Functional Characterization of a Testes-Specific α-Subunit Isoform of the Sodium/Potassium Adenosinetriphosphatase†

Gustavo Blanco,* Roger J. Melton, Gladis Sánchez, and Robert W. Mercer

Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

Received May 26, 1999; Revised Manuscript Received July 23, 1999

ABSTRACT: Different isoforms of the sodium/potassium adenosinetriphosphatase (Na,K-ATPase) α and β subunits have been identified in mammals. The association of the various α and β polypeptides results in distinct Na,K-ATPase isozymes with unique enzymatic properties. We studied the function of the Na,K-ATPase α4 isoform in *Sf*-9 cells using recombinant baculoviruses. When α4 and the Na pump β1 subunit are coexpressed in the cells, Na,K-ATPase activity is induced. This activity is reflected by a ouabain-sensitive hydrolysis of ATP, by a Na^+-dependent, K^+-sensitive, and ouabain-inhibitable phosphorylation from ATP, and by the ouabain-inhibitable transport of K^+. Furthermore, the activity of α4 is inhibited by the P-type ATPase blocker vanadate but not by compounds that inhibit the sarcoplasmic reticulum Ca-ATPase or the gastric H,K-ATPase. The Na,K-ATPase α4 isoform is specifically expressed in the testis of the rat. The gonad also expresses the β1 and β3 subunits. In insect cells, the α4 polypeptide is able to form active complexes with either of these subunits. Characterization of the enzymatic properties of the α4β1 and α4β3 isozymes indicates that both Na,K-ATPases have similar kinetics to Na^+, K^+, ATP, and ouabain. The enzymatic properties of α4β1 and α4β3 are, however, distinct from the other Na pump isozymes. A Na,K-ATPase activity with similar properties as the α4-containing enzymes was found in rat testis. This Na,K-ATPase activity represents approximately 55% of the total enzyme of the gonad. These results show that the α4 polypeptide is a functional isoform of the Na,K-ATPase both in vitro and in the native tissue.

The Na,K-ATPase or Na pump includes a family of isozymes, all of which hydrolyze ATP in a ouabain-sensitive and a Na^+- and K^+-dependent fashion (*1*−*4*). Structurally, the Na,K-ATPase isozymes consist of distinct forms of two major polypeptides, the α and the β subunits (*5, 6*). Three structural variants of the α (α1, α2, and α3) and β (β1, β2, and β3) subunits have been identified in vertebrates (*7*−*13*). Depending on the cell type, developmental stage, hormonal stimulation, and pathological state of the tissue, all Na,K-ATPase α and β polypeptides are expressed in a highly regulated manner (reviewed in refs *1*, *4*, *14*, and *15*). The particular expression and association of the different α and β isoforms in the cell results in Na,K-ATPase isozymes with unique kinetic properties (*16*−*22*). This difference in function suggests that rather than being redundant, the Na pump isozymes have specific roles. In 1987, evidence for the existence of an additional α subunit isoform was obtained. Shull and Lingrel (*23*) isolated a human gene, termed ATPIAL2, that exhibited sequence similarity with exons 3−6 of the Na,K-ATPase α subunit genes. However, it was not until recently that the cDNA representing this gene was isolated (*24*). This cDNA, designated α4, codes for a polypeptide consisting of 1028 amino acids with an identity of 76−78% to the other α isoforms of the Na pump. Interestingly, in rats, the α4 mRNA is selectively expressed in the testis (*24*). Because cells in the testes express the Na,K-ATPase α1 subunit and two different isoforms of the β polypeptide, β1 and β3 (*12, 25*), the possibility exists for α4 to form functionally active complexes with multiple β subunits. Recently, α4 polypeptides have been identified in the rat testes and have been expressed in mammalian cells (*26*). These studies indicate that the α4 isoform has properties consistent with that of a Na,K-ATPase catalytic subunit. However, whether the subunit can function as an ATPase, transport ions, or assemble with multiple β-subunit isoforms has not been determined.

In the past, we have studied the role of the Na,K-ATPase isoforms, using the baculovirus expression system (*17*−*19*). This system employs the *Autographica californica* virus to express foreign genes in *Sf*-9 insect cells, a cell line derived from the ovary of the fall armyworm, *Spodoptera frugiperda* (*27*). *Sf*-9 cells are able to produce the Na,K-ATPase α and β polypeptides and assemble them into active enzyme molecules. Moreover, *Sf*-9 cells contain very low levels of endogenous Na,K-ATPase activity, allowing the analysis of the expressed enzymes in an environment relatively free from contaminating Na,K-ATPase activity (*18, 19*). In this report, we study the function of the α4 polypeptide expressed in *Sf*-9 cells, in combination with the β1 and β3 isoforms. We demonstrate that both α4β1 and α4β3 are catalytically active isoforms of the Na,K-ATPase and characterize their enzymatic properties to Na^+, K^+, ATP, and ouabain.

† This work was supported by National Institutes of Health Grants GM 39746 and DK 45181 and by American Heart Association Grant 96012080.

* To whom correspondence should be addressed: Department of Cell Biology and Physiology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. Phone (314) 362-6922; Fax (314) 362-7463; e-mail gblanco@cellbio.wustl.edu.

10.1021/bi991207b CCC: $18.00
Published on Web 09/23/1999

MATERIALS AND METHODS

DNA and Viral Constructions. The cDNA corresponding to the rat Na,K-ATPase α4 was obtained from a λgt11 testis library purchased from Clontech (Palo Alto, CA). Oligonucleotides were designed from the published sequence and polymerase chain reaction (PCR)[1] was used to obtain five overlapping fragments. These fragments were then combined by overlap extension PCR and sequenced to confirm the integrity of the clone. The sequence obtained was in agreement with that published by Shamraj and Lingrel (*24*) except for a single nucleotide difference that resulted in a change in asparagine 26 to serine. The α4 cDNA was subcloned into the baculovirus expression vector pBluebac (Invitrogen Corp., Carlsbad, CA). The β3 cDNA was generously provided by R. Levenson (Penn State College of Medicine, Hershey, PA). Recombinant baculovirus preparation and selection were performed following the procedures recommended by the manufacturer. For α1, α2, α3, and β1 expression in insect cells, the recombinant viruses, prepared previously, were used (*18*, *19*).

Cells and Viral Infections. Sf-9 cells were grown in TNM−FH medium (defined in ref *27*; JRH Biosciences, Lenexa, KS), supplemented with 10% (v/v) fetal bovine serum, penicillin (100 units/mL), streptomycin (100 mg/mL), and Fungizone (0.25 mg/mL; complete medium). Infections of the cells were performed in serum-free medium for 1 h. After addition of complete medium, cultures were maintained for 72 h at 27 °C. For the determination of enzymatic activity, the cells in 150 mm Petri dishes were scraped from the plates, centrifuged at 1500*g* for 10 min, and washed twice in 10 mM imidazole hydrochloride (pH 7.5) and 1 mM EGTA. Cells were resuspended in the same solution and used for the assays. Before the determination of Na,K-ATPase activity, the intact cells were permeabilized with the ionophore alamethicin as described (*18*).

