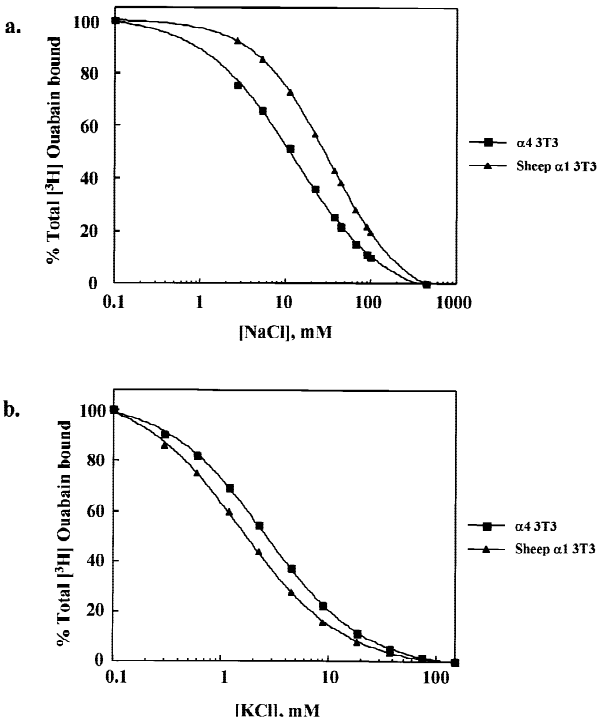

Fig. 4. Na^+ and K^+ competition curves. The amount of [^{3}H] ouabain bound to membrane preparations of α4 isoform expressing 3T3 cells (--■--) and sheep α1 expressing 3T3 cells (--▲--) is plotted against the concentration of (*a*) NaCl and (*b*) KCl present in the reaction. Data are fit and plotted as described in Materials and Methods. $IC_{50(Na)}$ values are as follows: α4 3T3 cells, 9.13 ± 1.81 mM and sheep α1 3T3 cells, 30.50 ± 3.68 mM. $IC_{50(K)}$ values are as follows: α4 3T3 cells, 2.14 ± 1.14 mM and sheep α1 3T3 cells 2.36 ± 0.60 mM.

intermediate of the Na,K-ATPase has the highest affinity for cardiac glycoside binding while the addition of either NaCl or KCl to the reaction changes this high affinity intermediate to either the E_1Na or E_2K forms, both of which have lower affinities for ouabain binding (Lingrel & Kuntzweiler, 1994). The ability for the enzyme to both bind cations and alter its conformation can be measured by looking at the amount of displaced [^{3}H] ouabain. Membrane preparations made from sheep α1 expressing 3T3 cells were used as a positive control in these assays.

Na^+ and K^+ competition for [^{3}H] ouabain binding are highly effective using α4 isoform expressing 3T3 cell membrane preparations (Fig. 4*a* and *b*). The calculated IC_{50} value for Na^+ inhibition of [^{3}H] ouabain binding in α4 3T3 cells was 9.13 ± 1.81 mM (Fig. 4*a*), while that for the K^+ inhibition of ouabain binding was 2.14 ± 1.14 mM (Fig. 4*b*). These results definitively demonstrate the ability of this high affinity ouabain receptor to bind both Na^+ and K^+ and to subsequently alter its conformation to that of a lower ouabain binding affinity intermediate.

Discussion

The information presented in this paper provides conclusive evidence that the previously cloned α4 isoform cDNA codes for a novel isoform of the Na,K-ATPase. Western analyses first demonstrate the presence of a unique 99 kDa protein, recognized by the α4 isoform-specific antibody αb4, in both rat testis and 3T3 cells expressing the rat α4 isoform cDNA. Biochemical studies involving ouabain binding competition assays next show that this protein exhibits three hallmark characteristics of an α subunit of the Na,K-ATPase: (i) a high affinity for binding cardiac glycosides such as ouabain, (ii) the ability to bind Na^+ and subsequently alter its conformation, and (iii) the ability to bind K^+ and subsequently alter its conformation.

