Different expression and activity of the $\alpha 1$ and $\alpha 4$ isoforms of the Na,K-ATPase during rat male germ cell ontogeny

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Abstract

Two catalytic isoforms of the Na,K-ATPase, $\alpha 1$ and $\alpha 4$, are present in testis. While $\alpha 1$ is ubiquitously expressed in tissues, $\alpha 4$ predominates in male germ cells. Each isoform has distinct enzymatic properties and appears to play specific roles. To gain insight into the relevance of the Na,K-ATPase α isoforms in male germ cell biology, we have studied the expression and activity of $\alpha 1$ and $\alpha 4$ during spermatogenesis and epididymal maturation. This was explored in rat testes at different ages, in isolated spermatogenic cells and in spermatozoa from the caput and caudal regions of the epididymis. Our results show that $\alpha 1$ and $\alpha 4$ undergo differential regulation during development. Whereas $\alpha 1$ exhibits only modest changes, $\alpha 4$ increases with gamete differentiation. The most drastic changes for $\alpha 4$ take place in spermatocytes at the mRNA level, and with the transition of round spermatids into spermatozoa for expression and activity of the protein. No further changes are detected during transit of spermatozoa through the epididymis. In addition, the cellular distribution of $\alpha 4$ is modified with development, being diffusely expressed at the plasma membrane and intracellular compartments of immature cells, finally to localize to the midregion of the spermatozoon flagellum. In contrast, the $\alpha 1$ isoform is evenly present along the plasma membrane of the developing and mature gametes. In conclusion, the Na,K-ATPase $\alpha 1$ and $\alpha 4$ isoforms are functional in diploid, meiotic and haploid male germ cells, $\alpha 4$ being significantly upregulated during spermatogenesis. These results support the importance of $\alpha 4$ in male gamete differentiation and function.

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Introduction

Mammalian spermatogenesis is a complex and highly coordinated event in which male germ cells undergo drastic changes in the seminiferous tubules to generate spermatozoa (Toshimori 2003). The process involves several steps and starts with the mitotic division of spermatogonia to produce spermatocytes. The spermatocytes then undergo meiotic divisions, to generate haploid round spermatids that enter the process of spermiogenesis to create spermatozoa (Dym 1994, de Rooij 2001, Toshimori 2003). Once produced, spermatozoa undergo in the epididymis final stages of differentiation that allow the gametes to become motile (Jones 1998). Spermatogenesis requires the synchronized regulation of expression of multiple proteins that are essential at specific stages of male germ cell differentiation, and for the function and fertility of the gametes (Hecht 1998, Eddy 2002). Several studies have explored gene expression profiles during different phases of spermatogenesis in an attempt to understand the role of specific proteins in the differentiation of male germ cells

(Fujii *et al.* 2002, Sha *et al.* 2002, Sluka *et al.* 2002, Tanaka *et al.* 2002, Pang *et al.* 2003, Yu *et al.* 2003, Guo *et al.* 2004, Shima *et al.* 2004, Wang *et al.* 2004, Small *et al.* 2005, Wrobel & Primig 2005). Because ion homeostasis is of major importance for the development and physiology of male germ cells, considerable attention has been focused on genes encoding different transporters of the plasma membrane of the cells (Hagiwara *et al.* 1984, Hettwer *et al.* 1985, Darszon *et al.* 1999, Salvatore *et al.* 1999, Calamita *et al.* 2001, Felix *et al.* 2002, Gong *et al.* 2002, Son *et al.* 2002, Tsevi *et al.* 2005). Despite this, the temporal and cell stage-specific expression of the Na,K-ATPase during spermatogenesis and gamete maturation has not been precisely characterized.

The Na,K-ATPase, or Na pump, is an enzyme of the plasma membrane that uses the energy from the hydrolysis of ATP to exchange cytoplasmic Na⁺ for extracellular K⁺ (Kaplan 2002). Its function plays a central role in maintaining cell volume and pH, keeping cell resting membrane potential, and providing the chemical energy for the secondary transport of other ions, solutes and water across

the cell membrane (Skou & Esmann 1992). Structurally, the Na,K-ATPase is an oligomer composed of two major polypeptides, the α and β subunits. The α subunit is a multispanning membrane protein responsible for the catalytic and transport properties of the enzyme. It contains the binding sites for the cations, ATP and cardiotonic steroid inhibitors, such as ouabain (Jorgensen *et al.* 2003). The β subunit is a single membrane-spanning polypeptide that is thought to help in the folding and delivery of the α polypeptide to the plasma membrane (Geering 2002). The Na,K-ATPase exists as multiple isozymes composed of different molecular forms or isoforms of the α ($\alpha 1$, $\alpha 2$, $\alpha 3$ and $\alpha 4$) and β ($\beta 1$, $\beta 2$ and $\beta 3$) subunits (reviewed in Blanco & Mercer 1998, Mobasher *et al.* 2000). Each Na,K-ATPase isozyme is characterized by a particular pattern of expression, and by unique functional properties that mainly depend on the type of α subunit that constitutes the enzyme. While $\alpha 1$ in association with $\beta 1$ is found in nearly every tissue and may function as the housekeeping Na,K-ATPase in the cell, the other α and β polypeptides are more restricted in their expression and appear to play tissue specific roles (Crambert *et al.* 2000, Mobasher *et al.* 2000, Segall *et al.* 2001).

The testis is characterized by the unique expression of the $\alpha 4$ polypeptide, being the isoform abundant in the male germ cells (Shamraj & Lingrel 1994, Underhill *et al.* 1999, Blanco *et al.* 2000). Besides $\alpha 4$, the ubiquitous $\alpha 1$ is also present in the male gonad (Shamraj & Lingrel 1994, Blanco *et al.* 2000). We have previously shown that $\alpha 4$ can associate with both the β subunits expressed in testis, $\beta 1$ and $\beta 3$ (Arystarkhova & Sweadner 1997) to render two catalytically competent Na,K-ATPases, $\alpha 4\beta 1$ and $\alpha 4\beta 3$, that have similar kinetic properties (Blanco *et al.* 1999). Nevertheless, the isozymes composed of $\alpha 4$ are functionally different from the other Na,K-ATPases. They have a high affinity for Na^+ , a low affinity for K^+ , an intermediate affinity for ATP, and a high sensitivity to ouabain (Blanco *et al.* 1999, Woo *et al.* 1999). The particular enzymatic properties of $\alpha 4$ suggest that the isoform plays a specific role in sustaining the ion gradients, membrane potential and excitability of male germ cells. In support of this, ouabain inhibition of $\alpha 4$ has been shown to impair sperm motility (Woo *et al.* 2000).