Antibodies. For the α4 isoform, a specific antiserum was raised at Quality Controlled Biochemicals Inc. (Hopkinton, MA). The antiserum was directed against a peptide (SEQKPRPTLRASNTNRQPK) corresponding to a sequence at the N-terminus of the α4 polypeptide. The purity of the peptide was determined by standard reverse-phase HPLC. The peptide was conjugated to the carrier keyhole limpet hemocyanin through a terminal cysteine and used to immunogenize rabbits. The effectiveness of immunization was tested with an enzyme-linked immunosorbent assay (ELISA), with bovine serum albumin-coupled peptide on the solid phase. The titer of the antiserum was approximately 30 000 and it specifically recognized the α4 polypeptide in immunoblots and immunocytochemistry at dilutions of 1:500 and 1:100, respectively. The α1 subunit was identified by use of an anti-αβ antiserum prepared against purified rat kidney Na,K-ATPase (poly αA) (*28*). For the α2 isoform, a monoclonal antibody (McB2) provided by Dr. Kathleen Sweadner (Massachusetts General Hospital) was used. The α3 isoform was detected by use of an antiserum made against a synthetic peptide derived from the N-terminal region of the α3 subunit (*29*).

Membrane Fraction Preparation. Adult rat testes were dissected and membrane fractions were prepared. For this, the tissue was homogenized on ice in 250 mM sucrose, 25 mM imidazole (pH 7.4), and 0.1 mM EGTA in a glass−glass homogenizer. The lysate was centrifuged at 1000*g* for 10 min. The pellet was resuspended, homogenized again, and recentrifuged at 1000*g* for 10 min. Both the supernatants of the first and second centrifugations were combined and centrifuged for an additional 30 min at 30 000*g*. The final pellet was resuspended in the original homogenization buffer. Na,K-ATPase activity in the testis membranes was approximately 3 μmol of P_i released (mg of protein)$^{-1}$ min^{-1} and comprised 7% of the total ATPase of the preparation. The same method was followed for the preparation of membranes from the insect cells.

PAGE and Immunoblot Analysis. Proteins were analyzed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and immunoblotting. After separation by SDS−PAGE (*30*), proteins were transferred to nitrocellulose (Hybond C+, Amersham Corp.) and immunoblotted as described previously (*17*) with an ^{125}I-labeled antiserum as the secondary antibody.

Immunocytochemistry and Confocal Microscopy. Sf-9 cells were plated in 24-well culture plates on 11 mm glass cover slips and infected with the α4 recombinant baculovirus. Forth-eight hours after infection, cells were treated with 100 μg/mL cycloheximide, an inhibitor of protein synthesis. This treatment clears the biosynthetic pathway of newly synthesized proteins, allowing the detection of the expressed polypeptide at their final cellular destination. Samples were then processed for immunocytochemistry as described (*28*), with the α4 antiserum as the primary antibody and a fluorescein isothiocyanate- (FITC-) conjugated goat anti-rabbit secondary antibody.

Biochemical Assays. Protein assays were performed with bicinchoninic acid/copper sulfate solution as described by the supplier (Pierce Chemical Co, Rockford, IL) after lysis of the cells in 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). Na,K-ATPase activity was assayed through determination of the initial rate of release of $^{32}P_i$ from [γ-^{32}P]ATP as described previously (*18*). The assay was performed with 10 μg of total membrane protein for testis and 20−50 μg for *Sf*-9 cells. Na,K-ATPase activity was measured in a final volume of 0.25 mL of medium containing 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, and 30 mM Tris-HCl (pH 7.4) ± 1 mM ouabain. Sodium azide (2.5 mM final concentration) was included in the mixture to inhibit mitochondrial ATPase. The assay was started by the addition of ATP with 0.2 μCi of [γ-^{32}P]ATP (3 mM final concentration). Following a 30 min incubation at 37 °C, the tubes were placed on ice and the reaction terminated by the addition of 25 μL of 55% trichloroacetic acid (TCA). Released $^{32}P_i-P_i$ was converted to phosphomolybdate and extracted with isobutyl alcohol. Radioactivity of 170 μL of the organic phase was measured by liquid scintillation counting. The ATP hydrolyzed never exceeded 15% of the total ATP present in the sample and hydrolysis was linear over the incubation time. For the analysis of activation by Na^+ and K^+, incubation media were the same as above except that for Na^+ dependency, the Na^+ concentration was varied from 2.5 to 122.5 mM. For K^+ stimulation,

[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; FITC, fluorescein isothiocyanate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; Sch-28080, 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)-imidazo-[1,2a]pyridine; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

the K^+ concentration was varied from 0 to 50 mM. Choline chloride was added so that the final concentration of Na^+ or K^+ plus choline totaled 150 mM. The ATP dependency was determined under saturating concentrations of all cations (120 mM Na^+, 30 mM K^+, and 3 mM Mg^{2+}). To determine the effect of different concentrations of ouabain on Na,K-ATPase activity, samples were preincubated with the indicated concentrations of ouabain for 20 min at 37 °C in the reaction medium and the reaction was started by the addition of ATP. Specific Na,K-ATPase activity was determined as the difference in ATP hydrolysis in the absence or presence of 1 mM ouabain for α1 or 3 μM for α4. In some experiments, certain ATPase inhibitors were used. Vanadate was added to inhibit P-type ATPases. Ca-ATPase activity was blocked with thapsigargin (Sigma Chemical Co., St. Louis, MO). To inhibit H,K-ATPase, 3-(cyanomethyl)-2-methyl-8-(phenylmethoxy)-imidazo[1,2*a*]pyridine (Sch-28080; provided by Astra-Hassle) was used.

For the phosphorylation of the Na,K-ATPase, the incorporation of P_i from [γ-^{32}P]ATP was performed on membrane fractions from uninfected and α4β1 infected insect cells. Phosphorylation was carried out at 0 °C in 0.5 mL of a medium containing 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EGTA, and 50 mM imidazole, pH 7.4, with or without 50 mM KCl or 1 mM ouabain as indicated. Membranes (250 μg of protein/tube) were preincubated in the indicated medium for 10 min at 37 °C to ensure binding of ouabain. Samples were transferred to 0 °C and allowed to equilibrate. The reaction was started by the addition of ATP−[γ-^{32}P]ATP to a final concentration of 10 μM. The reaction was stopped 30 s later with 1 mL of an ice-cold solution of 25% TCA, 10 mM unlabeled P_i, and 1 mM unlabeled ATP. Samples were filtered through glass fiber filters under vacuum and washed with 20 mL of ice-cold 5% TCA containing 10 mM P_i. Filters were transferred to scintillation vials and radioactivity was measured.