Western analyses have shown that the α4 isoform is similar in size to other previously identified Na,K-ATPase α isoforms. Monoclonal antibodies that specifically recognize the α1, α2 or α3 isoforms of the Na,K-ATPase have detected proteins of approximately 92 kDa, corresponding to the α1 isoform, and 94 kDa, corresponding to the α2 and α3 isoforms, matching well with the 99 kDa protein we have described here (Sweadner & Gilkeson, 1985; Urayama, Shutt & Sweadner, 1989; Arystarkhova & Sweadner, 1996). In addition, the α1 isoform-specific antibody α6F (University of Iowa Developmental Hybridoma Bank) reacts with a band of the same molecular size as that identified by αb4 in all of the cells tested here including untransfected 3T3 cells (Fig. 1b). The α4 isoform therefore displays a different expression pattern from that of the α1 isoform, but encodes a protein of very similar molecular size.

Ouabain binding competition assays revealed that this testis specific α isoform of the Na,K-ATPase has a high affinity for the cardiac glycoside ouabain. The ouabain binding affinity of the α4 isoform is slightly lower compared to that of other previously described cardiac glycoside sensitive rat α isoforms. For example, the rat α2 and rat α3 isoforms have K_D values for ouabain binding of 115 nM and 1.6 nM, respectively (O'Brien et al., 1994) while the rat α4 isoform described here has a K_D of 315 nM. The α4 isoform therefore has an affinity for ouabain similar to but distinct from the rat α2 isoform, while the rat α3 isoform's ouabain affinity more resembles that of the sheep α1 enzyme (1.5 nM) (Kuntzweiler et al., 1995*a,b;* Kuntzweiler et al., 1996). In contrast, the rat α1 isoform is typically described as having relatively low affinity for cardiac glycoside binding, with an IC_{50} value in the millimolar range (Price & Lingrel, 1988). Therefore, these experiments have determined that the rat α4 isoform cDNA produces a high affinity cardiac glycoside receptor that is also present in rat testis.

The presence of Na^+ and K^+ stimulated ATPase activity associated with the α4 isoform could not be mea-

sured in the 3T3 cell expression system employed here. This expression system is ideal for the initial characterization of the α4 isoform because it allows an isoform with a high affinity for ouabain to be produced and analyzed in a murine cell system without interference from that cell's endogenous, low affinity Na,K-ATPase (Kuntzweiler et al., 1995*a,b;* Kuntzweiler et al., 1996). However, these cells only express low levels of the α4 isoform presumably because the cells are selected for neomycin resistance and not for the function of the α4 isoform. This is also the case for the other α isoforms of the Na,K-ATPase (Kuntzweiler et al., 1995*a,b;* Kuntzweiler et al., 1996). The expression levels do allow cation affinities to be studied indirectly using ouabain binding competition assays. As a result of these ouabain binding competition studies, the α4 isoform has been determined to be a high affinity ouabain receptor that interacts with both Na^+ and K^+ and therefore exhibits three hallmark characteristics of a Na,K-ATPase. The cation affinities reported here for the α4 isoform correlate well with previously reported values for other α isoforms of the Na,K-ATPase (Jewell & Lingrel, 1991; Munzer et al., 1994; O'Brien et al., 1994). It is therefore highly unlikely that the α4 isoform is a member of another P-type ATPase family. The direct measurement of Na^+ and K^+ stimulated ATPase activity associated with the α4 isoform is currently being pursued by expressing this isoform in a system that does not have endogenous Na,K-ATPase activity.

In this paper we have demonstrated that the previously cloned putative fourth Na,K-ATPase isoform codes for a protein with characteristics similar to yet distinct from those of other previously described α isoforms, characterizing it as a novel isoform of this enzyme. It is also unique in its tissue-specific expression pattern and has so far only been detected in testis. Further investigation of the α4 isoform is under way in order to better understand both its biochemical characteristics and its functional role in testis.

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