Defining the expression profile, localization and function of the $\alpha 1$ and $\alpha 4$ isoforms of the Na,K-ATPase during male germ cell development is an essential step in understanding their relevance to the physiology of the spermatogenic cells and mature gametes. In the present work, we have investigated this in whole testis of the rat at different ages and in isolated rat spermatogenic cells. In addition, we have studied the Na,K-ATPase $\alpha 1$ and $\alpha 4$ isoforms in rat spermatozoa from the caput and caudal regions of the epididymis, since spermatozoa undergo maturational changes and gain the ability to swim forward during epididymal transit (Soler *et al.* 1994, Weissenberg *et al.* 1994). Our results show quantitative and qualitative developmental changes that primarily involve the $\alpha 4$ isoform.

The regulation of $\alpha 4$ during male germ cell ontogeny supports the relevance of the isoform in the physiology of the male gamete. A preliminary report of some of our findings has been published previously (Blanco 2003).

Materials and Methods

Animals, tissue, and cell preparations

Sprague Dawley rats, purchased from Harlan (Indianapolis, IN, USA), were used in this study as the source of whole testis and isolated male germ cells. Animal maintenance procedures used were in accordance with the guidelines approved by the University of Kansas Institutional Animal Care and Use Committee. Rats were killed by a saturated atmosphere of carbon dioxide, followed by cervical dislocation. For the experiments with whole testes, the gonads of rats of 1 week, 18 days and 2 months of age were dissected and decapsulated, and the tissue was homogenized on ice in 250 mM sucrose, 25 mM imidazole (pH 7.4) and 1 mM EGTA in a glass-glass homogenizer. Male germ cell isolation was performed by a modification of the unit gravity sedimentation method (Davis & Schuetz 1975, Bellvé *et al.* 1977a, 1977b). Spermatogonia were isolated from 10 day old testes, while spermatids and round spermatocytes were separated from adult testes. Briefly, testis samples were placed in DMEM/HAM's F-12 media. Adult testes were digested with 0.75 mg/ml collagenase/dispase (Roche), and 10-day old testes were treated with 0.75 mg/ml trypsin (Sigma) for approximately 15 min with continuous agitation at 33 °C in both cases. From this point, both samples were treated the same. Amounts of 1 mg/ml hyaluronidase and 0.1 mg/ml DNase (Sigma) were added, and the tissue was further incubated at 33 °C for 5–10 min with continuous shaking. Then, the dispersed seminiferous cords were allowed to sediment for 5 min, the supernatant was decanted, and the tubules were washed with DMEM/HAM's F12. After three washes, the seminiferous cords were dissociated with 0.75 mg/ml trypsin and 0.1 mg/ml DNase at 33 °C for another 30 min. Trypsin activity was stopped with 0.75 mg/ml trypsin inhibitor (Sigma), and the cell suspension filtered through a 40 μm nylon mesh. Cells were centrifuged, resuspended in a final volume of 20 ml of DMEM/HAM's F12 with 0.5% BSA at a density of 25×10^6 cells for adult rats, or 2×10^6 cells for 10-day-old rats, and loaded onto a continuous 2–4% gradient of BSA in DMEM/HAM's F-12 media. Cells were allowed to separate by unit gravity sedimentation for 2.5 h, and fractions (5 ml for 10-day-old testis and 10 ml for adult testis) were collected from the bottom of the separating apparatus. The cell type and purity in each fraction were assessed by Neumarski optics. Fractions with only the desired cell type and a purity of above 85% were pooled together. While this method provided spermatogonia, pachytene spermatocytes and round spermatids, mature spermatozoa were obtained from the caput and cauda of

adult rat epididymides. For this, the different regions of the tissue were minced with a razor blade and were gently stirred for 30 min at room temperature in PBS. Sperm was collected from the supernatant. Depending on the type of biochemical assays, cells were used either intact, or after homogenization in a glass-glass homogenizer. To further confirm the composition of the different spermatogenic cell populations obtained, RT-PCR of spermatogenic cell stage-specific markers were used (see Results). Thus, c-kit, which encodes for a transmembrane tyrosine kinase receptor, was predominantly found in spermatogonia, as has been previously described (Dym *et al.* 1995, Prasanth & Ali 2003, von Schonfeldt *et al.* 2004). Synaptonemal complex protein 1 (SCP-1), which is important in chromosome pairing during the meiotic prophase, was detected, as expected, in the spermatocyte fraction (Schmekel *et al.* 1996, Tureci *et al.* 1998). Finally, protamine 2 (PRM2), which is involved in nuclear DNA compaction, was mainly found in the samples containing spermatids and spermatozoa (Klemm *et al.* 1989, Tanhauser & Hecht 1989, Iuchi *et al.* 2001).

Biochemical assays

Protein was determined using a dye-binding assay based on the method of Bradford (Bio-Rad). Na,K-ATPase activity was assayed on cell homogenates through determination of the initial rate of release of $^{32}\text{P}_i$ from $\gamma[^{32}\text{P}]\text{-ATP}$, as previously described (Blanco *et al.* 1995). The ATPase activity of 10 μg total protein samples was measured in a final volume of 0.25 ml in medium containing 120 mM NaCl, 30 mM KCl, 3 mM MgCl_2 , 0.2 mM EGTA and 30 mM Tris-HCl (pH 7.4) with or without ouabain. The assay was started by the addition of ATP with 0.2 μCi $\gamma[^{32}\text{P}]\text{-ATP}$ (3 mM final concentration). After 30-min incubation at 37°C, the tubes were placed on ice, and the reaction was terminated by the addition of 25 μl 55% trichloroacetic acid. Released $^{32}\text{P}_i\text{-Pi}$ was converted to phosphomolybdate and extracted with isobutanol. Radioactivity in 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. Specific activity was determined as the difference in ATP hydrolysis between the absence and presence of 1 mM ouabain. Activity of $\alpha 4$ was that sensitive to 3×10^{-6} M ouabain, while the function of $\alpha 1$ corresponded to the difference in ATP hydrolysis between 3×10^{-6} M and 1 mM ouabain.

The transport activity of the Na,K-ATPase of the isolated male germ cells was determined by measuring the ouabain-sensitive uptake of ^{86}Rb , used as a tracer for K^+ (DeTomaso *et al.* 1994). For this, the isolated cells were centrifuged (2000 *g*) and resuspended at a density of 3×10^6 cells/ml in preincubation medium containing 150 mM NaCl, 25 mM Hepes (pH 7.4), 2.5 mM MgCl_2 , 1% BSA and 100 μM bumetanide. After 30 min on ice, cells were centrifuged and resuspended in flux medium

(50 mM NaCl, 7 mM KCl, 25 mM Hepes (pH 7.4), 2.5 mM MgCl_2 , 1% BSA and 100 μM bumetanide), in the absence and presence of 3×10^{-6} M and 1×10^{-3} M ouabain. After 5 min incubation at 37°C, the reaction was started by adding equal volume of flux medium containing ^{86}Rb (1 $\mu\text{Ci}/\text{ml}$, Amersham) in the absence or presence of ouabain. After 3 min incubation, 150 μl aliquots (1×10^6 cells) were removed and placed in Spin-X centrifuge tubes containing a 0.45 μm filter (Corning Inc., Acton, MA, USA). Samples were centrifuged for 10 s, and the cells in the filters were washed with 0.5 ml ice-cold 116 mM MgCl_2 and then underwent 30 s centrifugation at 10 000 *g*. Filters were excised from the tubes and transferred to scintillation vials to measure radioactivity. The ouabain-sensitive transport of ^{86}Rb into the cells was determined after 3 min incubation, in which transport was linear with time.