For the uptake of ^{86}Rb, 72 h infected cells in 60 mm plates were preincubated at room temperature in a medium containing 150 mM NaCl, 25 mM Hepes (pH 7.4), 2.5 mM $MgCl_2$, and 1% BSA. After 30 min, the medium was replaced with identical medium containing 0.1 mM bumetanide. After 10 min, the medium was aspirated and ^{86}Rb flux was started by adding 1.5 mL of 5 mM KCl, 150 mM NaCl, 25 mM Hepes (pH 7.4), 2.5 mM $MgCl_2$, 1% BSA, 0.1 mM bumetanide, and ^{86}Rb (3 mCi/mL; New England Nuclear, Boston, MA). At prescribed intervals, the medium was aspirated and the cells were washed three times with 3 mL of ice-cold 116 mM $MgCl_2$. After removal of final wash, the plates were allowed to air-dry. The cells were solubilized with 1.5 mL of 1% CHAPS and aliquots of 0.6 mL were counted by liquid scintillation.

Data Analysis. Curve-fitting of the experimental data was performed with a Marquardt least-squares nonlinear regression computing program (Sigma Plot, Jandel Scientific, San Rafael, CA), using the equations described before (*18*). Na^+ and K^+ activation curves were fitted according to a cooperative model for ligand binding. The Michaelis−Menten equation was used for the ATP dependency of Na,K-ATPase. For the ouabain inhibition of Na,K-ATPase activity, curve-fitting was performed with the assumption of one or two populations of Na,K-ATPase isozymes with different affinities for ouabain. Statistical comparison of the concentration−response curves of each isozyme was done by application of an *F* test as previously reported (*19*).

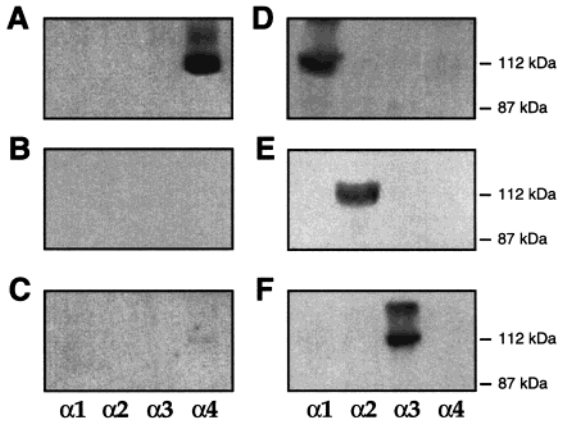

FIGURE 1: Detection of rat α4 polypeptide expressed in infected *Sf*-9 cells. Recombinant baculoviruses containing the cDNAs of the rat Na,K-ATPase α1, α2, and α3 isoforms and of the highly homologous α4 cDNA were used to infect insect cells. After 72 h, *Sf*-9 proteins (20 μg) were separated by SDS−PAGE and transferred to nitrocellulose. (A) Immunoblot analysis of the α4 polypeptide with an antiserum prepared against a synthetic peptide at the N-terminus of α4. (B) Rabbit preimmune serum used as a control. (C) Immunoblot with the anti-α4 antiserum previously bound to the synthetic peptide against which it was prepared. (D) Expression of the α1 isoform detected with an anti-αβ antiserum prepared against purified rat kidney Na,K-ATPase (poly αA). (E) Detection of the α2 isoform with a monoclonal antibody (McB2). (F) α3 expression as detected by an antiserum made against a synthetic peptide derived from the N-terminus of the α3 subunit (α3 synth pep). In all cases, an ^{125}I-labeled antibody was used as the secondary.

RESULTS

To explore the function of the Na,K-ATPase α-like subunit encoded by the α4 cDNA, the polypeptide was expressed in insect cells. For this, a recombinant baculovirus containing the α4 cDNA was prepared and used to infect *Sf*-9 insect cells. To confirm the expression of the virally induced polypeptide, proteins from infected cells were separated by SDS−PAGE and immunoblotted. To detect the α4 polypeptide, an antiserum was generated in rabbit to a sequence (SEQKPRPTLRASNTNRQPK) at the N-terminus of the protein that is not shared with the Na,K-ATPase α isoforms. The efficacy of the antiserum is shown in Figure 1. On immunoblots, the anti-α4 antiserum detects a protein of the proper size in cells infected with the α4 baculovirus (Figure 1A). In contrast, if the preimmune rabbit serum is used as the primary antibody (Figure 1B), no reactivity is seen. In addition, preincubation of the antiserum with the peptide against which it was made completely prevented binding to the α4 polypeptide (Figure 1C). The specificity of the anti-α4 antiserum is demonstrated by the lack of cross-reactivity to the other Na,K-ATPase α isoforms expressed in the *Sf*-9 cells (Figure 1D−F). Altogether, these results indicate that the insect cells are able to express the α4 isoform and that the anti-α4 antiserum specifically recognizes the polypeptide in those cells. To determine the cellular distribution of the α4 polypeptide, infected cells were analyzed by immunocytochemistry and confocal microscopy. For this, uninfected and α4 infected cells grown for 48 h were treated with cycloheximide, fixed, and probed with the anti-α4 antiserum. The α4 antibody was identified by use of an FITC-conjugated

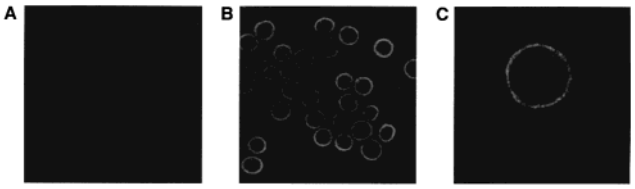

FIGURE 2: Localization of the α4 polypeptide in infected *Sf*-9 cells. Uninfected cells (A) and cells infected with α4 (B, C) were grown on coverslips. Forty-eight hours later, cells were preincubated in 100 μg/mL cycloheximide for 1 h and fixed as described. The α4 polypeptide was identified by use of a specific rabbit antiserum, followed by a fluorescein-conjugated anti-rabbit secondary antibody. The distribution of α4 was determined by confocal microscopy. In panel C, a closeup of a single cell expressing the α4 polypeptide is shown.

goat anti-rabbit secondary antibody. As shown in Figure 2, the antiserum only recognizes the Na,K-ATPase isoform in the baculovirus-infected cells. Also, as occurs with the other Na pump isoforms, α4 is localized to the plasma membrane of the cells. Thus, the insect cells are able to properly synthesize and deliver the α4 polypeptide to the surface of the cells.