Data analysis

Curve fitting of the experimental data was performed using a Marquardt least-squares, nonlinear regression computing program (Sigma Plot, Jandel Scientific, San Rafael, CA, USA). Dose-response relations for the inhibition of Na,K-ATPase by ouabain showed a biphasic response in whole testis and in all isolated cells. Curves were best fitted by applying the following equation that assumed the presence of two enzyme populations with different affinity for ouabain:

$$v = (F1/(1 + [I]/K_i)) + (F2/(1 + [I]/K_{ii}))$$

Where v is the Na,K-ATPase activity corresponding to a certain concentration of the inhibitor ouabain $[I]$, expressed as a fraction of activity in the absence of ouabain; $F1$ and $F2$ are the fractional amounts of each Na,K-ATPase isozyme; and K_i and K_{ii} are the concentrations of ouabain that give the half-maximal inhibition of each of the enzymes present in the sample. The validity of using a two-component versus a single-component model for ouabain binding was statistically supported by applying Snedecor's F test (Blanco *et al.* 1995). Results are expressed as the mean $\pm$ S.E.M. Statistical significance of the differences between experimental groups was calculated by Student's *t*-test, and statistical significance was defined as $P < 0.05$.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA from each cell type was isolated with TRIzol reagent according to the supplier's specifications (Invitrogen). Complementary DNA was prepared by reverse transcription of 2 μg total RNA with Superscript, as suggested by the supplier (Invitrogen). Briefly, reactions were carried out in a final volume of 20 μl containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 0.8 μmol dNTPs, 10 M dithiothreitol, 1 μg oligo(dT)12-18 primers (Invitrogen) and 200 units of reverse transcriptase (Superscript). After 1 h at 37°C, the enzyme was inactivated by boiling for 10 min at 95°C. The resulting first-strand cDNA was

Table 1 Characteristics of the primers used for RT-PCR analysis of Na, K-ATPase isoforms and other genes in male germ cells.

Gene targeted (accession no.)	Primer sequence	Primer position	Product size (bp)	Annealing conditions
α1 (NM_012504)	5'-AGC TGT ATC AGA ACA-3' (S)	40–1487	1447	55°C, 30 s
α2 (NM_012505)	5'-CTG CCG CCA CCA CTG GGG-3' (S)	33–1473	1440	65°C, 5 s
α3 (NM_012506)	5'-AAT ACC GAC TGC GTG CAG-3' (S)	136–1464	1328	60°C, 30 s
α4 (NM_022848)	5'-CTC AAG TCA ACA AGT CTA TT-3' (S)	1993–2928	935	60°C, 30 s
c-kit (NM_022264)	5'-GAG CTC AGA GTC TAG CGC AGC-3' (S)	17–867	850	57°C, 15 s
SCP-1 (NM_012810)	5'-AGT ATG AGC ACT CAG AAG ACT-3' (S)	1111–2132	1021	56°C, 10 s
PRM2 (NM_012873)	5'-GAC CAT GAG CGC GAG GAG CAG-3' (S)	57–296	239	62°C, 10 s
GAPDH (NM_017008)	5'-TGATGACATCAAGAGGTGTGGAG-3' (S)	1608–1847	239	60°C, 30 s

(S): sense primer; (AS): antisense primer.

subjected to amplification by primers specific for each of the Na,K-ATPase α isoforms, for the germ cell stage-specific markers (c-kit, SCP-1 and PRM2) and for glyceraldehyde phosphate dehydrogenase (GAPDH), an enzyme involved in glycolysis that is widely expressed in cells (Yu *et al.* 2003). The sequences of the primers used, their annealing properties and the size of the amplified cDNAs are described in Table 1. A volume of 1 µl DNA was added to 50 µl of PCR mixture containing 100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂, 200 µM dNTPs, 500 nmol of each primer and 2.5 units of Taq DNA polymerase. The conditions for PCR included a first cycle of 30 s at 94 °C, followed by 30 cycles of the following: 1. denaturation for 30 s at 94 °C; 2. an annealing step that varied depending on the primers used (see Table 1); 3. an elongation step for 50 s at 72 °C. Finally, an additional elongation step of 5 min was performed at 72 °C. The amplified DNA fragments were identified by electrophoresis in a 1% agarose gel stained with ethidium bromide.

Polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis

Protein from whole testis (70 µg total protein/lane) and isolated male germ cells (150 µg total protein/lane) was analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting. Proteins were separated in a 7.5% gel (Laemmli 1970), transferred to nitrocellulose and immunoblotted as described previously (Blanco *et al.* 1995). As a positive control, the Na,K-ATPase α1 and α4 isoforms produced in Sf-9 insect cells via baculoviral infection were used (Blanco *et al.* 1999). The Na,K-ATPase α1 and α4 isoforms were identified by polyclonal antisera raised against the N-terminal portions of each polypeptide (Blanco *et al.* 2000). The anti-α4 antiserum was purified at Covance Research Products (Denver, PA, USA) by affinity chromatography of the peptide against which the antibody was generated. Expression of GAPDH was determined by a monoclonal antibody from Abcam (Cambridge, MA, USA). Primary antibodies were then detected by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and chemiluminescence.

Immunocytochemistry and confocal microscopy

For immunocytochemical analysis, the isolated male germ cells were plated on 11 mm glass cover slips in 24-well tissue culture plates and centrifuged (3000 g for 3 min) to help them attach. Cells were fixed in 4% paraformaldehyde (buffered formalin phosphate; Fisher Scientific, Pittsburgh, PA, USA). Samples were then processed for immunocytochemistry, as previously described (Sanchez & Blanco 2004). Briefly, cells were permeabilized with 0.3% Triton X100 in 25 mM Hepes (pH 7.4), 150 mM NaCl and 1 mM EGTA (HBS). After blocking for 2 h at room temperature with 0.2% BSA and 2% normal goat

serum in HBS, the anti- $\alpha 1$ and anti- $\alpha 4$ antisera were applied overnight at 4°C at a dilution of 1:100. A fluorescein isothiocyanate (FITC)-conjugated goat antirabbit antiserum was used as the secondary antibody. To enhance the signal, an anti fluorescein/Oregon green, rabbit IgG fraction, conjugated to Alexa fluor 488 (Molecular Probes, Eugene, OR, USA), was used. Samples were also stained to visualize specific cell structures. For the nuclei, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) was used. For the acrosome and mitochondria, wheat germ agglutinin conjugated with Alexa Fluor 633 and MitoFluor Red 589 (Molecular Probes) was used respectively. Fluorescent digital images were obtained with a Zeiss LSM510 confocal microscope equipped with a UV laser (80 mW) for the excitation (364 nm, 50–100% laser power) and detection (band pass 385–470 nm filter; BP385–470) of the DAPI, with an argon/2 laser (25 mW) for the excitation (488 nm, 70% laser power) and detection (BP505–550) of the fluorescein, a HeNe laser (1 mW) for the excitation (543 nm, 100% laser power) and detection (BP560–615) of the MitoFluor Red 589, and a HeNe laser (1 mW) for the excitation (633 nm, 100% laser power) and detection (long pass 650 nm; LP 650) of the Alexa Fluor 633. Images were acquired in multitrack channel mode (sequential excitation/emission) with LSM510 (v 3.2) software and a Plan-Apochromat 63 $\times$ 1.4 Oil DIC objective with a frame size of 1024 $\times$ 1024 pixels and a zoom factor of 1 (field size of 0.146 mm $\times$ 0.146 mm) for mature sperm, or zoom factor of 2 (field size of 0.048 mm $\times$ 0.048 mm) for immature cells. Detector gain was set initially to cover the full range of all the samples and background corrected by setting the amplifier gain in comparison to the relevant control slides, and all images were then collected under the same photomultiplier detector conditions and pinhole diameter. Control slides were as follows: 1. mounted germ cells only, without the antibodies, used to check for autofluorescence; 2. single-color-stained samples to check for bleed through into all the other channels; 3. normal rabbit serum and secondary antibodies to check for nonspecific binding.

Results

Na,K-ATPase isoform activity in whole testis

We have previously shown that both of the α isoforms of the Na,K-ATPase, $\alpha 1$ and $\alpha 4$, expressed in rat testis are functionally active in the gonad and have different enzymatic properties (Blanco *et al.* 1999, 2000). The most conspicuous difference between the isoforms is the reactivity to ouabain, $\alpha 4$ being over 1000 times more sensitive to the compound than $\alpha 1$ (Blanco *et al.* 1999). In this manner, ouabain sensitivity can be used as a tool to distinguish the activity and relative composition of Na,K-ATPase isoforms in testis cells. To determine the functional status of the Na,K-ATPase α isoforms during male germ cell maturation, our first approach was to

determine the dose–response curves of ouabain inhibition of Na,K-ATPase activity in rat testes of different ages. Three time points in the sexual maturation of rats were chosen: 1. one week after birth, when the testes contain germ cells at the stage of spermatogonia; 2. 18 days of life, when preleptotene, leptotene, and pachytene spermatocytes are present, but the gonad has no haploid spermatids; 3. sexually mature animals, in which cells at all stages of spermatogenesis are found, and spermatozoa are fully differentiated (Marty *et al.* 2003). For each time point, tissue homogenates were prepared and ouabain inhibition profiles determined. As shown in Fig. 1, inhibition of Na,K-ATPase by the cardiotonic steroid exhibited a biphasic response at all ages. Table 2 shows the calculated inhibition constants (K_i) for each enzyme component. The K_i values in the nanomolar and millimolar range corresponded well with those reported previously for the $\alpha 1$ and $\alpha 4$ isoforms (Blanco & Mercer 1998, Blanco *et al.* 1999, 2000, Woo *et al.* 1999). These results confirm previous observations that testis express only the Na,K-ATPase $\alpha 1$ and $\alpha 4$ isoforms, but not $\alpha 2$ or $\alpha 3$ (Shamraj & Lingrel 1994, Underhill *et al.* 1999, Blanco *et al.* 2000). Interestingly, the relative contribution of each α isoform to the total Na,K-ATPase activity of the gonad varied with age. The percentile activity of $\alpha 4$ doubled

Figure 1 Dose–response curves for the ouabain inhibition of testes Na,K-ATPase activity from 1-week-old rats ($\blacktriangle$), 18-day-old rats ($\blacksquare$) and adult animals ($\bullet$). Specific activity was determined as described in Materials and Methods. Results are expressed as percentage of maximal activity in the absence of the inhibitor. Curves represent the best fit of the experimental data and indicate the presence of two enzyme components, one highly sensitive and the other resistant to ouabain. Each value is the mean, and error bars represent the S.E.M. of three experiments performed in triplicate. The F test indicated that the parameters describing each enzyme population are statistically different ($P < 0.001$).

between week 1 and day 18 after birth, and became almost half of the total Na,K-ATPase of the adult gonad (Table 2). While the relative functional levels of $\alpha 4$ increased with tissue maturation, the relative activity of $\alpha 1$ decreased.

Comparison of the absolute values of Na,K-ATPase activity indicated that the total ouabain-sensitive hydrolysis of ATP in the samples increased approximately twofold with tissue age. This increase was mainly dependent on the change in ATP hydrolysis sensitive to 3×10^{-6} M ouabain that represents the function of $\alpha 4$ (Fig. 2). Thus, $\alpha 4$ activity increases approximately sevenfold with development. Instead, the highly ouabain-resistant activity of the $\alpha 1$ isoform remained approximately constant.

Immunoblot analysis of Na pump α isoforms in whole testis

To determine the expression of the Na,K-ATPase $\alpha 1$ and $\alpha 4$ polypeptides, we performed immunoblots. For this, we used antisera made against the N-terminal domains of $\alpha 1$ and $\alpha 4$, which we have previously shown to be specific for the respective isoforms (Blanco *et al.* 1999). As a control of relative protein expression levels, parallel immunoblots were analyzed for the nonrelated protein GAPDH. The corresponding immunoblots are presented in Fig. 3. As shown, the $\alpha 1$ isoform was found at approximately similar levels in testis at the various ages studied. In contrast, the $\alpha 4$ polypeptide was substantially augmented in the gonad of adult versus prepubertal rats. The expression patterns of the Na,K-ATPase isoforms in testis agree with the functional assays, indicating that the $\alpha 4$ polypeptide is the isoform that changes during maturation of the male gonad.

Activity of α isoforms in isolated germ cell fractions

To determine more directly the functional expression of the Na,K-ATPase isoforms during development of the male germ cells, we used isolated spermatogenic cells and epididymal spermatozoa. Highly enriched fractions of spermatogonia, pachytene spermatocytes and round spermatids were isolated from prepubertal or adult testis. Spermatozoa were obtained from the caput and cauda of adult rat epididymides. Cells were homogenized, and dose–response curves for the ouabain inhibition

Table 2 Inhibition constants (K_i) and relative amounts of Na,K-ATPase $\alpha 1$ and $\alpha 4$ isoforms in rat testis of different ages.