The high homology of the α4 polypeptide with the other Na,K-ATPase α isoforms (*24*), and its ability to bind ouabain (*26*) suggest that the α4 polypeptide might function as a catalytic subunit of the enzyme. To test whether α4 displays the catalytic characteristics of a Na,K-ATPase, insect cells were infected with the α4 virus alone or in combination with a virus coding for the Na,K-ATPase β1 subunit. After 72 h, Na,K-ATPase activity was determined. In cells expressing the α4β1 polypeptides the Na,K-ATPase activity varied among samples and was approximately 3−6-fold higher than that of uninfected or α4-infected cells (data not shown). In addition, the ouabain-sensitive ATPase activity of α4β1 was greatly reduced when the concentration of Na^+ was lowered or when K^+ or Mg^{2+} were absent (Figure 3A). These results indicate that α4 is indeed a catalytically competent Na,K-ATPase subunit and that, as occurs with the other α isoforms, the presence of the β subunit is required for the normal activity of the enzyme (*31*−*33*).

During the reaction cycle, the α subunit of the Na,K-ATPase is phosphorylated by ATP at an aspartate at position 371. This residue is located in a highly conserved region between transmembrane domains 4 and 5 that is present in all Na pump α isoforms including the α4 polypeptide (*5*, *24*). Phosphorylation by ATP requires both Mg^{2+} and Na^+, while K^+ promotes dephosphorylation and release of the phosphate from the enzyme (reviewed in refs *2* and *34*). As shown in Figure 3B, membrane fractions from *Sf*-9 cells expressing α4β1 are able to incorporate phosphate from ATP in a ouabain-sensitive manner. Ouabain inhibits phosphorylation when it is allowed to bind to the enzyme before exposure to ATP. In addition, incorporation of P_i from ATP is facilitated by Na^+ and is sensitive to K^+. In the uninfected cells, phosphorylation by ATP can also be detected, however, at a level much lower than that of the α4β1 infected cells. This reflects the small amount of endogenous Na,K-ATPase present in the *Sf*-9 cells. These results demonstrate that the α4β1 isozyme is phosphorylated in a Na^+-dependent fashion and dephosphorylates in the presence of K^+, supporting the notion that α4 is a catalytic isoform of the Na pump.

To further investigate whether the α4β1 enzyme displays the ion-translocating characteristics of a Na,K-ATPase, we analyzed its ability to transport K^+. For this, we measured ouabain-sensitive K^+ uptake in infected *Sf*-9, cells using ^{86}-Rb^+ as a tracer for K^+ (*28*). As a control, cells expressing an unrelated transmembrane protein, the murine integrin-associated protein (IAP) were used. IAP is a 50 kDa multispanning membrane protein that copurifies with integrin $\alpha_v\beta_3$ and has been implicated in the regulation of ligand binding by the integrin receptor (*35*). As shown in Figure 3C, cells expressing the α4 and β1 polypeptides transport K^+ at a rate that is approximately 5-fold higher than the control cells (2.1 ± 0.2 nmol of K^+ (mg of protein)$^{-1}$ min^{-1} compared to 0.4 ± 0.2 nmol of K^+ (mg of protein)$^{-1}$ min^{-1}). Thus, as expected for a Na,K-ATPase, the α4 polypeptide exhibits an ATPase and K^+ transport activity that is sensitive to ouabain.

To further characterize the catalytic properties of α4, the ATPase activity of α4β1 produced in insect cells was assayed under various ionic conditions and with different inhibitors (Figure 3D,E). As expected for a P-type ATPase, the ouabain-sensitive α4 ATPase activity is inhibited by vanadate (*2*). In contrast, the activity of α4 is unaffected by thapsigargin or Sch-28080, compounds that are specific inhibitors of the sarcoplasmic reticulum Ca-ATPase (*36*) and gastric type H,K-ATPase (*37*, *38*) respectively (Figure 3D). To study the enzymatic requirement for H^+, the activity of α4 was determined at various pH values. As shown in Figure 3E, the hydrolysis of ATP by α4 is optimal at pH 7.2−7.4. In addition, even at low pH, the activity of α4 is dependent upon Na^+ (data not shown). The fact that the ability of α4 to hydrolyze ATP is highly dependent on Na^+ and is impaired by increasing concentrations of hydrogen precludes α4 being an H-ATPase. Also, the effect of several divalent cations on the function of α4 was evaluated. For this, we determined Na,K-ATPase activity of α4β1 in medium containing nonlimiting concentrations of Na^+, K^+, and Mg^{2+} with the addition of 1 mM Ca^{2+}, Cu^{2+}, Fe^{2+}, or Zn^{2+}. The ATPase activity of α4 was not activated but inhibited by all these divalent ions (data not shown), suggesting that they are not substrates of the enzyme. In summary, these results confirm that the α4 polypeptide functions as a catalytic subunit of the Na,K-ATPase.

The α4 polypeptide has been shown to be specifically expressed in the testis (*24*). The gonad also expresses the β1 and β3 isoforms of the Na,K-ATPase (*12*, *25*). Association among different isoforms of the α and β subunits of the Na pump has been reported (reviewed in ref *4*). Thus, the possibility exists that two structurally different isozymes, α4β1 and α4β3, are present in the testes. Coinfection of *Sf*-9 cells with viruses containing the cDNAs coding for the α4 and β3 isoforms resulted in the expression of Na,K-ATPase activity, indicating that α4β3 is also a catalytically competent isozyme of the Na,K-ATPase. To determine the affinity of the α4β1 and α4β3 isozymes to physiological ligands, activation curves of Na,K-ATPase activity by Na^+, K^+, and ATP were performed. The Na^+ dependency of Na,K-ATPase activity was determined at varying concentrations of Na^+ (2.5−122.5 mM) and constant saturating K^+ (30 mM). The obtained activation curves are shown in Figure 4A. The calculated values for the apparent affinities for Na^+ for α4β1