Isoform		Rat testis age		
		1 week	18 days	Adult
$\alpha 1$	(%)	86.6 $\pm$ 1.9	72.4 $\pm$ 1.8	45.0 $\pm$ 1.4
	K_i (M)	5.3 $\pm$ 5.5 $\times 10^{-5}$	7.2 $\pm$ 2.2 $\times 10^{-5}$	1.5 $\pm$ 1.3 $\times 10^{-4}$
$\alpha 4$	(%)	13.4 $\pm$ 2.8	27.6 $\pm$ 2.9	55.0 $\pm$ 2.8
	K_i (M)	1.4 $\pm$ 3.0 $\times 10^{-9}$	3.2 $\pm$ 2.8 $\times 10^{-9}$	1.6 $\pm$ 1.0 $\times 10^{-9}$

Parameters were calculated from dose–response curves for the ouabain inhibition of Na,K-ATPase activity. Values represent the mean $\pm$ S.E.M.

Figure 2 Total activity of the Na,K-ATPase and the $\alpha 1$ and $\alpha 4$ isoforms in rat testis of different ages. Specific activity was measured on rat testis homogenates at 7 and 18 days after birth and in adult animals. Total Na,K-ATPase represents the hydrolysis of ATP sensitive to 1 mM ouabain. Activity of $\alpha 4$ was determined as the hydrolysis of ATP sensitive to 3×10^{-6} M ouabain, while function of $\alpha 1$ was obtained by measuring the ATPase difference between 3×10^{-6} and 1×10^{-3} M ouabain. Values are the mean, and bars represent the S.E.M. of three experiments. The asterisks indicate values significantly different from corresponding ones at day 7 ($P < 0.001$).

Figure 3 Immunoblot analysis of Na,K-ATPase $\alpha 1$ and $\alpha 4$ isoforms in rat testis of different ages. Proteins from rat testis homogenates of 7 and 18 days after birth and adult animals were separated by SDS–PAGE (7.5% gel) and transferred to nitrocellulose, and the α polypeptides were detected with antisera that recognize the N-terminal portion of $\alpha 1$ and $\alpha 4$. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. As a positive control for the Na,K-ATPase $\alpha 1$ and $\alpha 4$ isoforms, the recombinant proteins produced in Sf-9 insect cells were used (control lane). Samples were also analyzed for expression of the ubiquitously expressed GAPDH.

of Na,K-ATPase activity were performed at saturating concentrations of Na^+ , K^+ , Mg^{2+} and ATP. As shown in Fig. 4A–D, all cell samples exhibited a heterogeneous response to ouabain. The calculated inhibition constants were compatible with the presence of the $\alpha 1$ and $\alpha 4$ isoforms (Table 3). The relative activity of the Na,K-ATPase isoforms varied depending on the cell type. Functional $\alpha 4$ was present in spermatogonia, and the percentile amounts of the isoform increased with differentiation of the cells. Maximal activity of $\alpha 4$ was found in spermatozoa from the caput and cauda of the epididymis, where the isoform contributed to two-thirds of the total Na,K-ATPase of the cells. Concomitantly, relative activity levels of $\alpha 1$ decreased (Fig. 4A–D and Table 3).

Gamete differentiation was accompanied by an approximately twofold increase in absolute values of total Na,K-ATPase activity; as shown in Fig. 5, this was primarily dependent on the higher activity of the Na,K-ATPase $\alpha 4$ isoform. While $\alpha 1$ changed only slightly (approximately 1.3-fold), $\alpha 4$ activity increased more than sevenfold, the major changes taking place with the development of the round spermatids into spermatozoa.

Na,K-ATPase isozyme-mediated ion transport in isolated germ cells

While determination of the ouabain-sensitive hydrolysis of ATP in cell homogenates gives an estimate of the activity of the Na,K-ATPase and its isozymes in the whole cells, it

Figure 4 Ouabain inhibition profile of Na,K-ATPase of isolated rat male germ cells. Spermatogonia (A), pachytene spermatocytes (B) and round spermatids (C) were obtained by unity gravity sedimentation, while spermatozoa (D) were obtained from the caput (□) and cauda (■) of the epididymis. Dose–response curves were determined on cell homogenates under saturating concentrations of all ligands and at the indicated ouabain concentrations. Results are expressed as percentage of maximal activity in the absence of the inhibitor. The biphasic curves represent the best fit of the experimental data to highly ouabain-sensitive and ouabain-resistant Na,K-ATPases, compatible with the presence of the $\alpha 4$ and $\alpha 1$ isoforms. Each value is the mean, and error bars represent the S.E.M. of three to five experiments. The F test indicated that the parameters describing the different enzyme population in each sample are statistically different ($P < 0.001$).

Table 3 Inhibition constants (K_i) and relative amounts of Na,K-ATPase $\alpha 1$ and $\alpha 4$ isoforms in rat male germ cells at different stages of development isolated by unit gravity sedimentation.

Isoform		Germ cell type				
		Spermatogonia	Spermatocytes	Spermatids	Caput spermatozoa	Caudal spermatozoa
$\alpha 1$	(%)	66.3 $\pm$ 3.1	51.0 $\pm$ 1.8	45.3 $\pm$ 2.9	34.8 $\pm$ 2.7	26.8 $\pm$ 2.5
	K_i (M)	4.8 $\pm$ 1.2 $\times 10^{-5}$	7.4 $\pm$ 1.5 $\times 10^{-5}$	4.2 $\pm$ 1.5 $\times 10^{-5}$	1.3 $\pm$ 0.6 $\times 10^{-5}$	6.2 $\pm$ 0.3 $\times 10^{-5}$
$\alpha 4$	(%)	33.7 $\pm$ 4.1	49.0 $\pm$ 2.5	52.8 $\pm$ 5.1	65.2 $\pm$ 5.9	73.2 $\pm$ 4.4
	K_i (M)	8.9 $\pm$ 7.8 $\times 10^{-9}$	7.3 $\pm$ 2.8 $\times 10^{-9}$	2.7 $\pm$ 2.5 $\times 10^{-9}$	2.8 $\pm$ 2.0 $\times 10^{-9}$	3.9 $\pm$ 1.9 $\times 10^{-9}$

Parameters were calculated from dose–response curves for the ouabain inhibition of Na, K-ATPase activity. Values represent the mean $\pm$ S.E.M.

does not provide specific information on the functional status of the Na pump molecules at the plasma membrane of the cells. This is relevant, since the activity of Na,K-ATPase at the cell surface is essential in maintaining the ion gradients and membrane potential of the cells. To determine the relative contribution of the $\alpha 1$ and $\alpha 4$ isoforms to the overall transport function of the Na,K-ATPase of male germ cells, we measured the ouabain-sensitive uptake of ^{86}Rb by the cells, which is used as a tracer for K^+ . This was performed in the absence and presence of two concentrations of ouabain, 3×10^{-6} and 1×10^{-3} M. As we have previously shown (Blanco *et al.* 1999) and from the results of Fig. 1, it is clear that these ouabain concentrations selectively inhibit the $\alpha 4$ and $\alpha 1$ isoforms respectively. The ^{86}Rb uptake corresponding to the total Na,K-ATPase and the relative contribution of each isoform to overall enzyme ion transport in spermatogonia, spermatocytes, spermatids, caput and caudal spermatozoa are presented in Fig. 6. As shown, the relative amount of ^{86}Rb uptake catalyzed by the $\alpha 4$ isoform increased as the cells developed. At the same time, the relative contribution of $\alpha 1$ to overall cell transport decreased. These observations correlate well with the percentile levels of $\alpha 1$

and $\alpha 4$ for the hydrolysis of ATP (Fig. 4). Altogether, these results indicate that the relative amounts of active Na,K-ATPase α isoforms at the plasma membrane are similar to and reflect those present in the whole cells.

α Isoform mRNA and polypeptides in isolated germ cells

To assess the expression pattern of the Na,K-ATPase α isoforms in male germ cells at the RNA level, we performed RT-PCR using the primers described in Table 1. As shown in Fig. 7A, cDNAs for only the $\alpha 1$ and $\alpha 4$ isoforms were amplified, indicating that these isoforms, and not $\alpha 2$ and $\alpha 3$, are being expressed in the cells. This agrees with our previous results from whole testis (Blanco *et al.* 2000). The specificity of the Na,K-ATPase primers used is indicated by the lack of cross-reactivity with cDNAs of α isoforms other than that containing the corresponding complementary sequence. In addition, PCR reactions performed on the samples in the absence of reverse transcription yielded no Na,K-ATPase isoform products, indicating the lack of genomic DNA contamination in the RNA isolation step (Fig. 7A, no RT). The mRNAs for $\alpha 1$ and $\alpha 4$ were identified at all stages of differentiation; however,

Figure 5 Total Na,K-ATPase activity and activity of the $\alpha 1$ and $\alpha 4$ isoforms in isolated rat male germ cells. Specific activity was measured on homogenates from the different cell types. Total Na,K-ATPase was determined as the hydrolysis of ATP sensitive to 1 mM ouabain. Activity of $\alpha 4$ was measured as the ATP hydrolysis sensitive to 3×10^{-6} ouabain, while function of $\alpha 1$ was obtained by the difference between 3×10^{-6} and 1×10^{-3} M ouabain. Values are the mean, and bars represent the S.E.M. of three to five experiments. Values significantly different from the corresponding ones of spermatogonia are indicated. * $P < 0.05$; ** $P < 0.001$.

Figure 6 Na,K-ATPase transport in isolated male germ cells. ^{86}Rb uptake (used as a tracer for K^+) was determined for the different cell types in the absence and presence of 3×10^{-6} and 1×10^{-3} M ouabain. The accumulation of isotope by the cells was measured over 3 min. Total uptake dependent on the Na,K-ATPase was determined as that sensitive to 1 mM ouabain. ^{86}Rb uptake of the $\alpha 4$ isoform was calculated as the difference observed between 0 and 3×10^{-6} ouabain, while ^{86}Rb uptake of $\alpha 1$ was estimated by subtracting the values at 1×10^{-3} M from those at 3×10^{-6} M ouabain. Total ^{86}Rb uptake values are expressed as percentage of maximal specific transport. Values are the mean and bars represent the S.E.M. of determinations performed in sextuplicate. Absolute values $\pm$ standard errors for maximal ^{86}Rb uptake in nmol/ μ g protein are shown in parenthesis over the corresponding bars. Asterisks indicate values significantly different from the corresponding ones of spermatogonia. * $P < 0.01$; ** $P < 0.001$.

relative to the ubiquitous GAPDH (Fig. 7B), their levels varied. While message for the $\alpha 1$ isoform was found to remain relatively constant in the various cell types, and slightly decreased in spermatozoa from the cauda of the epididymis, the mRNA for $\alpha 4$ was detected in small amounts in spermatogonia and peaked in pachytene spermatocytes and round spermatids. In addition, to assess the nature of the various cell fractions used, primers for amplification of several male germ cell specific markers were used. As shown in Fig. 7B, mRNA for c-kit was found in spermatogonia, SCP-1 mRNA was detected mainly in spermatocytes and the message for PRM2 was predominately found in postmeiotic cells, confirming the correlation of the Na,K-ATPase α isoform expression with the expected cell type.

To determine Na,K-ATPase expression at the protein level, immunoblot analysis of isolated male germ cell total proteins was performed for the $\alpha 1$ and $\alpha 4$ isoforms. Fig. 7C shows that both $\alpha 1$ and $\alpha 4$ isoforms were present at all stages of germ cell maturation. As positive controls, the corresponding Na,K-ATPase isoforms, exogenously expressed in Sf-9 cells, were included (Blanco & Mercer 1998). Relative to GAPDH, the expression level of the $\alpha 1$ isoform remained constant in the various cell types, whereas $\alpha 4$ increased during gametogenesis, becoming maximal in caput and caudal spermatozoa. These results are coincident with the developmental changes found in the functional assays, and show that the major changes correspond to those of the $\alpha 4$ isoform. The maximal expression of $\alpha 4$ in spermatozoa agrees with the raise in RNA levels for the isoform at earlier cell stages, such as pachytene spermatocytes and round spermatids (Fig. 7A).

Immunolocalization of Na,K-ATPase isoforms

Another important goal in the maturational analysis of the Na,K-ATPase of male germ cells is the localization of the isoforms in the isolated cells. We determined this by immunofluorescence staining, using the anti- $\alpha 1$ and anti- $\alpha 4$ antisera and FITC-conjugated secondary and tertiary antibodies. The confocal microscopy images obtained are shown in Fig. 8. Samples treated with normal rabbit serum, instead of the primary antibodies, were used as a control. In all cases, DAPI was included to stain the cell nuclei. As shown, the anti- $\alpha 1$ and anti- $\alpha 4$ antisera labeled the male germ cells differently. The $\alpha 1$ polypeptide appeared to be evenly distributed at the surface of spermatogonia, pachytene spermatocytes and round spermatids. In caput and cauda spermatozoa, $\alpha 1$ extended along the sperm flagellum, covering most of it, and was barely detected in the sperm head. In contrast, $\alpha 4$ was present not only at the plasma membrane, but also in intracellular compartments of spermatogonia, pachytene spermatocytes and round spermatids. The intracellular localization of $\alpha 4$ was more prominent in pachytene spermatocytes and in round spermatids. In the spermatozoa, from both the caput and cauda of the epididymis, $\alpha 4$ showed clear compartmentalization, localizing to the central region of the flagellum. Costaining of the caput and caudal spermatozoa with MitoFluor Red 589, a marker specific for mitochondria (Singh *et al.* 2003), showed a spatial correspondence with that of the $\alpha 4$ isoform along the midpiece of the flagellum (Fig. 9). Therefore, unlike $\alpha 1$, $\alpha 4$ is not present in the principal and end pieces of the sperm flagellum. Nevertheless, like $\alpha 1$, the label for $\alpha 4$ was almost absent in the sperm head and did not