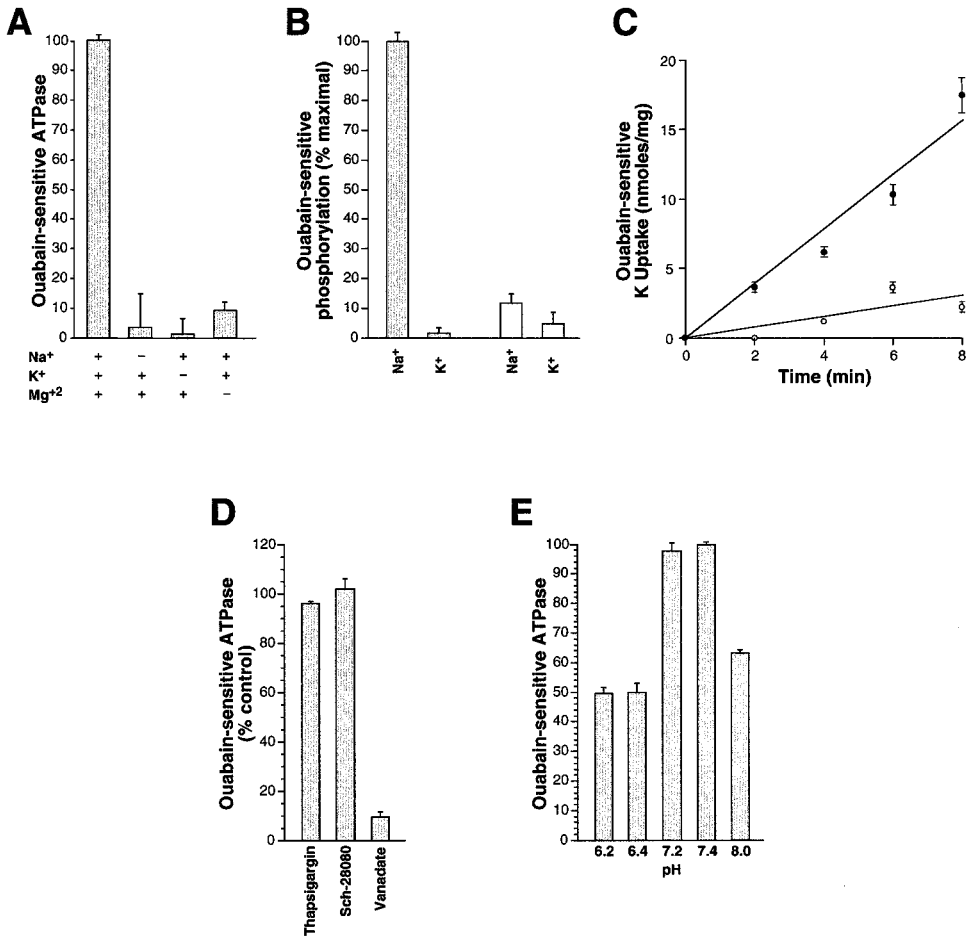

FIGURE 3: Functional activity and enzymatic properties of α4β1 expressed in insect cells. (A) Ouabain-sensitive hydrolysis of ATP in *Sf*-9 cells expressing α4β1. Na,K-ATPase activity of insect cells infected for 72 h with the α4 and β1 viruses was determined as described under Materials and Methods. The hydrolysis of ATP was measured in the presence of 120 mM NaCl, 30 mM KCl, and 3 mM $MgCl_2$; at 2.5 mM NaCl (−Na); or in medium in which the KCl or $MgCl_2$ was absent. The average V_{max} value for α4β1 was 0.6 ± 0.1 μmol of P_i mg^{-1} h^{-1}. Values are expressed as the percentage of the maximal ouabain-sensitive activity, obtained in the presence of all ions. (B) Ouabain-sensitive phosphorylation of membranes from uninfected (open bars) or α4β1 infected (solid bars) cells from ATP. A total of 250 μg of protein was phosphorylated with [γ-^{32}P]ATP. Phosphorylation was performed at 0 °C in 0.5 mL of a medium containing 1 mM $MgCl_2$, 0.1 mM EGTA, and 50 mM imidazole, pH 7.4 in the absence and presence of 1 mM ouabain and with or without 50 mM NaCl or KCl as indicated. Results are expressed as a percentage of the maximal ouabain-sensitive phosphorylation obtained in NaCl-containing medium. (C) Ouabain-sensitive K^+ uptake in *Sf*-9 cells expressing α4β1. K^+ uptake (with $^{86}Rb^+$ as a tracer for K^+) was measured in cells infected with viruses coding for α4β1 (●) or IAP (○). K^+ uptake was determined at the indicated time points in a medium containing 5 mM KCl, 150 mM NaCl, 25 mM Hepes (pH 7.4), 2.5 mM $MgCl_2$, 1% BSA, 0.1 mM bumetanide, and $^{86}Rb^+$. (D) Effect of inhibitors on α4β1 ATPase. Activity was measured in 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, and 30 mM Tris-HCl (pH 7.4) in the absence or presence of 30 μM thapsigargin, 100 μM Sch-28080, or 1 mM vanadate. Results are expressed as a percentage of the activity obtained in the absence of the inhibitors. (E) Effect of pH on α4β1 activity. Na,K-ATPase was determined in medium with saturating concentration of ligands, at the indicated pH. Results are expressed as a percentage of the activity obtained at pH 7.4. In all cases, results represent the mean and error bars the standard errors of the mean of two or three experiments performed in quadruplicate.

and α4β3 (13.5 ± 1.3 mM and 12.9 ± 0.6 mM, respectively) are within the range previously described for the other Na,K-ATPase isozymes (reviewed in ref *4*). To determine the requirement for K^+, Na,K-ATPase activity was measured at varying concentrations of K^+ (0−50 mM) with Na^+ fixed at 100 mM. The data obtained are presented in Figure 4B. The calculated $K_{0.5}$ values for K^+ of 5.9 ± 1.1 mM for α4β1 and 5.0 ± 0.3 mM for α4β3 are among the highest when compared to the other Na pump isozymes. To characterize the kinetics of the low-affinity ATP site, Na,K-ATPase activity was determined at various millimolar concentrations of ATP. The ATP requirements of the α4β1 and α4β3 isozymes are shown in Figure 4C. The calculated affinities of 0.19 ± 0.04 mM and 0.18 ± 0.04 mM, respectively, are intermediate between the K_m values for ATP of the Na,K-ATPase α2/α3 and the α1 isoforms. No statistical difference was found when the kinetics of α4β1 and α4β3 toward the various activators tested were compared. A summary of the enzymatic characteristics of the α4β1 and α4β3 isozymes as well as those of the other Na,K-ATPase α isoforms in association with the β1 subunit are shown in Table 1.

An important enzymatic property that differs among Na,K-ATPase isozymes is their sensitivity to inhibition by cardiotonic steroids (reviewed in ref *4*). To determine the sensitivity of the α4 isoform, we characterized the ouabain inhibition profiles of the Na,K-ATPase activity of the α4β1 and α4β3 isozymes. As shown in Figure 4D, both preparations yielded monophasic dose−response curves, indicating the presence of a single enzymatic species. As described previously for other Na,K-ATPase isozymes, the β subunit present did not significantly alter the ouabain sensitivity of the enzyme. Both α4β1 and α4β3 isozymes exhibited a high

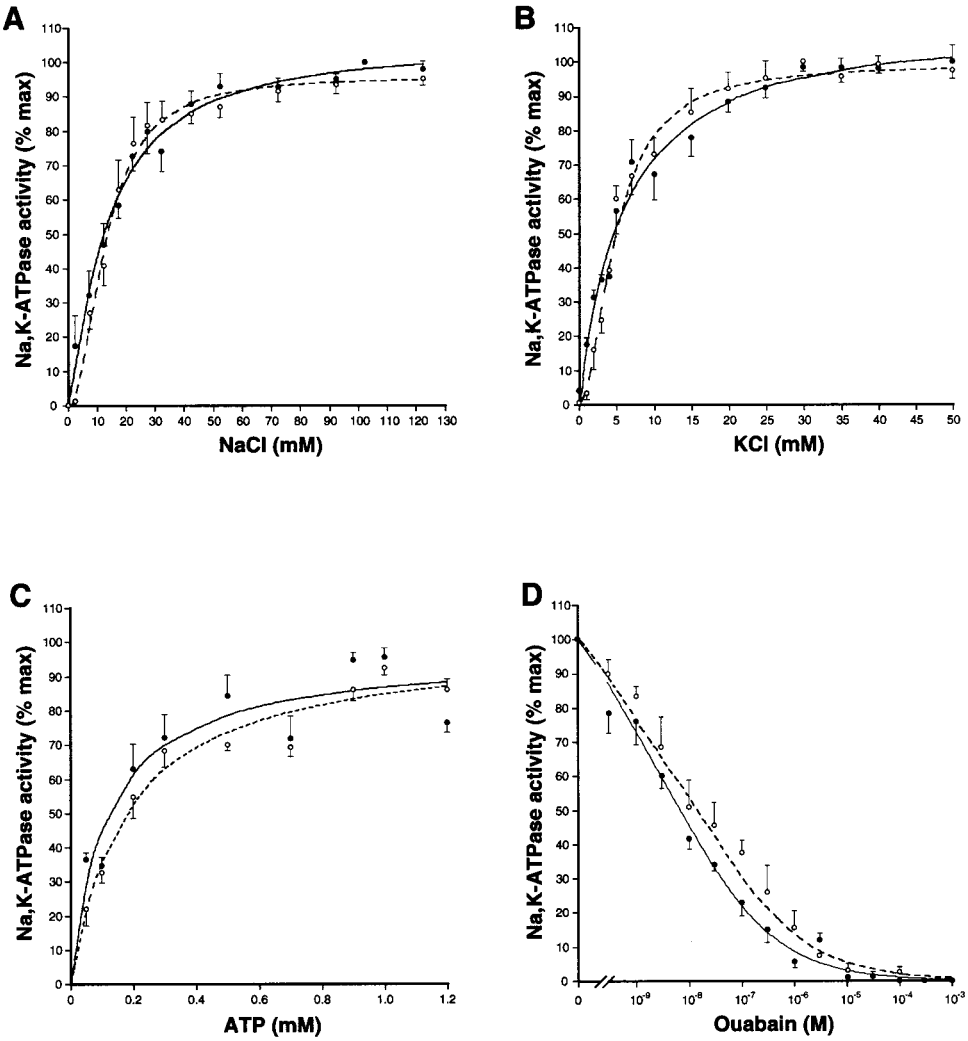

FIGURE 4: Kinetic characteristics of the Na,K-ATPase α4β1 (●) and α4β3 (○) isozymes expressed in insect cells. (A) Activation of Na,K-ATPase activity by Na^+. Hydrolysis of ATP was determined in a reaction medium containing 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 3 mM [γ-^{32}P]ATP−cold ATP, 30 mM Tris-HCl (pH 7.4), and NaCl as indicated, in the absence or presence of 1 mM ouabain. Ionic strength was kept constant with choline chloride. (B) Na,K-ATPase activation by K^+. Activity was measured in a reaction medium as described for panel A, except that NaCl was maintained constant at 100 mM NaCl and KCl was varied from 0 to 50 mM. (C) ATP stimulation of α4β1 and α4β3 isozymes. Na,K-ATPase activity was determined in a reaction medium containing saturating concentrations of all ions with varying concentrations of ATP. (D) Dose−response curves for the ouabain inhibition of Na,K-ATPase activity. Specific activity was determined after preincubation of membrane samples for 30 min at 37 °C in the reaction mixture containing 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl (pH 7.4), and the indicated ouabain concentrations. The reaction was started by the addition of [γ-^{32}P]ATP at a final concentration of 3 mM. Results in panels A, B, and C are expressed as percent of the maximal Na,K-ATPase activity obtained. In panel D, values are expressed as percentage of maximal activity in the absence of the inhibitor. Each value is the mean and error bars represent the standard errors of the mean of four to six experiments performed in triplicate.

Table 1: Kinetic Characteristics of the Rat Na,K-ATPase Isozymes Expressed in *Sf*-9 Insect Cells

isozyme	Na activation $K_{0.5}$ (mM)	K activation $K_{0.5}$ (mM)	ATP activation K_m (mM)	ouabain inhibition K_i (M)
α1β1	16.4 ± 0.7	1.9 ± 0.2	0.46 ± 0.10	$4.3 \pm 1.9 \times 10^{-5}$
α2β1	12.4 ± 0.5	3.6 ± 0.3	0.11 ± 0.01	$1.7 \pm 0.1 \times 10^{-7}$
α3β1	27.9 ± 1.3	5.3 ± 0.3	0.09 ± 0.01	$3.1 \pm 0.3 \times 10^{-8}$
α4β1	13.5 ± 1.3	5.9 ± 1.1	0.19 ± 0.04	$6.4 \pm 2.1 \times 10^{-9}$
α4β3	12.9 ± 0.6	5.0 ± 0.3	0.18 ± 0.04	$1.8 \pm 0.7 \times 10^{-8}$

[a] Apparent affinities ($K_{0.5}$), K_m, and inhibitor constants (K_i) were calculated from Na,K-ATPase dose−response curves for the indicated effectors. Values represent the mean ± standard error of the mean.

affinity for ouabain, with calculated K_I values of $(6.4 \pm 2.1) \times 10^{-9}$ M and $(1.8 \pm 0.7) \times 10^{-8}$ M, respectively.

In the testis, in addition to α4, the only other Na,K-ATPase α isoform detected is α1 (reviewed in ref *4*). The high ouabain affinity we encounter for the α4 subunit drastically differs from that of the rat Na,K-ATPase α1 polypeptide, which is very resistant to the inhibitor (Table 1) (*18*). This enzymatic difference between the α4 and α1 isoforms can be used to determine if the α4 polypeptide is functionally active in the native tissue. Therefore, we analyzed the ouabain inhibition of Na,K-ATPase activity in membrane preparations from rat testis. As depicted in Figure 5A, a heterogeneous dose−response curve was found in the testis, suggesting the presence of two isozymes with different