Figure 7 RT-PCR and immunoblot detection of Na,K-ATPase α isoform expression in isolated rat male germ cells. (A) Total RNA was subjected to reverse transcription and the obtained cDNA was then amplified by PCR using oligonucleotides specific for each isoform of the Na,K-ATPase (see Table 1). The cDNAs corresponding to the various α isoforms were used as a template control for the PCR reaction. As a control of the absence of genomic DNA, PCR amplification with Na,K-ATPase isoform-specific primers is shown for RNA from the different cell types before reverse transcription (no RT). (B) RT-PCR amplification of spermatogenic cell type-specific markers and GAPDH. The PCR reaction was carried out with primers specific for c -kit, SPC-1, PRM2 and GAPDH at the conditions shown in Table 1. (C) Immunoblot analysis of $\alpha 1$ and $\alpha 4$ isoforms in isolated rat male germ cells. Proteins from the different cell types were separated by SDS-PAGE (7.5% gel) and transferred to nitrocellulose, and the α polypeptides were detected with the anti- $\alpha 1$ and anti- $\alpha 4$ antisera, followed by horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. As a control for $\alpha 1$ and $\alpha 4$, the isoforms produced in Sf-9 insect cells were used (control lane). As an additional internal control, expression of GAPDH was included.

overlap with that of wheat germ agglutinin conjugated with Alexa Fluor, which labels the acrosome (Chan *et al.* 2002).

Discussion

The importance of the molecular heterogeneity of the Na,K-ATPase in the biology of male germ cells is not precisely understood. In the present work, we studied the expression and activity of the $\alpha 1$ and $\alpha 4$ isoforms of the enzyme and showed that they are differentially regulated during male germ cell development. Differentiation of the male gametes results in an approximately twofold increase in total Na,K-ATPase activity, and this primarily depends on the increase in activity of $\alpha 4$, which rises by approximately sevenfold. The relevance of this change is further supported by the ^{86}Rb transport experiments, which indi-

cate that ion transport catalyzed by $\alpha 4$ increases from approximately 25% in spermatogonia to approximately 75% in spermatozoa. The change in ATP hydrolysis and ^{86}Rb uptake associated with $\alpha 4$ significantly modifies the relative contribution of each α isoform to the overall activity of the Na,K-ATPase in the differentiating male germ cells, with a progressive increase in the $\alpha 4$ to $\alpha 1$ isoform ratio. Therefore, cell differentiation is accompanied by a differential usage of Na,K-ATPase isoforms, favoring the expression of enzyme molecules composed of $\alpha 4$ that may be functionally better adapted to the stage-specific requirements of the cells.

The functional developmental pattern of $\alpha 1$ and $\alpha 4$ correlates with the expression of mRNA and protein for the isoforms. This suggests that the $\alpha 4$ gene is transcriptionally upregulated in spermatocytes, and this is followed by

Figure 8 Immunolocalization of Na,K-ATPase $\alpha 1$ and $\alpha 4$ isoforms in male germ cells at different stages of development. Cells were fixed with paraformaldehyde and subjected to immunofluorescence, as described in Materials and Methods. The anti- $\alpha 1$ and anti- $\alpha 4$ antisera were used to detect the corresponding Na,K-ATPase α isoforms. A fluorescein isothiocyanate (FITC)-conjugated goat antirabbit antiserum was used as the secondary antibody. To enhance the signal, an anti-fluorescein/Oregon green, rabbit IgG, conjugated with Alexa Fluor 488 was used. Normal rabbit serum, followed by the secondary and tertiary antibodies, was used as a control. DAPI was included to stain the cell nuclei. Scale bar at the bottom right = 10 μ m.

a burst in protein synthesis days later during spermatid development. This pattern of induction places $\alpha 4$ with a cluster of male germ cell-specific genes that increase during or after meiosis, and that are essential for the structure, DNA organization, metabolism, motility and sperm–egg binding properties of spermatozoa (Shima *et al.* 2004). For some of these male germ cell genes, such as protamine and transition protein 2, induction of transcription is not followed by rapid translation of the mRNA, and

transcripts are instead stored after synthesis as ribonucleoproteins in a translationally repressed state until they are translated in the elongating spermatids (Steger 1999, Kleene 2005). The time lag we encounter between the expression of $\alpha 4$ mRNA and protein suggests that this regulatory mechanism could be operating for the isoform. This will provide the translationally quiescent mature spermatozoa with high levels of the Na,K-ATPase $\alpha 4$ polypeptide.

Figure 9 Colabeling of spermatozoa from the caput and cauda of the epididymis with the Na,K-ATPase anti- $\alpha 4$ antibody, mitochondrial and acrosome markers. Cells were prepared as described in Materials and Methods. The $\alpha 4$ isoform was detected with the anti- $\alpha 4$ antiserum, the acrosome was stained with wheat germ agglutinin conjugated with Alexa Fluor 633 and the mitochondria with MitoFluor Red 589, and DAPI was included to stain the cell nuclei. The images on the right represent the merging of all labels. Scale bar at the bottom right = 10 μm .

Although our functional and expression studies correlate well qualitatively, we find some quantitative discrepancies between the protein amounts and the catalytic or transport activity of the Na,K-ATPase. This is, for example, the case of the $\alpha 4$ isoform in spermatogonia, where mRNA and protein amounts are small and relatively low compared with the functional levels measured. This may just reflect the different sensitivity and intrinsic limitations of the techniques used to determine each parameter. Alternatively, the protein to function divergence suggests that other post-translational mechanisms are taking place in regulation of activity of the $\alpha 4$ isoform in a cell type-specific manner. In any case, the maximal expression of $\alpha 4$ in spermatozoa agrees with our previous observations indicating that the levels of the highly ouabain-sensitive Na,K-ATPase component of testes is drastically reduced in transgenic mice deficient in the Egr-4 transcription factor. The infertile phenotype of these mice results from maturational arrest of the male germ cells at an early spermatocyte stage (Blanco *et al.* 2000).

Our results also confirm previous work showing that expression of the Na,K-ATPase $\alpha 4$ isoform is upregulated during sexual maturation of the testis (Woo *et al.* 2000). However, in that study, $\alpha 4$ expression was determined in the whole gonad, and the changes were not detected until weeks 4 and 6 of age for mRNA and protein respectively. This placed expression of $\alpha 4$ at late stages of spermatogenesis, and only in the haploid cells. Our findings suggest an earlier appearance of $\alpha 4$ in development. Although they are at low levels, we find some expression and function of $\alpha 4$ in testis at postnatal day 7, where meiotic cells are absent (Figs 2 and 3), and in the spermatogonia isolated from 10-day-old animals (Figs 5 and 7). The discrepancy between our results and those of Woo *et al.* may depend on differences in the methods used in

each case to detect the Na,K-ATPase isoforms. Previous work has used Northern blot analysis and a different antibody for the immunoblot detection of $\alpha 4$ (Woo *et al.* 2000), whereas our approach included RT-PCR and functional assays that might be more sensitive. Moreover, the use of unit gravity sedimentation to obtain cell populations highly enriched in specific male germ cell types allows the determination of $\alpha 4$ expression without the interference caused by the presence of other testis cells.

Interestingly, we find mRNA for the Na,K-ATPase $\alpha 1$ and $\alpha 4$ isoforms in spermatozoa from the caput and cauda of the epididymis. Several other studies have reported the presence of RNA coding for other proteins in the terminally differentiated spermatozoon (reviewed in Kramer & Krawetz 1997, Miller *et al.* 1999). In general, RNA in the differentiated spermatozoa has been considered to be residual or nonfunctional, since it is unlikely to be used by the transcriptionally inactive gametes. Therefore, the message for the Na,K-ATPase $\alpha 1$ isoform, and that of the more abundant $\alpha 4$ isoform, are probably part of the spermatozoal remnant RNA.

The distinct regulation of each Na,K-ATPase isoform during gametogenesis supports the notion that $\alpha 1$, which is present in all tissues, functions as the isoform that maintains the basal Na^+ and K^+ transport in the cells, and that $\alpha 4$ plays germ cell-specific roles. Accordingly, $\alpha 4$ function has been shown to be necessary for normal sperm motility (Woo *et al.* 2000). However, the presence of $\alpha 4$ in both diploid and haploid male germ cells suggests that function of the isoform may be important not only for mature spermatozoa, but also for maintaining ion homeostasis through differentiation of the male gametes. For spermatogenesis to take place in an orderly manner, precise control at specific points of the process is required. This is achieved through different signaling mechanisms, among

which, changes in ion transport and in membrane potential play an important role. The existence of an isoform of the Na,K-ATPase with particular functional characteristics, such as $\alpha 4$, may be important in finely controlling Na^+ and K^+ electrochemical gradients to adjust cell ion content and membrane potential to the specific requirements of the different male germ cell types. The low affinity of $\alpha 4$ for extracellular K^+ (lower than $\alpha 1$) (Blanco *et al.* 1999) may correlate with the high K^+ environment the male germ cells face once they migrate into the adluminal compartment of the seminiferous epithelium (Muffly *et al.* 1985). Thus, at the high K^+ concentration of the testis tubules, the cation does not affect $\alpha 1$, but can regulate $\alpha 4$ function, influencing the ion gradients across the plasma membrane, and the membrane potential of the cells.

Once the spermatozoa mature and are released into the female tract, they face drastic changes in the ion concentrations, with K^+ becoming lower than Na^+ . Because of the high affinity of $\alpha 4$ for Na^+ (higher than $\alpha 1$) (Blanco *et al.* 1999), the influx of Na^+ into the cells preferentially stimulates $\alpha 4$, and the isoform works at near maximal levels. The higher activity of $\alpha 4$ will then be essential to provide the increased membrane excitability required for the demands of sperm motility. In this manner, the Na,K-ATPase $\alpha 4$ isoform may represent an important modulator of the basal ion homeostasis maintained by $\alpha 1$, both during spermatogenesis and for the function of the mature gametes.

Besides the developmental differences in expression of $\alpha 1$ and $\alpha 4$, we also found dissimilarities in the localization of the isoforms with male germ cell development. In contrast to the $\alpha 1$ isoform, which exhibits a homogeneous distribution on the surface of the immature cells and over the sperm flagellum, $\alpha 4$ shows a particular cell localization. In spermatogonia, pachytene spermatocytes and round spermatids, $\alpha 4$ is located at the plasma membrane, and also shows important levels in intracellular compartments. In other cell types, it has been shown that soon after synthesis in the endoplasmic reticulum, the Na,K-ATPase α subunit assembles with the β polypeptide to become active (Caplan *et al.* 1986, Gatto *et al.* 2000). These intracellular stores of Na,K-ATPase are important in maintaining the plasma membrane levels of the enzyme (reviewed in Therien & Blostein 2000, Teixeira *et al.* 2003), through regulatory mechanisms that involve the activation of protein kinases and phosphorylation of the Na,K-ATPase α subunit. Incorporation of phosphate in the α subunit results in translocation of the Na,K-ATPase between intracellular compartments and the plasma membrane, modifying the number of enzyme units and thus its activity at the cell surface (Yudowski *et al.* 2000, Budu *et al.* 2002). It is possible, then, that the $\alpha 4$ isoform we found in the intracellular compartment of spermatogenic cells are internal pools of the isoform waiting to be redistributed to the plasma membrane upon different stimuli. Although we have shown that $\alpha 4$ can assemble with both the $\beta 1$ and $\beta 3$ subunits when coexpressed in insect cells, β isoform expression and $\alpha\beta$ pairing in the male germ

cells are not known. It is plausible that preferential association of $\alpha 4$ with a particular beta subunit could generate pools of Na,K-ATPase with different cell localization. Experiments are now underway in our laboratory to explore this possibility.

In spermatozoa, $\alpha 4$ is confined to the midpiece of the cells, as shown by Woo *et al.* (2000). This suggests the existence of isoform-specific mechanisms for the targeting and particular retention of $\alpha 4$ at restricted domains of the plasma membrane of the male germ cells. Interestingly, little or no label for $\alpha 1$ or $\alpha 4$ was found in the sperm head. The localization of $\alpha 4$ in the midpiece of spermatozoa coincides with that of MitoFluor Red 589, which specifically labels the mitochondria in that region of the cells. This supports a primary role of the Na,K-ATPase in flagellar motility, and agrees with the notion that $\alpha 4$ at the plasma membrane and the subjacent mitochondria may work in close proximity. According to this model, the Na^+ gradient generated by $\alpha 4$ in the midpiece of the flagellum is coupled to the function of the Na^+/H^+ exchanger that works to clear the H^+ released to the cytoplasm by the mitochondria (Woo *et al.* 2002).

After they are produced in the testis and during their passage through the epididymis, spermatozoa undergo a series of changes that involve the reorganization of several plasma membrane components (Jones 1998). We did not find significant changes in expression and function of the Na,K-ATPase α isoforms when spermatozoa obtained from the caput and the cauda of the epididymis were compared. Altogether, our results suggest that the primary changes in $\alpha 1$ and $\alpha 4$ take place during spermatogenesis, and that the Na,K-ATPase isoforms are not subjected to further maturational processing in the epididymis.

In conclusion, this work represents the first study of the functional expression of the $\alpha 1$ and $\alpha 4$ isoforms of the Na,K-ATPase in isolated male germ cells during their ontogeny in the seminiferous tubules and along their maturation in the epididymis. The distinct developmental regulation of expression, localization and activity of $\alpha 4$, along with the unique kinetic properties of the polypeptide, supports an important role of this particular Na,K-ATPase isoform in the physiology of spermatogenic cells and the mature male gametes. Recently, the use of transgenic technology in mice has been a valuable tool in understanding Na,K-ATPase α isoform function (Shelly *et al.* 2004). Future studies in mice null or deficient in the $\alpha 4$ isoform will be important to confirm the role of the isoform in male gamete physiology and fertility.

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