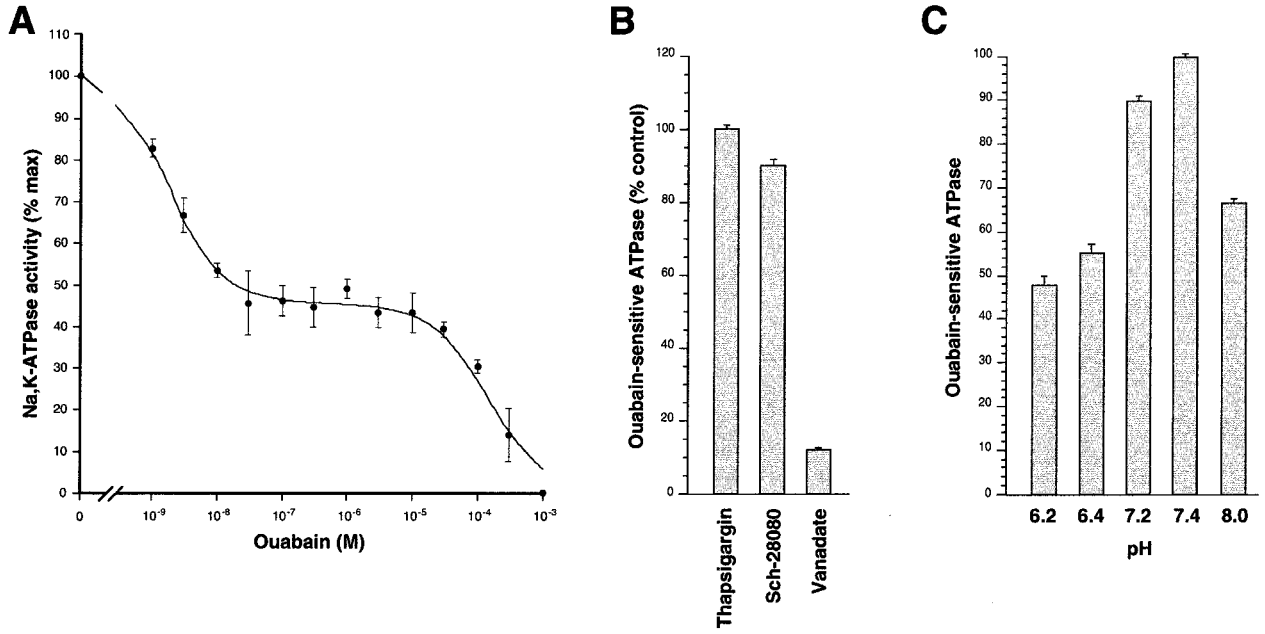

FIGURE 5: Enzymatic properties of the ouabain-sensitive ATPase of rat testis. (A) Dose−response curve for the ouabain inhibition of Na,K-ATPase from rat testis membranes. Na,K-ATPase activity was determined as in Figure 4D. Values are expressed as percentage of maximal activity in the absence of the inhibitor. Curves represent the best fit of the data assuming the presence of two enzyme populations with different ouabain affinities. Each value is the mean and error bars represent the standard errors of the mean of four experiments performed in triplicate. (B, C) Effect of inhibitors and pH on the ouabain-sensitive Na,K-ATPase from rat testis membranes. In all cases the reaction mixture contained 120 mM NaCl, 30 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 30 mM Tris-HCl (pH 7.4), and the indicated inhibitors (30 μM thapsigargin, 100 μM Sch-28080, or 1 mM vanadate), plus or minus 3×10^{-6} M ouabain. Results are expressed as a percentage of the activity obtained in the absence of inhibitors (B) or as a percentage of the activity at pH 7.4 (C). Each value is the mean and error bars represent the standard errors of the mean of three experiments performed in triplicate.

sensitivities to the cardiotonic steroid. One of them, corresponding to approximately 45% of the total Na,K-ATPase, has a calculated K_i of $(1.4 \pm 0.3) \times 10^{-4}$ M and very likely represents the Na pump composed of the α1 isoform. The second isozyme, consisting of approximately 55% of the total activity, exhibits a high affinity for the inhibitor, with a K_i of $(1.8 \pm 1.0) \times 10^{-9}$ M. Interestingly, the inhibition constant of this highly ouabain-sensitive component of the testis is similar to that found for the α4β1 and α4β3 isozymes expressed in the insect cells, strongly suggesting that the ouabain-sensitive Na,K-ATPase in the testes corresponds to the α4 isoform.

To further explore the catalytic properties of the α4 isoform of the native tissue, the effects of various ionic conditions and inhibitors on the Na,K-ATPase activity sensitive to low concentrations of ouabain was determined. As shown in Figure 5A, a ouabain concentration of 3×10^{-6} M completely inhibits α4 while leaving the α1 isozyme largely unaffected. Thus, the component of Na,K-ATPase activity sensitive to inhibition by 3×10^{-6} M ouabain represents the Na,K-ATPase isozyme composed of α4. Similar to the enzyme expressed in insect cells (Figure 3A), the activity of this ouabain-sensitive component of the testis is also highly dependent on Na^+, K^+, and Mg^{2+} (data not shown). In addition, the highly ouabain-sensitive component of the Na,K-ATPase has similar properties as the enzyme expressed in *Sf*-9 cells when assayed under different pHs and inhibitors (Figure 5B,C), or in the presence of Ca^{2+}, Cu^{2+}, Fe^{2+}, or Zn^{2+} (data not shown). Altogether, these results demonstrate that the extremely ouabain-sensitive Na,K-ATPase of the testis and the α4 isozyme produced in insect cells represents the same enzyme. Therefore, it appears in the testes that the α4 subunit is a functional isoform of the Na,K-ATPase.

DISCUSSION

In 1994, the cDNA of an α-like catalytic subunit of the Na,K-ATPase was isolated from rat testis (*24*). Despite being classified as a fourth isoform of the Na pump, the function of the polypeptide encoded by the α4 gene was unknown. Experimental evidence suggesting the role of α4 as a Na,K-ATPase subunit was recently reported (*26*). Nevertheless, a direct demonstration of the catalytic, as well as the transport properties of this novel polypeptide remained undetermined. The baculovirus expression system has been useful for the study of the different α and β isoforms of the Na,K-ATPase (*18*, *19*). In the present work we used this expression system to determine the function of the α4 polypeptide in *Sf*-9 cells. To follow the expression of the protein in insect cells, we prepared an antiserum that specifically recognizes the N-terminus of α4. Using this antiserum we demonstrate that the insect cells are able to express and deliver the polypeptide to the plasma membrane. Also, the insect cells are capable of properly assembling the α4 polypeptide with the Na,K-ATPase β1 isoform, as reflected by the expression of Na,K-ATPase activity. This is shown by (i) an increase in Na^+-, K^+-, and Mg^{2+}-dependent hydrolysis of ATP that is inhibited by ouabain, (ii) a Na-dependent phosphorylation from ATP that is sensitive to K^+ and inhibited by ouabain, and (iii) a ouabain-sensitive uptake of ^{86}Rb. Conversely, no Na,K-ATPase activity is induced in the cells if α4 is expressed alone. This same result has been observed with the α1, α2, and α3 isoforms of the Na,K-ATPase (*33*). Additional evidence supporting the notion that α4 is a catalytic subunit

of the Na,K-ATPase and not another P-type ATPase is provided by studies characterizing the cation requirements and inhibitor sensitivities of the enzyme. For example, the activity of the α4β1 enzyme is inhibited by Ca^{2+} and insensitive to thapsigargin, eliminating the possibility that the enzyme functions as a Ca-ATPase. The prospect that the α4 polypeptide functions as a subunit of a H,K-ATPase is also unlikely. The activity of α4β1 is highly dependent on Na^+, its maximal capability to hydrolyze ATP occurs at a pH of 7.2−7.4, and its activity is reduced by increased concentrations of hydrogen. More importantly, Sch-28080, a compound that inhibits members of the family of the gastric H,K-ATPases (*37, 38*) has no effect on the activity of α4β1. On the basis of their primary sequences a group of ATPases have been recently classified as the nongastric H,K-ATPases. These include the H,K-ATPase from toad bladder epithelium (*39*), the (Na,H),K-ATPase of rat distal colon (*40, 41*), and the human ATP1AL1-encoded protein (*42*−*44*). Structurally, the α subunits of these enzymes are equally distant from the Na,K-ATPase and the H,K-ATPase. Functionally, the nongastric H,K-ATPases are inhibited by high concentrations of ouabain (*40, 42, 44*). The toad bladder epithelium and the human ATP1AL1 (Na,H),K-ATPases are also inhibited by Sch-28080 (*39, 41*), and thus, their activity is different from that of the α4 polypeptide. On the other hand, the rat distal colon (Na,H),K-ATPase is only sensitive to ouabain with a K_i for the inhibitor of approximately 1 mM (*40*). The α4 polypeptide clearly differs from the colonic H,K-ATPase because of its high affinity for ouabain and inhibition by H^+. Thus, the α4 polypeptide is a catalytic subunit of the Na,K-ATPase and not an H,K-transporting ATPase. Because divalent ions such as Ca^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} are present in the testis where α4 is naturally expressed (*45, 46*), we determined whether α4 activity was linked to these cations. Ca^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} did not stimulate α4 activity, indicating that they are not substrates and therefore are not transported by the enzyme. Taken together, these results demonstrate for the first time that the α4 polypeptide is catalytically competent and exhibits enzymatic characteristics compatible with those of a Na,K-ATPase α subunit.

The expression of two different isoforms of the Na,K-ATPase β subunit, β1 and β3, in the testis allows for two structurally different Na pump isozymes, α4β1 and α4β3, in the gonad. We show that coexpression of α4 with either of the β subunits in the insect cells resulted in catalytically active Na,K-ATPases. This made it possible to characterize the kinetic properties of the α4β1 and α4β3 isozymes. Regardless of the β subunit present, the apparent affinities for Na^+ and K^+ and the K_m for ATP are similar for both isozymes. This is interesting, since the properties of α2 and α3 are modified depending on the β subunit present (*18, 19*). Also, differences in the enzymatic characteristics of the α1β1 and α1β3 complexes have been reported (*47*). The kinetic parameters of the enzymes containing α4 are within the range of those described for the other Na pump isozymes (reviewed in ref *4*). However, α4 is unique since its catalytic properties are only partially shared with each of the other Na,K-ATPase α isoforms. For example, the apparent affinity of α4 for Na^+ is relatively high and similar to that of α2. In contrast, the apparent affinity to K^+ is low and comparable to that of α3. Finally, the affinity for ATP is intermediate to that of the other Na,K-ATPase isoforms. The α4 isoform has a higher affinity for ATP than α1 but a lower affinity than α2 and α3 (*4*). In the testis, immunocytochemical studies have identified the Na,K-ATPase at the apical membrane of the Sertoli cells and at the basolateral membrane of the epithelial cells of the seminiferous tubules (*48*). These cells exchange fluid and solutes to create and maintain the appropriate medium for the proliferation and maturation of the germ cells (*49*−*51*). In the seminiferous tubules, the K^+ concentration is much higher than in the blood plasma. Thus, it is possible that the low apparent affinity for K^+ of α4 might be related to the unusual concentration of the cation in the testis.

A distinctive kinetic property of the rat Na pump isozymes is their difference in sensitivity to the cardiotonic steroids (reviewed in ref *4*). As shown, the α4β1 and α4β3 isozymes have a high affinity for ouabain. In fact, when compared to the other Na,K-ATPase isozymes expressed in insect cells, the ouabain sensitivity is the highest. High-affinity [^{3}H]ouabain binding has been found in 3T3 fibroblasts stably expressing the α4 cDNA (*26*). However, in that study, the ouabain affinity reported for α4 was lower. The differences in the membrane environment in each cell may be responsible for the disparity in results. The physiological relevance of the high ouabain sensitivity of the Na pump isozymes composed of α4 is unclear. Differences in ouabain affinity of the various Na,K-ATPases isozymes may have a role in the regulation of Na,K-ATPase activity (reviewed in ref *4*). This is supported by the finding of endogenous digitalis-like compounds in mammals (reviewed in refs *52*−*55*). The importance of this mechanism of enzyme modulation in the testis is unknown. Interestingly, the high affinity for ouabain of the α4 isoform may be related to the finding of endogenous digoxin-like immunoreactivity in seminal fluid of humans (*56*).

By determining the ouabain inhibition profile of Na,K-ATPase activity in rat testis, we found a Na^+- and K^+-dependent hydrolysis of ATP with a ouabain sensitivity similar to that of the α4β1 and α4β3 enzymes expressed by use of baculovirus. The tissue also exhibits the ouabain-resistant Na,K-ATPase activity corresponding to the α1 isoform. This is in agreement with previous studies that indicate that the testes express the mRNAs for only the α1 and α4 catalytic isoforms of the Na,K-ATPase (*24*). Although association of α1 with β3 might also result in active Na,K-ATPase in the gonad, the possibility that α1β3 represents the ouabain-sensitive component is unlikely, since the other β subunits do not greatly influence the sensitivity of the enzyme to the cardiotonic steroids. In agreement with this, the human Na,K-ATPase α1β3 isozyme, expressed in insect cells, has a ouabain affinity similar to that of the α1β1 enzyme (*47*). Consequently, the highly inhibitable ATPase activity of the gonad presumably corresponds to Na,K-ATPases composed of the α4 isoform. Comparison of other enzymatic properties of the ouabain-sensitive enzyme from testes with that of the α4β1 expressed in *Sf*-9 cells supports this identification. Both enzymes are inhibited by vanadate, insensitive to Ca-ATPase and H,K-ATPase blockers, and exhibit similar responses to pH, calcium, copper, iron, and zinc. Altogether, these characteristics confirm that the α4 isoform is a subunit of the Na,K-ATPase and that the subunit is catalytically competent in the native tissue. Although the exact role of the α4 isoform is unknown, the importance of

the subunit in the maintenance of ion homeostasis in the testis is reflected by the fact that its activity represents almost half of the total Na,K-ATPase of the gonad. Further characterization of the α4 isozyme may provide insights into the role of the Na,K-ATPase in the maintenance of ion homeostasis in the gonad.

ACKNOWLEDGMENT

We thank Dr. Robert Levenson for providing the cDNA of the Na,K-ATPase β3 isoform and Dr. Kathleen Sweadner for providing the anti-α2 (MCB2) monoclonal antibody.

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BI991207B